STUDIES OF THE ANTIDIABETIC ACTIVITY AND ORGAN-SPECIFIC TOXICITY OF AQUEOUS STEM BARK EXTRACT OF *ANNICKIA POLYCARPA*

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DECLARATION

I, Nathaniel Lartey Lartey (Department of Chemical Pathology, University of Ghana, Legon-Accra) hereby declare that this thesis is the outcome of my own research project under the supervision of Prof. Henry Asare-Anane (Department of Chemical Pathology, University of Ghana School of Biomedical and Allied Health Sciences, College of Health Sciences, Korle-Bu, Accra) and Prof. L. K. N. Okine (Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon, Accra). To the best of my knowledge, this thesis contains neither materials which has been accepted for the award of any degree or any material previously published by another author, except where due reference is made in the text of the thesis

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ABSTRACT

The antidiabetic effect of aqueous stem bark extract of *A. polycarpa* (AP) in male ICR mice and its sub-chronic toxicity in Sprague-Dawley rats were investigated. The effect of AP (20 mg/kg, 100 mg/kg, and 500 mg/kg) on fasting blood glucose, serum triglyceride and total cholesterol levels were determined over a period of 4 weeks. An oral glucose tolerance test was also conducted over 150 min post-glucose load. Organ specific toxicity of AP (20 mg/kg, 100 mg/kg, and 500 mg/kg) in Sprague- Dawley rats was also studied over a period of 3 months. Serum ALP, ALT, direct bilirubin and creatinine were measured at baseline and monthly intervals thereafter. Urinalysis was performed at baseline and at termination of treatments whilst hematological analysis was conducted at termination of treatments. Blood clotting time and pentobarbital-induced sleeping time were determined at termination of treatments. Phytochemical constituents and antioxidant activity of AP were also determined.

The AP, like glibenclamide and metformin, showed significant ($p<0.05$) hypoglycaemic, hypotriglyceridaemic and hypocholesterolaemic effects, which may possibly be due to increased secretion of insulin as a result of restoration of the morphology of beta cells of the pancreas that had become necrotic in the diabetic mice. AP showed significant antioxidant activity relative to the standard antioxidant, BHT evidenced by its ability to scavenge DPPH free radicals. This observed antioxidant activity may be due to the presence of phenolic compounds particularly flavonoids in AP.

The LD_{50} of AP in rats was greater than 5000 mg/kg with no observable signs of toxicity. AP showed selective organ specific toxicity of the lung in the alveolar areas which was characterized by interstitial fibrosis and alveolar septa thickening. AP had no adverse effect on the liver and kidney as shown by its lack of effect on serum ALP, ALT, direct bilirubin and creatinine and

urinalysis, as well as the absence of adverse effects on the morphology of the cells of these organs. No adverse morphological effects were also observed with the heart muscle cells. AP did not also affect blood clotting time, hematological parameters and pentobarbital induced sleeping time. These results indicate that AP has significant antidiabetic activity and provides scientific evidence to validate the anecdotal evidence of its use in the management of diabetes mellitus. However, its selective toxicity to the alveolar cells of the lung is a cause for worry and, therefore, care must be taken in its use in humans.

DEDICATION

To the almighty God, my mother, Miss Cynthia Korkor Tetteh and my beloved girlfriend, Ms. Josephine Nketsiah.

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LIST OF ABBREVIATIONS

ADA	American Diabetes Association
AGEs	Advanced glycation end products
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
AMP	Adenosine monophosphate
AP	Annickia polycarpa
ATP	Adenosine triphosphate
AUC	Area under the curve
BHT	Butylated hydroxytoluene
CPMR	Center for Plant Medicine Research
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
FBG	Fasting blood glucose
GA	Gallic acid
GAFCO	Ghana Agro Food Company Limited
GLIB	Glibenclamide
GLUT-2	Glucose transporter 2
G6PD	Glucose-6-phosphate dehydrogenase
HCl	Hydrochloric acid
HCT	Haematocrit
HGB	Haemoglobin
HLA	Human leukocyte antigen
ICR	Institute of cancer Research
IDF	International Diabetes Federation
IDDM	Insulin-dependent diabetes mellitus

i.v.	Intravenous
i.p	Intraperitoneal
LD_{50}	Median lethal dose
LYM	Lymphocytes
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean cell volume
MET	Metformin
MPV	Mean platelet volume
NADPH	Reduced nicotinamide dinucleotide phosphate
NIDDM	Non insulin-dependent diabetes mellitus
OGTT	Oral glucose tolerance test
PLT	Platelet
PKC	Pyruvate kinase C
QE	Quercetin
RBC	Red blood cell
RDVSD	Red blood cell distribution width
ROS	Reactive oxygen species
SEM	Standard error of the mean
STZ	Streptozotocin
TCHOL	Total cholesterol
TG	Triglycerides
WBC	White blood cell
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Diabetes mellitus is a metabolic disorder characterized mainly by hyperglycemia which results from deficiencies in the secretion of insulin, insulin action, or both. The clinical presentation of diabetes mellitus include frequent urination, excessive thirst, increased hunger, weight loss, fatigue, reduced concentration, numbness in the hands or feet, blurry vision, recurrent infections, slow-healing wounds, nausea and stomach pain (IDF, 2015). It is a prominent cause death worldwide, and was estimated that about 346 million adults were affected in 2011 (WHO, 2012) and projected that deaths resulting from diabetes will increase by two thirds between the year 2008 and 2030 (WHO, 2012).

Over the past decade, interest in the study of natural materials as a potential source of medicinal substances has been provoked (Rout *et al*., 2009) and these plant-based medicines are referred to as herbs. An estimated 60 and 95% of rural Africans are said to be reliant on traditional herbal preparations for their primary health care needs (Anyinam, 1995; WHO, 2000). Therefore, in developing countries with meager resources, medicinal plants have an ever important role to play in averting the complications of lifelong diseases like diabetes mellitus (Chauhan *et al*., 2010).

Antidiabetic herbal products are now very popular owing to the disadvantages and side effects that the allopathic drugs present. In Ghana, plants reported to possess antidiabetic properties include *Indigofera arrecta*, bark of *Myrianthus arboreus*, *Bridelia ferruginea*, unripe fruit of *Musa paradisiaca*, *Momordica charantia*, *Costus schlechteri* and *Ocimum canum* (Addy and Nyarko, 1988; Addy *et al*., 1992; Mills-Robertson, 1993; Hogarh, 1996). Others are yet to

receive scientific justification. The bark and leaves of *A. polycarpa* are thought to possess many alkaloids, most of them with structures such as berberine and protoberberines (Obeng, 2011). With respect to type 2 diabetes mellitus, these compounds are known to be anti-hyperglycemic, reduce insulin resistance, cause pancreatic β-cell regeneration, and reduce lipid peroxidation in mouse type 2 diabetes model (Lee *et al*., 2006). Thus leaf extract of *A. polycarpa* might be useful for the management of diabetes mellitus.

1.2 PROBLEM STATEMENT

The short term complications of diabetes mellitus which includes hyperglycaemia, if not well managed will result to long-term complications. The long-term complications of diabetes mellitus include progressive development of retinopathy with possible blindness, nephropathy that could result in renal failure and/or peripheral neuropathy with risk of foot ulcers, amputation, charcot joints (McCance *et al*., 1994; Stratton *et al*., 2000).

The estimated global prevalence of diabetes mellitus amongst adults in 2010 was 285 million (6.4%) and this value is projected to rise to around 439 million (7.7%) by 2030 (Shaw *et al*., 2010). The prevalence of diabetes mellitus in the Greater Accra Region of Ghana was found to be 6.3% (Amoah *et al*., 2002). Healthcare cost due to diabetes mellitus in Ghana ranged between USD 23,073.09 and USD 41,475.67 for the year 2010 (Zhang *et al*., 2010).

Management of diabetes mellitus usually involves the use of conventional drugs and herbal extracts. The uses of conventional drugs are accompanied by adverse side effects. Treatment with glibenclamide for example, is associated with drug-induced hypoglycaemia and cholestatic jaundice (Gangji *et al*., 2007). It also causes acute haemolysis in G6PD deficient individuals

(Meloni and Meloni, 1996). The cost of management of diabetes mellitus using conventional drugs is quite high. This high cost of management coupled with adverse side effects has caused people, particularly rural folks, to resort to the use of medicinal plants because they are cheaper and easily accessible. One main problem associated with the use of herbal preparations is lack of standardization, which may result in over dosage and possibly toxicity. Stem bark extract of *Cassia sieberiana* used in North-eastern Nigeria for the treatment of jaundice (Madusolumuo *et al*., 1999) has been proven to be hepatoxic at lower doses of 20-60 mg/kg and nephrotoxic at a higher dose of 180 mg/kg (Obidah *et al*., 2009). This is at variance with the popular assertion that plant extracts are non-toxic and thus the need to conduct toxicity studies on them. The safety and efficacy of most of these plants have not been scientifically proven and, therefore, put consumers at risk.

Discovery of traditional medicines which are more efficacious, display a rapid onset of drug action, produce little or no side effects, readily available and cheaper will prove beneficial in the search for better, affordable and more effective ways of managing diabetes mellitus in Ghana. There have been anecdotal claims of the use of *A. polycarpa* for the management of diabetes mellitus in Ghana. However, there is no available scientific data as to its use as a safe antidiabetic. There is, therefore, the need to conduct proper scientific investigation to evaluate its efficacy and safety as an antidiabetic, and to regulate its rational use.

1.3 JUSTIFICATION

Diabetes mellitus is increasing at a disturbing rate worldwide and particularly in urban areas of developing countries. This has been linked to overweight as a result of sedentary lifestyle, advanced age, urbanization, high incomes and level of education (Amoah *et al*., 2002; Amoah,

2003). The high cost of managing this chronic metabolic disease using orthodox drugs and the adverse side effects of treatment have caused many to resort to the use of medicinal plant products as alternatives. However, most of these herbal products have not been standardized for rational use in humans. Therefore, there is a need for the standardization and scientific validation of these products.

The bark of *A. polycarpa* possesses numerous phytochemicals with antioxidant and hypoglycaemic properties which may be effective in the management of diabetes mellitus and its complications. Research into *A. polycarpa* if found useful will help alleviate the financial burden of sufferers of diabetes because it will be cheaper and easily accessible and will possibly help reduce the complications of diabetes mellitus. This will also help reduce importation of orthodox drugs and possibly provide foreign exchange when exported.

1.4 HYPOTHESIS

Administration of aqueous extracts of *A. polycarpa* to diabetic animals will not reduce plasma glucose concentration and may be unsafe.

1.5 AIM

The study seeks to assess the antidiabetic activity and organ specific toxicity of aqueous extract of *A. polycarpa* in laboratory animals.

1.6 SPECIFIC OBJECTIVES

1. To determine the hypoglycaemic and hypo-lipidaemic effects of *A. polycarpa* in alloxan-induced diabetic mice.
2. To determine the effects of extract on the morphology of pancreatic cells of diabetic animals.
3. To determine the groups of phytochemicals, the total phenolic and flavonoid contents, and the antioxidant activity of the extract
4. To determine the LD_{50} of the extract, and assess its organ specific toxicity in sub-chronic toxicity studies.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 DIABETES MELLITUS

Diabetes mellitus (DM) is a metabolic disease and a serious health problem characterized by hyperglycemia, altered lipid, carbohydrate and protein metabolisms. It affects the quality of life of the patient, socially, psychologically and even physical ill health (Davis, 2006; Dewanjee *et al*., 2009). In urban Ghana, mainly obese patients of low socioeconomic status are affected by type 2 diabetes mellitus and often go with high blood pressure and abnormal blood lipid levels (Danquah *et al*., 2012). Type 2 diabetes mellitus affects at least 6% of adults and is linked to obesity and age. Factors such as urbanization, cultural change, and genetic susceptibility have been found to play roles in type 2 diabetes mellitus among Ghanaians (Cooper and Rotimi, 1997; Saleh *et al*., 2002; Banini *et al*., 2003).

2.2 CLASSIFICATION OF DIABETES MELLITUS

There are two main forms of diabetes (Types 1 and 2) and they differ in their pathogenesis, but both have hyperglycemia as a key diagnostic tool. In type 1 diabetes, hyperglycaemia is due to absolute insulin-lack. Hyperglycemia in type 2 diabetes is due to impairment in insulin secretion combined with or without impairment of insulin action (Lin and Sun, 2010). Insulin resistance typically precedes the onset of type 2 diabetes and is commonly accompanied by other cardiovascular risk factors such as dyslipidemia, hypertension, and pro-thrombotic factors (Gray *et al*., 1998).

2.2.1 Insulin-Dependent Diabetes Mellitus

This type of diabetes mellitus (IDDM) also referred to type 1 diabetes mellitus, commonly manifests clinically by a sudden onset of symptoms. It is further grouped into autoimmune and idiopathic diabetes mellitus. Patients are susceptible to ketosis and require injection with insulin to sustain life (Willis *et al*., 1996). It accounts for only 5–10% of those with diabetes and is due to autoimmune damage of the pancreatic beta cells (World Health Organization, 1999; American Diabetic Association, 2005). Characteristically, this type of disease is common in juveniles, and was formerly referred to as juvenile diabetes. On the other hand, recognition and occurrence of symptoms can be seen for the first time at any age. Therefore, it is inappropriate for diagnosis to be centered on age at onset. This type of diabetes can also be recognized in a pre-ketosis-prone stage in addition to the ketosis-prone stage. Abnormal immune responses and autoimmunity are also thought to play an etiologic role, and islet cell antibodies are frequently present at diagnosis in this type of diabetes (Irvine, 1979). Insulin resistance is not a contributing factor in its pathogenesis (Atkinson and Maclaren, 1994).

Idiopathic diabetes mellitus has been described commonly in African-Americans and Asians as well as other ethnic groups (Imagawa *et al*., 2000; Tan *et al*., 2000). Generally these patients have perpetual insulinopenia, and they are predisposed to ketoacidosis, but have no evidence of autoimmunity (McLarty *et al*., 1990) or HLA association. Using insulin therapy for glycaemic control in most patients with idiopathic diabetes is better as compared to either oral hypoglycaemic agents or diet therapy alone and that long-term glycaemic control is well sustained (Pinero-Pilona *et al*., 2001).

2.2.2 Non-Insulin Dependent Diabetes Mellitus

The second type of diabetes, non-insulin dependent diabetes mellitus (NIDDM) or type 2 diabetes mellitus, frequently presents with reduced symptoms linked to the metabolic abnormalities due to diabetes mellitus. Patients suffering from this type of diabetes do not rely on insulin to avert hyperglycaemia and are often not susceptible to ketosis. On the other hand, they may need insulin to correct symptomatic, or persistent, fasting hyperglycemia if the use of diet or oral agents proves futile. There is mainly resistance to insulin, that is, diminished capacity of insulin to act on peripheral tissues and cause glucose to be taken up into tissues (Defronzo *et al*., 1992; Lillioja *et al*., 1993). Severe stress triggered by infections or trauma may cause patients to develop ketosis.

Characteristically, insulin levels may be normal with slight insulinopenia, or beyond normal levels of insulin linked with insulin resistance. Genetic predisposition together with environmental factors influences the onset of non-insulin dependent diabetes mellitus. Too much calories consumption which leads to gain in weight and obesity is possibly a vital element in its pathogenesis. Almost all patients suffering from type 2 diabetes mellitus have been found to be obese, and the obesity factor intensifies resistance to insulin (Bogardus *et al*., 1985; Campbell and Carlson, 1993). Weight loss improves hyperglycemia and glucose intolerance.

2.2.3 Other Types of Diabetes Mellitus

Diabetes mellitus is found to be associated with other disorders and conditions that presents with many medical symptoms not usually related with diabetes. The simultaneous occurrence of glucose intolerance and other features have been identified to be etiologically associated. In others, the regularity of co-occurrence suggests that there may be causal association. Therefore,

this type has been classified according to the known or suspected etiologic associations. For instance, diabetes mellitus may occur as a secondary condition to the following: pancreatic disease; endocrine diseases such as Cushing's syndrome, acromegaly, pheochromocytoma, somatostatinoma, glucagonoma, and primary aldosteronism; drugs, hormones, and chemical administration that result in hyperglycemia.

2.3 COMPLICATIONS OF DIABETES MELLITUS

Diabetes mellitus patients are prone to developing a number of serious health problems. Consistent hyperglycaemia can expose patients to conditions which will affect important parts of the body including the heart and blood vessels, kidneys, nerves, eyes and teeth. Furthermore, people living with diabetes also have a greater risk of developing infections (Shah and Hux, 2003).

2.3.1 Cardiovascular Disease

This affects the circulatory system which includes the heart and blood vessels and may result in lethal complications such as coronary artery disease which might lead to heart attack and stroke (Barrett-Connor *et al.*, 1991). This is the commonest reason for death in people with diabetes. A heightened blood pressure, hypercholesterolemia, hyperglycaemia and other risk factors contribute to increasing the risk of cardiovascular disease.

2.3.2 Diabetic Nephropathy

This is due to damage to small blood vessels in the kidney which renders it less efficient or could result in kidney damage. The prevalence of diabetic nephropathy in type 2 diabetic patients is about 30–40% (Adler *et al.*, 2003).

2.3.3 Diabetic Neuropathy

Diabetes mellitus can cause impairment in nerve function all over the body as a result of hyperglycaemia and high blood pressure. The end result of this is erectile dysfunction, digestion problems, and problems with many other functions. The extremities, predominantly the feet are usually the affected areas (Watkins, 1998).

2.3.4 Diabetic Retinopathy

Diabetes predisposes patients to some form of eye disease which causes reduced vision or blindness. Consistent hyperglycaemia, together with high blood pressure and high cholesterol, are the key causes of retinopathy. Sorbitol or glucitol is a polyhydroxyalcohol derived from glucose and metabolized to fructose under normal metabolic conditions. Cataract accompanied by other lesions in the eye occurs as a result of sorbitol deregulation in the eye (Ononogbu, 1988).

2.3.5 Pregnancy Complications

Women with any type of diabetes during pregnancy risk a number of complications if they do not carefully monitor and manage their condition. Hyperglycaemia during pregnancy can result in the foetus being overweight (Cox, 1994). This has the potential of causing delivery problems, shock to the child and mother, and an abrupt fall in blood glucose levels for the child after birth. Even though glucose tolerance of most women with gestational diabetes mellitus normalizes after delivery, type 2 DM might develop later on in life (Kim *et al*., 2002).

2.4 OXIDATIVE STRESS IN DIABETES

Oxidative metabolism is vital for the continued existence of cells. This results in free radicals generation and other reactive oxygen species that cause oxidative changes. Oxidative stress is said to occur when there is an excess of free radicals in the body than it can neutralize. This occurs either when antioxidant production in the body is reduced or when there is an excess of free radicals. There is a rise in free radical generation in diabetes mellitus and these results in oxidative stress.

Hyperglycaemia leads to an increase in free radical concentrations in plasma (Hammes *et al*., 1997, Cimato *et al*., 2008). Free radical generation is stimulated by unrestrained hyperglycaemia which occurs via the following ways (Giacco and Brownlee, 2010; Bajaj and Khan, 2012); increased glycolysis (Vaag *et al*., 1992); stimulation of sorbitol pathway (Di Naso *et al*., 2011); glucose autoxidation (Wolff *et al*., 1991); protein kinase C (PKC) dependent activation of NAD(P)H oxidase (Inoguchi *et al*., 2003); increased hexosamine pathway flux (Rolo and Palmeira, 2006), increased intracellular formation of AGEs (advanced glycation end products) (Giacco and Brownlee, 2010); increased expression of receptor for AGEs and its activating ligands (Giacco and Brownlee, 2010); and non-enzymatic protein glycation (Ceriello *et al*., 1992).

Hyperinsulinaemia and insulin resistance are two important factors that lead to decline in physical fitness and a change in distribution of fat (De-Fronzo and Ferrannini, 1991). Free radical generation has been implicated in high blood insulin levels (Ceolotto *et al*., 2004). Also, prolonged exposure of human adipocytes to insulin resulted in accumulation of hydrogen peroxide (H_2O_2) *in vitro* (Krieger-Brauer and Kather, 1992). Intra-peritoneal injection of dextrose in rats which led to increased insulin concentration has been linked to increased free

radical generation (Habib *et al*., 1994). Fasting hyperinsulinaemia is known to be a seal of insulin resistance (De-Fronzo and Ferrannini, 1991) and an association exists between plasma free radical concentration and insulin resistance (Ceriello and Pirisi, 1995; Ceriello, 2000).

Oxidative stress thus plays a role both in the development and the progression of diabetes mellitus and its complications. Hence diabetic patients have insufficient antioxidant defenses (Martin-Gallan *et al*., 2003). Any regimen for diabetes mellitus management should, therefore, have antioxidant activity to reduce diabetic complications.

2.5 MANAGEMENT OF DIABETES MELLITUS

2.5.1 Insulin

Insulin is the backbone of treatment in type 1 diabetes but is also used in type 2 diabetes when oral hypoglycaemic agents become ineffective. Pancreases from pigs, cows and fish were the first source of insulin for medical use in human beings. The efficacy of insulin from these sources owes to the fact that they are closely identical to human insulin (amino acid difference for bovine insulin is two whilst that for porcine is one). Insulin is apparently a protein which has been conserved over a long period of time. Human insulin is now manufactured for widespread clinical use using genetic engineering techniques.

2.5.2 Oral Hypoglycaemic Agents

2.5.2.1 Sulphonylureas

These drugs were discovered by chance when it was noted that a sulphonamide derivative used for treating typhoid, caused marked hypoglycaemia. Sulphonylureas increases insulin secretion

by closing the ATP-sensitive K channel on the β-cell membrane resulting in its depolarization and release of insulin. Examples include glibenclamide and chlorpropamide (not safe in renal impairment; can cause prolonged hypoglycaemia in patients with renal impairment). Meglitinides have a close link with sulfonylureas; their augmentation of insulin release is shorter and stronger, and is often taken together with meals to enhance the insulin response after each meal.

2.5.2.2 Biguanides

They improve insulin sensitivity by increasing glucose utilisation by skeletal muscle and also decrease hepatic gluconeogenesis. The exact mechanisms by which they cause these effects are not well understood but a study has indicated that they probably work by increasing cellular AMP-kinase levels (Zhou *et al.,* 2001). Metformin is the most widely known and used drug in this class and is frequently prescribed to overweight patients as it also promotes weight loss.

2.5.3 Medicinal Plants

Hypoglycaemic herbs are commonly used as non-prescription management for diabetes mellitus (Yin *et al.,* 2008). These medical plants may be working in a way to correct derangements in metabolism as well as delay the complications of DM. Drugs isolated from hypoglycaemic herbs have exhibited antidiabetic activity with enhanced efficacy as compared to synthetic oral hypoglycemic agents. As a result, search for new antidiabetic drugs from medicinal plants should be promoted, as these plants have been acknowledged as essential sources of new drugs (Lim *et al*., 1990). Aqueous extract of *Terminalia paniculata* bark has possible antidiabetic activity in diabetic rats. *In vitro* study results scientifically supported the *Terminalia paniculata* bark *in vivo* antidiabetic activity. Furthermore, aqueous extract of *Terminalia paniculata* bark contains active

biomarkers which may possibly be responsible for the antidiabetic activity of *Terminalia paniculata* bark (Ramachandran *et al*., 2012).

2.5.3.1 Selected Antidiabetic plants

In recent years, antidiabetic herbal preparations have gained much recognition in view of the disadvantages and side effects that the allopathic drugs present. Many herbal preparations have claims of antidiabetic properties, some of which have been scientifically investigated and confirmed. For example in Ghana, *Indigofera arrecta*, *Bridelia ferruginea*, unripe fruit of *Musa paradisiaca*, *Momordica charantia*, bark of *Myrianthus arboreus, Costus schlechteri* and *Ocimum canum* have all been reported to possess antidiabetic properties (Ayensu, 1978; Addy and Nyarko, 1988; Addy *et al*., 1992; Mills-Robertson, 1993; Hogarh, 1996). The aqueous extract of *Psidium guajava* leaves has a good effect to lower blood glucose (Deguchi *et al*., 1998). Still others are yet to receive scientific validation.

2.5.3.2 *Annickia polycarpa*

This is a small to medium-sized tree that grows up to 20 m tall. Its stem is typically straight and cylindrical and up to 40 cm in diameter, with no buttresses. Its bark is usually smooth to slightly rough or fissured. Other features of the bark are green to blackish, inner bark fibrous, bright yellow. Geographically, it is found in West Africa, stretching from East (Sierra Leone) through Nigeria and Western Cameroon. Its wood has variety of uses including: house building, light flooring, interior trim, and furniture such as making cabinet and also appropriate for ship building. Furthermore, the bark is used for making various traditional medicines. A bark decoction has been effective in treating ulcers, leprosy, sores and ophthalmia in Ivory Coast. The bark also promotes healing and has been used to treat fever including malaria. Bark decoctions

are also effective in treating jaundice in Sierra Leone (Obeng, 2011). The bark and leaves are thought to contain berberine and protoberberines. These compounds may be the reason for the incredible antitrypanosomal activity proven in experiments (Atindehou *et al*., 2004). Quinine and dihydroquinidine isolated from the bark validates the traditional use of the bark against malaria in West Africa. Bark extracts exhibited antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Klebsiella pneumoniae*. The berberine alkaloids are thought to be reason behind the dyeing characteristics of the bark (Obeng, 2011).

2.6 ANIMAL MODELS OF DIABETES MELLITUS

2.6.1 Pharmacological Induction of Diabetes

Streptozotocin (STZ, 69%) and alloxan (31%) are certainly the most commonly used diabetogenic chemicals in the induction of diabetes in animals. These animal models have been advantageous for the study of multiple facets of the condition. The diabetogenic effect of both chemicals is seen when administered via the following routes: intraperitoneally; intravenously; parenterally and subcutaneously. Factors which determine the dose of these drugs are: the species of the animal; nutritional status and route of administration.

Different states that are comparable to types 1 and 2 diabetes mellitus or even glucose intolerance may be induced depending on the dosage used in administering these agents, (Lenzen *et al*., 1996; Mythili *et al*., 2004). The effect of these chemicals is as a result of free radical formation and their mechanisms of action are different (Federiuk *et al*., 2004; Lei *et al*., 2005).

2.6.1.1 Alloxan

Alloxan monohydrate is reduced to form dialuric acid. These two are involved in a cycle of events that generates free radicals. These radicals are transformed to hydrogen peroxide and in the process there is an increase in calcium concentrations in the cytosol of the pancreas thus leading to the destruction of the beta cells of the pancreas (Szudelski, 2001). The choice of the effective dose needed to induce diabetes is quite narrow such that even a slight increase in dose is bound to cause death of many animals. This loss of animals due to overdosage is likely being as a result of tubular cell necrotic toxicity (Lenzen *et al*., 1996). Commonly utilised intravenous dosage in rats is 65 mg/kg body weight; however the effective dose must be higher in intraperitoneal (i.p.) or subcutaneous routes of administrations (Federiuk *et al*., 2004). A typical example is that an intraperitoneal dosage below 150 mg/kg body weight might be inadequate to cause successful induction of diabetes (Katsumata *et al*., 1992). Doses vary in mice species between 100 mg/kg to 200 mg/kg body weight via the intravenous route (Machado *et al*., 2001; Miranda *et al*., 2006).

2.6.1.2 Streptozotocin

Streptozotocin (STZ) uses the glucose transporter-GLUT2 to enter the pancreatic beta-cell and causes alkylation of deoxyribonucleic acid (DNA), activation of poly adenosine diphosphate ribosylation and release of nitric oxide. Pancreatic beta-cells are destroyed by necrosis due to STZ action (Mythili *et al*., 2004). The frequently used dose of STZ to induce insulin-dependent diabetes in adult rats is 60 mg/kg (Patel *et al*., 2006), nevertheless greater than 60 mg/kg doses are also used. Administration of a similar or higher dose of STZ intraperitoneally is also efficacious; however single doses lower than 40 mg/kg might possibly be less effective (Katsumata *et al*., 1992). The diabetic state of rats is confirmed if tail blood glucose

concentrations of fed animals are found to be more than 11.1–16.7 mM, 2 days after STZ administration. It has been observed that in adult mice, STZ given in low doses repeatedly induces an insulin-dependent diabetes mellitus that is relatively similar to the autoimmune forms of type 1 diabetes (Rees and Alcolado, 2005). Alternatively, administration of a single dose ranging between 60 mg/kg and 100 mg/kg body weight (Lei *et al*., 2005; Sharma *et al*., 2006) can also result in insulin-dependent diabetes mellitus that comes without the autoimmune feature (Yu *et al*., 2000). Streptozotocin does not only cause damage to the beta cells of the pancreas but can also lead to kidney damage (Valentovic *et al*., 2006), dysfunction of the endothelial system and inflammation (Lei *et al*., 2005). This is the main potential problem with STZ administration.

2.6.2 Surgical Models of Diabetes

Alternative procedure for inducing diabetes is the removal of the pancreas (pancreatectomy). Partial pancreatectomy has been used to induce diabetes but large resection (more than 80% in rats) is necessary to obtain mild to moderate hyperglycaemia. When this happens, small additional resection can lead to significant hypoinsulinaemia (Masiello, 2006). Findings of euglycaemia, hyperinsulinaemia and hyperglycaemia have been used to investigate relative uptake of glucose in various tissues of rats that have undergone 90% pancreatectomy (Choi *et al.*, 2004). These experimental findings make it possible to evaluate the ability of a compound to improve insulin secretion or sensitivity by peripheral tissues. Complete pancreatectomy is also used but comes with some limitations including the fact that high level of technical expertise is needed and it may cause loss of counter regulatory reaction to hypoglycemia by the pancreas.

2.6.3 Genetic Models of Diabetes

These models make it possible to assess the effect of a natural product in an animal without the interference of side effects caused by chemical drugs like alloxan and STZ. Certain strains like Ob/Ob mouse might sustain euglycaemia because the pancreatic beta cells respond strongly to match insulin resistance. Contrarily, the db/db mouse develops rapid hyperglycemia rapidly because they are unable to sustain increases in secretion of insulin necessary in life. Limit to food intake decreases the incidence of hyperglycaemia and obesity peculiar to this strain. One other model is the diabetic Goto-Kakizaki rat which is a type 2 diabetes mellitus model that comes from breeding over several generations of non-diabetic Wistar rats that are glucose-intolerant (Chen and Wang, 2005).

In this technique, rodents may be made to over (transgenic) or under (knockout)-express proteins believed to be involved in glucose metabolism. The db/db mouse of the C57BL/Ks strain is a typical example of a genetically diabetic animal; diabetes in the mouse resembles NIDDM in man because it is characterized by hyperglycaemia, obesity and insulin resistance. Diabetes in these mice is as a result of single autosomal recessive gene with complete penetrance allowing matings that will produce predictable numbers of afflicted offsprings. There is hyperinsulinaemia at 14 days which may be connected with the primary action of the gene (Coleman and Hummel, 1969). The high costs put limitation on their use to study modes of actions of potential therapeutic agents (Meiton, 2006).

2.7 ASSESSING THE ANTIDIABETIC ACTIVITY OF MEDICINAL PLANTS

The effectiveness of any antidiabetic plant could be investigated by either *in vivo* or *in vitro* biological assays. *In vivo* assay uses intact, whole organism or parts of the animal subjected to

perfusion techniques, to maintain as much as possible the integrity of the tissue whereas *in vitro* assay involves the use of selected tissue or cells. The main advantage of *in vivo* assay over *in vitro* assay is that observations are made that reflects the true situations in the animals. *In vitro* assay on the other hand can be used to investigate the possible mode of action of a test plant. However, it is always imperative to carry out an *in vivo* assay first in cases where not much information is known about the supposedly antidiabetic plant before proceeding on to using an *in vitro* assay.

2.8 TOXICITY STUDIES

2.8.1 Acute Toxicity

This test is conducted on all chemicals that are of any biologic interest. It involves administering the compound to animals on a single occasion. The test aims at determining the symptomatology resulting from administration of a test compound and to define the order of lethality of the compound. Distinct tests are required to determine the effects of skin and eye contact (topical or local toxicity; corrosion, irritancy and sensitization) and the effects on internal organs of a substance that is inhaled, swallowed, absorbed through the skin or injected.

Acute toxicity studies may be performed in three ways in rodents. The first type is done to establish the maximum dose after which the animals recover wholly from all effects of the chemical (i.e. the maximum tolerated dose) and the minimum lethal dose of the chemical. The second type of study aims at establishing the target organ(s) for toxicity and animals are given a single dose. The last type of study is usually meant to determine the precise LD_{50} or median lethal dose (Pascoe, 1983; Poole and Leslie, 1989; Timbrell, 2002)

Table 2.1: Classification of toxicity based on LD_{50} dose ranges

Category	LD_{50} (mg/kg)
Extremely toxic	1 or less
Highly toxic	1 to 50
Moderately toxic	50 to 500
Slightly toxic	500 to 5000
Practically non-toxic	5000 to 15000
Relatively harmless	More than 15000

Source: Loomis and Hayes, 1996

2.8.2 Sub-Chronic Toxicity

This test involves the use of rodents and non-rodents to study the toxic effects of a substance in a 90 day period. The test substance is administered orally for a period between 45-90 days within which changes in the following parameters are monitored; monthly biochemical and cardiovascular parameters changes, behavioral changes, and weekly body weight. After completing the period of administration of the test substance, the experimental animals are sacrificed followed by histological examination of all tissues to determine changes in cellular morphology. Variations in all the measured and observed parameters between control and test animals should be little and the allowed weight variation range is ±20%. The study protocol may include a satellite group serving as both a control group and a high-dose group (Muralidhara *et al*., 2001).

2.8.3 Chronic Toxicity

This is the ability of a test compound to cause lethal consequences in animals, humans and other organisms after numerous exposures that occurs over a prolonged period of time or over a substantial fraction of an animal's or human's lifetime (Health and Safety, 2004). This test investigates the dangerous effects caused by foreign substances upon repeated introduction over an extended period of time (Poole and Leslie, 1989). The dose levels of substances used usually range from the therapeutically effective to doses close to the maximum non-lethal dose as proven in acute toxicity studies (Poole and Leslie, 1989; Loomis and Hayes, 1996). To determine organ toxicity, clinical chemistry parameters together with haematological parameters are measured at the baseline of the study and when the study is terminated. The animals are sacrificed at the end of the study and tissues found to be abnormal together with major organs like the kidneys, liver, lungs, heart, and spleen of the animals are subjected to full histopathological analysis (Poole and Leslie, 1989; Timbrell, 2002).

2.9 ROUTES OF ADMINISTRATION

This is a term used to denote the path by which drugs or compounds are taken into the body of animals or humans. In the assessment of toxicity of a compound in animals, a number of routes could be used but the oral route or intraperitoneal injection is the most frequently used (Poole and Leslie, 1989).

2.9.1 Intra-Peritoneal Injection

In this approach, the animal is laid on its back followed by shaving the abdomen. The abdominal wall is now punctured using a suitable syringe and needle after it has been cleansed. The injection is normally done in a way in order to reduce the risk of perforation of abdominal

viscera. The depth of penetration must not surpass 5 mm (Waynforth, 1980; Poole and Leslie, 1989). This approach usually makes available information about local and systemic toxicity of a drug or compound.

2.9.2 Oral Administration

This is a form of drug administration which makes use of the gastrointestinal tract, a flexible tube which goes through the body, starting at the mouth and ending at the stomach. The oral route is perhaps one of the most commonly used means by which a chemical substance enters the body. Though the gastrointestinal tract lies within the body, its contents are exterior to the body fluids. The systemic effect of most orally administered substances occurs after absorption from the mouth or the gastrointestinal tract.

Administration of substances that undergo fast absorption in the gastrointestinal tract exposes the liver to significant amounts which would not happen when other routes were used (Loomis and Hayes, 1996). Compounds that are known to be hepatotoxic are thought to be more toxic when administered orally in a frequent manner; however administering these same compounds by other routes other than orally may produce less toxic effects (Waynforth, 1980; Loomis and Hayes, 1996).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Plant Material

The stem bark of *A. polycarpa* were collected from Begoro, Eastern Region, Ghana (6°23′N 0°23′W) and authenticated by Mr. H. Blagogee, of the Plant Production Department(PDD), Centre for Plant Medicine Research (CPMR), Mampong-Akuapem in the Eastern Region of Ghana, and deposited with the herbarium of the PDD.

3.1.2 Experimental Animals

Male Sprague-Dawley rats (180-200 g) and ICR mice (20-30 g) were obtained from the Animal Unit, CPMR. The animals were housed in stainless steel metal cages with wood shavings as bedding and fed on powdered feed obtained from GAFCO (Tema, Ghana) and allowed free access to sterilized distilled water. The experimental animals were in an environment of 22 ± 2ºC temperature and 70 ± 4% humidity under alternating 12 hour period each of light and darkness. They were kept at most 6 animals in a cage sized 20.3 cm width × 28.7 cm length × 17.3 cm height in order to allow appreciable space for movement. The ICR mice and Sprague-Dawley rats were used for the antidiabetic and sub-chronic toxicity studies, respectively. All animals used in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 1996).

3.1.3 Reagents and Chemicals

Triacylglycerol, cholesterol, alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine, direct bilirubin and glucose assay kits were obtained from ELI Tech, Puteaux, France. Glibenclamide and metformin were obtained from BLISS GVS PHARMA Limited, India. Diphenylpicrylhydrazyl was obtained from Sigma Chemical Company, St. Louis, MO, USA. Urine test strips (UroColor^{TM}10) were supplied by Standard Diagnostics Inc. Kyonggi-do, S. Korea**.** All other chemicals and reagents used were obtained from FLUKA, Zofingen, Switzerland.

3.2 METHODS

3.2.1 Preparation of Plant Material

Fresh stem bark of *A. polycarpa* were air-dried at 25^{0}C for 1 week and then milled into powdered form in a laboratory mill. The powdered leaves were weighed and boiled in water (20% w/v) for thirty minutes, allowed to cool and filtered through cotton wool. The filtrate was concentrated by a rotary evaporator and lyophilized into powder by a freeze dryer (EYELA, Tokyo Rikakikai Co. Ltd, Japan). The freeze dried extract (subsequently referred to as AP in this study) was weighed and stored in a desiccator at 4-8^{0}C. The % yield of extract from plant material was calculated.

3.2.2 Qualitative Phytochemical Screening

Phytochemical tests were performed on AP to determine the presence of saponins, cyanogenic glycosides, alkaloids, flavonoids, reducing sugars, phenolic substances, polyuronides, anthracenosides, triterpenes and phytosterols according to standard protocols. For each test, a small amount (1-2 g) of AP was reconstituted in distilled water/other solvent before use.

Saponins

A small volume (2 ml) of extract was shaken with few millilitres of distilled water in a test tube and the mixture observed for the presence of a froth which does not break readily upon standing but persist for at least 15 min (Tiwari *et al*., 2011).

Cyanogenic glycosides

A small volume (5 ml) of the extract was added to 1 ml of chloroform in a test tube and corked with a yellow picric paper. The mixture was heated on a water bath for few minutes. . A colour change from picric yellow to brown or red was a positive test (Sofowora, 1993).

Alkaloids

About 3 drops of 25% ammonium hydroxide was added to 10 ml of the extract in a separating funnel followed by the addition of 5 ml chloroform. The extract was then collected into a test tube and then heated on a water bath to evaporate both water and solvent. A small volume (5 ml) of 2 N hydrochloric acid was added to the resulting slurry, followed by 2 drops of Mayer's reagent and observed for a yellow coloured precipitate (Tiwari *et al*., 2011).

Flavonoids

Extract was treated with few drops of 10% sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of 2 N HCl, indicates the presence of flavonoids (Tiwari *et al*., 2011).

Reducing Sugars

Equal volumes of Fehling's solution A and B (0.5 ml) was added to 2 ml of extract and heated on a water bath for 10 min. The mixture was then observed for a brick red precipitate (Sofowora, 1993).

Phenolic Substances

About two drops of 5% ferric chloride solution was added to a small volume (3 ml) of the extract and observed for a dark green colouration (Tiwari *et al*., 2011).

Polyuronides

A volume (2 ml) of extract was filtered into 5 ml of acetone and observed for chalky white precipitate (Sofowora, 1993).

Anthracenosides

A 5 ml of the extract was transferred into a separating funnel and 3 drops of diethyl ether added to it, shaken gently and allowed to stand for about 10 min. A volume (2 ml) of 25% ammonium hydroxide was added to 2 ml of the diethyl ether portion and shaken gently. A red colour of the alkaline solution indicates the presence of anthracenosides (Oseni and Alphonse, 2011).

Triterpenes

An amount (2 g) of the extract was refluxed in chloroform for an hour and filtered. The filtrates were treated with few drops of concentrated sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes (Tiwari *et al*., 2011).

Phytosterols

An amount (2 g) of the extract was refluxed in chloroform for an hour and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Few drops of concentrated sulphuric acid were then added. Formation of brown ring at the junction indicates the presence of phytosterols (Tiwari *et al*., 2011).

3.2.3 Quantitative Phytochemical Screening

3.2.3.1 Determination of total phenolic content

The total phenolic content of the extract was determined by the Folin-Ciocalteau method (Singleton *et al*., 1999). A volume of 100 µl of 5% Folin-Ciocalteau reagent was added to 20 µl of 0-5 mg/ml of increasing concentration of the extract followed by 80 µl of 7.5% Na_2CO_3 in a well plate and mixed. Mixture was incubated at room temperature in the dark for 1 hour and the absorbance measured at 765 nm using the infinite M200Pro micro titer plate reader (Tecan, Austria). Gallic acid at concentration range of 0-5 mg/ml was used to plot the standard calibration curve from which the concentration of phenol in each extract concentration was estimated. The total phenolic content of extract was expressed in mg of Gallic acid equivalents (GAE) /g of the extract.

3.2.3.2 Determination of total flavonoids

Various concentrations of extract in distilled water were prepared by serial dilution; (10, 5, 2.5, 1.25, 0.625, 0.3125, 0.1563, 0.0781, 0.0391 mg/ml). A volume (100 µl) of 2% $AlCl_3$ was added to 100 µl of the extract (0.0391 – 5 mg/ml), followed by incubation in the dark for 10 min at room temperature. Absorbance of the solution was measured at a wavelength of 415 nm using

the Infinite microtiter plate reader (Tecan, Austria). Quercetin (0-5 mg/ml) was used as a standard whilst distilled water was used as a blank. A calibration curve for quercetin was plotted from which the unknown concentration of flavonoids in each extract concentration was estimated. The total flavonoid content of extract was expressed in mg of quercetin equivalents (QE)/g of extract.

3.2.4 Antidiabetic Studies

3.2.4.1 Induction of diabetes in mice

The diabetic state was induced in the ICR mice by a single intraperitoneal injection of alloxan monohydrate (150 mg/kg) (Aruna *et al*., 1999). Alloxan was first weighed individually for each animal according to the body weight and then dissolved in 0.5 ml saline (0.9% NaCl) just prior to administration to animal. Two days after alloxan administration, the fasting blood glucose (FBG) of mice was determined and those with FBG $\geq$ 7.8 mM were classified as diabetic and, therefore, included in the study. Treatment with AP commenced 48 h after establishing the diabetic state of animals.

3.2.4.2. Treatment of animals

The alloxan-induced diabetic mice were divided into six (6) treatment groups of five (5) animals each. The first three groups were treated daily with AP of increasing dosage (20, 100 and 500 mg/kg). Members of the 4th group were treated daily with the maximum therapeutic dose of glibenclamide (2.5 mg/kg) whereas the 5th group received daily the maximum therapeutic dose of metformin (50 mg/kg). The last group received equivalent volume of water (0.5 ml) and served as the diabetic control group. Five non-diabetic littermates were put in a 7^{th} group, which

received equivalent volume of water (0.5 ml) and represented the non-diabetic control. The animals were treated for a period of four weeks by oral gavage. *Annickia polycarpa* or standard drugs were reconstituted in sterilized distilled water before administration to the animals. The body weight of the animals was determined weekly.

3.2.4.3 Blood sampling/Glucose determination

At baseline, and the end of weeks 1, 2, 3 and 4, blood samples were collected by tail bleeding of the animals after an overnight fast (8 hours) into heparinized tubes and centrifuged at 4500g for five minutes to obtain plasma The plasma was pipetted into fresh tubes and its glucose concentration determined by the glucose oxidase method following the manufacturer's (ELI Tech) protocol with Biosystems A25 Chemistry Analyzer (Biosystems S.A, Barcelona, Spain).

3.2.4.4 Serum biochemical analyses

Serum total cholesterol and TAG were determined following the manufacturer's (ELI Tech) protocol with Biosystems A25 Chemistry Analyzer (Biosystems S.A, Barcelona, Spain) at termination of treatments.

3.2.4.5 Oral glucose tolerance test

The oral glucose tolerance test was conducted on the same animals at the termination of treatments. The animals of all treatment groups were fasted for 8 hr and the FBG determined at baseline. Each animal was given an oral glucose load (2 g/kg), by administering about 0.5 ml/animal (depending on body weight) of glucose solution. Blood samples were collected by tail bleeding at 30, 60, 90, 120 and 150 min post-glucose load. Blood glucose levels were measured using the glucose oxidase method.

3.2.4.6 Histology of pancreas

Animals were killed at the end of the study by cervical dislocation. Pancreas was removed, washed with cold saline and preserved in 10% buffered formalin. Blocks from tissues were routinely processed and embedded in paraffin. Thin sections were cut using microtome and stained with hematoxylin and eosin for histopathological evaluation by light microscopy (Gomeri, 1950).

3.2.5 Assessing Antioxidant Activity

3.2.5.1 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

A fresh solution of DPPH in 80% methanol (0.5 mM) was prepared. Various concentrations (0.625, 1.25, 2.5 mg/ml) of extracts were prepared by serial dilution of an initial stock solution of 10 mg/ml in methanol. 100 µl of DPPH solution was added to 100 µl of each extract in a 96 microlitre well plate. Plate was shaken to uniformly mix the solution and kept in the dark for 30 min. The absorbance was read at 517 nm using infinite M200Pro microtiter plate reader (Tecan, Austria). Butylated hydroxytoluene (BHT) at concentrations as extract in methanol was used as positive control (standard) and 80% methanol as blank.

The % DPPH scavenging activity of AP was calculated using the formula below:

% DPPH scavenging effects = (Ac -At)/Ac * 100

Where: Ac = Absorbance of control, As = Absorbance of the standard/extract.

A graph of percentage DPPH scavenging activity was plotted against the various concentrations.

3.2.5.2 Ferric ion reducing ability

The ferric ion reducing ability of AP was determined in accordance with the method of Yen and Chen (1995) with Gallic acid, BHT and quercetin as standards. 0.5 ml of increasing concentration (0-5mg/ml) of AP was added to 0.5 ml of distilled water and mixed with 1.25 ml of sodium phosphate buffer (0.2 M, pH=6.6), followed by 1.25 ml of 1% potassium ferric cyanide. After incubation at 50^{o}C for 20 min, 1.25 ml of 10% trichloroacetic acid was added to samples and centrifuged at 3000 x g for 10 min. 100 µl of distilled water and 100 µl of 0.1% $FeCl_3$ were added to100 µl of AP in a plate. Absorbance of the resulting solutions was read at 700 nm. A blank was prepared by adding 0.5 ml of distilled water to 1.25 ml of sodium phosphate buffer and 1.25 ml of potassium ferricyanide. A graph of absorbance against the concentrations was plotted for each extract and standard.

3.2.6 Toxicity Studies

3.2.6.1 Acute toxicity study

A single oral dose at 5000 mg/kg (2 ml/rat) of AP was administered to six rats. Physical signs of toxicity and death of the rats were observed over a period of 24 hours. Animals that survived were observed for an additional period of 13 days for any symptoms toxicity such as pilo-erection, and defects in lachrymatory, locomotory and respiratory activities.

3.2.6.2 Sub-chronic toxicity studies

The animals were divided into four groups of six each and placed into four separate metal cages. Animals belonging to group one served as control group and were given sterilized distilled water for a period of three months. The remaining groups 2, 3 and 4 were treated with 20 mg/kg, 100

mg/kg and 500 mg/kg, respectively of AP suspended in sterilized distilled water for three months. The animals in each group were weighed on day zero (baseline) and weekly thereafter for 3 months.

Blood sampling/Clotting time

Blood samples of rats in each group were taken by tail bleeding (at baseline and monthly thereafter) into serum separator tubes, centrifuged at 4000 x g for 5 minutes and serum stored at -40ºC for biochemical analyses. Other blood samples were collected into separate tubes pre-coated with trisodium citrate for haematological analyses within 24 hrs. Clotting times of other blood samples in Eppendorf tubes were also determined.

Urinalysis

Urine samples of the rats in each treatment group produced as a result of involuntary discharges by the groups were collected on clean ceramic tiles at the start of the study (baseline) and monthly thereafter for three months. Analysis of urine for glucose, bilirubin, ketones, specific gravity, pH, proteins, urobilinogen, nitrate, blood and leukocytes were done using urine reagent strips UroColor^{TM} 10 (Standard Diagnostic Inc., Korea).

Serum biochemical analyses

Serum alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin (direct) and creatinine of samples were determined following the manufacturer's (ELI Tech) protocol with Biosystems A25 Chemistry Analyzer.

Haematological analyses

Red blood cell (RBC) count, white blood cell (WBC) count, haematocrit (HCT), haemoglobin (HGB), mean cell volume (MCV) and platelet count (PLT), mean corpuscular hemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), lymphocytes (LYM), red blood cell distribution width (RDWSD), Mean platelet volume (MPV) of blood were determined with Haema-screen 18 (Hospitex Diagnostics, Italy) in accordance with established protocol.

Pentobarbital-induced sleeping time

The sleep evaluation method was based on potentiation of pentobarbital-induced sleeping time. Briefly, the four (4) animals in each treatment group were given a single dose of pentobarbital (40 mg/kg) to induce sleep. The time the animals lost righting reflex till the time they gained righting reflex was determined. The interval between these two times was taken as the pentobarbital-induced sleeping time (Nyarko *et al*., 1999).

Mean organ wet weights

The animals were weighed and sacrificed at the end of the study by cervical dislocation. The body organs (kidneys, liver, lungs and heart) of each rat were excised, washed in ice-cold 1.15% KCl solution, dried and weighed. Organ wet weights were expressed as g% ((g of organ weight/ g of body weight) x100%).

Histopathological analyses

Portions of the liver, kidneys, lungs, spleen and heart were fixed in 10% formalin. All procedures were carried out at temperature of 0-4^{0}C. They were dehydrated in varying concentrations of ethanol (70-100%) and then cleared in xylene before embedding in paraffin wax. Micro-sections

(about 4 μm) were prepared, mounted on slides and stained with haematoxylin and eosin (H&E) and examined under a light microscope (Baker and Silverstone, 1985).

3.2.7 Statistical Analyses

Results were presented as mean ± SEM. for fasting plasma glucose level and biochemical parameters of 4-6 observations. Statistical analysis was conducted using one-way analysis of variance followed by Dunnett post hoc analysis using the statistical software program, Graph Pad Prism 5.0. Statistical significance was considered at $P<0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 QUALITATIVE PHYTOCHEMICAL ANALYSIS

The preliminary phytochemical screening of the aqueous stem bark extract of *Annickia polycarpa* (AP) obtained (5% yield = 100 g extract/ 2000g plant material x 100) is shown. The groups of phytochemicals present were saponins, reducing sugars, phenolic compounds, alkaloids, and flavonoids. Polyuronides, cyanogenic glycosides, anthracenosodes, triterpenes and phytosterols were absent (Table 4.1).

Table 4.1 Phytochemical constituents of aqueous stem bark extract of *A. polycarpa*

CONSTITUENT	AP
Saponins	+
Reducing sugars	+
Phenolic compounds	+
Polyuronides	−
Cyanogenic glycosides	−
Alkaloids	+
Anthracenosides	−
Flavonoids	+
Triterpenes	−
Phytosterols	−

(−): absent; (+): present; AP: *A. polycarpa* extract

4.2 QUANTITATIVE PHYTOCHEMICAL ANALYSIS

The phenolic and flavonoid contents of AP increased with increasing concentration, reaching a plateau at 2 mg/ml (Figs. 4.1 and 4.2). The total phenolic and flavonoid contents of AP were 62.19 ± 4.15 mg Gallic acid/g extract and 662.8 ± 24.2 mg Quercetin Equivalents/g extract, respectively.

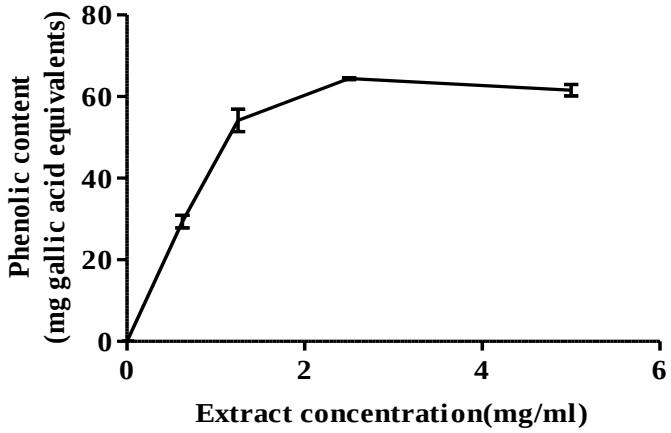

Fig 4.1: Phenolic content of aqueous stem bark extract expressed as mg gallic acid equivalents per gram of extract in increasing extract concentration. Values are means ± S.E.M. of n = 3 AP: *A. polycarpa* extract.

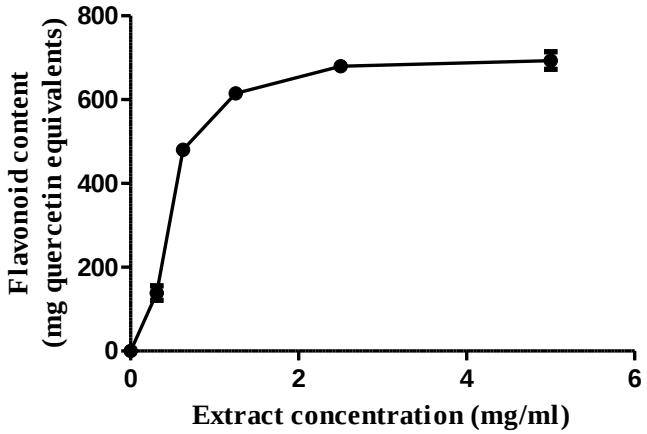

Fig 4.2: Flavonoid content of aqueous stem bark extract expressed as mg quercetin equivalents per gram of extract in increasing extract concentration. Values are means ± S.E.M. of n = 3 AP: *A. polycarpa* extract

4.3 ANTIOXIDANT ACTIVITY

4.3.1 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) Free Radical Scavenging Activity

The DPPH free radical scavenging activity of AP and standard antioxidant BHT indicated that at the concentrations of AP used it showed concentration dependency whilst BHT did not (Fig. 4.3). The free radical scavenging activity of BHT at the highest concentration of 2.5 mg/ml was 75.04% whereas that of extract AP at the same concentration was 45.07%.

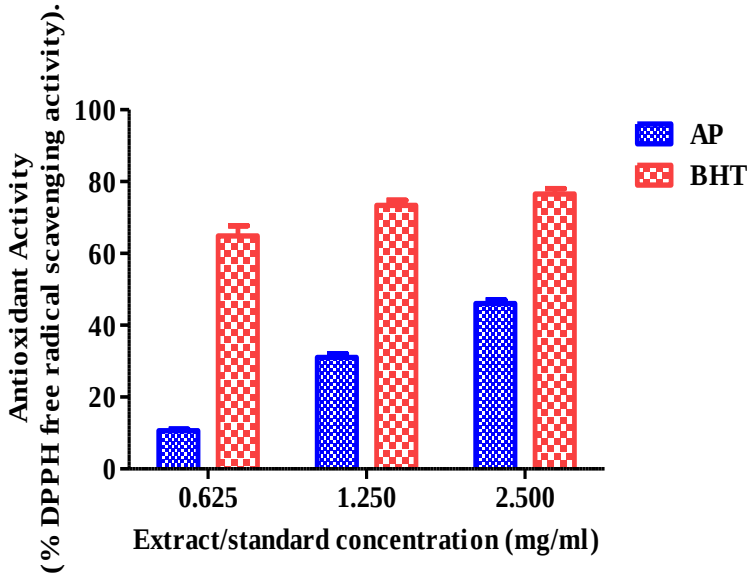

Fig. 4.3: DPPH free radical scavenging activity of varying concentrations of AP and the standard BHT. Results are means ± S.E.M of n = 3. AP = *A. polycarpa* extract; BHT = Butylated hydroxytoluene.

4.3.2 *In vitro* Reducing Activity

There was a concentration-dependent increase in ferric reducing ability in respect of Gallic acid and quercetin, reaching a plateau after the concentration of 1.25 mg/ml. BHT and AP at varying concentrations did not show much ferric ion reducing capability (Fig. 4.4).

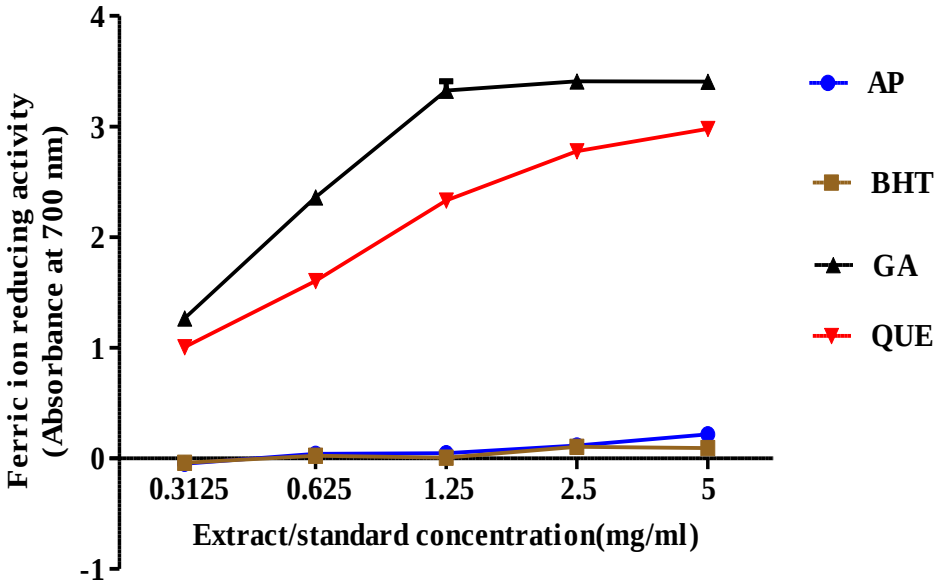

Fig. 4.4: The ferric ion reducing activity of quercetin, Gallic acid, butylated hydroxytoluene (BHT) and aqueous extract of *A. polycarpa* (AP) at varying concentrations. Results are means ± S.E.M of n = 3.

4.4 FASTING BLOOD GLUCOSE LEVELS

The fasting blood glucose (FBG) level in alloxan-induced diabetic mice was two-fold that of the non-diabetic controls at baseline (Fig.4.5a). The FBG levels in the diabetic and normoglycaemic control mice were maintained over the period of study. FBG levels in diabetic animals were significantly reduced ($p<0.05$) by AP over time reaching near normoglycaemic control value by week 4 as seen with the standard drugs metformin and glibenclamide. This is depicted in the area under the curve (Fig. 4.5b).

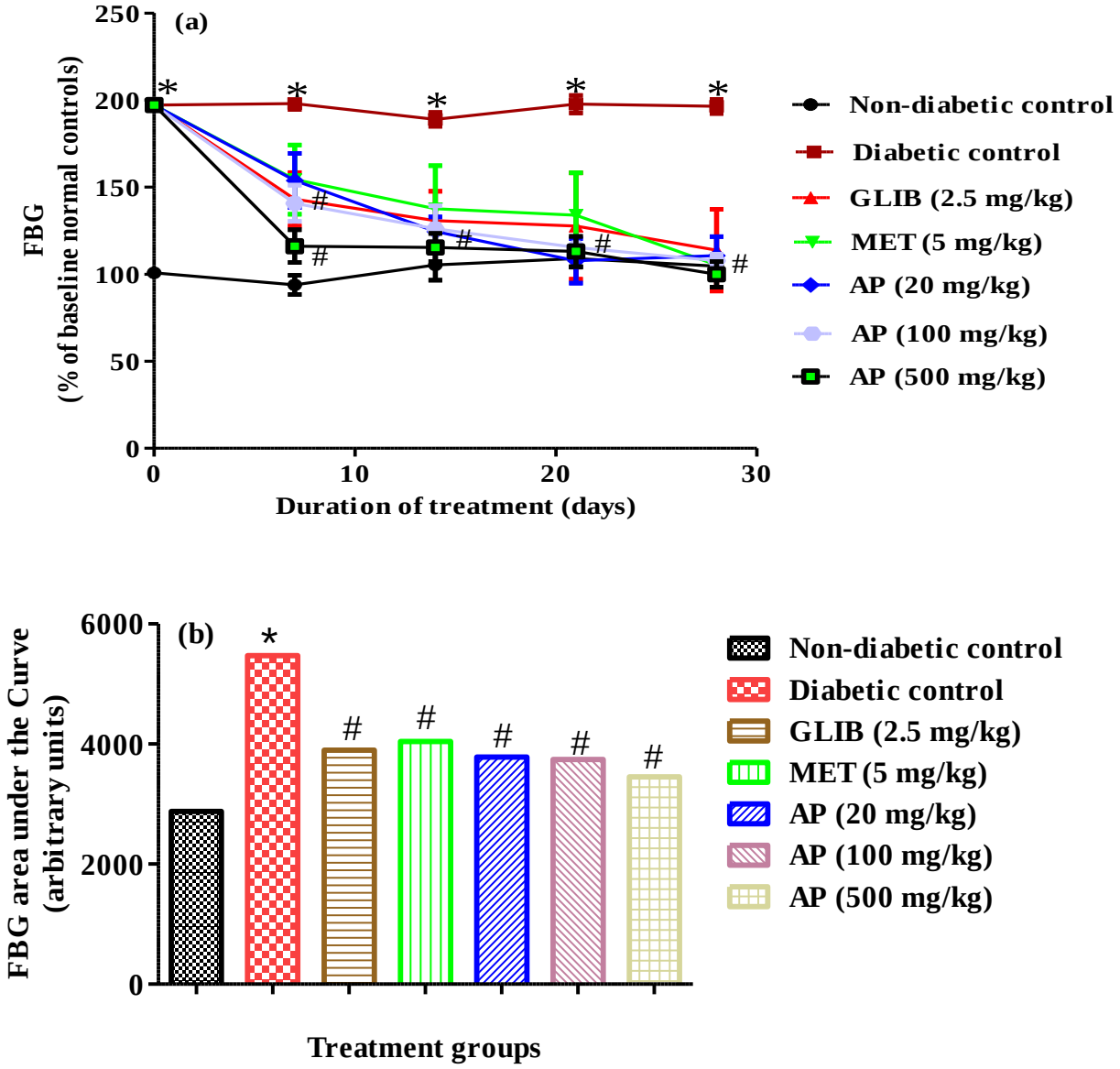

Fig. 4.5: Effect of AP and standard drugs on fasting blood glucose levels. (a): line graph; (b): area under the curve (AUC). Values are percentages of baseline non-diabetic control fasting blood glucose (4.3 ± 0.1 mmol/L). Results are mean ± S.E.M of n=4. Glib = glibenclamide; Met= metformin; AP = *A. polycarpa* extract. * Value significcantly ($p<0.05$) different from non-diabetic control. # Value significantly ($p<0.05$) different from diabetic control.

4.5 SERUM TOTAL CHOLESTEROL AND TRIACYLGLYCEROL

Serum total cholesterol and triacylglycerol levels were significantly ($p<0.05$) greater in the diabetic controls (27%) in comparison to the non-diabetic controls (Fig. 4.6). Treatment of diabetic mice with AP significantly ($p<0.05$) decreased total cholesterol levels (17%) whereas glibenclamide and metformin significantly ($p<0.05$) reduced it by 21-22%. Serum triacylglycerol level of diabetic mice was significantly ($p<0.05$) higher (83%) than that of normoglycaemic animals.. Glibenclamide and metformin significantly ($p<0.05$) reduced the triacylglycerol levels (95-98%) in diabetic mice to below non-diabetic control levels. AP also significantly reduced triacylglycerol (50-90%) levels in diabetic mice to near non-diabetic control levels.

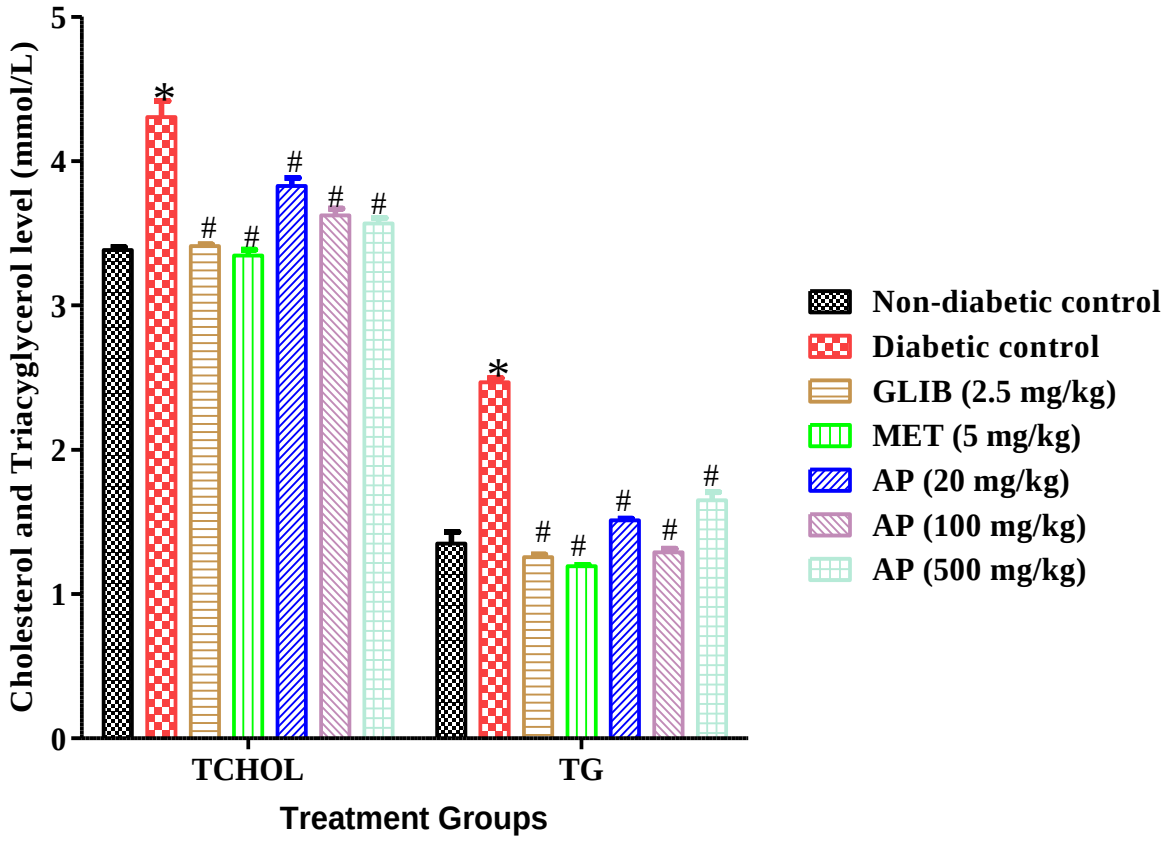

Fig 4.6: Effect of AP/standards on serum cholesterol and triacylglycerol levels. Results are means ± S.E.M. of n=3. TCHOL= total cholesterol; TG= triacylglycerol; AP = *A. polycarpa* extract. *Value significantly ($p<0.05$) different from non-diabetic control. #Value significantly ($p<0.05$) different from diabetic control.

4.6 CHANGES IN MEAN BODY WEIGHT

There was a gradual increase in mean body weights across all animal treatment groups over the period of study. The changes in body weights of extract/drug-treated diabetic animals appeared to have reached near normal control value but significantly higher than the diabetic control value at termination of treatments (Fig. 4.7).

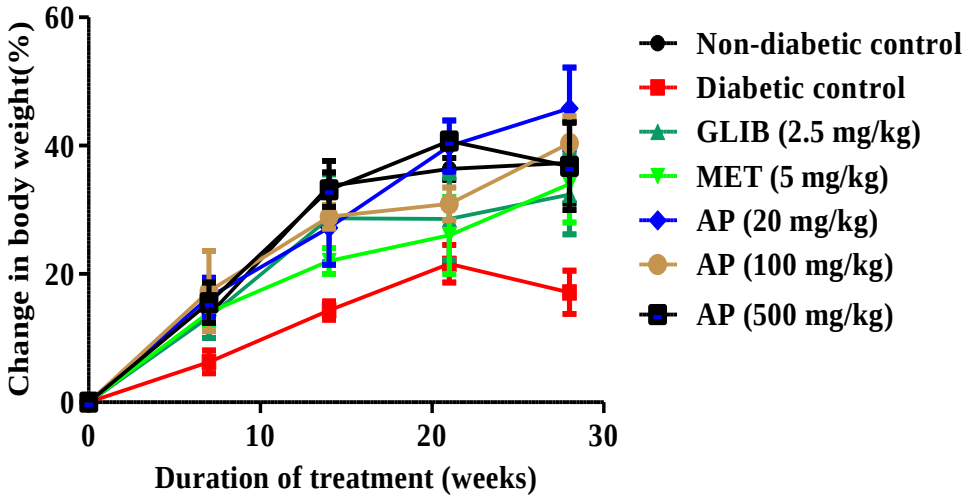

Fig 4.7: Weekly percentage changes in body weight of alloxan-induced diabetic mice over duration of treatment with AP or standard drugs. Values were expressed as percentage change in baseline non-diabetic control group of weight 27.6 ± 0.6 g. Glib = glibenclamide; Met= metformin; AP = *A. polycarpa* extract. Results are mean ± S.E.M of n=6

4.7 ORAL GLUCOSE TOLERANCE TEST

At baseline, the blood glucose level of untreated diabetic controls was 3-fold that of non-diabetic controls whilst those of other treatment groups were in between these two control groups. Administration of an oral glucose load to diabetic control mice caused about a 3-fold increase in baseline blood glucose level within 30 min with a gradual decline to above baseline level at 150 min. The blood glucose of non-diabetic controls increased 2.5-fold in 30 min and reduced to baseline levels by 60 min and remained at that level up to 150 min. The blood glucose levels of diabetic mice treated with the standard drugs and AP responded in a similar fashion as the non-diabetic controls after the glucose load (Fig. 4.8a). The general effects of treatments on the management of the glucose load over the period of study are depicted in the AUC (Fig. 4.8b), which showed that the AP/standard drugs significantly reduced the marked increase in blood glucose level in diabetic mice after the glucose load.

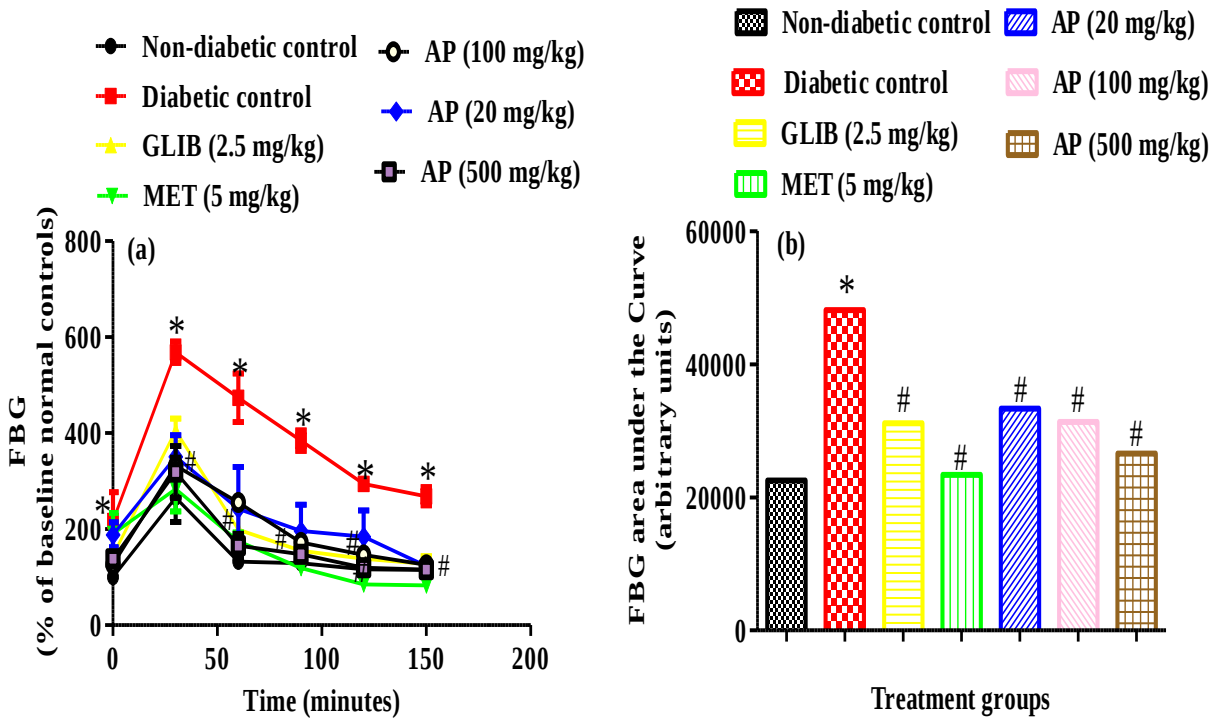

Fig 4.8: Effect of *A. polycarpa*, glibenclamide and metformin on fasting blood glucose levels after an oral glucose load. (a): line graph; (b): area under the curve (AUC). Values are percentages of baseline normal control fasting blood glucose of (4.5 ± 0.2) mmol/L. Glib = glibenclamide; Met= metformin; AP = *A. polycarpa* extract. Results are mean ± S.E.M of n=6. *Value significantly ($p<0.05$) different from non-diabetic control. #Value significantly ($p<0.05$) different from diabetic control.

4.8 HISTOLOGY OF THE PANCREAS

The micrographs of pancreas at termination of treatment of alloxan-induced diabetic mice with AP, glibenclamide and metformin are shown in (Fig. 4.9). The diabetic control group showed necrosis of the beta cells whilst the non-diabetic control group showed intact pancreatic beta cells. Treatment with metformin, glibenclamide and AP facilitated restoration of the morphology of the beta cells of the pancreas that had become necrotic in the diabetic mice.

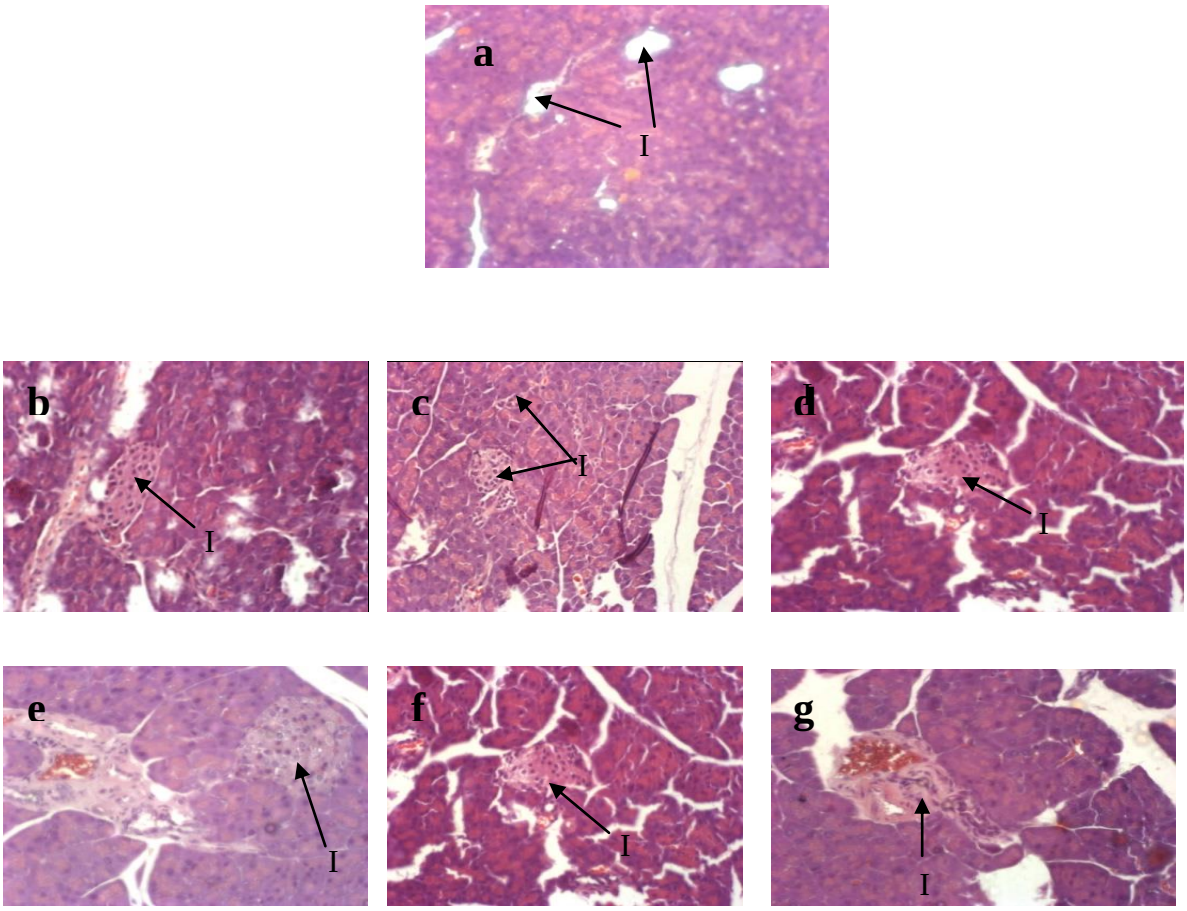

Fig. 4.9: Histological appearance of mice pancreas at termination of treatments for (a) diabetic control animals, (b) non-diabetic control animals, (c) diabetic animals treated with glibenclamide, (d) diabetic animals treated with metformin, (e) diabetic animals treated with treated with AP (20 mg/kg), (f) diabetic animals treated with AP (100 mg/kg) and (g) diabetic animals treated AP (500 mg/kg) showing necrosis of pancreatic beta cells in diabetic mice and their restoration in other treatment groups compared to non-diabetic controls. AP= *A. polycarpa* extract. (I). Magnification: x100

4.9 ACUTE TOXICITY

The effect of a single oral dose of 5000 mg/kg AP administered to six animals showed no deaths within 24 hr, and no physical signs of toxicity as evidenced by normal breathing and locomotion, and absence of signs of tremors, convulsions, salivation, diarrhoea, lethargy, excessive lachrymatory activity and pilo-erection over the 14-day period of study.

4.10 CHRONIC TOXICITY

4.10.1 Changes in Rat Body Weight

There were significant increases ($p<0.05$) in rat mean body weight changes with time over the experimental period for all animal treatment groups but no significant differences ($p>0.05$) between the AP treatment groups and control (Fig. 4.10).

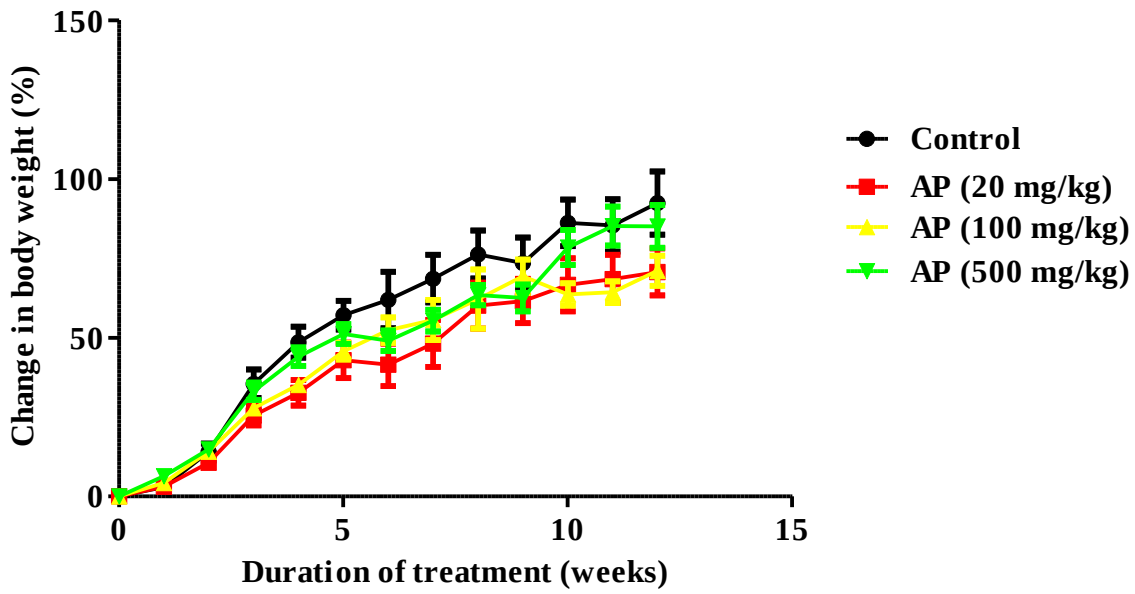

Fig 4.10: Percentage changes in body weight of Sprague-Dawley rats over duration of treatment with AP. Values are expressed as percentage change in baseline control value of 131.5 ± 1.2 g. Results are mean ± S.E.M of n=6. AP= *A. polycarpa* extract.

4.10.2 Mean organ wet weight

The effect of treatment with AP on organ wet weights expressed as a percentage of body weight showed that there were no significant ($p > 0.05$) differences in the wet weights of organs (heart, kidney, liver, lung and spleen) between the AP-treated groups and controls (Table 4.2). .

Table 4.2: Effect of treatment with AP on organ/body weight

	Organ wet weights (%)			
Parameters	Treatment Groups			
	Control	AP (20 mg/kg)	AP (100 mg/kg)	AP (500 mg/kg)
Heart	0.29 ± 0.01	0.27 ± 0.01	0.29 ± 0.01	0.28 ± 0.01
Kidney	0.49 ± 0.03	0.51 ± 0.03	0.49 ± 0.01	0.49 ± 0.02
Liver	2.9 ± 0.23	2.84 ± 0.12	2.94 ± 0.14	2.67 ± 0.27
Lung	0.66 ± 0.06	0.61 ± 0.02	0.58 ± 0.03	0.73 ± 0.11
Spleen	0.25 ± 0.02	0.22 ± 0.02	0.23 ± 0.01	0.23 ± 0.02

Values are means ± S.E.M. of n=6. AP= *A. polycarpa* extract

4.10.3 Hematological Analyses

All hematological parameters measured at termination of treatments indicated that there were no significant differences ($p>0.05$) in value between AP-treated groups and the control animals (Table 4.3).

Table 4.3: Effect of AP on haematological parameters at termination of treatments

	Treatment Groups			
Parameters	**Control**	**AP (20 mg/kg)**	**AP (100 mg/kg)**	**AP = 500mg/kg**
WBC (10^9/l)	12.87 ± 1.90	14.57 ± 1.10	12.50 ± 0.93	14.57 ± 1.09
RBC (10^{12}/L)	8.78 ± 0.32	8.80 ± 0.42	8.46 ± 0.18	8.83 ± 0.30
HGB (g/dl)	14.70 ± 0.10	15.43 ± 0.67	15.00 ± 0.46	14.90 ± 0.26
HCT (%)	46.97 ± 0.82	49.00 ± 2.52	47.23 ± 1.37	47.33 ± 1.11
MCV (fl)	53.60 ± 1.27	55.67 ± 0.43	55.83 ± 0.47	53.63 ± 0.64
MCH (pg)	16.80 ± 0.55	16.57 ± 1.19	17.70 ± 0.17	16.87 ± 0.29
MCHC (g/dl)	31.30 ± 0.35	31.53 ± 0.29	31.77 ± 0.15	31.50 ± 0.20
PLT (10^9/l)	1193 ± 102	922.0 ± 6.0	1020 ± 116	990.7±87.6
LYM(%)	67.00 ± 1.35	67.76 ± 1.47	69.93 ± 2.60	68.33 ± 4.33
LYM#	8.60 ± 1.21	11.93 ± 0.99	8.70 ± 0.66	9.83 ± 0.20
RDWSD (fl)	30.60 ± 0.20	28.70 ± 1.03	24.30 ± 4.62	28.40 ± 0.29
MPV (fl)	7.47 ± 0.07	8.20 ± 0.10	7.85 ± 0.15	7.87 ± 0.23

(RBC= Red blood cell count, WBC= white blood cell count, HCT= hematocrit, HGB= hemoglobin, MCV= mean cell volume, PLT= platelet count mean, MCH= corpuscular hemoglobin, MCHC= mean corpuscular hemoglobin concentration, LYM= lymphocytes, RDWSD= Red blood cell distribution width, MPV=Mean platelet volume). Values are mean ± SEM of n=3. AP= *A. polycarpa* extract.

4.10.4 Urinalysis

Dipstick urinalysis of animals at baseline and control and test animals at termination of treatments showed no differences in urine parameters measured at baseline and controls at termination of study, as well as absence of differences in these parameters between AP-treated animals and controls at termination of study (Table 4.4).

Table 4.4: Effect of treatment with AP on urine parameters at baseline and termination of treatments

Parameter	Baseline	Treatment groups at termination			
			AP		
		Control	20 mg/kg	100 mg/kg	500 mg/kg
Glucose (mg/dl)	-	-	-	-	-
Bilirubin (mg/dl)	-	-	-	-	-
Ketones (mg/dl)	-	-	-	-	-
Specific gravity (g/dl)	1.015±0.003	1.030±0.003	1.03±0.003	1.023±0.007	1.030±0.003
Blood	-	-	-	-	-
pH	6.93 ± 0.30	6.70±0.1	6.67±0.09	6.77±0.03	6.77±0.03
Protein (g/dl)	+(26.25±3.75)	+(26.67±1.67)	+(26.67±1.67)	+(28.33±1.67)	+(28.33±1.67)
Urobilinogen (mg/dl)	N	N	N	N	N
Nitrites	-	-	-	-	-
Leucocytes (WBC/µL)	-	-	-	-	-

AP= *A. polycarpa* extract; N= normal; (-) = absent; (+) = present. Values are mean ± SEM of n=3.

4.10.5 Serum Biochemical Analyses

4.10.5.1 Serum alkaline phosphatase

The serum ALP level was significantly ($p<0.05$) increased at week 4 with the 500 mg/kg dose of AP and returned to near control level between weeks 8 and 12. The other AP-treated (20 and 100 mg/kg) groups did not significantly ($p>0.05$) change ALP levels over the duration of study. At the termination of treatments there were no significant differences between AP-treated animals and the control (Fig. 4.11a). This is confirmed by the area under the curve (Fig. 4.11b).

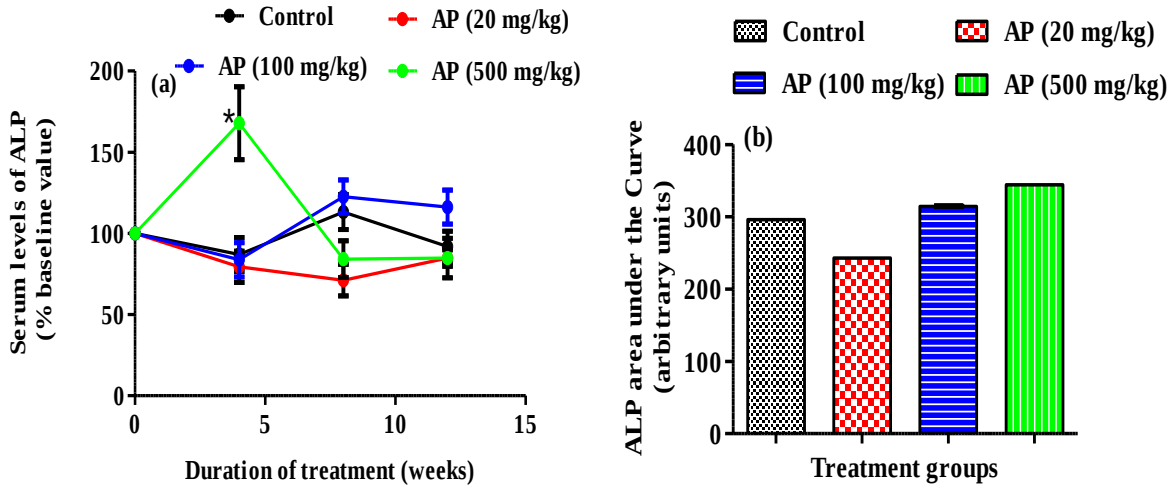

Fig 4.11: Effect of AP on serum levels of ALP over duration of treatments. (a): line graph; (b): area under the curve (AUC) as arbitrary units. Values are percentages of baseline control (440.4 ± 29.2 U/L). Results are mean ± SEM n = 6. * Value significantly ($p<0.05$) different from control group. AP= *A. polycarpa* extract

4.10.5.2 Serum alanine aminotransferase

There was a general increase in serum ALT levels with duration of treatments with all the animal treatment groups. However, there were no significant ($p>0.05$) differences in AP-treated animals compared to the control (Fig. 4.12a). This is depicted by the area under the curve (Fig. 4.12b)

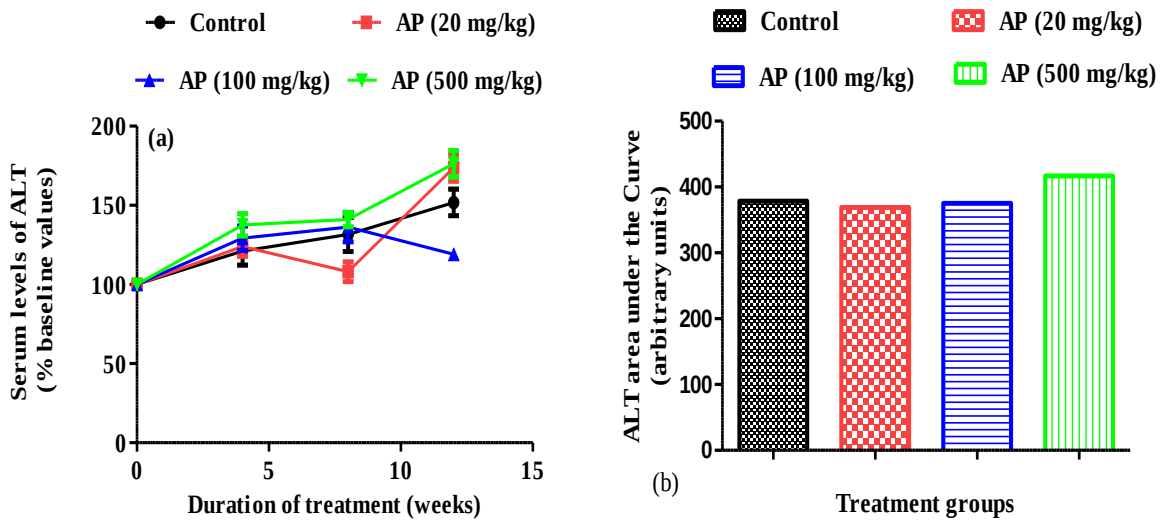

Fig 4.12: Effect of AP on serum levels of ALT over duration of treatments. (a): line graph; (b): area under curve (AUC) in arbitrary units. Values are percentages of baseline control (53.52 ± 4.57 U/L). Results are mean ± SEM of n = 6. * Value significantly ($p<0.05$) different from control group. AP= *A. polycarpa* extract

4.10.5.3 Serum direct bilirubin

There were slight variations in serum direct bilirubin levels with all animal treatment groups over the period of study. However, there were no significant differences in the AP-treated animals compared to the control (Fig. 4.13a). The overall results showed that AP had no effect on serum direct bilirubin levels as depicted by the AUC (Fig. 4.13b)

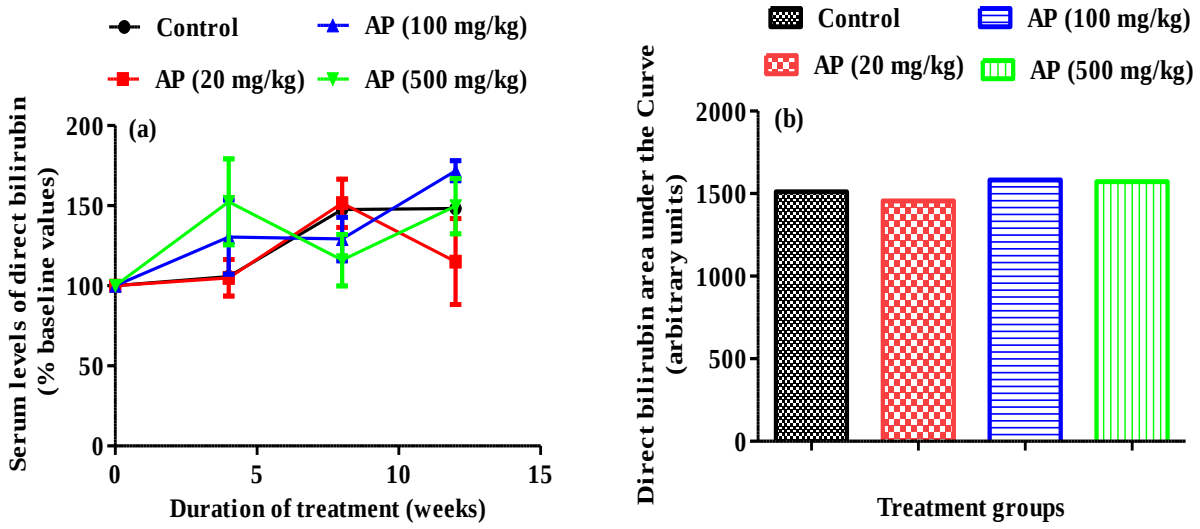

Fig 4.13: Effect of AP on serum levels of direct bilirubin over duration of treatments. (a): line graph; (b): area under the curve (AUC) as arbitrary units. Values are percentages of baseline control (0.23 ± 0.02 mg/dl). Results are means ± SEM of n = 6. AP= *A. polycarpa* extract.

4.10.5.4 Serum creatinine

Graph shows a steady decline in serum creatinine levels in all animal treatment groups within 4 weeks significantly different ($p<0.05$) from baseline control value. This decline continued with the 500 mg/kg AP-treated group up to termination of treatment. However, serum creatinine levels in the other AP-treated groups remained fairly constant thereafter till the termination of study. It was only at week 4 that the creatinine levels of the 100 and 500 mg/kg AP-treated groups were significantly higher ($p<0.05$) than the control (Fig. 4.14a). The overall effect was that AP did not affect serum creatinine levels as depicted by the AUC (Fig. 4.14b).

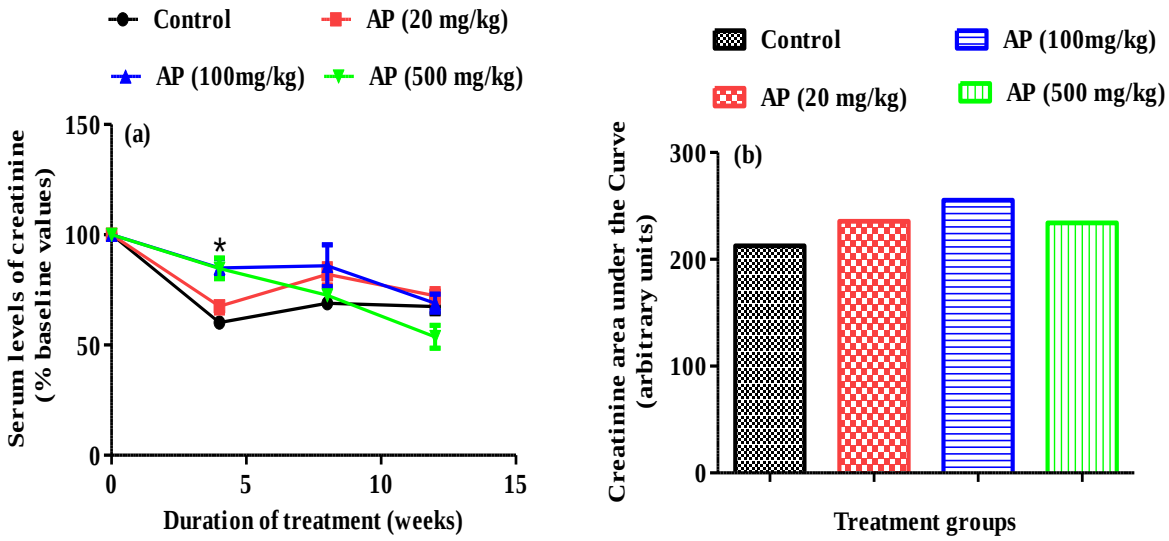

Fig 4.14: Effect of AP on serum levels of creatinine over duration of treatments (a): line graph; (b): area under the curve (AUC) as arbitrary units. Values are percentages of baseline control of (0.97 ± 0.05) mg/dl. Results are mean ± SEM of 6 determinations. * Value significantly ($p<0.05$) different from control group. AP= *A. polycarpa* extract.

4.10.6 Blood Clotting Time

The blood clotting times of animals treated with AP were not significantly different ($p>0.05$) from control animals at termination of treatments (Fig. 4.15).

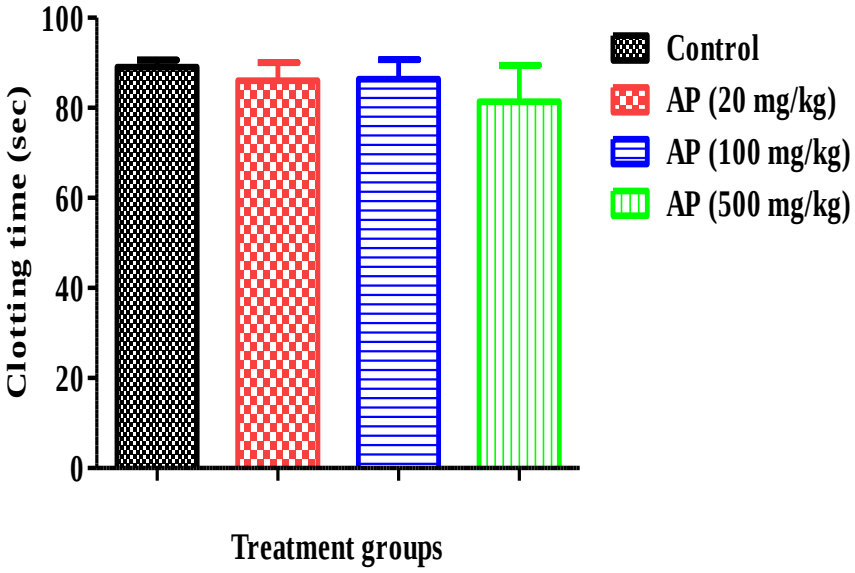

Fig. 4.15: Effect of *A. polycarpa* extract (AP) on blood clotting time. AP= *A. polycarpa*. Results are means ± S.E.M of n=4.

4.10.7 Pentobarbital-Induced Sleeping Time

There were no significant differences ($p>0.05$) in the sleeping time between AP-treated animals and controls at termination of treatments (Fig. 4.16).

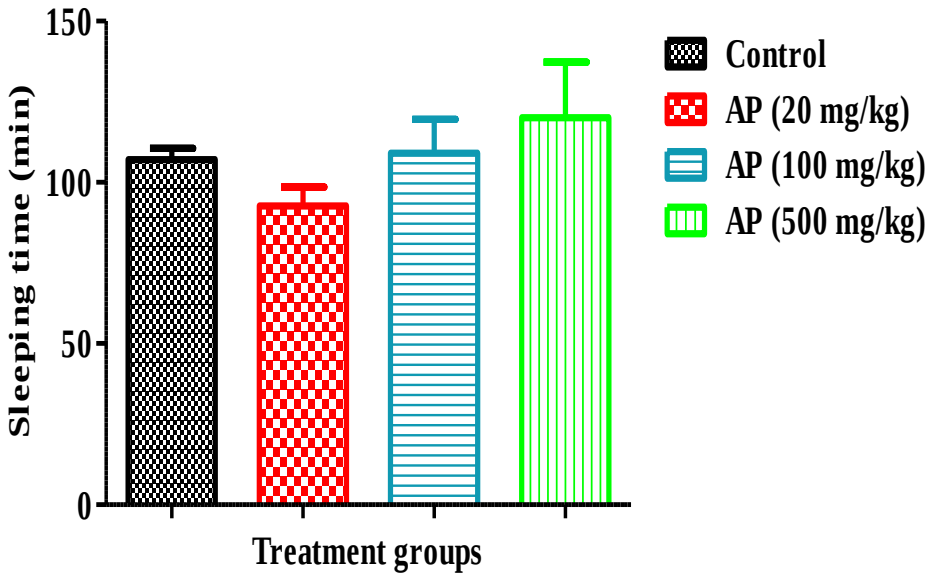

Fig. 4.16: Effect of *A. polycarpa* extract (AP) on pentobarbital- induced sleeping time. Results are means± S.E.M of n=4.

4.10.8 Histopathological Analyses

The micrographs of liver, heart, kidney lungs at termination of treatment of rats with AP shows no observable morphological differences between the organs of the AP treated rats and the controls except for slight morphological changes in the lung of the AP-treated groups characterized by interstitial fibrosis and thickening of alveolar septa, with normal Clara cells lining the bronchiolar epithelium (Figs. 4.17- 4.20)

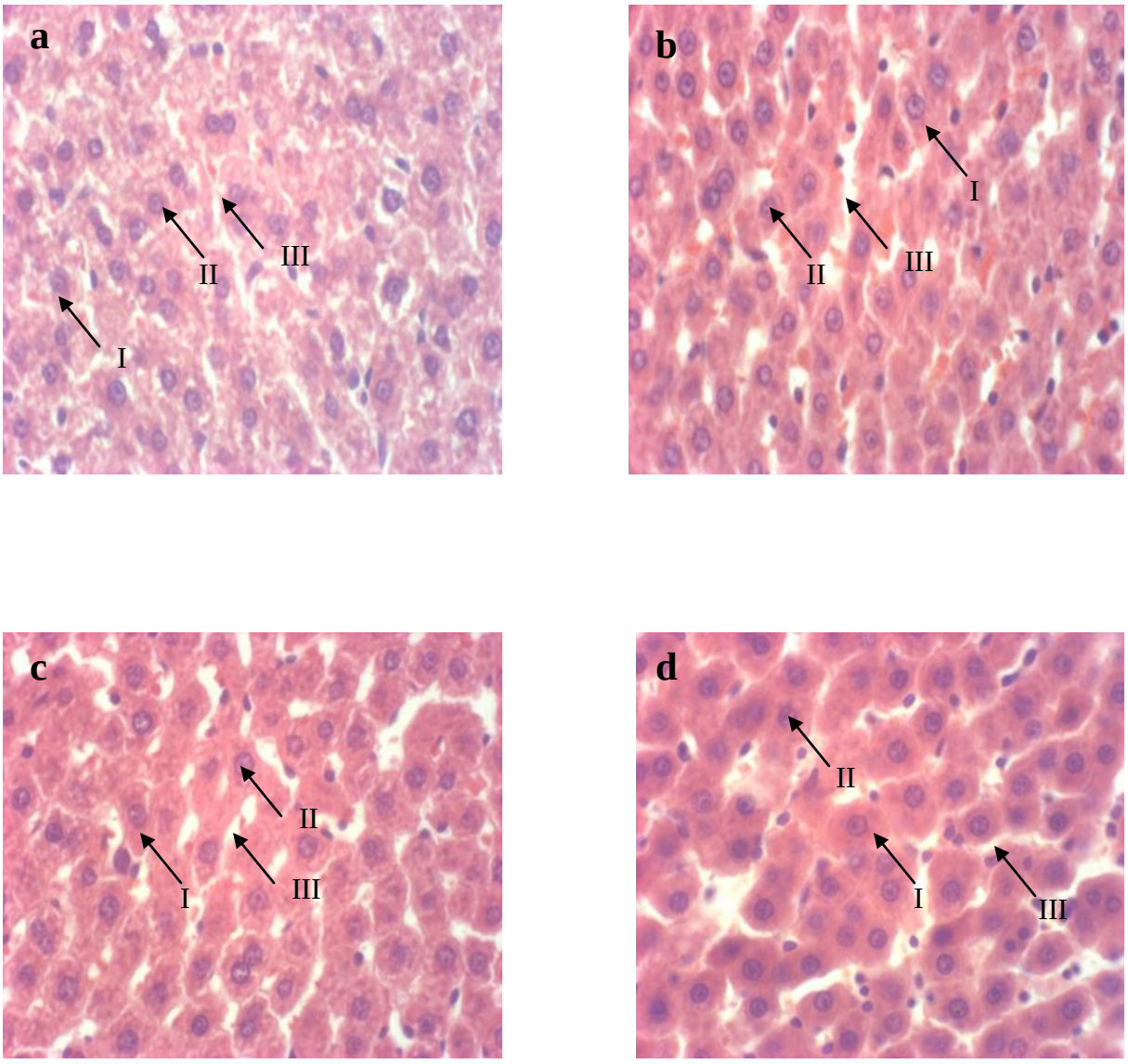

Fig. 4.17: Histological appearance of rat liver at termination of treatments for (a) control animals, (b) animals treated with AP (20 mg/kg), (c) animals treated with AP (100 mg/kg) and (d) animals treated with AP (500 mg/kg) showing normal hepatocyte (I), nuclei (II) and interstitial spaces (III). AP= *A. polycarpa* extract. Magnification: x200

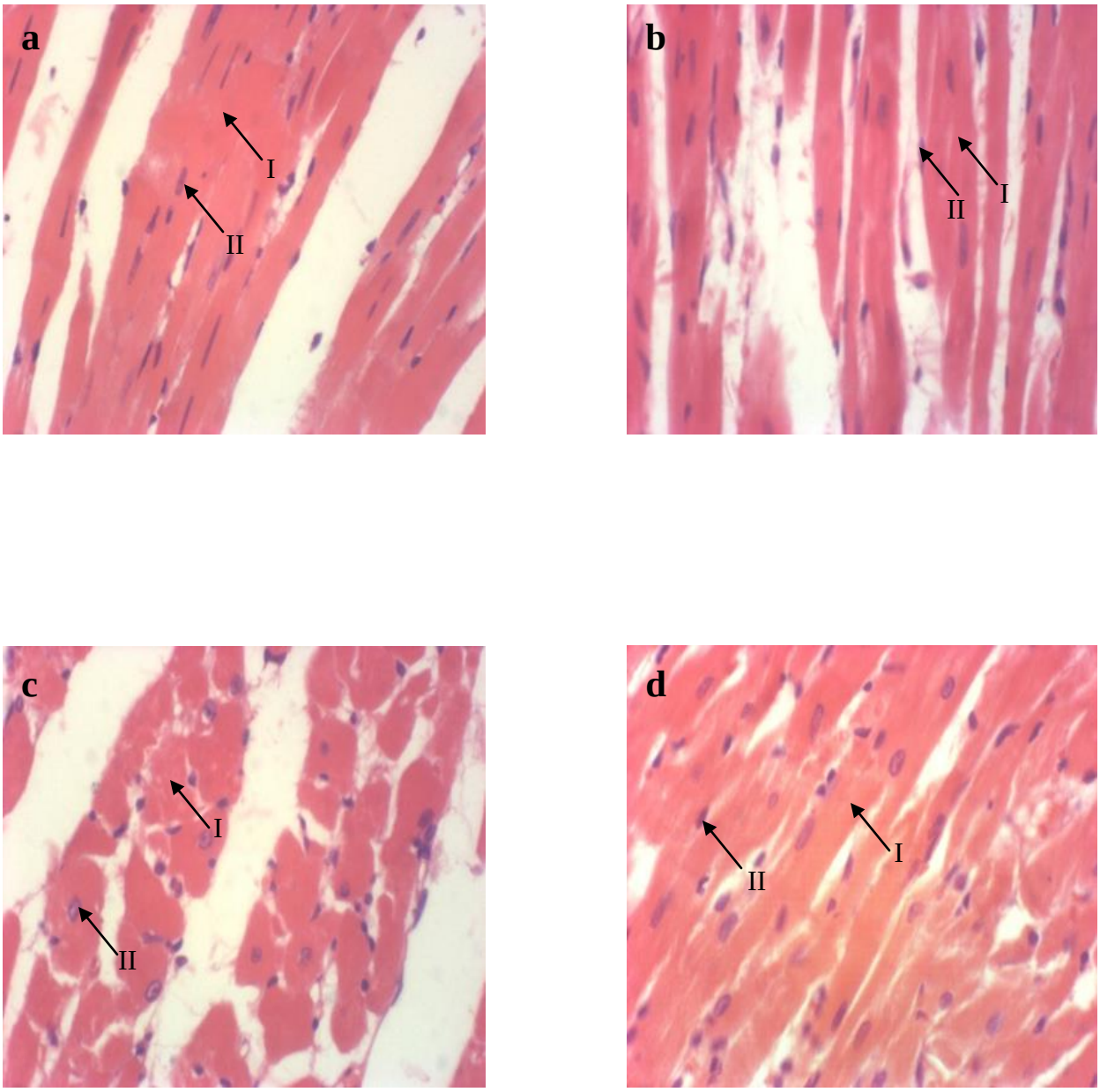

Fig. 4.18: Histological appearance of rat heart muscle at termination of treatments for (a) control animals, (b) animals treated with AP (20 mg/kg), (c) animals treated with AP (100 mg/kg and (d) animals treated with AP (500 mg/kg) showing no differences in morphology of (I) cardiac muscle fibres and (II) nuclei. AP= *A. polycarpa* extract. Magnification: x200

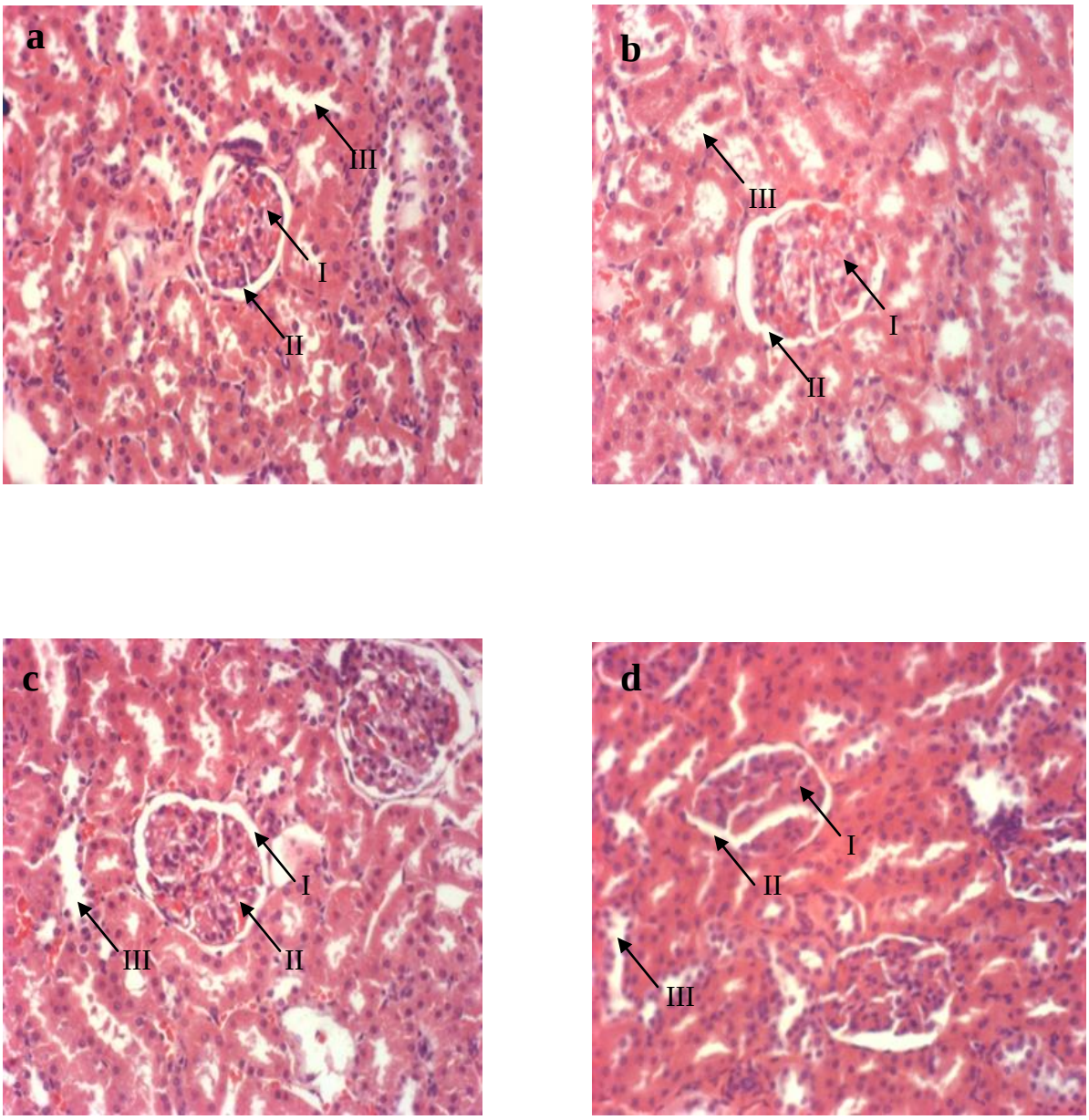

Fig. 4.19: Histological appearance of rat kidney at termination of treatments for (a) control animals, (b) animals treated with AP (20 mg/kg), (c) animals treated with AP (100 mg/kg) and (d) animals treated with AP (500 mg/kg) showing no difference in appearance of (I) glomerulus, (II) bowman's capsule and (III) renal tubules. AP= *A. polycarpa* extract. Magnification: x100

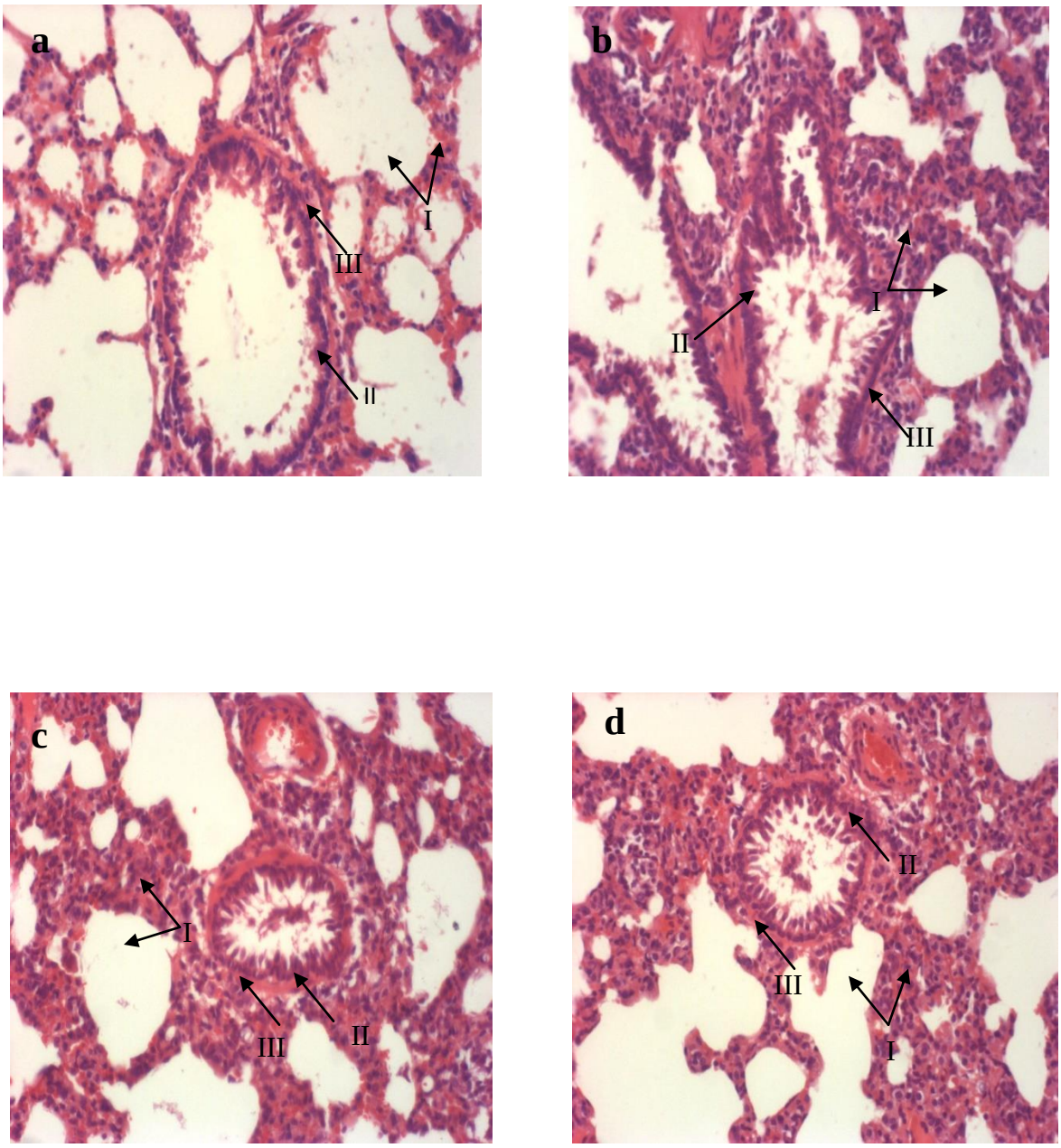

Fig. 4.20: Histological appearance of rat lung at termination of treatments for (a) control animals showing normal alveolar areas (I) with Clara cells (II) lining the bronchiolar epithelium (III), (b) animals treated with AP (20 mg/kg), (c) animals treated with AP (100 mg/kg) and (d) animals treated with AP (500 mg/kg) showing a dose-dependent alveolar damage characterized by interstitial fibrosis and thickening of alveolar septa with normal Clara cells (II) lining the bronchiolar epithelium (III). AP= *A. polycarpa* extract. Magnification: x100

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

The study was conducted to evaluate the antidiabetic effect of aqueous stem bark extract of *Annickia polycarpa* (AP) in alloxan-induced diabetic mice, as well as determine its organ-specific toxicity in Sprague-Dawley rats with the view to validating the anecdotal evidence of its use in the management of diabetes mellitus in humans.

Diabetes mellitus is a medical condition characterized by chronic hyperglycemia caused by a relative or absolute deficiency of insulin or resistance to the action of insulin at the cellular level (Krishnaveni *et al*., 2010). Alloxan-induced hyperglycaemia is considered as a suitable experimental model to study the hypoglycaemic effect of antidiabetic agents in type 2 diabetes mellitus. In alloxan-induced diabetes mellitus, there is the destruction of the beta cells thus resulting in low production of insulin and consequent hyperglycaemia (Sabu and Kuttan, 2002).

In this study, male ICR mice were made diabetic by a single intraperitoneal injection with alloxan (150 mg/kg body weight) followed by treatment for 4 weeks with AP and the standard drugs, glibenclamide and metformin. Treatment of alloxan-induced diabetic mice with the extract reduced the glucose levels significantly ($p<0.05$) over the period of study reaching near control levels by termination of treatments (Fig. 4.5), suggesting that the extract possesses hypoglycaemic activity. The hypoglycaemic effect of the extract was comparable to that of glibenclamide and metformin at the doses used. Metformin acts by increasing glucose absorption into peripheral tissues and glibenclamide acts by increasing insulin production. The hypoglycaemic effect displayed by AP may possibly be due to increased secretion of insulin as a

result of restoration of the morphology of beta cells of the pancreas that had become necrotic in the diabetic mice as also shown by glibenclamide (Fig. 4.9). The presence of alkaloids and phenolic compounds especially flavonoids in the extract may be responsible for the observed hypoglycaemic effect of the extract (Loew and Kaszkin, 2002; Anjaneyulu and Chopra, 2004). The glucose tolerance test further revealed that the extract improved the handling of the oral glucose load significantly in a way similar to that of glibenclamide and metformin (Fig. 4.8).

Diabetes mellitus is usually accompanied by dyslipidaemia characterized by hypertriglyceridemia and hypercholesterolemia (Maghrain *et al*., 2004), which may result in increased chances of developing coronary heart diseases. This normally occurs due to insulin insufficiency since insulin is involved in the stimulation of the enzyme lipoprotein lipase which breaks down triglycerides. Lipolysis is not repressed when insulin is deficient; however an upsurge in the breakdown of fats and other lipids eventually results in hyperlipidemia (Maghrain *et al*., 2004). Treatment of alloxan-induced diabetic mice with AP and standard drugs helped reverse the incidence of hypercholesterolemia and hypertriglyceridemia (Fig 4.6). This further goes to support the possible stimulation of insulin release by the extract/standard drugs, thereby inhibiting lipolysis and thus lowering serum lipid levels.

Diabetes mellitus leads to the generation of excess quantities of free radicals due to hyperglycaemia and hyperinsulinaemia (Ceolotto *et al*., 2004; Cimato *et al*., 2008) and this result in oxidative stress. Oxidative stress plays a role in the progression of diabetes mellitus and its complications. Diabetic patients have insufficient antioxidant defenses (Martin-Gallan *et al*., 2003) and for that matter any treatment regimen for diabetes mellitus should of necessity have antioxidant activity to reduce diabetic complications. Antioxidants are compounds that show reducing activity. They keep the components of cells and biomolecules from oxidation by

donating an electron/hydrogen atom to free radicals/reactive oxygen species (ROS) such as superoxide, hydroxyl, and peroxy radicals. Studies have proven the advantageous role antioxidants play in diabetes (Maritim *et al*., 2003; Liu *et al*., 2006). Plant phenolics are essential due to the fact that they possess hydroxyl groups that confer scavenging ability. The plant extract (AP) was shown to contain the following groups of phytochemicals: saponins; reducing sugars; phenolic compounds; alkaloids; and flavonoids (Table 4.1) and the extract was found to contain appreciable quantities of phenolic compounds particularly flavonoids (Figs. 4.1 and 4.2).

The antioxidant effect of flavonoids has been shown to be the possible reason why they prevent glycation (Wu and Yen, 2005). Studies have shown that flavonoids are highly effective scavengers of most oxidizing molecules which include singlet oxygen and various free radicals (Bravo, 1998) implicated in several diseases. The antioxidant activity of the extract may involve the scavenging of free radicals as seen with DPPH (Fig. 4.3), thereby protecting cells from the damaging effect of the free radicals and successive complications. The antioxidant activity of a substance is also measured by its reducing ability by donating a hydrogen atom which helps disrupt free radical chains thereby breaking them (Gordon, 1990). The extract appeared to have little to no reducing ability (Fig. 4.4) confirming that the antioxidant activity of the extract is as a result of the scavenging of free radicals.

The acute toxicity studies show a lethal dose (LD_{50}) of AP in rats greater than 5000 mg/kg, with no physical signs of toxicity, an indication that it is relatively safe (Loomis and Hayes, 1996). Adverse changes in body weight have been used as an indicator of toxicity of drugs and chemicals (Hilaly *et al*., 2004). The normal growth of AP-treated animals in this study, therefore, suggests absence of toxicity (Fig. 4.9). Adverse changes in mean organ weight are a reflection of organ specific toxicity (Wooley, 2003). Absence of effect of AP on mean organ weights suggests

that they were not oedematous as a result of defects in osmoregulation (Curtis *et al.*, 1999) or hypertrophic as a result of lack of control of cell growth and division (Greaves, 2000). In this study, AP did not cause significant changes in mean organ weight (table 4.2) and therefore the extract may not have these adverse effects.

Blood is an essential circulatory tissue that consists of red and white blood cells and platelets suspended in plasma and functions to maintain homeostasis (Isaac *et al.*, 2013). Red blood cells serve as carriers of haemoglobin which transports oxygen to tissues (Chineke *et al*., 2006). A reduced red blood cell count suggests that oxygen and carbon dioxide transport will be impaired (Isaac *et al.*, 2013). White blood cells help to fight infections and as a result reduced white blood cell count makes one susceptible to infections (Soetan *et al*., 2013). Blood platelets are associated with blood clotting and for that matter low platelet concentration will lead to long clotting time leading to blood loss. The lack of effect of AP in platelet count is reflected in its lack of effect on blood clotting time. Some haemotoxicants for example paracetamol and some phytochemicals are known to cause reduction in red blood cell counts and also alter haemoglobin concentration with subsequent anaemia in experimental animals (Mullick *et al.*, 1973; Patrick-Iwuanyanwu *et al.*, 2007). Oral administration of AP for three months did not result in a significant difference between haematological parameters of the control and treatment groups. This presupposes that the extract has no direct effect on the blood cells or bone marrow or indirect effect on erythropoiesis.

The liver is a key organ in the body involved in metabolism, storage and excretion of exogenous compounds as well as metabolism of endogenous compounds. It is the powerhouse of the body (Rej, 1978). When there is a toxic insult to the liver by toxicants, the liver cells are damaged and enzymes such as alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase

in the liver cells are released into the blood (Kesari *et al*., 2007), thus increasing the serum levels of these enzymes. Toxic insult to the liver also affects the biosynthetic ability of the liver cell thus protein synthesis may be impaired. Liver is the site of synthesis of blood clotting factors, which are protein in nature hence damage to the liver will prolong blood clotting time (prothrombin time). This study showed that AP did not cause the elevation of these serum enzyme levels and also did not affect the blood clotting time, an indication of the absence of hepatocellular damage. These observations are corroborated by the absence of adverse changes in the morphology of liver cells and lack of effect on pentobarbital induced sleeping time in AP treated animals. Damage to the liver cells may result in the reduction of cytochrome P_{450} levels, thus leading to an increase in pentobarbital induced sleeping time as a result of reduced metabolism of pentobarbital (Shin, 1989).

The kidneys produce erythropoietin which is involved in erythropoiesis. Damage to the kidney impairs erythropoiesis and blood cell formation. Damage to the glomerulus causes increase in serum creatinine and urea levels as well as the presence of albumin in urine. The renal tubules are responsible for concentrating urine, regulation of ion excretion and the production of acidic urine. There were no significant differences in urine protein and pH between baseline values and termination values for control and extract-treated groups. The presence of protein in urine at baseline may be due to low renal threshold for protein. The extract did not cause any changes in the serum and urine parameters mentioned above and did not affect blood cell formation. This seems to suggest that the extract may not be nephrotoxic. These results are corroborated by the morphology of the kidney which shows no adverse effects on the Bowman's capsule, glomerulus and renal tubules (Fig. 4.19).

The heart is a muscular organ which functions by pumping blood to all parts of the body supplying tissues with oxygen and nutrients. The lung is the site for gaseous exchange in the body. AP did not cause any morphological changes in the heart muscle indicating that it may not be cardiotoxic (Fig. 4.18). However, in the case of the lung; AP appears to cause alveolar cell damage, without causing bronchiolar cell damage, which appears to be dose-dependent (Fig. 4.20) and characterized by the thickening of the alveolar septa and interstitial fibrosis. This may affect the diffusion of oxygen across the alveoli with its attendant effect on degree of oxygen transport and thus cellular metabolism. The extract thus appears to be mildly pneumotoxic so care should be taken in its use by determining its therapeutic index.

5.2 CONCLUSIONS

In conclusion, it may be said that *A. polycarpa* extract has significant antidiabetic activity and this may be due to its ability to cause the regeneration of beta cells of the pancreas and thus the stimulation of insulin secretion. The plant extract also possesses appreciable antioxidant activity possibly through the scavenging of ROS and thus may help in overcoming some of the advanced complications of diabtetes mellitus. The presence of alkaloids and phenolic compounds for example flavonoids may be responsible for both the hypoglycaemic and antioxidant effects observed. However, AP showed organ specific toxicity with respect to the lung, where it caused selective damage to alveolar cells. These results validate the anecdotal evidence of its use in the management of diabetes mellitus, but care must be taken in its use because of its selective lung toxicity.

5.3 RECOMMENDATIONS

- The use of other animal models of diabetes to investigate the antidiabetic potential of AP is highly recommended.

- Further studies should be conducted to determine the possible mode(s) of action of this extract.
- Lower doses of the extract should be considered to see whether it has a high therapeutic index (high efficacy relative to toxicity).
- Toxicity studies should be conducted in other animal species.

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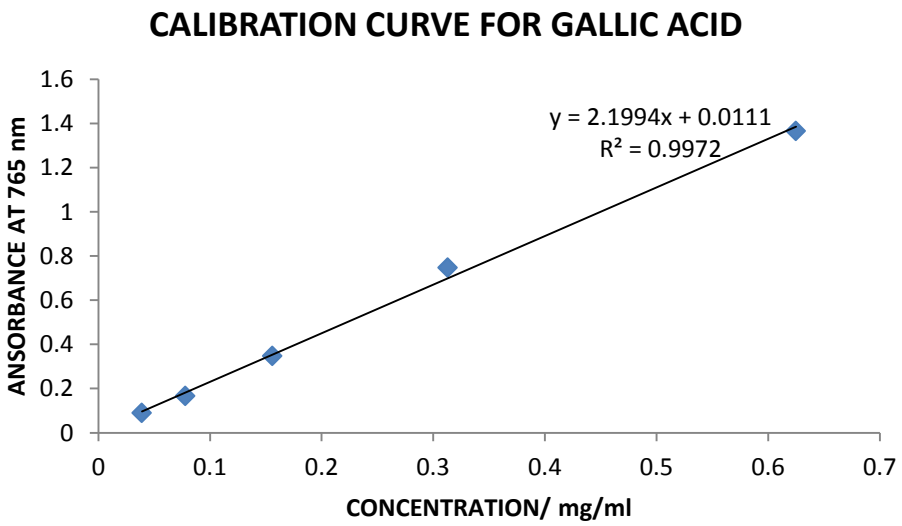
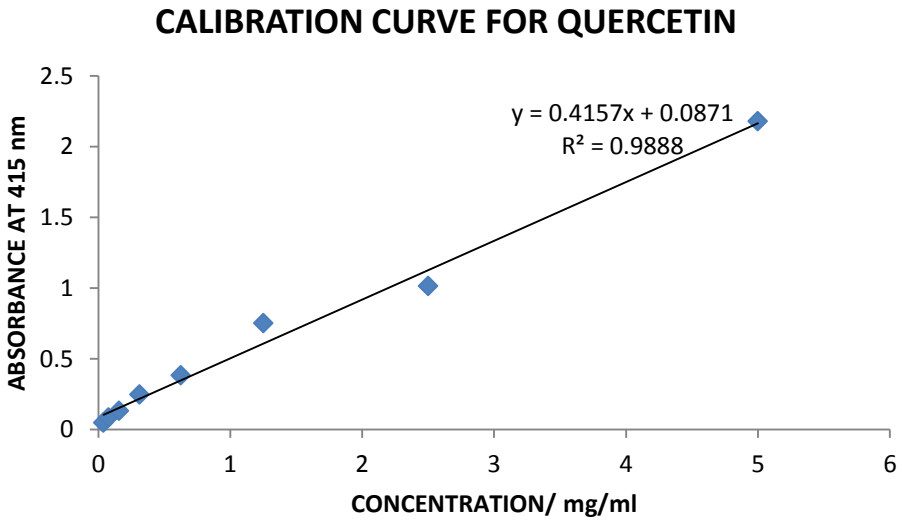
APPENDICES



Appendix 1. Standard calibration curve for Gallic acid for the determination of the phenolic content of the extract.



Appendix 2. Standard calibration curve for quercetin used for the determination of flavonoid content of the extract

Appendix 3. Fasting blood glucose levels of mice for 4 weeks

WEEK	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
0	4.33±0.09	8.50±0.11	8.2±0.17	8.33±0.85	9.33±0.68	8.30±0.44	8.60±0.21
7	4.07±0.24	8.00±0.23	6.2±0.67	7.17±1.31	6.67±0.68	6.10±0.45	5.03±0.41
14	4.57±0.38	7.9±0.35	5.67±0.73	6.27±1.37	5.40±0.36	5.47±0.57	5.00±0.35
21	5.73±0.59	7.70±0.65	5.53±1.32	5.80±1.06	4.67±0.55	5.00±0.12	4.90±0.38
28	4.53±0.23	7.50±0.36	4.93±1.02	5.87±1.20	4.80±0.47	4.67±0.17	4.33±0.32

Group 1= Non-diabetic control group; Group 2 = Diabetic control group; Group 3= Glibenclamide treated group (2.5 mg/ml); Group 4= Metformin treated group; Group 5 = AP (20 mg/kg); Group 6 =AP (100 mg/kg); Group 7 =AP (500 mg/kg); AP = *Annickia polycarpa*. Results are mean ± S.E.M of n=6

Appendix 4. Blood glucose levels of mice after an oral glucose load

WEEK	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
0	4.5±0.2	9.8±0.6	6.5±0.5	8.6±1.9	8.5±2.8	5.7±0.4	6.25±0.5
30	12.0±2.3	25.7±3.3	18.3±2.1	12.8±6.3	15.8±5.4	15.0±2.4	14.5±2.9
60	6.0±0.7	21.4±2.1	9.0±0.8	7.9±8.4	10.9±7.5	11.6±1.1	7.5±2.3
90	5.8±0.6	17.4±1.0	7.0±0.3	5.4±8.5	8.9±6.9	7.8±0.4	6.65±1.6
120	5.3±0.5	13.3±0.7	6.3±0.4	3.9±5.5	8.3±5.3	6.6±0.6	5.4±0.9
150	5.2±0.5	12.1±0.7	5.7±0.3	3.8±4.3	5.5±3.7	5.7±0.5	5.2±0.6

Group 1 = Non-diabetic control group; Group 2= Diabetic control group; Group 3= Glibenclamide treated group (2.5 mg/ml); Group 4= Metformin treated group; Group 5 = AP (20 mg/kg); Group 6 =AP (100 mg/kg); Group 7 =AP (500 mg/kg); AP = *Annickia polycarpa*. Results are mean ± S.E.M of n=6

Appendix 5. Weight of diabetic/ non-diabetic mice for 4 weeks following treatment

WEEK	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
0	27.6±0.6	24.0±1.7	24.6±0.4	24.3±0.6	24.0±1.2	24.8±1.5	23.0±0.7
1	31.2±0.9	29.6±1.8	31.2±0.3	31.5±1.0	32.0±1.2	32.3±1.5	32.0±1.1
2	36.9±1.5	31.5±2.9	35.3±0.9	33.7±5.0	35.1±2.2	35.6±1.5	36.7±0.8
3	37.5±1.8	33.4±2.7	35.3±0.9	34.8±5.5	38..4±1.8	35.9±1.7	38.6±0.9
4	37.8±1.4	32.3±2.3	36.4±1.6	37.0±4.5	40.0±1.9	38.6±1.8	37.5±1.4

Group 1= Non-diabetic control group; Group 2= Diabetic control group; Group 3= Glibenclamide treated group (2.5 mg/ml); Group 4= Metformin treated group; Group 5 = AP (20 mg/kg); Group 6 =AP (100 mg/kg); Group 7 =AP (500 mg/kg); AP = *Annickia polycarpa*. Results are mean ± S.E.M of n=6

Appendix 6. Weights of rats for 12 weeks following treatment

WEEK	CONTROL	AP (20 mg/kg)	AP (100 mg/kg)	AP (500 mg/kg)
0	131.5±1.18	164.33±1.31	152.33±0.67	142.5±1.96
1	135.33±1.54	169.33±1.74	158.83±1.58	151.67±2.53
2	149.83±3.17	181.83±3.89	173.83±1.64	163.50±2.08
3	178.00±5.09	206.33±6.08	180.00±14.11	189.50±2.09
4	191.67±6.31	218.17±7.66	205.20±2.87	205.00±2.62
5	202.67±5.82	235.00±10.31	221.20±3.28	215.17±2.95
6	208.83±11.47	232.50±11.49	231.20±5.38	212.17±2.83
7	217.50±9.66	243.50±12.49	236.00±8.86	221.17±3.27
8	225.00±9.51	261.80±13.38	246.00±13.35	232.67±2.73
9	223.83±10.38	264.20±12.98	257.00±7.57	231.33±4.27
10	240.17±9.44	272.60±15.36	248.40±4.99	254.00±6.95
11	239.17±10.65	275.60±14.05	249.40±4.92	263.50±7.30
12	248.17±12.83	279.20±13.78	259.60±6.87	263.33±8.27

AP= *Annickia polycarpa.* Results are mean ± S.E.M of n=6

Appendix 7. Serum ALP levels in rats over the duration of treatment

WEEK	CONTROL	AP (20 mg/kg)	AP (100 mg/kg)	AP (500 mg/kg)
0	356.50±27.78	510.83±53.68	448.50±63.35	578.75±144.29
4	383.20±46.37	349.50±42.46	369.00±46.48	739.17±98.74
8	498.33±47.65	313.60±43.00	540.20±45.54	370.40±49.62
12	404.75±41.47	373.80±53.50	512.00±46.16	373.08±22.40

AP= *Annickia polycarpa.* Results are mean ± S.E.M of n=6

Appendix 8. Serum ALT levels in rats over the duration of treatment

WEEK	CONTROL	AP (20 mg/kg)	AP (100mg/kg)	AP (500 mg/kg)
0	50.30±6.65	65.77±7.32	53.08±2.36	71.97±1.77
4	70.98±5.18	72.63±3.58	75.76±4.51	80.67±4.17
8	77.08±6.27	63.30±3.69	79.85±5.49	81.10±9.79
12	88.97±4.93	93.70±6.55	84.85±4.97	943.10±8.45

AP= *Annickia polycarpa.* Results are mean ± S.E.M of n=6

Appendix 9. Serum creatinine levels in rats over the duration of treatment

WEEK	CONTROL	AP (20 mg/kg)	AP (100mg/kg)	AP (500 mg/kg)
0	0.93±0.07	1.06±0.06	0.81±0.04	1.13±0.17
4	0.58±0.02	0.65±0.03	0.82±0.04	0.82±0.05
8	0.67±0.02	0.80±0.05	0.83±0.09	0.70±0.02
12	0.65±0.03	0.70±0.03	0.67±0.04	0.52±0.05

AP= *Annickia polycarpa.* Results are mean ± S.E.M of n=6

Appendix 10. Serum levels of direct bilirubin in rats at over the duration of treatment

WEEK	CONTROL	AP (20 mg/kg)	AP (100 mg/kg)	AP (500 mg/kg)
0	0.22±0.04	0.28±0.04	0.20±0.03	0.20±0.06
4	0.24±0.03	0.24±0.03	0.30±0.05	0.35±0.06
8	0.34±0.03	0.34±0.03	0.29±0.03	0.26±0.04
12	0.34±0.03	0.26±0.06	0.39±0.03	0.34±0.04

AP= *Annickia polycarpa.* Results are mean ± S.E.M of n=6