ANTIDIABETIC ACTIVITIES OF THE METHANOLIC ROOT BARK EXTRACT OF *AFZELIA AFRICANA* IN ALLOXAN-INDUCED DIABETIC MICE

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TITLE PAGE

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POST GRADUATE STUDIES OF THE UNIVERSITY OF NIGERIA IN PARTIAL FULFILMENT FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE (VETERINARY PHARMACOLOGY AND TOXICOLOGY)

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CERTIFICATION

Dr. Odo Rita Ifeoma (Nee Ezeme), a post graduate student in the Department of Veterinary Physiology and Pharmacology and with registration number PG/MSc/08/48311 has satisfactorily completed the requirements for course and research work for the degree of Master of Science (M.Sc.) in Veterinary Pharmacology and Toxicology. The work embodied in this dissertation is original and has not been submitted in full or part for any other diploma or degree of this or any other University.

……	……	……	…….
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(Supervisor)	**Date**	**(Head of Department)**	**Date**

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Prof. C. N. Uchendu		**Prof. P. A. Onyeyili**	**Date**
(Dean of the Faculty)	**Date**	**(External Examiner)**	

DEDICATION

This work is dedicated to my beloved husband ï Mr. Odo Pius and my children ï Miracle, Benita, Divine Favour and Faith.

ACKNOWLEDGMENT

I appreciate the Almighty God for His continuous providence, protection and guidance. I express my heart felt gratitude to my husband and children for their love, care and prayer.

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ABSTRACT

The methanolic root bark extract of *Afzelia africana* was tested for antidiabetic activities *in-vivo*. The acute toxicity of the extract was tested in mice and the result showed that the extract

has low toxicity. Investigation on the phytochemical constituents of the plant extract revealed the presence of flavonoids, tannins, alkaloids, steroids and saponins.

The plant extract was tested for antidiabetic activities in alloxan - induced diabetic mice at doses; 62.5 mg/kg, 125 mg/kg, 250 mg/kg, 500 mg/kg and 1000 mg/kg over a period of 6 hours. The extract was found to have antidiabetic activity. Optimum activity was noted at the dose of 250 mg/kg and 6 hours post treatment. The antidiabetic activity of the extract did not differ significantly ($p>0.05$) from that of glibenclamide.

Column and thin layer chromatography revealed the presence of five fractions in the plant extract with fraction 3 found to be the active fraction.

The free radical scavenging activity of the active fraction determined by *in- vitro* DPPH (1, 1-diphenyl 2-picryl hydrazine) and FRAP (Fehlingôs reducing antioxidant power) Methods revealed that it has an antioxidant activity.

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CHAPTER ONE

INTRODUCTION

Diabetes mellitus, commonly referred to as diabetes (as it will be in this article) was first identified as a disease associated with ñsweet urineò, and excessive muscle loss in the ancient world. An elevated level of blood glucose (hyperglycemia) leads to spillage of glucose into the urine, hence the term sweet urine.

It is a metabolic disease characterized by high blood glucose levels, either, because the body does not produce enough insulin or because the body cells do not properly respond to insulin that is produced (Rother, 2007). Insulin is a hormone produced by the beta cells of the pancreas. Its primary function is to transport glucose to cells. When the blood glucose is elevated (for example after eating food), insulin is released from the pancreas to normalize the glucose level. If this function cannot be met as in diabetic cases glucose accumulates in the blood leading to many complications (Rother, 2007).

The discovery of a role for the pancreas in diabetes is generally ascribed to Joseph Von Mering and Oskar Minkowski, who in 1889 found that dogs whose pancreas were removed developed all the signs and symptoms of diabetes and died shortly afterwards (Von Mering and Minkowski, 1890). The classical symptoms of Diabetes mellitus are polyuria, polydipsia, polyphagia and weight loss (Cooke and Plotnick, 2008). Diabetes mellitus is a relatively common endocrinopathy in dogs and cats. It is more important in these species, though it has been reported in horses, dairy cattle, pigs and sheep (Aiello *et al.,* 1998). In dogs and cats, most spontaneous cases occur in middle aged animals. In dogs, females are affected twice as often as male and the incidence appears to be increased in certain breeds of dogs such as miniature poodles, Dachshunds, Shnauzers, Terriers and Beagles, but any breed may be affected (Aiello *et al.,* 1998).

In humans, diabetes mellitus is a major cause of death worldwide. In the year 2000, according to WHO, at least 171 million people worldwide suffered from diabetes, or 2.8% of the population (WHO, 1999). Type 2 diabetes is by far the most common, affecting 90 to 95% of the U.S diabetic population. The incidence of diabetes in developing countries follows the trend of urbanization and lifestyle changes, perhaps most importantly a ñWestern-styleò diet. This has suggested an environmental (i.e. dietary) effect.

From an economic perspective, the National Diabetes Information Clearing house estimates that diabetes costs $132 billion in the United States alone every year. The *per capita* cost resulting from diabetes in 1997 amounted to $10,071.00; while health care costs for people without diabetes incurred a *per capita* cost of $2,699.00. During the same year, 13.9 million days of hospital stay were attributed to diabetes while 30.3 million physician office visits were diabetes related. These numbers reflect only the population in the United States. Globally, the statistics are staggering. The American Diabetes Association cites the 2003 assessment of the National Centre for Chronic Disease Prevention and Health Promotion that one in three Americans born after the year 2000 will develop diabetes in their lifetime (Narayan *et al.,*2003). According to the American Diabetes Association, approximately 18.13% (8.6 million) of Americans aged 60 and older have diabetes (Seniors and Diabetes, 2006). Diabetes mellitus prevalence increases with age and the number of older persons with diabetes are expected to grow as the elderly population increases in number. The National Health and Nutrition Examination Survey (NHANES III) demonstrated that, in the population over 65 years old, 18% - 20% have diabetes, with 40% having either diabetes or its precursor form of unpaired glucose tolerance (Harris *et al*., 1998).

1.1 STATEMENT OF THE PROBLEM

- Ethnomedical products are sometimes exaggerated and based on trial and error hence the need to justify the practice of traditional medicine.
- Despite the availability of synthetic drugs, diabetes has remained a major cause of death world wide (The Diabetes Control and Complications Trial Research Group, 1993), thus there is need to search for a more potent antidiabetic agent of natural origin.
- Plant drugs are frequently considered to be less toxic more free from side effects than synthetic agents.
- Plant drugs are cheaper to obtain than synthetic agents as they are readily available in nature.

1.2 RESEARCH OBJECTIVES

1. To validate the ethnomedical use of methanolic root-bark extract of *Afzelia africana* in the treatment of diabetes mellitus.
2. To establish the toxicity of the extract on laboratory animals.

CHAPTER TWO

LITERATURE REVIEW

2.1 DEFINITION OF DIABETES MELLITUS

Diabetes mellitus, simply describes a higher than normal glucose level in the blood (WHO,1999). Blood carries glucose to every cell in the body. The cells use glucose as a fuel source. Glucose enters a cell and gets metabolized, giving energy to the cell. It is similar to the way a car burns gasoline in order to run. For all cells, glucose is the main source of fuel. If glucose canôt get into the cell properly, it starts to accumulate in the blood. This results in higher amounts in the blood (hyperglycemia). In the fasting state (no food for at least eight hours), a normal level of glucose in the blood is considered to be less than 6.4 mMol/L, when glucose accumulates to levels of greater than 6.4 mMol/L (Iwalewa *et al*., 2008), in the fasting state, diabetes mellitus is confirmed. There are different types of diabetes, but all have common abnormality of higher than normal glucose level in the blood.

2.2 CLASSIFICATION OF DIABETES MELLITUS

The 1980 WHO Expert committee proposed two major classes of Diabetes Mellitus as follows:

1. Insulin Dependent Diabetes Mellitus (IDDM) or Type 1 diabetes

Type 1 diabetes is characterized by loss of insulin producing beta cells of the islets of Langerhans in the pancreas leading to absolute insulin deficiency. This type of diabetes can be further classified as immune mediated or idiopathic. The majority of Type 1 diabetes is of the immune mediated nature, where beta cell loss is a T-cell mediated autoimmune attack (Rother, 2007). Type 1 diabetes can affect children or adults but was traditionally termed ñjuvenile diabetesò because it represents a majority of the diabetic cases in children.

2. Non Insulin dependent Diabetes Mellitus (NIDDM) or Type 2 diabetes

Type 2 diabetes is characterized by insulin resistance which may be combined with reduced insulin secretion. The defective responsiveness of body tissues to insulin is believed to involve the insulin receptor. In the early stage of Type 2 diabetes, the predominant abnormality is reduced insulin sensitivity. At this stage hyperglycemia can be reversed by a variety of measures and medications that improve insulin sensitivity or reduce glucose production by the

liver. As the disease progresses, the impairment of insulin secretion occurs, and therapeutic replacement of insulin may sometimes become necessary in certain patients (Rother, 2007).

2.3 OTHER TYPES

- Diabetes caused by genetic mutations leading to defects in beta cellsô function.
- Diabetes caused by genetic defects in insulin action.
- Diabetes caused by diseases that cause extensive damage to the pancreas e.g. chronic pancreatitis and cystic fibrosis.
- Diseases associated with extensive secretion of insulin-antagonistic hormones.
- Diabetes caused by drugs or chemicals such as nicotinic acid, glucocorticoid and thyroid hormone which impair insulin secretion.
- Gestational diabetes mellitus which occurs when pregnant women who have never had diabetes before, have a high glucose level during pregnancy. It occurs in 2% -5% of pregnant women and disappears after delivery.

2.4 SYMPTOMS OF DIABETES

- Tiredness: cells need glucose for energy, and the glucose is not getting to them. In fact, the cells are starving and cannot work efficiently. This causes tiredness.
- Glycosuria: Blood is filtered by the kidney. If there is excess glucose in the blood, this also goes to the kidneys. The kidneys can re-absorb and recycle normal amounts of glucose, as the body does not want to lose it in the urine. However, when the glucose rises above the ñrenal thresholdò, the kidney begins to excrete the excess and there is glucose in the urine (glycosuria).
- Polyuria**:** More urine is produced due to inability of the renal epithelium to concentrate the urine against the osmotic attraction hence frequent urination.
- Polydipsia: The increased urination causes dehydration, leading to increased thirst.
- Weight loss: This directly results from the loss of glucose in the urine. Glucose is a sugar, and represents calories to the body. Losing glucose in the urine means losing calories in the urine. This is like eating a piece of bread and having some of the calories from it lost in the urine. The body is not using all the calories in the diet and as the cells starve, weight is lost. Furthermore, uncontrolled (high) glucose boosts the metabolic rate, making it easier to lose weight.

- Blurred vision**:** This is directly due to a physical change in the shape of the lens of the eye. When the glucose in the blood goes up, extra glucose gets into the lens of the eye. When this happens, water again follows it (very similar to the water following the glucose as it passes through the kidney). When extra water enters the lens, it alters the shape of it. The lens looses its ability to focus light for the eye, which is perceived as blurred vision. This is often a most annoying symptom to the newly diagnosed diabetic person, and it must be emphasized that any visual change that occurs with high blood sugars is diabetic, i.e., it corrects with correction of the blood glucose. However, this takes time, and it may be as long as 6-8 weeks after blood glucose has been corrected that vision returns to its baseline, and stays there. Eyeglasses should not be prescribed when the blood glucose level is high.
- Polyphagia: The cells are starving because of inassimilable carbohydrate. Furthermore high blood glucose boosts the metabolic rate hence continuous quest for food.
- Moodiness: The brain does not like an environment of high glucose, and moodiness is a very common symptom of high sugars. Decreased ability to concentrate is also common for the same reason.
- Muscle cramps**:** As discussed under weight loss, muscle mass is lost when glucose level is uncontrolled. There is some tissue breakdown, and there is considerable loss of ñElectrolytesò. These are salts that are present in all tissue breakdown, and there is considerable loss of ñelectrolytesò. These are salts that are present in all tissues of the body, and are abundant in muscle. When they are lost, the muscle becomes very prone to a cramping pain.

- Numbness or Tingling of the Hands or Feet**:** As the glucose goes up, more of it passes into the nerve tissue. This radically alters the electrolytes in the nerves, as well as changing many other functions, and even structures of molecule (protein and enzyme) in the nerve. When the nerve is so affected, it gives false messages to the brain, and this is perceived as numbness, tingling, or even pain.

2.5 CAUSES OF DIABETES

- Old age

Insulin production decreases because of age-related impairment of pancreatic beta cells. Additionally, insulin resistance increases because of the loss of lean tissue and the accumulation of fat, particularly intra abdominal fat, and the decreased tissue sensitivity to insulin, Glucose tolerance progressively declines with age, leading to a high prevalence of type 2 diabetes and post-challenge hyperglycemia in older population (Harris *et al.,* 1998)

- Life style

A number of lifestyle factors are known to be important to the development of type 2 diabetes. In one study, those who had high levels of Physical_activity, a healthy diet, did not smoke, and consumed alcohol in moderation had an 82% lower rate of diabetes. When a normal weight was included the rate was 89% lower. In this study a healthy diet was defined as one high in fiber, with a high polyunsaturated to saturated fat ratio, and a lower mean glycemic index [Mozaffarian *et al*., 2009), Obesity has been found to contribute to approximately 55% type 2 diabetes, (Centre for Disease Control and Prevention, 2004) and decreasing consumption of saturated fats and trans fatty acids while replacing them with unsaturated fats may decrease the risk (Riserus *et al.,* 2009). The increased rate of childhood obesity in between the 1960s and 2000s is believed to have lead to the increase in type 2 diabetes in children and adolescents. (Arlan and Janet, 2003)

Environmental toxins may contribute to recent increases in the rate of type 2 diabetes. A positive correlation has been found between the concentration in the urine of bisphenol A, a constituent of some plastics, and the incidence of type 2 diabetes (Lang *et al.,* 2008).

- Medical conditions

Subclinical cushingôs syndrome (cortisol excess) may be associated with DM type 2 (Iwasaki *et al*., 2008). The percentage of subclinical cushingôs syndrome in diabetic population is about 9% (Chiodini *et al.,* 2005). Diabetic patients with a pituitary microadenoma can improve insulin sensitivity by removal of these microadenomas (Taniguchi *et al*., 2008).

Hypogonadism is often associated with cortisol excess, and testosterone deficiency is also associated with diabetes mellitus type 2, (Saad and Georen, 2009), although the exact mechanism by which testosterone improve insulin sensitivity is still not known.

- Genetics

Both type 1 and type 2 diabetes are partly inherited. Type 1 diabetes may be triggered by certain infections, with some evidence pointing at Coxsackie B4 virus. There is a genetic element in individual susceptibility to some of these triggers which has been traced to particular HLA genotypes (i.e. the genetic ñselfô identifiers relied upon by the immune system). However, even in those who have inherited the susceptibility, type I diabetes mellitus seem to require an environmental trigger. There is a stronger inheritance pattern for type 2 diabetes. Those with first-degree relatives with type 2 have a much higher risk of developing type 2, increasing with the number of those relatives. Concordance among monozygotic twins is close to 100%, and about 25% of those with the disease have a family history of diabetes. Genes significantly associated with developing type 2 diabetes, include TCF7L2, PPARG, FTO, KCNJ11, NOTCH2, WFSI, CDKALI, IGF2BP2, SLC30A8, JAZFI, AND HHEX (Lyssenko *et al*., 2008). KCNJ11 (potassium inwardly rectifying channel, subfamily J, member 11), encodes the islet ATP-sensitive potassium channel Kir6.2, and TCF7L2 (transcription factor 7-like 2) regulates proglucagon gene expression and thus the production of glucagon-like peptide-1 (Rother, 2007). Moreover, obesity (which is an independent risk factor for type 2 diabetes) is strongly inherited (Walley *et al*., 2006). Monogenic forms, e.g. MODY, constitute 1-5% of all cases (National Diabetes Information Clearinghouse, 2008). Various hereditary conditions may feature diabetes, for example myotonic dystrophy and Friedreichôs ataxia. Wolframôs syndrome is an autosomal recessive neurodegenerative disorder that first becomes evident in childhood. It consists of diabetes insipidus, diabetes mellitus, optic atrophy, and deafness, hence the acronym DIDMOAD (Barret, 2001). Gene expression promoted by a diet of fat and glucose as well as high levels of inflammation related cytokines found in the obese results in cells that ñproduce fewer and smaller mitochondria than is normal,ò and are thus prone to insulin resistance.

2.6 PREVENTION OF DIABETES

Type 1

Type 1 diabetes risk is known to depend upon a genetic predisposition based on HLA types (particularly types DR3 and DR4), an unknown environmental trigger (suspected to be an infection, although none has proven definitive in all cases), and an uncontrolled autoimmune response that attacks the insulin producing beta cells (Daneman, 2006). Some research has suggested that breastfeeding decreased the risk in later life (Borch-Johnson *et al.,* 1984); various

other nutritional risk factors are being studied, but no firm evidence has been found (Virtanen and Knip, 2003). Giving children 2000 IU of Vitamin D during their first year of life is associated with reduced risk of type 1 diabetes, though the causal relationship is obscure (Hypponen *et al*., 2001)

Children with antibodies to beta cell proteins (i.e. at early stages of an immune reaction to them) but no overt diabetes, and treated with vitamin B-3 (niacin), had less than half the diabetes onset incidence in an antibodies as above, but who received no vitamin B3 (Elliott *et al.,* 1996).

Type 2

- Lifestyle

Type 2 diabetes risk can be reduced in many cases by making changes in diet and increasing physical activity (Riserus *et al.,* 2009). The American Diabetes Association (ADA) recommends maintaining a healthy weight, getting at least 2½ hours of exercise per week (several brisk sustained walks appear sufficient), having a modest fat intake, and eating sufficient fiber (e.g, from whole grains). The ADA does not recommend alcohol consumption as a preventive, but it is interesting to note that moderate alcohol intake may reduce the risk. There is inadequate evidence that eating foods of low glycemic index is clinically helpful despite recommendations and suggested diets emphasizing this approach (Bantle *et al*., 2006). Diets that are very low in saturated fats reduce the risk of becoming insulin resistant and diabetic (Barnard, 2007). Study group participants whose ñphysical activity level and dietary, smoking, and alcohol habits were all in the low-risk group had an 82% lower incidence of diabetesò (Mozaffarian *et al*., 2009). In another study of dietary practice and incidence of diabetes, ñfoods rich in vegetable oils, including non-hydrogenated margarines, nuts, and seeds, should replace foods rich in saturated fats from meats and fat-rich dairy products. Consumption of partially hydrogenated fats should be minimized (Riserus *et al*., 2009).

There are numerous studies which suggest connections between some aspects of Type II diabetes with ingestion of certain foods or with some drugs. Breastfeeding may also be associated with the prevention of type 2 of the disease in mothers (Stuebe *et al*., 2005).

- Medications

Some studies have shown delayed progression to diabetes in predisposed patients through prophylactic use of metformin (Knowler *et al*., 2002), rosiglitazone (Gerstein *et al.,* 2006), or valsartan (Kjeldsen *et al.,* 2006). In patients on hydroxychloroquine for rheumatoid arthritis,

incidence of diabetes was reduced by 77% though causal mechanisms are unclear (Wasko *et al.,* 2007) Lifestyle interventions are however more effective than metformin at preventing diabetes regardless of weight loss. (Mozaffarian *et al.,* 2009).

2.7 COMPLICATIONS OF DIABETES MELLITUS

Diabetes without proper management can cause many complications. Acute complications are;

- Diabetic ketoacidosis (DKA): This is an extreme state of metabolic dysregulation characterized by the smell of acetone on the patientôs breath, a rapid deep breathing known as Kusmaul breathing, polyuria, nausea, vomiting, abdominal pain and any of many altered states of consciousness or arousal (such as hostility and mania or equally confusion and lithargy). In severe DKA, coma may follow, progressing to death. DKA is a medical emergency and requires immediate hospitalization.
- Hyperosmolar nonketotic state: This is more common in type 2 diabetes and is mainly the result of dehydration due to loss of body water.

Serious long-term complications are retinopathy, cardiovascular disease and chronic renal failure.

2.8 DIAGNOSIS OF DIABETES

The only way to prove if diabetes is present or absent is by checking the blood glucose levels. High blood glucose is the abnormality common to all types of diabetes. There are several techniques used to measure blood glucose levels:

- Fasting blood glucose**:** The Blood glucose level is measured after fasting for twelve hours or more. If the level is greater than 6.4 mMol/L (Iwalewa *et al.,* 2008), diabetes mellitus is comfirmed.
- Glucose tolerance test**:** If diabetes is suspected, but canôt be proven by a fasting glucose test, a Glucose Tolerance Test is done. For this test, fasting blood glucose level is taken, after which a sweet drink that contains 75 grams of glucose is given. Blood glucose is rechecked after two hours. If the blood glucose level is greater than 11.0 mMol/L**,** then the diagnosis of diabetes is made.

- Random glucose**:** Finally, diabetes is diagnosed by taking blood glucose reading at random, even after a meal. If the level is greater than 11 .0 mMol/L, the diagnosis of diabetes can also be made.

2.9 MANAGEMENT OF DIABETES

Diabetes mellitus is a chronic disease which is difficult to cure. Management concentrates on keeping blood glucose level as close to normal as possible without presenting undue patient danger. This can be achieved through the following:

- Diet, exercise and change in lifestyle:

The American Diabetes Association (ADA) recommends maintaining a healthy weight, getting at least 2 ½ hours of exercise per week (several brisk sustained walks appear sufficient), having a modest fat intake, and eating sufficient fibre (e.g from whole grains, ADA also recommends cessation of smoking and alcohol consumption as managemental factors.

- Insulin replacement therapy
- Oral hypoglycemic agents

- sulfonylureas such as tolbutamide, acetohexamide, tolazamide and chlorpropamide (first generation agents); gliburide (glibenclamide), glipizide and gliclazide (second generation agents).
- Biguanides (made up of Metformin, Phenformin and Buformin).
- Thiazolidenediones (made up of ciglitazone, pioglitazone, englitazone and troglitazone)
- Incretin mimetics

- Pancreas transplants have been tried with limited success in type I diabetes mellitus, gastric by pass surgery has been successful in many with morbid obesity and type 2 diabetes mellitus and gestational diabetes usually resolves after delivery.

2.10 MECHANISMS OF ACTION OF ANTIDABETIC COMPOUNDS

Antidiabetic compounds are medications that work to lower blood glucose concentrations. They exert their useful effects through the following ways:

1. By Increasing Insulin levels in the body

Since the absolute lack of insulin (in type 1 diabetes) or the relative lack of insulin (in type 2 diabetes) is the basis of diabetes, insulin replacement or augmentation is an obvious approach to therapy.

Individual agents:

- Insulin- Diabetes mellitus type 1 is a disease caused by absolute lack of insulin, hence insulin replacement must be used in type 1, which, must be injected or inhaled (Lebovitz and Harold, 2004). By changing certain amino acids in the insulin molecule or changing how it is combined with carrier molecules, many ways have been discovered to speed up or slow down insulinõs glucose lowering effects. As a result, there are several forms of insulin in the market. Some act immediately after they are injected while others act slowly over many hours. Examples are *insulin glargine* (lantus, a slow insulin) and insulin lispro (Humalog, a fast insulin).
- Sulfonylureas – are oral hypoglycaemic medications.They cause hypoglycaemia by stimulating insulin release from pancreatic beta cells. They are insulin secretagogues which their effect are initiated by binding to and blocking an ATP sensitive K^+ channel of the pancreatic beta cells, which recently has been cloned hence triggering insulin release (Proks *et al*.,2002). They are not used for people with gestational diabetes or type 1 diabetes, they are only used in type 11 diabetes.

Drugs in this class are divided into 2 groups or generations of agents. All members of this class are substituted arylsuphonylureas. They differ by substitution at the para position on the benzene ring and at one nitrogen residue of the urea moiety. The first generation agents are tolbutamide, acetohexamide, Tolazamide and chlorpropamide while the second generation agents are glyburide (gilbenclamide), glipizide and gliclazide. The second generation agents are considerably more potent than the first generation agents and have fewer side effects (Proks *et al.,* 2002). All may cause weight gain. The primary side effect is hypoglycaemia.

- Meglitinides

Meglitinides lower blood glucose level by stimulating the pancrease to release insulin, much like the sulfonylureas do (Blickle, 2006) and are often called ñshort acting secretagoguesò. They

block the potassium channels as sulfonylurease, but at a different binding site (Rendell, 2004). By closing the potassium channels of the pancreatic beta cells, they open the calcium channels, hence enhancing insulin secretion (Rendell, 2004). They are taken with or shortly before meals to boost the insulin response to each meal. Examples are repaglinide (Prandin) and Nateglinide (starlix). Adverse reactions are weight gain and hypoglcaemia.

- Incretin mimetics

Incretin mimetics act in the pancrease to release stored insulin, but differ from sulfonylureas and meglitinides because they only have this effect when glucose levels are high. This class of drugs must be injected to be effective. Exenatide (Byetta) is very similar to the incretin known as Glucagon Like Peptide-1 (GLP- 1), Incretin mimetics are also associated with weight loss and may be associated with increases in the number of insulin-producing beta cells in the pancreas (Chia and Egan, 2008).

2. By Increasing the Sensitivity of Target Organs to Insulin

Among oral antidiabetic drugs, insulin sensitizers are the largest category . Important abnormality in diabetes, especially type 2 diabetes is that insulin does not work as well as it should. For a given amount of insulin, blood sugar does not decrease as it should. This is termed insulin resistance or a decrease in insulin sensitivity. Drugs that overcome this problem are called insulin sensitizers.

- Thiazolidinediones

Thiazolidinediones, also known as ñglitazonesò, increase insulin sensitivity in liver, skeletal muscles and adipose tissues. They appear to augment insulin action in insulin resistant animals by increasing the no of glucose transporters (Tack and Smits, 2006). Drugs in this class change the pattern of gene and protein expression in some cells that helps make organs such as the liver and muscle more sensitive to effects of insulin (Tack and Smits, 2006). They bind to $PPAR_Y$, a type of nuclear regulatory protein involved in transcription of genes regulating glucose and fat metabolism. These PPARs act on Peroxysome Proliferation Responsive Elements (PPRE) (Rendel, 2004). The PPREs influence insulin sensitive genes which enhance production of mRNAs of insulin dependent enzymes. The final result is better use of glucose by the cells. Examples of drug in this class are rosiglitazone (Avandia), Pioglitazone (Actos) and troglitazone (rezulin).

- Biguanides

Biguanides increase glucose uptake by the peripheral tissues and reduce hepatic glucose output due to inhibition of gluconeogenesis. Metformin, a biguanide is the most commonly used agent for type 2 diabetes in children and teenagers and unlike other antidiabetic drugs, it does not cause weight gain. Examples of drugs in this class are metformin (Glucophage), phenformin and buformin.

3. By Decreasing Glucose Influx

Antidiabetic drugs in this category work by slowing down the rate at which nutrients especially glucose move from the intestinal lumen to the blood.

Individual agents:

- Alpha glucosidase inhibitors

These agents slow the digestion of starch in the small intestine by inhibiting the action of intestinal brush border alpha glucosidase hence the glucose from the starch enters the blood stream more slowly, and can be matched more effectively by impaired insulin response. Drugs such as Miglitol (Glyset) (Campbell *et al.,* 2000) and acarbose (Precose) are in this class. They have unpleasant side effects such as flatulence and bloating, but acabose has been shown to provide significant cardiovascular benefits (Hanefeld and Schaper, 2008).

- Amylinomimetics

These work by replicating the effects of amylin, a hormone from the pancreas that is released along with insulin. Pramlintide (simlin) is the only drug in these class. The main effect of pramlintide is slowing the rate at which food leaves the stomach, this results in lower postprandial spikes in blood glucose concentrations (Pullman *et al*., 2006).

6. By Decreasing the Liver's Glucose Output.

Glucose can enter the blood from the intestine or it can be released from storage pools in the liver during times when intestines are relatively empty.

Individual agents:

- Incretin mimetics (discussed above). Drugs such as axenatide act on the liver to reduce the amount of glycogen that is converted to glucose.
- Dipeptidyl Peptidase (iv) (DPP-4) Inhibitors, work by enhancing or prolonging the effect of a substance in the body called Glucagon Like Peptide -1 (GLP-1), and in this way they indirectly inhibit the conversation of glycogen to glucose.
- Insulin has many effects, including a direct effect on the liver to reduce the amount of glycogen turned into glucose.
- Thiazolidinediones, metformin and amylinomimetics also work in the liver to decrease glucose output.

5. By Sequestering Bile Acids

Drugs that sequester or capture bile acid were developed to reduce high levels of low density lipoprotein in people with hypercholestrolemia but have recently been shown to reduce blood glucose levels in patients with type 2 diabetes. This reinforces the idea that many of the bodyôs control systems overlap (Goldfine, 2008). The drug colesevelam was recently approved by the Food and Drug Administration (FDA) to help control blood glucose in this condition (Goldberg *et al*., 2008).

2.11 IDENTIFICATION OF *AFZELIA AFRICANA* PLANT

Many plants have been found to posses active principles useful for treating diabetes mellitus (Mohammed and Javed, 1990). Plant drugs are frequently considered to be less toxic and more free from side effects than synthetic chemical agents (Mohammed and Javed, 1990). Indeed along with dietary measures, preparations of plants formed the basis of treatment of diabetes mellitus until the introduction of insulin in 1922, (Rother, 2007).

Relevant literature on plants which have been used in ethnomedicine and for which antidiabetic activity has been scientifically documented by use of experimental methods for the period between 1907 and 2009 have been reviewed .

Afzelia afric*a*na is one of the many plants used for antidiabetic therapy in folk medicine on which no scientific work has been done. It is hoped that this work will promote studies on plants with reputed antidiabetic activities which have not been thoroughly scientifically investigated and from which the active principles will be identified as this is a logical way of searching for new drugs for the treatment and management of diabetes mellitus.

Afzelia africana belongs to the family Fabaceae. Its English name is ñAfrican mahogamyò. Its Ivorian name is ñlingueò. It is called ñKankalgaò in More, ñLingahiò in Fulfulde and ñOluò in Nsukka [Nigeria).

Scientific Classification

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Fabales
Family:	Fabaceae
Genus:	*Afzelia*
Species:	*Afzelia africana*
Binomial name:	*Afzelia africana* (Smith)

Description of the plant:

Afzelia africana is a large tree with a spreading crown; its height varies from 10-20 m and in west Burkina Faso mean average height was found to be 10 m for a tree with a 36 cm diameter. Scaly grey-brown bark, with a pink or red slash. Twigs glabrous, with lenticels. Glabrous leaves up to 30 cm long with 7-17 pairs of leaflets elliptic or ovate 5-15 x 3-9cm with an obtuse or sub-acuminate tip. Petioles 0.4-1.0 cm long. Nerves protruding, with 8-12 pairs of narrow, secondary connecting nerves. Flowers with one single white petal, with red striae, 1.5cm long set in terminal panicles up to 20cm long. Pods flattened 12-17 x 5-8 x 3.5cm, glabrous, black, woody, persistent bursts open at maturity spreading the seeds. Seeds poisonous with a sweet edible aril. Tree associated with ectomycorrhizal fungi (Petit , 2000).

Habitat

The plantôs habitat includes the humid and dry forests, savanna and forest galleries (Petit, 2000).

Soil

It is found in well watered sites with a deep sandy soil; it can adapt to lateritic soils (Petit, 2000).

Distribution

It is usually found in Nsukka, parts of Benue and Benin (Nigeria) where it grows in the wild. It is also found in Burkina Faso, Cameroon, Sierra Leone, Chad, Congo, Guinea Bissau, Senegal, Togo, Uganda, Ghana and Mali .

Propagation

Propagation by seeds is easy and natural regeneration is good. However, species are not very resistant to fire and is becoming rare.

Products and Uses

The wood is hard, heavy, durable, termite-proof, light brown to red-brown in colour, excellent timber, difficult to work, though, used in carpentry, furniture making, canoe and house building, sawdust irritating producing sneezing (Petit, 2000). Its Ethnomedical uses are: as antiinflammatory (Akah *et al*., 2007), analgesic (Akah *et al.,* 2007), anti-hemorrhagic (Petit, 2000), laxative, emetic, emmenagogic and aphrodisiac (Petit, 2000), antimicrobial (Agbelusi *et al*., 2007), Trypanocidal (Atawodi, 2005). The foliage is good cattle forage, particularly before the regrowth of grass in the early rainy season. Wild animals browse the arils, and antelopes eat the young shoots. Pods are rich in ashes used for making soap. As the leaves are rich in nitrogen they are used to enrich the soil. Although locally thought to be inhabited by spirits, it is the hunterôs favourite tree.

CHAPTER THREE

MATERIALS AND METHODS

3.1 PLANT COLLECTION AND IDENTIFICATION

Fresh samples of the plant were collected from Ogbadigbo Local Government Area of Benue state in the month of September 2008, by a local herbalist who claimed to use the plant in the management of Diabetes mellitus. The fresh plant samples were identified as *Afzelia africana* by Mr. A.O Ozioko, a taxonomist in the Department of Botany, University of Nigeria, Nsukka.

3.2 MATERIALS

(a) Animals

- Matured male mice weighing between 27-32 g were used for the experiments. The mice were between the ages of 12 and 16 weeks. They were sourced from Altran Nig. Ltd.
- Ethical rules guiding the use of animals for laboratory experiments were strictly adhered to.

(b) Equipments:

- Double Beam Spectrophotometer (P. Y E. UNICAM)
- Analytical Weighing Balance ((Scientific and instrument company England).
- Electronic weighing balance (Mettler, Toledo Ltd, Switzerland).
- Water Bath (Techmel, Texas, U.S.A)
- Rotary Evaporator (Buchi, Switzerland)
- Refrigerator (Haier Thermocool)
- Adjustable Micropipette (Volac UK)
- UV light Machine (YLN, China)
- Stainless Steel Cages
- Scissors
- Thin Layer Chromatographic Plates.
- Glass Wool
- Glucose Test Strips (Accu Chek Adavantage II)
- Electronic Glucometer (Accu Chek Adavantage II)
- Gastric tube
- Feeding Troughs and Drinkers
- Mortar and Pestle

- Milling Machine (A.H Thomas Philadelpha)
- Bunsen Burner
- Oven (GallenKamp, England)
- Cotton wool
- Crocodile clip
- Capillary tube

(c) Glass/Plastic Wares

- Measuring Cylinder (Technico, England)
- Flat Bottomed Flask
- Glass Funnel (Pyrex)
- Beakers and test tubes (Pyrex)
- Graduated cylinder (Pyrex)
- Column Glass Tube (Pyrex)
- Test Tube Racks
- Glass rod (Pyrex)
- Syringes and needles

(d) Drugs And Chemicals

- Glibenclamide
- Alloxan monohydrate (Sigma Chemical Co, U.S.A)
- Methanol (Sigma Aldrich, U.K.)
- Ethylacetate (Sigma Aldrich, U.K.)
- Chloroform (Sigma Aldrich, U.K.)
- Hexane (Sigma Aldrich, U.K.)
- Silica gel (Lab. Tech. Chemicals)
- 1, 1-diphenl-2-picryl hydroxyl (DPPH)
- Ascorbic Acid
- Sodium Acetate
- Glacial Acetic Acid
- Tripyridyltriazine (T P T Z)
- Hydrochloric Acid
- Ferric Chloride

- Wagnerôs Reagent
- Meyerôs Reagent
- Draggendorffôs Reagent
- Sodium Hydroxide
- Fehling Solutions I and II
- Olive oil
- Pig Blood

(e) Physiological Solution

- Distilled Water

3.3 METHODS

3.3.1 PREPARATION OF THE PLANT EXTRACT

- The fresh root bark of *Afzelia africana* was dried under mild sunlight. It was pulverized using mortar and pestle into coarse particles. The material was finely ground with a grinding machine.
- Cold extraction of the powdered root bark was performed using 80% methanol with intermittent shaking at intervals of 2 hours for 48 hours.
- The extract was filtered with whatman No.1 filter paper and concentrated using rotary evaporator.
- The percentage yield of the extract was determined as follows $\frac{w^1}{w} \times \frac{100}{1}$

Where w^1 = weight of concentrated extract

w = weight of dry pulverized root bark

3.3.2 ACUTE TOXICITY TEST

Five groups of animals with five mice per group was used for the experiment. Groups I, II, III, IV, and V were given the extract *per os* at the dose of 200 mg/kg, 400 mg/kg, 800 mg/kg, 1600 mg/kg and 2500 mg/kg respectively. Signs of toxicity such as excitement, dullness, sedation, anaesthesia, wretches, pupilary dilation and death were watched out for within 48 hours.

3.3.3 DOSE RESPONSE EFFECT

Diabetes was induced in 7 groups of mice with 6 mice per group, using the method described by Venugopal *et al.,* (1998). The mice were fasted overnight and fasting blood sugar (FBS) was determined after which the mice were injected with alloxan monohydrate dissolved in distilled water at a dose of 150 mg/kg body weight, intraperitoneally. Eight days post injection of alloxan, diabetes was confirmed in alloxanized mice with fasting blood sugar (FBS) levels above 6.4 mMol/L (Iwalewa *et al.,* 2008). The blood glucose levels were determined by dropping the blood samples obtained from cut tail tip of the mice on glucose test strips, the test strip was then inserted into an electronic glucometer which displayed the blood glucose level on a screen. The alloxan induced diabetic mice were treated as follows:

Groups 1-5 were treated *per os* with the extract at the doses of 62.5 mg/kg, 125 mg/kg, 250 mg/kg, 500 mg/kg and 1000 mg/kg respectively while groups 6 and 7 were treated with glibenclamide (standard antidiabetic drug) and distilled water respectively. Fasting blood sugar (FBS) levels were determined at 0 hour (before treatment) and at 1 hour, 3 hours and 6 hours post treatment, hence the dose that produced optimum activity was determined. Weight of mice were measured before induction of diabetes and after induction of diabetes has been comfirmed hence decrease in weight was determined.

3.3.4 PHYTOCHEMICAL ANALYSIS OF THE PLANT

A small portion of the dry extract was used for phytochemical tests for compounds which include tannins, flavonoids, alkaloids, saponins, steriods and glycosides in accordance with the methods of Trease and Evans (1983) and Harborne (1998).

a. Test for the presence of tannins

Three mililitres of 1g of the extract dissolved in 10 ml of distilled water was placed into three test tubes each. A few drops of lead subacetate was added into the first test tube while 5 ml of dilute sulphuric acid *2%* was added to the second tube. Into the third tube was added a few drops of ferric chloride. Distilled water in the fourth test tube was used as a negative control. Brownish precipitate, yellowish colour and bluish black precipitate with lead subacetate, dilute sulphuric acid and ferric chloride solution respectively indicates the presence of tannins.

b. Test for the Presence of Alkaloids

To 1ml of 1 g of the extract dissolved in 10 ml of distilled water in three test tubes labeled I to III was added a few drops each of Wagner's reagent , Meyer's reagent and Draggendorffôs reagent respectively . Distilled water was used as a negative control. Intense yellowish colour, slight yellowish colour and dirty yellowish precipitate with Wagnerôs reagent, Meyerôs reagent and Draggendorffôs reagent respectively indicates presence of alkaloids.

c. Test for the presence of Flavonoids

The extract (1 ml of 1 g of the extract dissolved in 10 ml of distilled water) was diluted to 5 ml with distilled water in a test tube. One mililitre of 20% sodium hydroxide was added and distilled water was used as a negative control. Intense yellow colour with sodium hydroxide indicates the presence of flavonoids.

d. Test for the Presence of Glycosides

The extract (5 ml of 1 g of the extract dissolved in 10 ml of distilled water) in a test tube was added 2 ml of dilute sulphuric acid, heated, cooled and neutralized with equal volume of sodium hydroxide solution.The mixture was boiled. Five mililitres of fehling's solutions 1 and II mixture was then added and heated for 5 minutes and cooled. Brick red precipitate at the bottom of the test tube indicates the presence of glycosides. Distilled water was used as negative control.

e. Test for the presence of Saponins

i. Frothing test:- Three mililitres of 1 g of the extract dissolved in 10 ml of distilled water in a test tube was diluted to 5 ml with distilled water. The solution was shaken vigorously for 30 seconds and allowed to stand. Much foaming indicates the presence of saponins.

ii. Emulsifying test:- One drop of olive oil was added to the solution in one and shaken vigorously. Emulsification indicates the presence of saponins. Distilled water was used as a negative control.

iii. Hemolysis test:- Five mililitres of 10% dilute solution of pig blood was put into two test tubes. One mililitre of 1 g of the test extract dissolved in 10 ml of distilled water was added in the first test tube and 1 ml of distilled water was added to the second test tube. The two test tubes were shaken properly. Haemolysis in the first test tube indicates the presence of saponins.

f. Test for presence of steroids

Plant extract (0.5g) was dissolved in 3 ml of chloroform. Concentrated sulphuric acid was carefully added to form lower layer. Reddish brown colour at the interface indicates the presence of steroids. Distilled water was used as a negative control.

3.3.5 FRACTIONATION OF THE PLANT EXTRACT

The plant extract was fractionated using the method of Stahl (1969). A plug of glass wool was put at the bottom end of a vertical glass column (tube) to prevent solid materials from contaminating the fractions to be collected.

Hexane and silica gel (stationary phase) were mixed and pre-loaded into a column above the plug of glass wool and left overnight for the silica gel to pack properly.

The plant extract (7g) was measured and dissolved with methanol, then 28 g of silica gel was measured and mixed with the methanolic extract (weight of extract and silica gel were in the ratio of 1:4). After this the extract/silica gel mixture was concentrated by stirring it until the mixture became powdery.

At this point, the hexane used to mix the silica gel was drained off. The extract to be analyzed by column chromatography was applied at the top of the column. The liquid solvents (the eluents) were passed through the column slowly by gravity (gravity column chromatography) as follows:

Table 1: Gradient solvents used in the separation of the extract.

	Hexane (ml)	chloroform (ml)	ethylacetate (ml)	Methanol (ml)
1	100	-	-	-
2	80	20	-	-
3	60	40	-	-
4	40	60	-	-
5	20	80	-	-
6	-	100	-	-
7	-	80	20	-
8	-	60	40	-
9	-	40	60	-
10	-	20	80	-
11	-	-	100	-
12	-	-	80	20
13	-	-	60	40
14	-	-	40	60
15	-	-	20	80
16	-	-	-	100

Equilibrium was established between the solute adsorbed on the adsorbent and the eluting solvent flowing down the column. The different components of the sample had different affinities for the mobile and stationary phases, and emerged from the stationary phase at different times hence separation was achieved.

The individual fractions (eluents) were collected (10 ml per test tube) sequentially in labeled test tubes as the solvent dripped from the bottom of the column.

3.3.6 THIN LAYER CHROMATOGRAPHY (TLC)

Thin layer chromatography was conducted on the fractions gotten from column chromatograph using the method of Ergon (1969)

Suitable solvent mixture of chloroform, ethylacetate and methanol in the ratio of 3:2:1 was put in a beaker. The beaker was lined with some filter paper soaked in the solvent mixture and covered, to ensure that the atmosphere in the beaker was saturated with solvent vapour.

A pencil line was drawn near the bottom of a TLC plate about one centimeter from the base, a small drop of fraction from each test tube was placed on the drawn line at different points and labeled with pencils. When the spot of the fractions dried the filter paper was removed from the solvent mixture on the beaker and the TLC plate was stood at an angle 60^0 in a shallow pool of the solvent mixture such that only the very bottom of the plate below the spots was in the solvent mixture. The solvent mixture, or the eluent, was the mobile phase, and slowly rose up the TLC plate by capillary action. As the solvent mixture moved past the spots that were applied, equilibrium was established for each component between the molecules of that component which were adsorbed on the solid and the molecules which were in solution. The fractions differed in solubility and in strength of their adsorption to the adsorbent and some factions were carried farther up the plate than others. When the solvent mixture reached close to top of the plate, the plate was removed from the beaker of solvent mixture and the position of the solvent was marked with pencil before it had a chance to evaporate. The plate was dried, visualized using ultraviolet (UV) light and the distance traveled by the various fractions were marked by drawing a pencil circle around them. The plate was then sprayed with vanyl sulphuric acid and heated in an oven at 110^0c and the fractions which could not be developed with UV light were developed and the distance they traveled were marked by drawing a pencil circle around them. The retention factor (R_f) value for each fraction was calculated using the formula:

R_f = Distance traveled by the fraction

Distance traveled by the solvent.

Fractions with the same R_f values were pooled together and concentrated in a rotary evaporator.

3.3.7 ANTIDIABETIC ACTIVITIES OF THE FRACTIONS

Diabetes was induced again using 7 groups of mice with 6 mice per group according to the method of Venugopal *et al.,* (1998). The 5 fractions obtained were used to treat 5 different groups of mice at the dose of 50 mg/kg each dissolved in distilled water and administered *per* os using gastric gavage, to determine the fraction that is responsible for the antihyperglycaemic activity of the plant. Groups 6 and 7 were treated with glibenclamide (2 mg/kg dissolved in distilled water) and distilled water (10 ml/kg) respectively *per os* using gastric gavage and maximum volume of 0.2 ml was administered to the mice. Fasting blood glucose levels were determined at O hour (before treatment) and at I, 3 and 6 hours post treatment. Weight of the mice were measured before induction of diabetes and when diabetes was comfirmed hence decrease in weight was determined. The fraction responsible for the antidiabetic activities of the root bark extract of *Afzelia africana* was then determined.

3.3.8 ESTIMATION OF THE ANTIOXIDANT ACTIVITIES OF THE ACTIVE FRACTION OF *AFZELIA AFRICANA*

The free radical scavenging activity of the active fraction was analyzed by DPPH (1,1-diphenyl -2-picryl hydrazyl) assay using the method of Mensor *et al.,* (2001) and FRAP (fehlingôs reducing Antioxidant power) using the method of Benzie and Strain, (1999).

DPPH ASSAY

DPPH assay was performed using the method of Mensor *et al.*, (2001). Two mililitres of the test fraction, at concentrations of 10 ε/ml, 50 εg/ml, 100 εg/ml, 200 εg/ml and 400 εg/ml each was mixed with 1 ml of 0.5 mM DPPH (in methanol) in a cuvette. The absorbance at 517 nm was taken at O min and after 30 minutes of incubation in the dark at room temperature. The experiment was done in triplicate. One mililitre of methanol plus 2.0 ml of the fraction was used as blank while 1.0 ml of the 0.5 mM DPPH solution plus 2.0 ml of methanol was used as the negative control. Ascorbic acid was used as reference standard. The percentage antioxidant activity was calculated as follows: % Antioxidant Activity (AA) =

100 ï (Abs sample ï Abs blank) x 100) / Abs control.

FRAP ASSAY

FRAP Assay was performed using the method of Benzie and Strain, (1999). The experiment was conducted at room temperature, where the reduction of a ferric tripyridyltraizine (fe^{3+} - TPTZ) to the ferrous form (blue colour) was monitored for 0 - 4 mins absorbance change at 593 nm. The FRAP reagent contains A + B + C in the ratio of 10: 1:1.

A = 0.775 g sodium acetate. $3H_2O$ + 4 ml Glacial Acetic acid, made up to 250 ml with distilled water

B = 0.0781g of TPT2 dissolved in 0.03646 g HCl made up to 25 ml with distilled water.

C = 0.13515 g of $FeCl_3 . 6H_2O$

Standard = 0.0044 g of Ascorbic acid in 25 ml of MeOH.

One hundred microlitres of the test fraction, at concentrations of 10 εg/ml, 50 εg/ml, 200 εg/ml, 400 εg/ml and ascorbic acid each was mixed with 3 ml of FRAP reagent in triplicate. The absorbance was read at 593 nm at O minute and at 4 minutes after placing the samples in water bath at 37^0C. FRAP value of the sample was calculated as follows:

FRAP value of sample (εm) =

$$\frac{\text{Change in absorbance of sample from 0 ï 4 minutes}}{\text{Change in absorbance of standard from 0 ï 4mins}} \times \text{FRAP value of the standard (1000εm)}$$

3 .3 .9 PHYTOCHEMICAL ANALYSIS OF THE ACTIVE FRACTION

A small portion of the fraction was used for phytochemical tests for compounds which include tannins, flavonoids, alkaloids, saponins, steriods and glycosides in accordance with the methods of Trease and Evans (1983) and Harborne (1998).

a. Test for the presence of tannins

One mililitre of 0.1 g of the fraction dissolved in 10 ml of distilled water was placed into three test tubes each. A few drops of lead subacetate was added into the first test tube while, 5 ml of dilute sulphuric acid *2%* was added to the second tube. Into the third tube was added a few drops of ferric chloride. Distilled water in the fourth test tube was used as a negative control. . Brownish precipitate, yellowish colour and bluish black precipitate with lead subacetate, dilute sulphuric acid and ferric chloride solution respectively indicates the presence of tannins.

b. Test for the Presence of Alkaloids

To 1ml of 0.1 g of the fraction dissolved in 10 ml of distilled water in three test tubes labeled I-III was added a few drops each of Wagner's reagent , Meyer's reagent and Draggendorffôs reagent respectively . Distilled water was used as a negative control. Intense yellowish colour, slight yellowish colour and dirty yellowish precipitate with Wagnerôs reagent, Meyerôs reagent and Draggendorffôs reagent respectively indicates presence of alkaloids.

c. Test for the presence of Flavonoids

The fraction (1 ml of 0.1 g of the fraction dissolved in 10 ml of distilled water) was diluted to 5 ml with distilled water in a test tube. One mililitre of 20% sodium hydroxide was added and water was used as a negative control.. Intense yellow colour with sodium hydroxide indicates the presence of flavonoids

d. Test for the Presence of Glycosides

To 1 ml of 0.1g of the fraction dissolved in 10 ml of distilled water in a test tube was added 2 ml of dilute sulphuric acid, heated, cooled and neutralized with equal volume of sodium hydroxide solution.The mixture was boiled. Five mililitres of fehling's solutions 1 and II mixture was added and then heated for 5 minutes and cooled. Brick red precipitate at the bottom of the test tube indicates the presence of glycosides. Distilled water was used as negative control.

e. Test for the presence of Saponins

i. Frothing test:- One mililitre of 0.1 g of the fraction dissolved in 10 ml of distilled water was diluted to 5 ml with distilled water. The solution was shaken vigorously for 30 seconds and allowed to stand. . Much foaming indicates the presence of saponins.

ii. Emulsifying test:- One drop of olive oil was added to the solution in one and shaken vigorously. Emulsification indicates the presence of saponins. Distilled water was used as a negative control.

iii. Hemolysis test:- Five mililitres of 10% dilute solution of pig blood was put into two test tubes. One mililitre of 0.1 g of the test fraction dissolved in 10 ml of distilled water was added in the first test tube and 1 ml of distilled water was added to the second test tube. The two test tubes were shaken properly. Haemolysis in the first test tube indicates the presence of saponins.

f. Test for presence of steroids

The fraction (0.05g) was dissolved in 3 ml of chloroform. Concentrated sulphuric acid was carefully added to form lower layer. Reddish brown colour at the interface indicates the presence of steroids. Distilled water was used as a negative control.

3.3.10 Statistical Analysis

Data obtained from the study was analyzed using one way analysis of variance (ANOVA) followed by Duncan Post hoc test. Differences in values less than a probability of 0.05 were considered significant.

CHAPTER FOUR
RESULTS

4.1 The Extract:

The aqueous methanolic extract of *Afzelia africana* was dark brown in colour and odourless. The percentage yield of the extract was calculated as 15.6%

4.2 Phytochemical Analysis

Investigations on the phytochemical constituents of the plant extract revealed the presence of flavonoids, tannins, alkaloids, saponins and steroids (table 1).

4.3 Acute toxicity test

Acute toxicity test revealed that the extract administered at varying doses (200, 400, 800 1600 and 2500 mg/kg) did not produce death in the treated mice. Clinical sign of dullness was observed in animals treated with 1600 and 2500 mg/kg (Table 2).

4.4 Dose Response Effect.

Eight days post induction of diabetes with alloxan monohydrate at the dose of 150 mg/kg ,the mice showed fasting hyperglycaemia and general weight loss (table 3) which were typical of Diabetes mellitus.

The antidiabetic property of the extract was investigated at five dose levels (62.5 mg/kg, 125 mg/kg, 250 mg/kg, 500 mg/kg and 1000 mg/kg) . There were reductions in fasting blood glucose levels of the mice treated with the extract doses (Table 3). The animals treated with 250 mg/kg of the extract showed the highest decrease in blood glucose level post treatment (Table 4). Glibenclamide significantly ($p<0.05$) reduced the blood glucose level when compared to the extract treatment.There was also a decrease in blood glucose of the groups treated with distilled water, however, this decrease was not significant ($p>0.05$).

4.5 Column Chromatography and Thin Layer Chromatography

These revealed the presence of 5 fractions in the plant extract.

4.6 Antidiabetic Activities of the Extract Fractions.

Treatment of diabetic mice with the various fractions of the extract showed that fraction 3 (Table 5) showed a high reduction in blood glucose level. This observed reduction was time dependent.The other fractions were not effective in reducing the blood glucose in the treated mice. The mean percentage FBS reduction by the extract fractions is shown in Table 6.The

fraction 3 produced 75.5±217% blood glucose reduction and was shown to be significantly ($p<0.05$) similar to glibenclamide with 90.20±2.06% reduction.

4.7 Phytochemical Analysis of The Active Fraction

The phytochemical analysis of the active fraction (Fraction 3) revealed the presence of flavoniods.

4.8 The antioxidant activities of the Active Fraction

Figure 1 shows the free radical scavenging activities of the active Fraction (F3) of *Afzelia africana* using FRAP method, while, figure 2 shows the free radical scavenging activities of active Fraction (F3) of Afz*eli*a *africana* using DPPH method. The fraction was shown to have antioxidant activities which in the study was observed to be concentration dependent. The antioxidant activities of the highest concentration (400 µg/ml) of the extract was lower than that of vitamin C a known free radical scavenger. FRAP Method showed that at concentrations of 10 µg/mg, 50 µg/ml, 100 µg/ml, 200 µg/ml and 400 µg/ml, the antioxidant activities (µm) were 0.26, 0.28, 1.25, 1.43 and 1.60 respectively while the FRAP value of the standard (Ascorbic acid) was 2 µm. The FRAP value of the standard was significantly ($p<0.05$) higher than the value of the fraction at 10 µg/ml and 50 µg/ml but not at 100 µg/ml 200 µg/ml and 400 µg/ml. The DPPH method revealed that the antioxidant values of the fraction (%) were 13, 14, 60,63 and 77 at concentrations of 10 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml and 400 µg/ml respectively while the percentage antioxidant activities of the standard were 75,77,80, 83 and 87 respectively at the same concentrations. The DPPH values of the standard were significantly ($p<0.05$) higher than that of the fraction at 10 µg/ml and 50 µg/m/ but not at 100 µg/ml, 200 µg/ml an 400 µg/ml.

Table 2: Phytochemistry of the methanolic root bark extract of *Afzelia africana*

Phytochemical constituents	Tests	Presence/Absence
Tannins	Lead subacetate	+
	Sulphuric acid	+
	Ferric chloride	+
Alkaloids	Wagnerôs reagent	+
	Meyerôs reagent	+
	Draggendorffôs reagent	+
Flavonoids	Sodium hydroxide	+
Saponins	Frothing test	+
	Emulsifying test	+
	Haemolytic test	+
Glycosides	Sulphuric acid, sodium hydroxide, Fehlingôs solution I and II.	-
Steroids	Sulphuric acid	+

Investigations on the phytochemical constituents of the plant extract revealed the presence of flavonoids, tannins, alkaloids, saponins and steroids .

Table 3: Acute Toxicity Test of *Afzelia africana* Extract in Mice.

Groups	Dose of Extract (mg/kg)	Signs of Toxicity
I	200	Nil
II	400	Nil
III	800	Nil
IV	1600	Dullness
V	2500	Dullness

The result of the acute toxicity test of the extract in mice is represented in Table 3. The result revealed that the extract administered at varying doses (200, 400, 800 1600 and 2500 mg/kg) did not produce death in the treated mice. Clinical sign of dullness was observed in animals treated with 1600 and 2500 mg/kg.

Table 4: The Effect of *A. Africana* Methanolic Extract in Alloxan-induced Diabetic Mice

Extract/Drug (mg/kg)	Groups	Mean Decrease In weight post induction of diabetes	Fasting blood glucose level (Mmol/L) (Mean ± SEM)			
			0 hr	1 hr post treatment	3 hrs post treatment	6 hrs post treatment
62.5	1	5.80±1.11	13.12 ±2.47^{a}	12.74 ±1.43^{a}	10.06 ± 0 .77^{a}	9.44 ±0.99^{b}
125	2	3.00±1.14	9.82 ± 2.17^{a}	6.42 ±1.34^{a}	4.96 ±1..51^{a}	4.68 ±1..56^{b}
250	3	2.80±1.16	17.78 ±1..59^{a}	11.38 ± 2.82^{ab}	10.34 ±3.44^{bc}	3.78 ± 0.85^{c}
500	4	3.40±0.81	23.7 ± 1.96^{a}	22.58 ± 2.72^{ab}	19.68 ±2.49^{ab}	13.64 ±4.45^{b}
1000	5	6.80±2.17	18.36 ±3.47	17.44 ±4.05	18.28 ±3.99	13.02 2±.78
Glibenclamide (2mg/kg)	6	3.20±0.80	20.20 ±2.38^{a}	12.82 ±1.38^{b}	5.80 ±1.07^{c}	1.98 ±0.47^{d}
Distilled water	7	2.40±0.80	12.72 ±2.64	11.80 ±2.52	11.58 2±.52	8.96 ±2.45

a,b,c,d, Different supercripts indicate difference between the mean ($p<0.05$).

O hr = before treatment.

The result of the effect of *Afzelia africana* methanolic extract in alloxan-induced diabetic mice is represented in Table 4. Eight days post induction of diabetes with alloxan monohydrate at the dose of 150 mg/kg ,the mice showed fasting hyperglycaemia and general weight loss (table 3) which were typical of diabetes mellitus. The antidiabetic property of the extract was investigated at five dose levels (62.5 mg/kg, 125 mg/kg, 250 mg/kg, 500 mg/kg and 1000

mg/kg) . There were significant time related reductions in fasting blood glucose levels of the mice treated with the extract doses- 62.5, 125, 250 and 500 mg/kg and glibenclamide but not with 1000 mg/kg and distilled water.

Table 5: Mean Percentage FBS Reduction of the Extract, Glibenclamide and Distilled water in Diabetic Mice.

(Mean ± SEM)

Dose of extract / glibenclamide (mg/kg)	Mean FBS reduction [%]
62.5	22.62 ± 7.37[a]
125	55.78 ± 4.18[b]
250	78.94 ± 6.33[c]
500	42.78 ± 13.91[bd]
1000	28.50 ± 5.29[ad]
Glibenclamide (2)	90.70± 2.06[c]
Distilled water	3.54 ± 1.21[a]

a,b,c,d, Different superscripts indicate difference between the mean ($p<0.05$)

The result of the mean percentage FBS reduction of the extract, glibenclamide and distilled water in diabetic mice is represented in Table 5. The result revealed that the animals treated with 250 mg/kg of the extract showed the highest decrease in blood glucose level post treatment. Glibenclamide significantly ($p<0.05$) reduced the blood glucose level when compared to the extract treatment.

Table 6: Antidiabetic Activities of the Fractions of *A. Africana* Extract on the Mean Fasting Blood Glucose Levels of Alloxan induced diabetic Mice.

Extract/Drug dose (mg/kg)	Groups	Mean decrease in weight post induction of diabetes	Fasting blood glucose level (Mmol/L) (Mean ± SEM)			
			0hr	1 hr post treatment	3 hrs post treatment	6 hrs post treatment
Fraction 1 (50)	1	3.20±0.80	11.26 ± 2.43	11.16 ±2.43	11.02 ± 2.41	8.88 ±0.90
Fraction 2 (50)	2	7.40±2.09	12.86 ±1.83	12.46 ±1.79	12.28 ±1.84	12.22 ±2.44
Fraction 3 (50)	3	4.20±1.39	20.84 ± 4.45^{a}	14.22 ±2.90^{ab}	10.28 ±3.01^{b}	4.16 ±2.17^{c}
Fraction 4 (50)	4	3.00±0.63	20.42 ±3.77	20.28 ± 2.66	19.70 ± 2.60	19.64 ± 2.68
Fraction 5 (50)	5	4.00±1.45	9.00 ± 1.03	8.84 ±1.01	8.76 ±1.00	8.40 ±1.30
Glibenclamide (2)	6	5.80±1.11	20.20 ±2.38^{a}	12.82 ± 1.38^{b}	5.80 ± 1.07^{c}	1.98 ± 0.47^{d}
Distilled water	7	3.00±2.50	14.46 ±2.90	14.24 ± 2.70	13.98 ±2.94	13.38 ±2.30

a,b,c,d, Different superscripts indicate difference between the mean ($p<0.05$)

Antidiabetic activities of the Fractions of *A. africana* extract on the mean fasting blood glucose levels of alloxan induced diabetic mice is represented in table 6. The result revealed that the treatment of diabetic mice with the various fractions of the extract showed that fraction 3 had the highest reduction in blood glucose level. This observed reduction was time dependent. The other fractions were not effective in reducing the blood glucose in the treated mice.

Table 7: Mean Percentage FBS Reduction by the Extract Fractions ,Glibenclamide and Distilled water.

Dose of fractions / glibenclamide (mg/kg)	Mean FBS reduction [%]
Fraction 1 (50)	3.70 ± 1.30^{a}
Fraction 2 (50)	5.90 ± 0.11^{a}
Fraction 3 (50)	75.50 ± 2.17^{b}
Fraction 4 (50)	3.80 ± 1.68^{a}
Fraction 5 (50)	3.60 ± 1.30^{a}
Glibenclamide (2)	90.20 ± 2.06^{b}
Distilled water	3.40 ± 0.40^{a}

a,b,c,d, Different supercripts indicate difference between the mean ($p<0.05$)

FBS = Fasting Blood Sugar.

The mean percentage FBS reduction by the extract fractions is shown in Table 7.The result revealed that fraction 3 produced 75.5±217% blood glucose reduction and was shown to be significantly ($p<0.05$) similar to glibenclamide with 90.20±2.06% reduction.

Table 8: Phytochemical Analysis of the Active Fraction

Compound	**Reaction**
Tannins	Negative
Alkaloids	Negative
Flavonoids	Positive
Saponins	Negative
Steroids	Negative
Glycosides	Negative

The phytochemical analysis of the active fraction is represented in Table 7. The result of the phytochemical analysis of the active fraction (Fraction 3) revealed that it is a flavoniod.

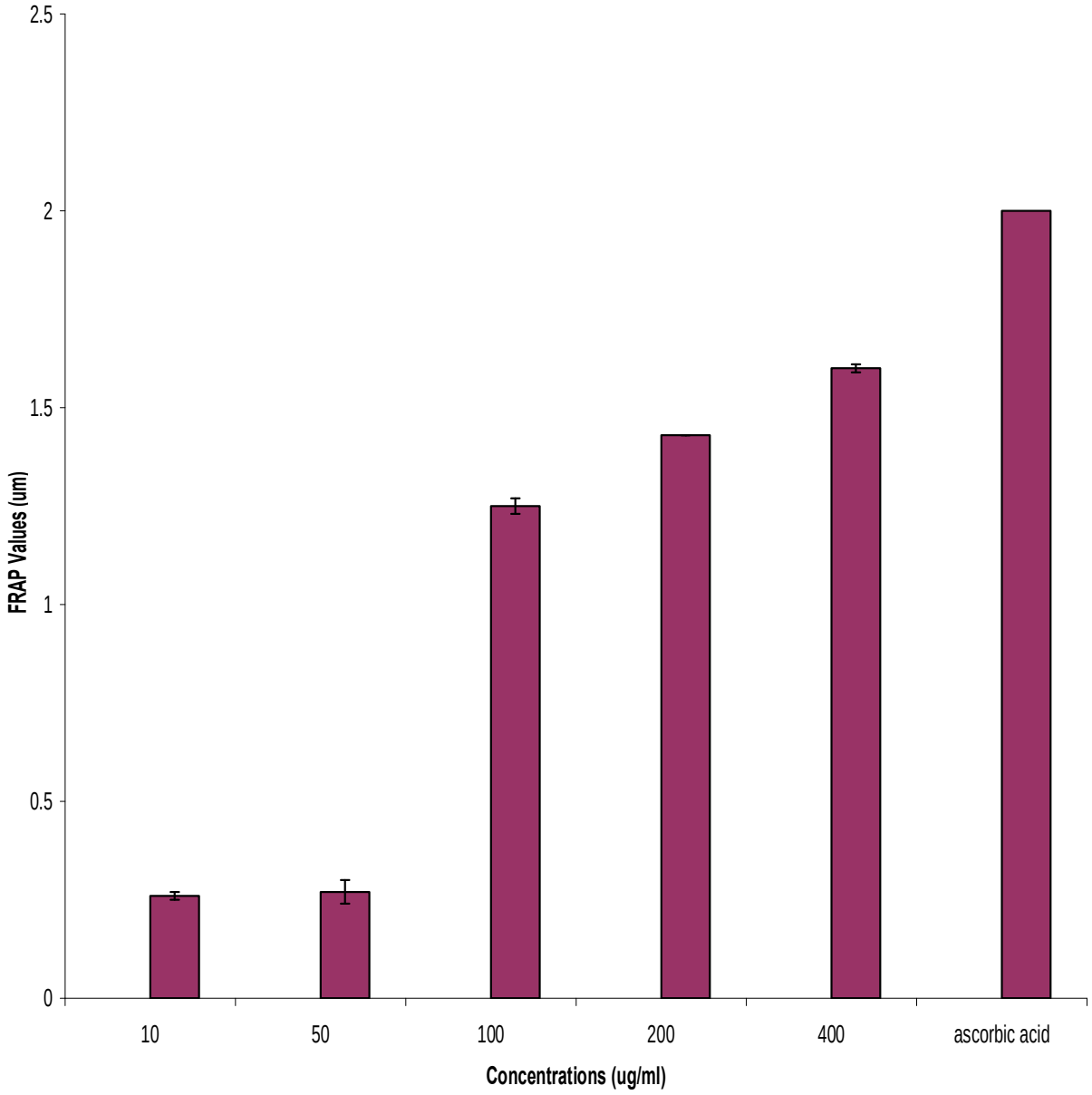

Fig 1: The *in-vitro* Antioxidant Effect of Fraction 3 at Different Concentrations Compared with Ascorbic acid using FRAP The Assay

The antioxidant effect of fraction 3 using FRAP assay is represented in Figure 1. The result revealed that fraction has antioxidant activities which in the study was observed to be concentration dependent. The antioxidant activities of the highest concentration (400 µg/ml) of the extract was lower than that of vitamin C, a known free radical scavenger.

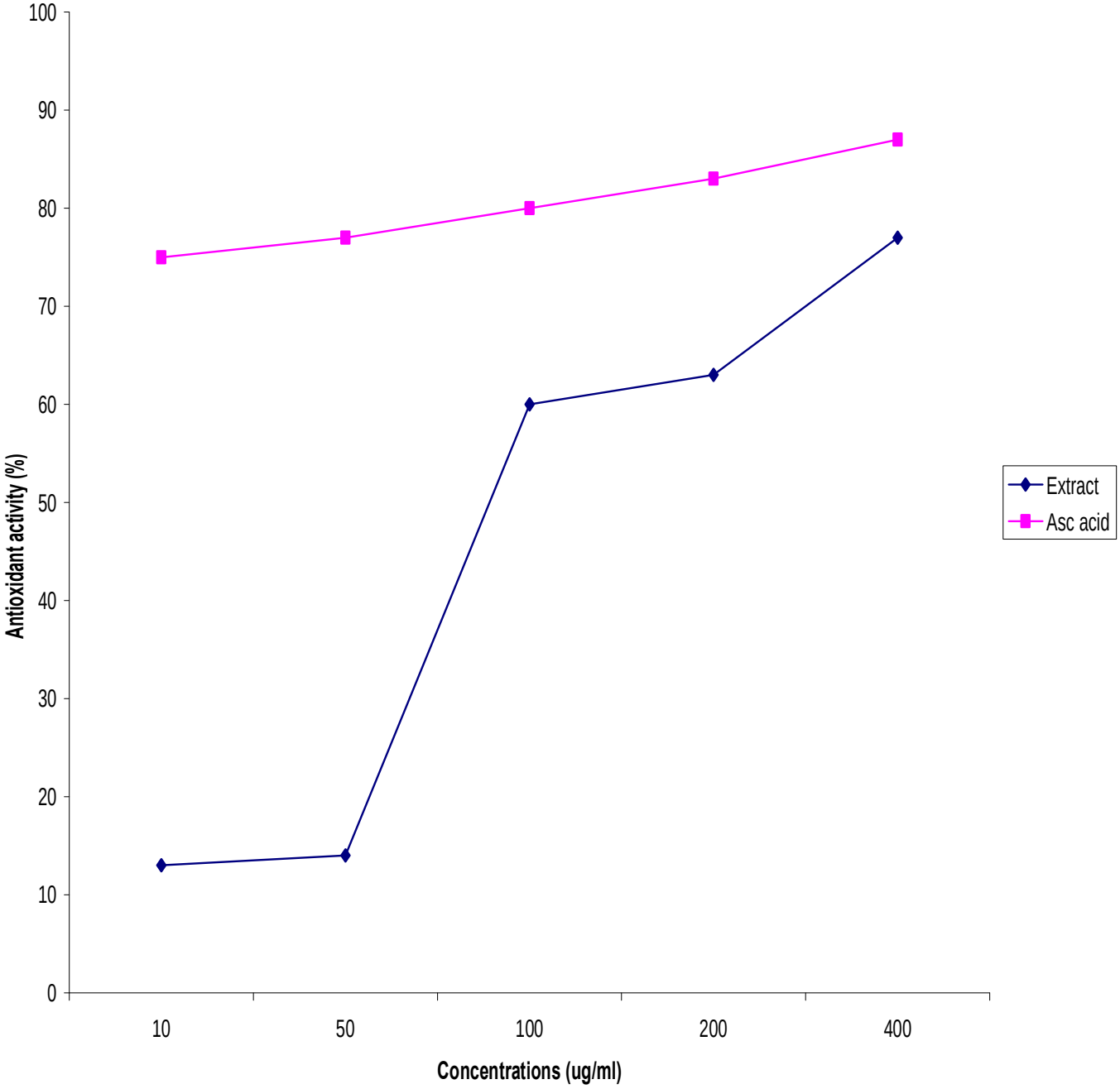

Fig 2: The Antioxidant Effect of Fraction 3 at Different Concentrations using *in-vitro* DPPH Assay

The antioxidant effect of fraction 3 using DPPH assay is represented in Figure 2. The result revealed that fraction 3 has antioxidant activities which in the study was observed to be concentration dependent. The antioxidant activities of the highest concentration (400 µg/ml) of the extract was lower than that of vitamin C.

CHAPTER FIVE

DISCUSSION

There were no deaths recorded in all the doses in acute toxicity test, however, in 1600 mg/kg and 2500 mg/kg, the extract doses produced dullness which is a sign of central nervous system depression. This may indicated that the plant extract has a wide margin of safety and that the dose must be below 1600 mg/kg.

Eight days post induction of diabetes mellitus the observed general weight loss (Table 3) may be suggestive that diabetes mellitus had been successfully induced. Alloxan monohydrate is known to induce diabetes mellitus eight days post administration by destroying virtually all the functional beta cells of the pancreatic islets (Rother, 2007).

Conclusive evidence that diabetes mellitus had been successfully induced was shown by tremendous increase in mean FBS levels of the mice eight days post induction (Table 3).

The extract was observed to have antidiabetic activities. The FBS values were reduced by the various doses of the extract. The extract produced an optimum activity at the dose of 250 mg/kg.

There were time related decreases in the FBS levels by all the doses with optimum activity occurring at the 6 hours post treatment. Hence the dose ï response effect of the extract on diabetic mice revealed that the dose, 250 mg/kg showed optimum activity and did not differ significantly ($p>0.05$) from glibenclamide at the sixth hour post treatment. The percentage FBS reduction by the extract at 250 mg/kg and glibenclamide (2 mg/kg) were 78.9% and 90.7 respectively.

The column and thin layer chromatography revealed the presence of five fractions in the extract. There were time related decreases in the FBS levels by the fraction 3 with optimum activity occurring at the sixth hour post treatment (Table 5). The percentage FBS reduction of fraction 3 was 75.5 % while that of glibenclamide was 90.2 %. The percentage FBS reduction by fraction 3 did not differ significantly ($P > 0.05$) from percentage FBS reduction by glibenclamide at six hours post treatment.

Investigation on the phytochemical constituents of the plant extract revealed the presence of flavonoids, tannins, saponins, alkaloid and steroids (table1).Test to ascertain the phytochemical constituents of Fraction 3 revealed that it is a flavonoid, and this may be responsible for the antidiabetic activity of *Afzelia africana* root bark. Flavonoids exhibit a wide

range of biological activities which include antimicrobial, anti-inflammatory, analgesic, antiallergic and antioxidant properties (Hodek *et al.,* 2002). Flavonoidôs ability of scavenging hydroxyl radicals, superoxide anion radical and lipid peroxyl radical highlights many of the flavonoid health promoting functions, which are important for managing diseases associated with oxidative damage such as diabetes mellitus (Ferguson, 2001). Flavonoids in human diet may reduce the risk of various cancers, as well as prevent menopausal symptoms (Hodek *et al*., 2002). In addition to the antidiabetic activities exhibited by flavonoids, they also exhibit antitrypanosomal and antileishmanial activities (Tasdemir *et al*., 2006). Epidermiological studies suggest that the consumption of flavonoids is effective in lowering the risk of coronary heart disease (Rice Evans and Miller, 1996).

The free radical scavenging activity of Fraction 3 determined by DPPH and FRAP tests confirmed that fraction 3 has an antioxidant activity. Antioxidants scavenge free radicals in the body and this wards off diseases and promotes general well being. Specific health benefits of antioxidants are anti-ageing of cells, greatly reduced incidence of cancers, glaucoma and mascular degeneration prevention, reduced risk of cholesterol oxidation and heart disease, stronger immunity, helps in controlling diabetes, relief from allergies, relief from asthma, relief from stroke, relief from gum disease, relief from arthritis, reduces high blood pressure and others.

CONCLUSION

In conclusion the acute toxicity test showed that the plant extract has a wide margin of safety. The plant extract also has antidiabetic activity. The dose response effect of the extract on diabetic mice revealed that the 250 mg/kg dose showed optimum activity and did not differ significantly ($p > 0.05$) from glibenclamide (2 mg/kg) at six hours post treatment. The percentage FBS reduction by fraction 3 (75.5%) did not differ significantly ($p > 0.05$) from percentage FBS reduction by glibenclamide (90.2%) at six hours post treatment.

Investigation on the phytochemical constituents of Fraction 3 revealed that it is a flavonoid. Fraction 3 was revealed to have an antioxidant effect, which is important in the management of diabetes mellitus since diabetes mellitus is associated with oxidative damages.

The result obtained in this study appears to justify the ethnomedical use of the plant in the management of diabetes mellitus.

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