INHIBITION OF SOME KEY ENZYMES LINKED TO DIABETES BY *ANNONA SENEGALENSIS* LEAF EXTRACT *IN VITRO*

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ZARIA

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DEPARTMENT OF BIOCHEMISTRY,
FACULTY OF LIFE SCIENCES,
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA

September, 2018

DECLARATION

I declare that: This research dissertation, entitled "**Inhibition of some Key Enzymes Linked to Diabetes by *Annona senegalensis* Leaf Extract *In vitro"*** has been carried out by me in the Department of Biochemistry except where otherwise indicated, is my original research. This dissertation has not been submitted for any degree or examination at any other university, it does not contain people's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from them and also does not contain structures, text or graphics copied and pasted from the Internet, unless been acknowledged, and the source being detailed in the dissertation and in the References sections.

_____ _____ _____

Name of studentSignature Date

CERTIFICATION

This Dissertation entitled **Inhibition of some Key Enzymes Linked to Diabetes by *Annona Senegalensis* Leaf Extract *In vitro*** meets the regulations governing the award of the degree of Master of Science in Biochemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Prof. I. A. Umar — Signature _____
Chairman, Supervisory Committee — Date _____

Dr. I. A. Aimola — Signature _____
Member, Supervisory Committee — Date _____

Prof. M. N. Shuaibu — Signature _____
Head of Department — Date _____

Prof. S. Z. Abubakar — Signature _____
Dean, School of Postgraduate Studies — Date _____

DEDICATION

This work is dedicated to my parents, for their love which has been my driving force till now and forever.

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ABSTRACT

Annona senegalensis Pers(Annonaceae) known as Wild Custard Apple is locally used in treatment of diabetes in Nigeria. This study was aimed at investigating the possible compounds present in the leaf extracts of *A. senegalensis* and their inhibitory potentials on the activities of α-amylase, α-glucosidase and aldose reductase. The leaf sample was extracted sequentially in n-hexane, ethyl acetate and ethanol and the effect of the extracts on the activities of some enzymes was tested. The most active extract was further fractionated using column chromatographyand the fractions obtained were used to determine the inhibitory activities of some enzymes to find the most active fraction. The possible bioactive compounds were determined by Gas Chromatography Mass Spectroscopy (GC-MS). Ethanol extract had significantly higher ($p<0.05$) inhibitory activities with lower IC_{50} values against the activities of α-amylase: 204.04±6.38 µg/ml, α-glucosidase: 97.91±2.40 µg/ml and aldose reductase: 119.58±12.85 µg/ml compared to other extracts. The most active fraction-**F** from the ethanolic extract had significantly higher ($p<0.05$) α-amylase and α-glucosidase inhibitory activities with IC_{50} values 237.14±31.19 µg/ml and α-88.25±0.59 µg/ml respectively while fraction-**B** had higher aldose reductase inhibitory activity with IC_{50} value 83.18±0.03µg/ml. The kinetics of the enzymes' activities of the fraction-**F** is a competitive inhibitor for α-amylase with V_{max}: 27.03µmol/min, K_m: 0.24% and k_i: 8.46µg/ml and non-competitive inhibitor for α-glucosidase V_{max}: 1.10 µmol/min, K_m: 3.7 mmol/L, and k_i: 1.26µg/mL. Phenolic compounds were found in fraction-**F**. 1, 2-Benzenedicarboxylic acid butyl octyl ester, 1, 2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester, Hexadecanoic acid, methyl ester and Methyl 9-methyltetradecanoate were found in fraction-**F**. The data of this study suggest

that *A. senegalensis* possess inhibitory activities on against α-amylase, α-glucosidase and aldose reductase activities; and therefore could be attributed to the presence of compounds identified.

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LIST OF ABBREVIATIONS

ABBREVIATION	MEANING
ABU	Ahmadu Bello University
ADA	American Diabetes Association
AA	Alpha amylase
AG	Alpha glucosidase
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AR	Aldose reductase
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CFRD	Cystic fibrosis related diabetes
CHO	Carbohydrate
CVD	Cardiovascular disease
DBA	Diabetic acarbose
dH_20	Distilled water
DKA	Diabetic ketoacidosis
DM	Diabetes mellitus
DMSO	Dimethyl sulphoxide
DNS	3,5-dinitrosalicylic acid
FFA	Free fatty acid
GC-MS	Gas chromatography mass spectroscopy
GDM	Gestational diabetes mellitus
GIIS	Glucose induced insulin secretion
GLUT-4	Glucose transporter type 4
IDDM	Insulin dependent diabetes mellitus
IDF	International Diabetes Federation
ISs	Insulin secretagogues

MODY	Maturity onset diabetes of the young
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
NIDDM	Non-Insulin Dependent Diabetes Mellitus
NIST	National institute of standards and technology
PKA	Protein kinase A
PNPG	Paranitrophenyl-α-D-glucopyranoside
RT	Retention time
SD	Standard deviation
SPSS	Statistical Package for the Social Sciences
T2D	Type 2 diabetes
TIC	Total ion chromatogram
TLC	Thin layer chromatography
TZDs	Thiazolidines
WHOWorld Health organization	

CHAPTER ONE

1.0 INTRODUCTION

The term diabetes mellitus (DM) refers to a metabolic disorder linked to defective insulin secretion, insulin insensitivity and/or both. It is characterized by chronic hyperglycemia with multiple etiological complications and is associated with alterations in the metabolisms of carbohydrates, fats and proteins (ADA, 2014).Diabetes mellitus is classified into two major types, Type 1 and 2. Type 1 is also referred to as Insulin Dependent Diabetes Mellitus (IDDM) mostly characterized by absence of circulating insulin; plasma glucagon is inappropriately elevated, and the pancreatic β-cells fail to respond to all insulinogenic stimuli (ADA, 2014).Type 2 diabetes also called adult-onset diabetes or Non Insulin Dependent Diabetes Mellitus (NIDDM) is mainly a problem of a "target-organ" disorder resulting in ineffective insulin action that can secondarily disrupt the regulation of hepatic glucose synthesis, muscle glucose uptake and adipose tissue lipolysis (Karam, 1998). Thus insulin resistance is the fundamental pathogenic factor for NIDDM and is one of the targets for antidiabetic medication.Recent information showed that the number of people with DM has massively increased; becoming one of the most important public health challenges globally (Nasli-Esfahani *et al.,* 2017). Uncontrolledhyperglycemia can lead to progressive secondary complications in DMsuch as nephropathy, neuropathy, retinopathy and cardiovascular diseases.Presently, various options are available for control of DM and its associated complications. Among the treatment options is the control of postprandial hyperglycemia via inhibition of glycoside hydrolases (α-glucosidase and α-amylase), resulting in an aggressive delay of carbohydrate digestion to absorbable monosaccharides (Zhang *et al.,* 2017). On the other hand, aldose

reductase inhibitors from various pharmacological approaches have received much attention because of its involvement in the pathophysiology of diabetic complications including cataract (Halder*et al.,* 2003 andOsadebe*et al.,* 2010).Aldose reductase is foundin almost all mammalian cells, but at high levels in organs such as the cornea, lens, retina, kidney, myelin sheath and sciatic nerves, which are affected by diabetic complications(Kim*et al.,* 2010).Though it is important that glycaemic control should be achieved as rapidly as possible to minimize the impact of glucose toxicity, it is also necessary to provide therapy to control other related complications stated above (ADA, 2009).However,conventional oral hypoglycaemic drugs such as the sulphonylureas, biguanides and α-glucosidase inhibitors,such as acarbose and miglitolused in diabetes treatment are known to pose unwanted adverse consequences, such as diarrhea and abdominal pains(Naveen and Baskaran, 2017). This stimulates the increasing interest on plant-derived products since fewer side effects are associated with the use of plant-derived products (Atanasov*et al.,* 2015).Numerous traditional herbal drugs have been indicated for their effectiveness in treatment of diabetesand related disorders. In Nigeria, hundreds of plants are used traditionally for the management of diabetes mellitus locally (Shinkafi *et al.,* 2015). These plants contain active components which confer the hypoglycaemic activity: substances like phenolics, steroids, alkaloidsand some steroidal-saponins (Evans, 1999). Interestingly, *Anonna senegalensis*Pers (Annonaceae) is among the most promising plants used locally in treatment of DM in Nigeria and other African countries (Etuk and Mohammed 2009; Karou*et al.,* 2011,Ahmoho*et al.,* 2012, Diallo *et al.,* 2012, Laleye*et al.,* 2015 and Shinkafi *et al.,*2015). In Nigeria, polyherbal antidiabetic remedy containing

A.senegalensis leaf extract as an active ingredient was reported to ameliorate hyperglycaemia and diabetic- induced complications (Okine *et al.,* 2005).

1.1 Statement of Research Problem

The prevalence of diabetes is increasing annually; affecting more than 415 million people globally with 14 million people in Africa and the projection is at well above 642 million by 2040 globally (IDF, 2015). However, the current estimation also suggests that in Nigeria there were more than 1.56 million cases of diabetes in 2015. With the current statistics DM not only takes a heavy toll on lives around the world but imposes a serious financial burden on the sufferers and their family members (Nasli-Esfahani *et al.,* 2017). These geometric increases in the number of diabetics in recent times cannot be dissociated from unhealthy life style, urbanization, aging and ravaging influence of free radicals (ADA, 2015).The foregoing therefore makes the search, that the current treatment options maybe inefficient or not accessible to anti-hyperglycemic properties that could also decrease postprandial hyperglycemiaand other diabetic complications.

1.2 Justification of the Study

Like other parts of the world, diabetes has also become a serious issue in Africa.It is quite obvious that the prevalence of DM and its complications are increasing at an alarming rate, especially among the black race.The International Diabetes Federation's (IDF) Diabetes Atlas estimates that, more than two thirds of people with diabetes in Africa are undiagnosed (IDF, 2015). Among the types of diabetes, T2D is known to have the highest prevalence, which is between 90% - 95% of all diabetes cases (IDF, 2015). Traditional medicinal plants with their various biological constituents have been used in treatment of diabetes, because of their perceived effectiveness and minimal side effects in clinical experience and relatively low costs.African custard-apple plant (*A. senegalensis*) is an important herb used traditionally in many parts of Nigeria and the world to treat array of diseases including DM (Shinkafi *et al.*, 2015). However, very few or virtually no work has been done to validate the traditional claim of *A. senegalensis* leaf in DM treatment. Therefore, our present study is designed to investigate the *in vitro*inhibitory potential of *A.senegalensis* leaf on some key intestinal carbohydrate-digesting enzymes linked to diabetes to validate the ethnobotanical usage of the plant in DM treatment.

1.3 Aim and Objectives

1.3.1 Aim

The aim of this study is to investigate the inhibitory effect of *A. senegalensis* leaf extracts on the activities of some key enzymes linked to diabetes *in vitro*.

1.3.2 The Specific Objectives are

1. To determine the inhibitory effect of crude *A.senegalensis* leaf extract/fraction on α-amylase, α-glucosidase and aldose reductase activities.

2. To separate the inhibitory agent using bioassay guided fractionation protocol

3. To determine the inhibition kinetics of the purified fraction on α-amylase and α-glucosidase

4. To determine the possible compounds present in the most active fraction using GC-MS analysis.

1.4 Research Hypothesis

A.senegalensis leaf extractpossess inhibitory potential against α-amylase, α-glucosidase and aldose reductase enzymes important in the pathophysiology of diabetes mellitus.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Diabetes Mellitus

Diabetes mellitus (DM) is a group of metabolic disorders characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (ADA. 2014). The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is defective action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action (IEC, 2009). Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycemia. Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss,sometimes with polyphagia, and blurredvision. Impairment of growth and susceptibilityto certain infections may also accompanychronic hyperglycemia(ADA, 2014). Acute,life-threatening consequences of uncontrolleddiabetes are hyperglycemia withketoacidosis or the nonketotic hyperosmolarsyndrome.Long-term complications of diabetesinclude retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral

neuropathy with risk of foot ulcers, amputations, autonomic neuropathy causing gastrointestinal,genitourinary and cardiovascular symptoms (Craig *et al.,* 2009).

2.1.1 Classification of diabetes mellitus

Assigning a type of diabetes to an individual often depends on the circumstances present at the time of diagnosis, with individuals not necessarily fitting clearly into a single category. For example, some patients cannot be clearly classified as having type 1 or type 2 diabetes. Clinical presentation and disease progression may vary considerably in both types of diabetes.The traditional paradigms of type 2 diabetes occurring only in adults and type 1 diabetes only in children are no longer accurate, as both diseases occur in both groups. Occasionally, patients with T2D may present with diabetic ketoacidosis (DKA). Children with type 1 diabetes typically present with the hallmark symptoms of polyuria/polydipsia and occasionally with DKA.The onset of type1 diabetes maybe variable in adults and may not present with the classic symptoms seen in children. However, difficulties in diagnosis may occur in children, adolescents and adults, with the true diagnosis becoming more obvious over time (Genuth*et al.,* 2003).

Type 1 diabetes mellitus:This form, previously called "insulindependent diabetes mellitus" or "juvenile-onset diabetes isduetocellular-mediatedautoimmune destruction of the pancreatic β-cells (ADA, 2015). Autoimmune markers include islet cell autoantibodies, autoantibodies to insulin, autoantibodies to GAD (GAD65), autoantibodies to the tyrosine phosphatasesIA-2andIA-2b,andautoantibodies to zinc transporter 8 (ZnT8). Type 1 diabetes is defined by the presence of one or more of these autoimmune markers. The disease has strong HLA associations, with linkage to the DQA and DQB genes. These HLA-DR/DQ alleles can be either predisposing or protective (Ziegler and Nepom,

2010).The rate of β-cell destruction is quite variable, being rapid in some individuals (mainlyinfantsandchildren)andslowin others (mainly adults). Children and adolescents may present with ketoacidosis as the first manifestation of the disease. Others have modest fasting hyperglycemia that can rapidly change to severehyperglycemiaand/orketoacidosis with infection or other stress. Adults may retain sufficient β-cell function to prevent ketoacidosis for many years; such individuals eventually become dependent on insulin for survival and are at risk for ketoacidosis. At this latter stage of the disease, there is little or no insulin secretion, as manifested by low or undetectable levels of plasma C-peptide. Immune-mediated diabetescommonly occurs in childhood and adolescence, but it can occur at any age, even in the 8th and 9th decades of life (ADA, 2009).Autoimmunedestructionof β-cellshas multiple genetic predispositions and is also related to environmental factors that are still poorlydefined. Although patients are not typically obese when they present with type 1 diabetes, obesity should not preclude the diagnosis. These patients are also prone to other autoimmune disorders such as Graves' disease, Addison's disease, celiac disease, autoimmune hepatitis, and pernicious anemia.

Type 2 diabetes mellitus: This form was previously referred to as "non-insulin-dependent diabetes mellitus" or "adult onset diabetes. Types 2 diabetes encompasses individuals who have insulin resistance and usually relative (rather than absolute) insulin deficiency (Genuth*et al.,* 2003). Initially, and often throughout their lifetime, these individuals may not need insulin treatment to survive.There are various causes of T2D. Although the specific etiologies are not known, autoimmune destruction of β-cells does not occur, and patients do not have any of the other known causes of diabetes. Most, but not all, patients with T2D are obese. Obesity itself causes some degree of insulin resistance. Patients

whoarenotobesebytraditionalweight criteria may have an increased percentage of body fat distributed predominantly in the abdominal region (ADA, 2009).Ketoacidosis seldom occurs spontaneously in T2D; when seen, it usually arises in association with the stress of another illness such as infection. T2D frequently goes undiagnosedformanyyearsbecausehyperglycemia develops gradually and at earlier stages is often not severe enough for the patient to notice the classic diabetes symptoms. Nevertheless, such patients are at an increased risk of developing macrovascular and microvascular complications.Whereas patients with T2D may have insulin levels that appear normal orelevated, thehigherblood glucose levelsinthesepatientswouldbeexpected to result in even higher insulin value and their β-cell function been normal. Thus, insulin secretion is defective in these patients and insufficient to compensate for insulin resistance. Insulin resistance may improve with weight reduction and/or pharmacological treatment of hyperglycemiabutis rarely restoredtonormal (Droumaguet*et al.*, 2006). The risk of developing T2D increases with age, obesity, and lack of physicalactivity.Itoccursmorefrequentlyin women with prior GDM, in those with hypertension or dyslipidemia, and in certain racial/ethnic subgroups (African American, American Indian, Hispanic/ Latino, and Asian American). It is often associated with a strong genetic predisposition, more so than type 1 diabetes. However,thegeneticsof T2D is poorly understood (Droumaguet*et al.,* 2006).

*Gestational Diabetes Mellitus (GDM):*For many years, GDM was defined as any degree of glucose intolerance that was first recognized during pregnancy, regardless of whether the condition may have predated the pregnancy or persisted after the pregnancy. This definition facilitated a uniform strategy for detection and classification of GDM, but it was

limited by imprecision (Metzger *et al.,* 2008).The ongoing epidemic of obesity and diabetes has led to more T2D inwomenofchildbearingage,resultingin an increase in the number of pregnant womenwithundiagnosed T2D. Because of the number of pregnant women with undiagnosed T2D, it is reasonable to test women with riskfactorsfor T2D at their initial prenatal visit, using standard diagnostic criteria. Women with diabetes in the first trimester would be classified as having T2D. GDM is diabetes diagnosed in the second or third trimester of pregnancy that is not clearly diabetes.

Other specific types of diabetes:

*Maturity-Onset Diabetes of the Young (MODY):*MODY is characterized by impaired insulin secretion with minimal or no defects in insulin action. It is inherited in an autosomal dominant pattern. Abnormalities at six genetic loci on different chromosomes have beenidentifiedtodate.Themostcommon formis associatedwithmutationsonchromosome12inahepatictranscriptionfactor referred to as hepatocyte nuclear factor (HNF)-1a. A second form is associated with mutations in the glucokinase gene on chromosome 7p and results in a defective glucokinase molecule. Glucokinase converts glucose to glucose-6-phosphate, the metabolism of which, in turn, stimulates insulin secretion by the β-cell. The less common forms of MODY result from mutationsinothertranscriptionfactors,including HNF-4a, HNF-1b, insulin promoter factor (IPF)-1, and NeuroD1(ADA, 2009).

*Neonatal Diabetes:*Diabetesdiagnosedinthe first6monthsof life has been shown not to be typical autoimmune type 1 diabetes. This so-called neonatal diabetes can either be transient or permanent. The most common genetic defectcausingtransientdiseaseisadefect on ZAC/HYAMI imprinting, whereas permanent neonatal diabetes is most commonly a defect

in the gene encoding the Kir6.2 subunit of the β-cell KATP channel.Diagnosingthelatterhasimplications, since such children can be well managed with sulfonylureas (IEC, 2009).

*Cystic Fibrosis–Related Diabetes (CFRD):*CFRDisthemostcommonin people with cystic fibrosis, occurringin about20% of adolescents and40–50% of adults. Diabetes in this population is associated with worse nutritional status, more severe inflammatory lung disease, and greater mortality from respiratory failure. Insulin insufficiency related to partial fibrotic destruction of the islet mass is the primary defect in CFRD. Genetically determined function of the remaining β-cells and insulin resistance associated with infection and inflammationmayalsoplayarole.Whilescreening for diabetes before the age of 10 years can identify risk for progression to CFRD inthosewithabnormalglucosetolerance, there appears to be no benefit with respecttoweight,height,BMI,orlungfunction compared to those with normal glucose tolerance,10 years of age. The use of continuous glucose monitoring may be more sensitive than OGTT to detect risk for progression to CFRD, but this likely needs more evidence(Droumaguet*et al.*, 2006).

Genetic defects in insulin action: There are unusual causes of diabetes that result from genetically determined abnormalities of insulin action. The metabolic abnormalities associated with mutations of the insulin receptor may range from hyperinsulinemia and modest hyperglycemia to severe diabetes. Some individuals with these mutations may have acanthosis nigricans. Women may be virilized and have enlarged, cystic ovaries. In the past, this syndrome was termed type A insulin resistance. Leprechaunism and the Rabson Mendenhall syndrome are two pediatric syndromes that have mutations in the insulin receptor gene with subsequent alterations in insulin receptor function and extreme insulin

resistance. The former has characteristic facial features and is usually fatal in infancy, while the latter is associated with abnormalities of teeth and nails and pineal gland hyperplasia. Alterations in the structure and function of the insulin receptor cannot be demonstrated in patients with insulin resistant lipoatrophic diabetes. Therefore, it is assumed that the lesion(s) must reside in the post receptor signal transduction pathways (Droumaguet*et al.,* 2006).

Diseases of the exocrine pancreas: Any process that diffusely injures the pancreas can cause diabetes. Acquired processes include pancreatitis, trauma, infection, pancreatectomy and pancreatic carcinoma. With the exception of that caused by cancer, damage to the pancreas must be extensive for diabetes to occur; adrenocarcinomas that involve only a small portion of the pancreas have been associated with diabetes. This implies a mechanism other than simple reduction in β-cell mass. If extensive enough, cystic fibrosis and hemochromatosis will also damage β-cells and impair insulin secretion. Fibrocalculouspancreatopathy may be accompanied by abdominal pain radiating to the back and pancreatic calcifications identified on x-ray examination. Pancreatic fibrosis and calcium stones in the exocrine ducts have been found at autopsy (ADA, 2015).

Endocrinopathies: Several hormones (e.g, growth hormone, cortisol, glucagon and epinephrine) antagonize insulin action. Excess amounts of these hormones can cause diabetes. This generally occurs in individuals with pre-existing defects in insulin secretion and hyperglycemia typically resolves when the hormone excess is resolved. Somatostatinoma and aldosteronoma-induced hypokalemia can cause diabetes, at least in part, by inhibiting insulin secretion. Hyperglycemia generally resolves after successful removal of the tumor (Droumaguet*et al.,* 2006).

Drug or chemical-induced diabetes: Many drugs can impair insulin secretion. These drugs may not cause diabetes by themselves, but they may promote diabetes development in individuals with insulin resistance. In such cases, the classification is unclear because the sequence or relative importance of β-cell dysfunction and insulin resistance is unknown. Certain toxins such as vacor (a rat poison) and intravenous pentamidine can permanently destroy pancreatic β-cells. Such drug reactions fortunately are rare. There are also many drugs and hormones that can impair insulin action; examples include nicotinic acid and glucocorticoids. Patients receiving α-interferon have been reported to develop diabetes associated with islet cell antibodies and in certain instances, severe insulin deficiency (Droumaguet*et al.,* 2006).

Viruses and infections: A virus cannot cause diabetes on its own, but people are sometimes diagnosed with type 1 diabetes during or after a viral infection, suggesting a link between the two. Also, the onset of type 1 diabetes occurs more frequently during the winter when viral infections are more common. Viruses possibly associated with type 1 diabetes include coxsackievirus B, cytomegalovirus, adenovirus, rubella, and mumps. Scientists have described several ways these viruses may damage or destroy beta cells or possibly trigger an autoimmune response in susceptible people. For example, anti-islet antibodies have been found in patients with congenital rubella syndrome, and cytomegalovirus has been associated with significant beta cell damage and acute pancreatitis—inflammation of the pancreas. Scientists are trying to identify a virus that can cause type 1 diabetes so that a vaccine might be developed to prevent the disease (ADA, 2015).

Other genetic syndromes associated with diabetes: Many genetic syndromes are accompanied by an increased incidence of diabetes. These include the chromosomal

abnormalities of Down syndrome, Klinefelter syndrome and Turner syndrome. Wolfram's syndrome is an autosomal recessive disorder characterized by insulin deficient diabetes and the absence of β-cells at autopsy. Additional manifestations include diabetes insipidus, hypogonadism, optic atrophy and neural deafness (Droumaguet*et al.*, 2006).

2.1.2 Prevalence of Diabetes

The prevalence and incidence of type 2 diabetes, representing 90% of all cases of diabetes, are increasing rapidly throughout the world. It has been estimated that the number of people with diabetes is expected to rise from 415 million in 2015 to 642 million by 2040 if no urgent action is taken (IDF, 2015).

Moreover, the prevalence of diabetes is higher in men than women, but there are more women with diabetes than men.The most important demographic change to diabetes prevalence across the world appears to be the increase in the proportion of people greater than 65 years of age (Ogurtsova*et al.*, 2015).

All nations, rich and poor are suffering the impact of the diabetes epidemic. The impact is worse in those countries that are socially and economically disadvantaged. Diabetes threatens the achievement of the Millennium Development Goals (MDGs), increases the risk of developing tuberculosis and is closely linked with other infections (IDF, 2011).

Globally in 2013, it is estimated that almost 382 million people suffer from diabetes with a prevalence of 8.3%. North America and the Caribbean are the regions with the highest prevalence of 11% having 37 million people with diabetes followed by the Middle East and North Africa with a prevalence of 9.2% having 35 million people with diabetes.

Western Pacific is the region with higher number of people living with diabetes (138 million), however its prevalence is 8.6%, close to the prevalence of the World (IDF, 2013).

An estimated 14.2 million adults aged 20-79 have diabetes in the Africanregion, representing a regional prevalence of 2.1-6.7%. The Africa region has the highest proportion of undiagnosed diabetes; over two thirds (66.7%) of people with diabetes are unaware they have the disease. The majority (58.8%) of people with diabetes live in cities, even though the population in the region is predominantly (61.3%) rural. Diabetes in adults is in general much higher on islands in the Africa Region, compared to the mainland. The highest prevalence is found in the Seychelles (17.4% age-adjusted comparative prevalence, 17.4% raw prevalence), followed by the island of Reunion (15.8% age-adjusted, 18.2% raw) and Comoros (9.9% age-adjusted, 7.5% raw). Some of Africa's most populous countries have the highest numbers of people with diabetes, including South Africa (2.3 million), Democratic Republic of Congo (1.8 million), Nigeria (1.56 million) and Ethiopia (1.3 million). Nearly half of all adults with diabetes in the region live in these four countries (IDF, 2015).

Nigeria with over 250 tribes and different culture and food values, the prevalence values has not been uniform though the International Diabetes Federation recorded that there were more than 1.56 million cases of diabetes in Nigeria (IDF, 2015) and the World Health Organization suggest that Nigeria has the greatest number of people living with diabetes in Africa.

2.1.3. Postprandial Hyperglycemia

Postprandial phase is characterized by a rapid and large increase in blood glucose levels and the possibility that these postprandial "hyperglycemic spikes" may be relevant to the

pathophysiology of late diabetes complications are recently receiving much attention. A large number of epidemiologic studies have documented the strong link between chronic hyperglycemia typically reflected by glycosylated hemoglobin (A1C) and long term morbidity and mortality in patients with diabetes. Results from a cohort of 879 individuals with type 1 diabetes who were followed for 20 years indicated that A1C was significantly associated with all cause and cardiovascular mortality (Bash *et al.*, 2008).Several studies have demonstrated the effectiveness of targeting postprandial glucose to decrease the risk of diabetes complications. The Campanian postprandial hyperglycemia study compared the effects of repaglinide and glyburide on PPG, carotid intimamedia thickness and markers of systematic vascular inflammation in 175 patients with type 2 diabetes. After 12 months, peak PPG was 148 mg/dL in the repaglinide group versus 180 mg/dL in the glyburide group. Regression of carotid intimamedia thickness (a decrease greater than 0.020 nm) was observed in 52% of patients in the repaglinide group versus 18% of those in the glyburide group. Reductions in C-reactive protein and IL6 were significantly greater with repaglinide than with glyburide. These results show that targeting postprandial glucose can promote atheroma regression in patients with type 2 diabetes (Esposito *et al.*,2004).

Alpha Amylase

Alpha Amylase (EC 3.2.1.1) (α-1,4- glucan-4-glucanohydrolase) is an endo-acting enzyme that hydrolyses alpha bonds of α-(1,4) glycosidic linkages of polysaccharides, such as starch and glycogen, yielding glucose and maltose (Maureen, 2000). It is the major form of amylase found in humans and other mammals (Voet, 2005). Alpha amylase inhibitors such as acarbose, miglitol, voglibose, nojirimycin and 1- deoxynojirimycin, also known as carbo- blockers (Kim *et al.*, 2000) prevent degradation of complex dietary carbohydrates to

oligosaccharides anddissacharides. Theses inhibitors are indirectly helpful in weight loss due to ability to preventsugar assimilation, through inhibition of starch hydrolysis (Kim *et al.,* 2000).

Alpha-glucosidase

Alpha-glucosidase (EC3.2.1.20), maltase, glucoinvertase, glucosidosucrase, maltaseglucoamylase,alpha-glucopyranosidase, glucosidoinvertase, alpha-D-glucosidase, alpha glucoside hydrolase, alpha-1,4-glucosidase, alpha-D-glucoside glucohydrolase is a glucosidase located in the brush border of the small intestine that acts upon 1,4-alpha bonds (Bruni*et al,*1970).The membrane-bound intestinal alpha-glucosidases hydrolyze oligosaccharides, trisaccharides, and disaccharides to glucose and other monosaccharides in the small intestine. Alpha-glucosidase inhibitors are saccharides that act as competitive inhibitors of enzymes (especially alpha glucosidase) needed to digest carbohydrates. Currently, three (3) drugs are therapeutically used as anti-glucosidases: acarbose, miglitoland voglibose (DeFronzo, 1999). These drugs have greater glycemic control over hyperglycemia in diabetes mellitus type 2, particularly with regard to postprandial hyperglycemia, by interfering with the rate of digestion of dietary carbohydrate. Therefore, less glucose is absorbed because the carbohydrates are not broken down into glucose molecules. In diabetic patients, the short-term effect of these drugs therapies is to decrease current blood glucose levels: the long-term effect is a small reduction in glycosylated hemoglobin (glycohemoglobin or hemoglobin A1C) level (Samantha *et al.,* 2008).

Aldose Reductase

Aldose reductase(AR: EC 1.1.1.21) is a monomeric reduced nicotinamide adenine dinucleotide (NAD) phosphate (NADPH)-dependent enzyme and a member of aldo-keto

reductase superfamily which catalyzes glucose to sorbitol and sorbitol dehydrogenase, the second enzyme of the pathway, further converts sorbitol to fructose (Chylack and Friend, 1990). High levels of AR activity are present in the eye lens and under hyperglycemicconditions, this pathway accounts for more than 30% of the glucose utilized (Gonzalez *et al.,* 1984). However, severaldrugs with varying AR-inhibiting efficacy (*e.g*,sorbinil, statil and tolrestat) have been synthesized and tested (Pfeifer*et al.,* 1997). AR inhibitors, in addition to preventing diabetic and galactosemic cataracts (Varma and Kinoshita,1976), ameliorated some of the features of diabetic nephropathy (Passariello*et al.,* 1993) and neuropathy (Jaspan*et al.,* 1986).

2.1.4. Complications of Diabetes Mellitus

Diabetic retinopathy

During hyperglycemia, reduction of glucose to sorbitol by aldose reductase constitutes the first and the rate-limiting step of the polyol pathway that converts glucose to fructose via sorbitol dehydrogenase (SDH). Inthis pathway both NADPH and NAD are consumed as cofactors for the enzymes aldose reductase and sorbitol dehydrogenase (Figure 2.1). Osmotic stress due to accumulation of sorbitol and oxidative stress due to changes in the ratio of NADPH/NADP and reduced NAD (NADH)/NAD are the major cause of retinopathy complications of secondary diabetes. (Srivastava *et al*., 2005)

Diabetic neuropathy

Diabetic neuropathy, a life-threatening complication involves both peripheral and autonomic nerves; affecting almost half of the diabetic population (Chawla *et al.,* 2016). The risk of developing of diabetic neuropathy is directly proportional to both the duration and magnitude of hyperglycemia. In addition, some individuals may also possess genetic

facets that influence their predisposition in developing such complications (Fowler, 2008). Peripheral neuropathy in diabetes appears in several forms depending on the site, manifesting as sensory, focal/multifocal, and autonomic neuropathies. Diabetic neuropathy has resulted in more than 80% amputations after foot ulceration or injury (Chawla *et al.*, 2016).

Diabetic nephropathy

The pathogenic mechanisms underlying diabetic nephropathy involve generation of reactive oxygen species (ROS), accumulation of advanced glycation end products (AGE), and activation of intracellular signaling molecules such as protein kinase C (PKC) (Cade, 2008). PKC activation, increased polyol flux, and hexosamine formation are also linked to oxidative stress in promoting macrovascular complications through multiple mechanisms (Chawla *et al.,* 2015).

Vascular Complication

Oxidative stress, caused by the overproduction of ROS plays an important role in the activation of other pathogenic pathways involved in diabetic complications which in turn lead to the development of micro and macro vascular complications (Chawla *et al.,* 2016). It also inactivates two critical anti-atherosclerotic enzymes, endothelial nitric oxide synthase, and prostacyclin synthase (Folli*et al.,* 2011). ROS-mediated cellular damage may be a form of pathologic “memory” in the microvasculature that persists even after glucose normalization.

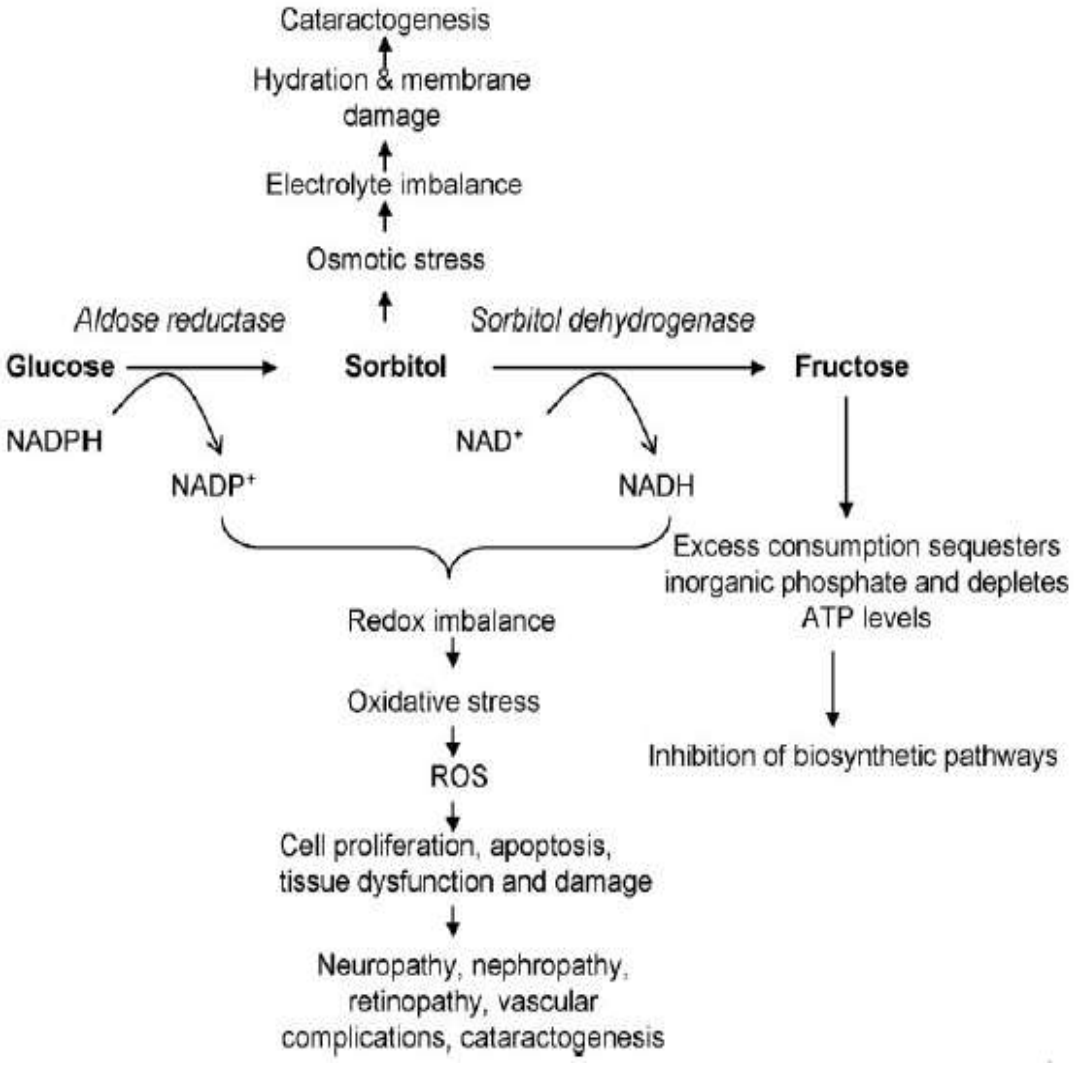


Figure 2.1: Aldose Reductase and Diabetic Complications (Srivastava *et al*., 2005)

2.1.5. Therapeutic agents for treatment options

There are several anti-hyperglycemicand glucose lowering agents that are used in the treatment of DM. They act differentlyto maintain lowblood glucose. Most of them are orally administered, except for insulin and insulin preparations which are administered parenterally (Warjeet, 2011). Most of these anti-diabetic drugs have been tried on experimental models like rodents, in which diabetes has been induced by pharmacological, surgical or genetic manipulations to examine anti-diabetic effects (Fröde and Medeiros, 2008). Depending on their mechanism of action, anti-diabetic drugs or agents used in the treatment of DM are classified into several groups such as: insulin and insulin secretagogues, insulin sensitizers, suppressors of hepatic glucose production, alpha glucosidase, aldose reductase inhibitors and so on.

Insulin and Insulin Secretagogues

Most diabetic patients may eventually develop progressive pancreatic beta-cell failure and impaired insulin secretion, and as such may need insulin to control hyperglycemia (Hamaty, 2011). Insulin as a drug is an injectableanalogue of the normal physiological insulin with similar function of promoting cell uptake of glucose, glucose disposal and storage of glucose as glycogen, thus lowering blood glucose level. Therapeutic insulin is divided into two types depending on the mode of action; long-acting (basal) insulin, which is usually taken at bed time to maintain normal blood glucose and rapid acting (prandial or bolus) insulin which is usually injected after a meal to control postprandial blood glucose rise (Hamaty, 2011). Insulin secretagogues (ISs) are diabetic medications that are used to remedy impaired insulin secretion in T2D patients. They help the pancreas to produce and secrete insulin for blood glucose homeostasis (Patel *et al*., 2012). Some existing commonly

used insulin secretagogues are the sulphonylureas like glyburide, gliclazide and glipizide, which are sometimes used in combination with other hypoglycemic agents. However, it has been reported that this class of ISs are associated with hyperinsulinemia; risk of hypoglycemia; inadequate glycemic control (in T2D patients); gradual failure in beta-cell function; and weight gain, while meglitinides like repaglinde, which is another class of ISs and a prandial glucose regulator is believed to have better glycemic control and lower risk of hypoglycemia than sulphonylureas (Davies, 2002).

Insulin Sensitizers

Insulin resistance and insensitivity in the body, especially in the muscles are major metabolic defects associated withT2D.Insulin produced cannot adequately induce blood glucose uptake or disposal in these tissues, which contributesto hyperglycemia. Insulin sensitivity improvement drugs, such as thiazolidines (TZDs)and biguanides like metforminwork by improving the insulin insensitivity and insulin dependent glucose uptake in muscles and other tissues, hence improving metabolic control in T2D patients (Klip and Leiter, 1990; Sirtori andPasik, 1994 and Hauner, 2002). It has been reported that TDZs can reduce serum glucose, insulin and triglyceride level and also increase blood glucose uptake when used to treat T2D patients (Kahn *et al*.,2000). Hauner (2002) reported that the mechanism of action of TDZs involves a Peroxisome proliferator-activated receptor *gamma* (PPAR-γ)-dependent transcription control of genes involved in glucose and fat metabolism in adipose and muscle tissues, which are promoted via an endocrine signal from adipocytes. Other anti-diabetic drugs belonging to this class include bigaunides (e.g. metformin); troglitazone, rosiglitazone and pioglitazone have been hypothesized to have potential therapy against T2D related complications such ascardiovascular diseases

(Hauner, 2002; Charbonnel*et al*., 2004). Despite this, some TDZs like rosiglitazone are believed to be associated with some adverse effects like risk of weight gain, coronary heart disease, heart attack and some other vascular disease (Hussein *et al*., 2004). Metformin treatment has been reported to be associated with vitamin B12deficiency and increased risk of lactic acidosis especially in individuals with renal or CVD (Bailey and Turner, 1996; Liu*et al*., 2006). Studies conducted by Ekström*et al*.(2012) showed lower risk of CVD and acidosis or serious infection in metformin treated T2D patients than some oral glycemic drugs and insulin, concluding that the beneficial effects of metformin outweighs the risk of adverse effects.

Suppressors of Hepatic Glucose Production

Hepatic glycogenolysis and gluconeogenesis are two glucagon-dependent glucose production processes that serves as major sources of glucose for body metabolic functions, during fasting and when blood glucose drops. The inability of insulin to suppress these processes and the hormonal and enzymatic factors that promote them is a major etiological factor in the development of hyperglycemia in diabetic patients (Postic*et al*., 2004). These processes, which are cyclic AMP (cAMP) dependent,are believed to be major targets for some classes of anti-diabetic drugs (Miller*et al*., 2013).Glucagon binding to cell membrane receptors causes the activation of adenylatecyclase, and subsequent cAMP production, which activates protein kinase A (PKA). Activated PKA phosphorylates and activates target proteins that signals or up regulates hepatic glucose output (Jiang and Zhang, 2005).Anti-diabetic drugs belonging to this class acts by down regulating glycogenolysis and gluconeogenesis, which are major processes that increase hepatic glucose output. Although the molecular mechanism behind the anti-diabetic action of biguanides have

been previously suggested to be via the enhancement of glucose disposal and activation of the enzyme AMP-activated protein kinase (AMPK), a cellular glucose uptake inducer; (Goodazi and Bryer-Ash, 2005) recent studies have reported biguanides to be a suppressor of hepatic glucose production (Viollet*et al*., 2012), and metformin an antagonist of glucagon action (Miller *et al*., 2013).According to Miller *et al*. (2013) metformin treated mice exhibited accumulation of AMP (an adenylatecyclase inhibitor) in the liver cells and increased PKA activity and target protein phosphorylation, indicatingsuppression or blocking of glucagon-dependent hepatic glucose output and hypoglycemic function.

Enzyme Inhibitors

This class of oral glycemic drugs works by preventing postprandial hyperglycemia (Reuser and Wisselaar, (1994). They delay the digestion of carbohydrates-like starch and sucrose, and also delay the subsequent absorption of absorbable monosaccharides like glucose resulting from carbohydrate digestion (Bisschoff, 1994; Patel *et al*., 2012). It is believed that α-glucosidase inhibitors are saccharides that competitively inhibit carbohydrate hydrolyzing enzyme like α-glucosidase and α-amylase (Horii *et al*., 1986; Bischoff, 1995; Kim*et al*., 1999). Studies have shown that*Acarbose:* is an oral α-glucosidase and α-amylase inhibitor for use in the management of T2D mellitus (Wang *et al.*, 2014). It is chemically known as O-4,6- dideoxy-4-[[(1S,4R,5S,6S)-4,5,6-trihydroxy-3-(hydroxymethyl)-2-cyclohexen-1-yl]amino]-α-D glucopyranosyl - (1→4) – O – α - D-glucopyranosyl - (1→4) - D-glucose. The antihyperglcemic action of acarbose results from a competitive, reversible inhibition of pancreatic α-amylase and a membrane bound intestinal α-glucosidase hydrolase enzyme. Acarbose is shown to reduce and slow down the intestinal absorption of glucose, which subsequently minimize the postprandial rise of

blood glucose and insulin concentration (Wang *et al.,* 2014).These effects and further beneficial effects of acarbose against nephropathy, neuropathy, retinopathy, endothelial dysfunctions and CVD problems make it a potential therapy for prevention of T2D complications (Bischoff, 1995; Standl and Schnell, 2012). It was first extracted from the culture broths of actinomycetes and was applied in clinical studies for more than 10 years (Coniff and Krol, 1997; Scheen, 1998). It reversibly inhibits α-glycosidases that exist in the brush-border of the small intestinal mucosa (Clissold and Edwards, 1988).

Sorbinil: is believed to be among the strongest of the aldose reductase inhibitors, though their anti-cataractous activities in animal studies remain controversial.

Sodium Glucose co-transporters 2 inhibitors

Sodium glucose co-transporters 2 (SGLT2) inhibitors represent a new strategy in the treatment of diabetes. These drugs inhibit glucose reabsorption from renal tubules, thereby promoting urinary glucose excretion and decreasing plasmaglucoselevels.Thesedrugshaveauniquemechanismof action that is independent of pancreatic β-cell function or modulation of tissue insulin sensitivity. Thus, they have the potential to be used as combination therapy with other oral anti-diabetic drugs as well as insulin. A number of SGLT2 inhibitors are in various phases of preclinical and clinical development, and dapagliflozin is the furthest advanced compound (Misra, 2012).

Glucose absorption at the enterocytes, reabsorption at the renal tubules, transport across the blood–brain barrier and uptake and release by all cells in the body is mediated by two groups of transporters. These include glucose transporters (GLUTs)andsodium-glucoseco-transporters (SGLTs) (Wood and Trayhurn, 2003). GLUTs are facilitative or passive transporters that transport glucose along the concentration gradient. They belong to the

solute carrier family 2 (SLC2) gene family, which has 13 members: GLUT1-12 and the H^+-myoinositolco-transporters. GLUTs are expressed in every cell of the body. SGLTs transport sodium and glucose into cells using the sodium gradient produced by sodium/potassium ATPase pump at the basolateral cell membrane. These transportersbelongtothesolutecarrierfamily5(SLC5)gene family,whichhasninememberswithknownfunctions.Of these, SGLT1 and SGLT2 are primarily responsible for renal glucose reabsorption (Nair and Wilding, 2010).SGLT2playsamajorroleinrenalglucosereabsorptionand accounts for approximately 90% of renal glucose reabsorption.TheevidenceforSGLT2beingamajorpathwayforrenal glucose reabsorption has been derived from the genetic studies of individuals with familial renal glycosuria. Mutations in the SLC5A2 gene encoding SGLT2 lead to familial renal glycosuria. This benign disorder is inherited as an autosomal recessive trait and is characterized by isolatedpersistent glycosuria with normal blood glucose levels and normaloralglucosetolerancetest results (Nair and Wilding, 2010).

AnumberofSGLT2inhibitorsareinvariousstagesofclinical development for the treatment of diabetes.SGLT2 inhibitors interfere with the function of SGLT2 in proximal convoluted tubules of kidney and induce glycosuria.Animal studies and clinicaltrialshaverevealedthatSGLT2inhibitionbenefitsthe diabetic state by lowering plasma glucose levels, decreasing glucotoxicity and reducing plasma insulin and glycosylated hemoglobinlevels (Boldys and Okopien, 2009). Reductionintheplasmaglucoselevel improves liver sensitivity to insulin. This suppresses hepatic glucose production, leading to an improvement in the diabetic state.By causing glycosuria, SGLT2 inhibitors not only reduce plasma glucose levels,but also cause a net loss of calories from the body and

maintain overall negative energy balance.Inaddition,theseinhibitorsalsohaveabloodpressure lowering effect, which might be related to their mild diuretic and weight-reducing action (Kipnes, 2010). As compared to currently used antidiabetic drugs, SGLT2 inhibitors do not stimulate insulin secretion or pose the risk of hypoglycemia or cause gastrointestinal side effects (Boldys and Okopien, 2009). The convenience of oral administration is another advantage of this new class of antidiabetic drugs. The novel mechanism of action suggests their potential use in combination with other antidiabetic agents to exert additive or synergistic effects in lowering glucoselevelsin T2D mellitus. Moreover, these drugs present so many advantages among which is the prevention of blood pressure elevation in hypertension prone patients because of reduced sodium reabsorption thereby causing a decrease in the activity of the renin-angiotensin-aldosterone system.Despite interesting reports on several potential beneficial effects of these drugs, they are still not without adverse effects.Common side effects include symptoms of thirst, urinary tract infections and mycotic genital infections. Less common side effects are hypoglycemia, dehydration, hypovolemia and serum cholesterol and creatinine elevations (Tahrani*et al.*, 2013).

2.2. Medicinal Plants

The abundant tropical biodiversity and frontiers found in the rainforest belt and coastal area have endowed Nigeria as well as other tropical countries with a wide floristic diversity. Many of these plants found in our tropical rain forest areas in Nigeria are associated with some potential medicinal properties and are being exploited for their medicinal values by traditional herbal practitioners in the preparation of herbal medicines in the treatment of various ailments and diseases (Alqasim, 2013). Today, some pharmaceutical drugs are derived from excellent ingredients in medicinal plants

(e.ggalegine and diosgenin). In folk medicine, many natural formulations have the potential to treat many diseases and disorders.

2.2.1 *Anonna senegalensis*

Anonna senegalensis; (Annonaceae) Family. Generally known as ‘African custard-apple’ and usually known as ‘’Gwandardaji’ in Hausa, “Abo” in Yoruba, “Uburuocha” in Igbo(Plate I). The plant is widespread throughout savannah or sub-tropical regions of Nigeria and used in the treatment of DM (Shinkafi *et al.,* 2015).

Plate I:*Annona senegalensis* Pers. in its natural habitat in photo at Mando Kaduna

2.2.2 Habitat

This plant is common and quite wild in the shrubbery, open bush and along rivers and streams in Nigeria particularly in Oyo, Enugu, Kaduna, Kano, Bauchi, Jigawa, Katsina and Niger Stateand it is cultivated in some countries in the world includingSwaziland and India and also in parts of Africa such asCameroon, Congo, Guinea, Kenya, Mali, Mozambique, Senegal, Sierra Leone, South Africa, Sudan, and Tanzania.

2.2.3 Scientific Classification

Kingdom: Plantae, Subkingdom: Tracheobionta, Superdivision: Spermatophyta,Division: Magnoliophyta, Class: Magnoliopsida, Subclass: Magnoliidae, Order: Magnoliales, Family: Annonaceae, Genus: *Annona,* Species: *A. senegalensis*

2.2.4Ethnopharmacological Benefits

The fruit obtained from this plant is widely used locally in the treatment of two common energy deficiency syndromes known as kwashiorkor and marasmus,the plant is also reported to be of great medicinal value and it's use in native medicine includes headache, body ache (Arnold and Gulumian 1984 and Chhabra*et al.,* 1987)andeyelid swelling (Klaus *and* Adala,1994). The stem bark of *A.senegalensis* is used by some populations all over Africa, in treating guinea worm, diarrhea, gastroenteritis, snake bites, toothache, respiratory infections and malaria (Obi *et al.,* 2003). Globade(2009) reported the use of the leaf locally in the treatment of diabetes. A decoction of the roots is used to stop chest colds, venereal diseases, stomach ache and dizziness (Awa *et al.,* 2012)

2.2.5Biological activities of *A. senegalensis*

Phytochemical Compounds

The phytochemical screening of *A. senegalensis* revealed the presence of flavonoids, alkaloid, steroids or triterpenes glucids, coumarines, tannis, and saponins (Awa *et al.*, 2012).

Antioxidant Activity

The antioxidant activity and drug detoxification activity of *Annona senegalensis* leafwas reported to decrease significant superoxide ion, hydrogen peroxide (H2O2), 2, 2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) and ferric ion (Yeo *et al.*, 2011). The responsible chemical constituent of antioxidant activity may be due to the presence of flavonoids with known properties of free radical scavenging effect in the extracts.

Antidiarrheal Activity

Oral administration of stem bark methanol extract of *A. senegalensis* was reported to demonstrate decreased intestinal transit time via decreasing the spontaneous contractions of the intestinein treatment of diarrhea related disorders (Suleiman *et al.,* 2008).

Antimicrobial Activity

The antimicrobial activities of the ethanolic and aqueous leaf extracts of *A. senegalensis* were significant against pathogenic bacterial strains *Staphylococcus aureus, Shigellaflexneri, Salmonella paratyphi*and *Pseudomonas aeruginosa*(Johnson and Olatoye, 2002). Whereas methanolic extract also showed significant antimicrobial activity against clinical isolates of *S. enteriditis, S. dysenteriae*and *E. coli* (Awa *et al.,* 2012).

Cytotoxic and antiparasitic activity

Theethanolic seed extracts of *A. senegalensis*was reported to have cytotoxic and antiparasitic activity against human epidermoid carcinoma (KB), normal (VERO) cell lines and trypanosome respectively (Sahpaz*et al.,* 1994). Yeo *et al.* (2011) reported significant decrease in the number of inflammatory cells of asthma and cough by the leaf extract of *A. senegalensis.*

Antimalarial and Anti-snake venom activity

The methanol leaf extract of *A.senegalensis* possess antimalarial activity against *Plasmodium berghei* with chemosuppression activity(Ajaiyeoba*et al.,* 2006). Adzu*et al.* (2005) further reported the reduction in the induced hyperthermia directly detoxified snake venom in themethanol root barkextract of the *A. senegalensis* on the brine shrimp (*Artemia saline* Leach) against cobra (*Najanigricotlisnigricotlis*Wetch) in rats.

Antihelminticand anti-nociceptive activity

Alawa *et al.* (2003) reported the significant reduction in the egg hatch and larval recoveryas the concentration increases of the methanolic leaf extract of *A.senegalensis* in*Haemonchuscontortus*eggs.However,methanolic leafextract of *A.senegalensis* exhibited antinociceptive activity in the hot plate test, acetic acid writhing test, and the late phase of formalin induced nociception(Adzu*et al.*,2003).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals, Reagents and Equipment

Porcine pancreatic α-amylase, yeast α-glucosidase, NADPH, DL-glyceraldehyde,dimethyl sulphoxide (DMSO),3,5-dinitrosalicylic acid, di-basic sodium phosphate, paranitrophenyl-α-D-glucopyranoside, acarbose, and Quercetin all were purchased from Sigma Aldrich, Germany and starch was collected from Department of Biochemistry Ahmadu Bello University, Zaria and bovine Aldose reductase was extracted from the eye lens of cattle slaughtered at Zango Abattoir, Samaru, Zaria, Nigeria.

3.1.2 Plant Material Collection and Identification

Fresh leafsample of*A.senegalensis* was obtained from Mando, IgabiLocal Government Area, Kaduna State in the month of June, 2016.The sample was identified at the Herbarium unit of Department of Botany, Ahmadu Bello University (A.B.U.), Zaria. A voucher number V/No: (1709) was collected in the Herbarium for future reference.

3.2 METHODS

3.2.1 Preparation of Plant Material

The *A. senegalensis*leaf sample used for this researchwasplucked from the stem, washed and then dried under shade until constant weight was obtained. The dried samples were then grounded into powder in a laboratory using amortar and pestle. The powdered part was then kept in a closed plastic container and was used in the experiment.

3.2.2 Crude Extraction

The powdered dried leaf (25g) of *A.senegalensis* was extracted sequentially for 48 hours in 500ml of three (3) different solvents (n-hexane (HEX), ethyl-acetate (EtOAc) and ethanol (EtOH)) respectively, after which they were filtered using muslin cloth and Whatman filter paper. The extracts were then concentrated using a rotary evaporator.

3.2.3 Phytochemical Screening

Liebermann-Burchard test for steroids: The sample portion, 2 ml of acetic acid will be added and the solution followed by the addition of concentrated H_2SO_4 carefully. Colour development from violet to blue or bluish-green indicates the presence of steroids(Sofowora, 1993).

Bontragers Test for Anthraquinones:The sample of the fraction-f of *A. senegalensis* extract was mixed with 10% ammonium hydroxide solution. The presence of Anthraquinones was indicated by the pink or red color is presences.

*Test for alkaloids:*A small amount of sample portion was mixedwith Dragendorff's reagent; occurrence of orange-red coloredis taken as positive (Sofowora, 1993).

*Test for Flavonoids:*The sample was mixed with of 1% aluminum chloride solution was in methanol was added. Formation of yellow color indicated the presence of Flavonoids,

*Ferric chloride test for Phenols:*2ml of 2% solution of $FeCl_3$was mixed with sample of the fraction-F of *A. senegalensis* extract.A blue-green or black coloration indicated the presence of phenols (Sofowora, 1993).

3.2.4 Experimental Design:The design of the experiment is presented in Figure 3.1

3.2.5 α-amylase (E.C.3.2.1.1) Inhibitory Effects

The α-amylase inhibitory effect of the extracts/fractions was carried out using a modified method of McCue and Shetty (2004).

*Principle:*Starch + H_2O $\xrightarrow{\alpha\text{-amylase}}$ Reducing sugars (Maltose)

Reducing sugars + dinitrosalicyclic acid ⟶ pink solution

*Procedure:*Porcine pancreatic α-amylase was used for this assay. A solution of enzyme (1 mg/ml) was prepared using 0.02 M sodium phosphate buffer (pH 6.9).Starch solution (1%)was also prepared using the above mentioned buffer, which was used as substrate. Solutions of the extracts were prepared by using equal volume of distilled water and dimethyl sulphoxide (DMSO). Different concentrations of the extracts ranging from 30-240 µg/ml for the assay were prepared from the stock solution. Then, 150 µL of 30-240 µg/ml of extract was added to 150 µL of the α-amylase solution, after which 150 µL of 1% starch solution was added and incubated at 25°C for 30 minutes. After that300 µL of dinitrosalicylic acid (DNS) was added to terminate the reactionand the tubes were then incubated in boiling water bath for 5 minutes and cooled to room temperature. Then3ml of distilled water was also added to the reaction mixture and the absorbance was measured at 540 nm using a Shimadzu mini 1240 spectrophotometer.

A control was prepared using the same procedure replacing the extract with distilled water. The α-amylase inhibitory activity was then calculated as percentage inhibition. Acarbose was used as positive control. All the reactions were carried out in triplicates. Percentage inhibition was calculated as:% Inhibition = [(Abs control-Abs extracts)/Abs control] x 100.

EXPERIMENTAL DESIGN

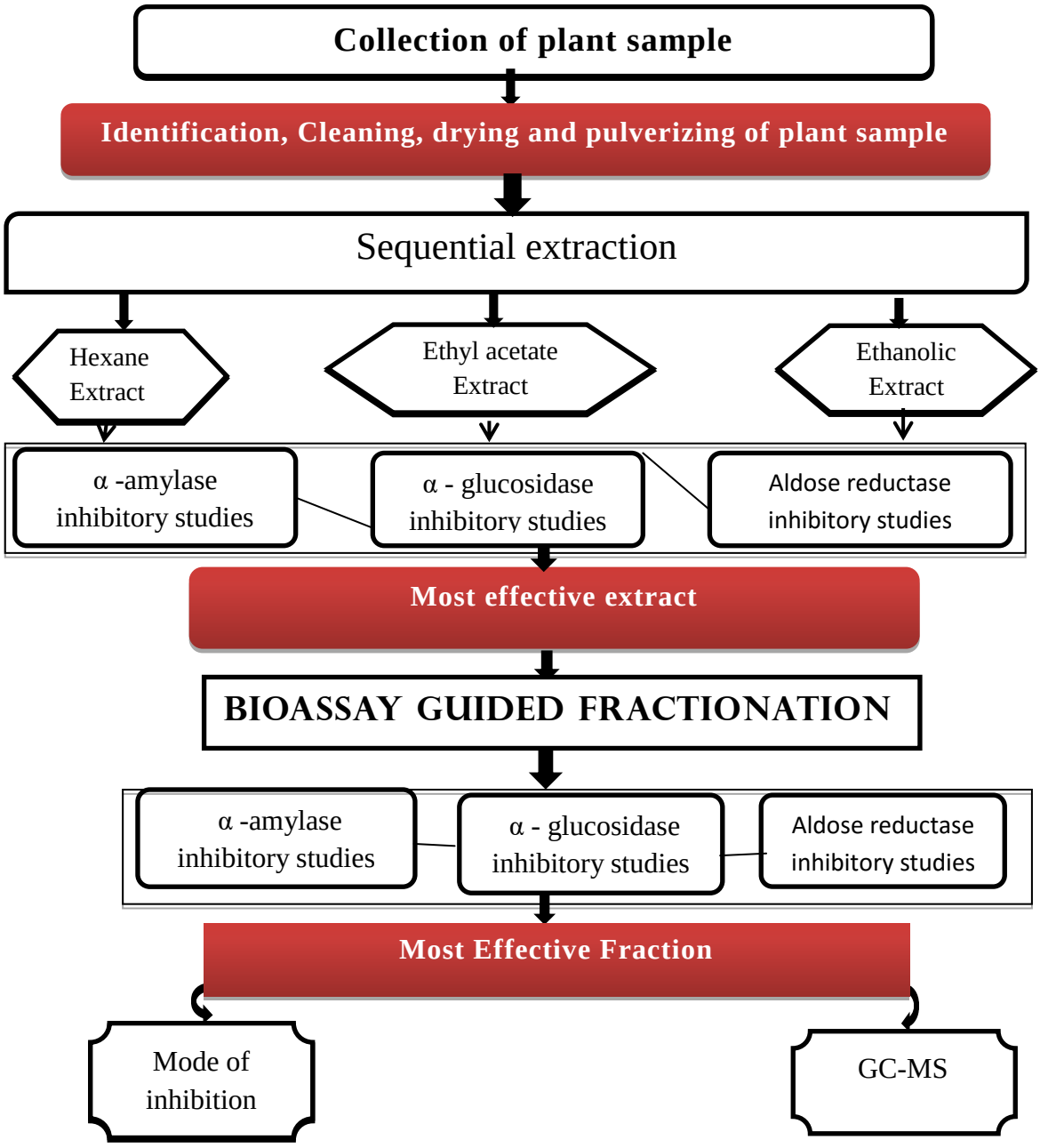


Figure 3.1: Experimental Design of the *Invitro* Studies of the Inhibitory Effect of Leaf Extracts of *A. senegalensis*on the Activities of α-Amylase, α-Glucosidase and Aldose Reductase

Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically using the concentration/inhibition plots.

3.2.6 α-Glucosidase (E.C.3.2.1.20) Inhibitory Effects

The inhibitory effect of the plant extracts/fractions on α-glucosidase activity was determined according to the method described by Kim *et al*. (2005)

Principle: *p*-Nitrophenyl-α-D-Glucopyranoside $\xrightarrow{\alpha\text{-glucosidase}}$ α-D-Glucose + *p*-Nitrophenol

*Procedure:*α-glucosidase type 1 from *baker's yeast* was used for the assay. The substrate solution *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) was prepared in 100 mM phosphate buffer, pH 6.9. The substrate (1 ml) was prepared by weighing 6.025 mg of *p*NPG into ameasuring cylinder and making up the volume to the 1 ml mark with phosphate buffer pH 6.9. Then, 200µL of α-glucosidase (1U/ml) was added to test tubes containing 80µL of different concentrations ranging from 30-240 µg/ml of the extract.After which 120µL of 5.0 mM (*p*NPG) was then added to start the reaction. The reaction mixture was incubated at 37ºC for 1hour and stopped by adding 4 ml of 0.1 M Na_2CO_3. The absorbance was determined by measuring the yellow colored p-nitrophenol released from *p*NPG at 400 nm using a Shimadzu UV mini 1240 spectrophotometer. The blank solution was prepared by adding the Na_2CO_3 to the reaction mixture before adding the enzyme. The results were expressed as percentage of the negative control in which the extract was replaced with DMSO and distilled water mixture in equal volume. Acarbose was used as the positive control. All the reactions were conducted in triplicates. Percentage inhibition was calculated as:

% Inhibition = [(Abs control - Abs extract)/Abs control] x 100.

Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically using the concentration/inhibition plots.

3.2.7 Aldose Reductase (E.C. 1.1.1.21)Inhibitory Effects

Preparation of Bovine eye lens Homogenate

The bovine lens homogenate was prepared according to the method of Hayman and Kinoshita (1965) with some modifications (Jung *et al.*, 2011).Non cataractous transparent lenses were enucleated through posterior approach and pooled and homogenate was prepared with three volumes of 0.1M phosphate buffer saline (pH 6.2). The homogenate was centrifuged at 10,000rpm for 20 minutes at 4˚C. The resulting supernatant containing the aldose reductase was collected and used for the assay procedure.

Assay procedure: The solutions of plant extracts were prepared by dissolving 2 mg of the extract in 50µL DMSO (5%) and made up to 1 mL, with distilled water this was used in preparing 30-240µg/ml different concentrations. Test sample contained 0.4 mLof the enzyme (rAR), 0.2 mL of 0.3 mM NADPH, 10 mM of the substrate (DL-glyceraldehyde) (0.2mL)and the inhibitor (plant extract) (0.2 mL) making a volume of 1 ml. The negative control sample contained the same volume of 0.3 mM NADPH, 10 mM substrate, enzyme preparation and 5% DMSO instead of test inhibitor while the blank solution contained double distilled water instead of substrate and the final volume was made up to 1 ml using 100mM phosphate buffer saline (pH 6.2). Aldose reductase activity was assayed spectrophotometrically by measuring the decrease in absorbance of NADPH at 340nm using a Shimadzu UV mini 1240 spectrophotometer at the beginning and after substrate addition for 2 minutes at 30 seconds interval. The bioassays were carried out in triplicates and the average inhibitory activity was determined.

Quercetin, a known aldose reductase inhibitor was used as positive control to compare the plant extracts inhibitory activity. The inhibitory activity of the extracts was calculated using the following formula

$$\% \, ARI = \frac{\Delta \, Abs \, (Neg. Ctrl.) - \Delta \, Abs. \, (extract)}{\Delta \, Abs \, (neg. Ctrl.)} \times 100$$

Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically using the concentration/inhibition plots.

3.2.8Bioassay Guided Fractionation

Thin Layer Chromatographic Analysis (TLCA)

In this procedure, precoated TLC plates were used to carryout thin layer chromatography by one way ascending technique. Capillary tubes were used to manually apply spots of *A. senegalensis*extract on the TLC plate and the chromatogram was developed in an air tight chromatographic tank at room temperature employing different solvent systems. The spots were visualized under UV (254-366nm) and by use of spray (10% sulphuric acid) followed by heating in an oven at 110^{o}C for 5 minutes. The various TLC solvent systems used to develop the plates were: HEX 100%, HEX and EtOAc (9:1, 8:2 and 7:3), EtOAc 100%, EtOAc and methanol(MeOH) 9:1, chloroform ($CHCl_3$)and MeOH 9:1 and $CHCl_3$ 100%.

Column Chromatography of Ethanolic Fraction

The extract with higher inhibitory action (EtOH extract (4g)) of *A.senegalensis* leaf was adsorbed onto silica gel (60-120 mesh size) and chromatographed over silica gel packedGlass column (750 cm x 3.5cm)with sintered disc at the bottom using solvent systems of increasing polarity HEX:EtOAc and EtOAc:MeOH with increments of 10% in each step and finally washed with MeOH 100% to yield 85 fractions of 20 ml each and were pooled together base on similarity profile and number of spots to give 7 major fractions.Then the inhibitory potentials of the pooled fractions were tested on α-amylase, α-glucosidase and aldose reductase activities.

3.2.9 Mechanism of α-glucosidase and α-amylase Inhibitions

Fraction-**F** was found to be the most active fraction among the fractions collected; it was therefore subjected to kinetics experiments to determine its type of inhibitionon α-amylase and α-glucosidase.The experiment was conductedaccording to the protocols as described above at a constant concentration of sample fraction(240 µg/mL) with variable concentration of substrate for the both α-amylase and α-glucosidase (0.125–1 % starch and 0.625–5 mmol/l of *p*NPG respectively), with or without the presence of sample fraction. The varying concentration of p-nitrophenol and maltose were used to construct calibration curves,(appendix A and B respectively); used to determine the initial rate of reactions, for the α-glucosidase and α-amylase inhibition assays, respectively. The initial velocity data obtained were used to construct Lineweaver-Burk plots to determine the kinetics constant (K_m and V_{max}) of the enzyme as well as the k_i (inhibition binding constant as a measure of affinity of the inhibitor to the enzymes) and the type of inhibition for both enzymes.

3.2.10 Gas chromatography-Mass spectrometry analysis

The fraction that had the best α-amylase and α-glucosidase inhibitory activity (Fraction-F) was subjected to Gas chromatography-Mass spectrometry (GC-MS) analysis (Model; QP 2010 series, Shimadzu, Tokyo, Japan) equipped with a VF-5ms fused silica capillary column of 30 m length, 0.25 mm diameter, and 0.25 mm film thickness. The carrier gas was ultra-pure helium at a flow rate of 0.7 mL min^{-1} and a linear velocity of 37 cm s^{-1}. The injector temperature was set at 250 °C. The initial oven temperature was 60 °C, which was programmed to 280 °C at the rate of 10 °C min^{-1} with a hold time of 3 min. Injections of 2 µL were made in the split less mode with a manual split ratio of 20:1. The mass spectrometer was operated in the electron ionization mode at 70 eV and electron multiplier

voltage at 1859 V. Other MS operating parameters were as follows: ion source temperature 230 °C, quadrupole temperature 150 °C, solvent delay 4 minute and scan range 50-700 amu. The spectrum of the compounds was compared with the spectrum of National Institute of Standard and Technology (NIST) library database. Compounds were identified by direct comparison of the retention times and interpretations of mass spectrum of fraction were conducted using the database of National Institute of Standard and Technology (NIST) library having more than 62,000 spectral patterns.

3.2.11 Statistical analysis

The results of triplicate determinations were presented as mean ± standard deviation (SD). The analysis of variance (ANOVA) (SPSS version 20.0) was used to compare the means of the groups. Post-hoc tests were performed using Duncan's Multiple Range Test; a probability level of less than 5% ($P < 0.05$) was considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Percentage Yield of *A. senegalensis* Leaf Extracts Recovered:

The percentage yield recovered from the *A. senegalensis* leaf extracts of HEX, EtOAc and EtOH(1.90, 1.88 and 18.67) respectively is presented in Table 4.1. With Ethanolic extract had the highest percentage yield.

4.2 Inhibition of α-Amylase, α-Glucosidase and Aldose Reductase by the Extracts

The data on the *in vitro* inhibition of α-amylase, α-glucosidase and aldose reductase by various extracts derived from*A.senegalensis* leaf is presented inFigures 4.1, 4.2, 4.3, with ethanolic extract showing the highest inhibitory effect when compared to the other extracts.

It was observed from Table 4.2that EtOH extract had significantly ($p<0.05$) lower IC_{50} (204.04±6.38 and 97.91±2.40)compared to HEXextract (359.96±14.16and 4145.43±136.94) and EtOAc extract (617.72 ±62.93 and 253.57±36.30) for α-amylase and α-glucosidase inhibitory activities respectively. However, the IC_{50}(204.04±6.38 and 97.91±2.40) recorded for the EtOHextract was significantly ($p<0.05$) highercompared to the standard acarbose(151.31±4.01 and121.29±0.10)on α-amylase and α-glucosidase respectively (Table 4.2). Similarly, the IC_{50} (119.58±12.85) exhibited by the EtOH extract on aldose reductase was found to be significantly ($p<0.05$) lower compared to HEX extract (253.23±6.62) and EtOAc extract (355.22±67.84) although higher than that of standard quercetin (96.41±15.67) (Table 4.2).

Table 4.1: Percentage Yield of *A. senegalensis*LeafExtracts Recovered

Solvent	**Yield (%)w/w**
n-Hexane	1.90
Ethyl acetate	1.88
Ethanol	18.67

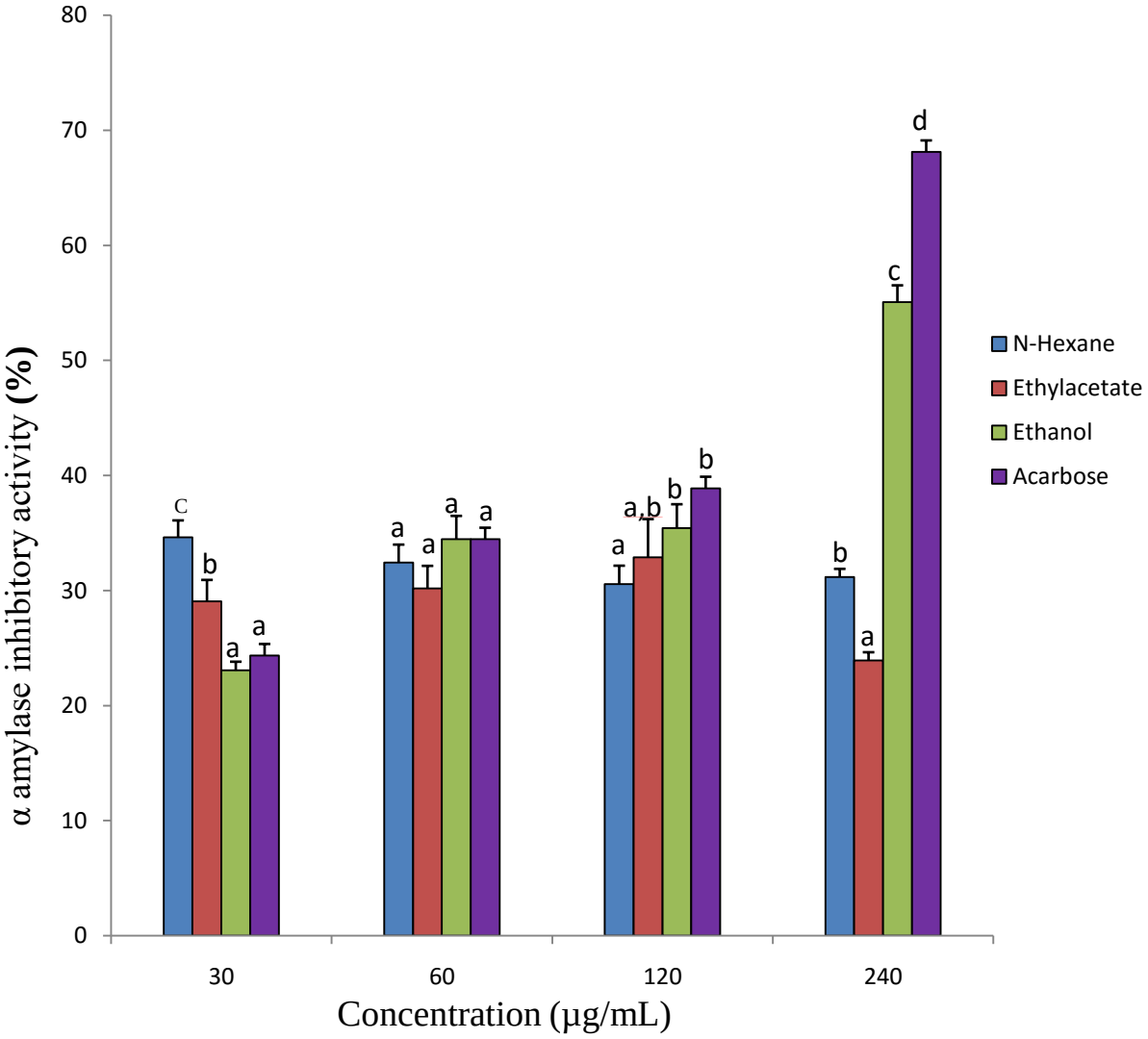


Figure4.1:The Effect of *A.senegalensis* Leaf Extracts on *In vitro* α-Amylase Activity

Data are presented as mean ± SD of triplicate analysis. Different letters presented above the bars for a given concentration for each extract are significantly different from each other (Duncan's multiple range *posthoc*tests,$p < 0.05$);the same letters stand for non-significant difference

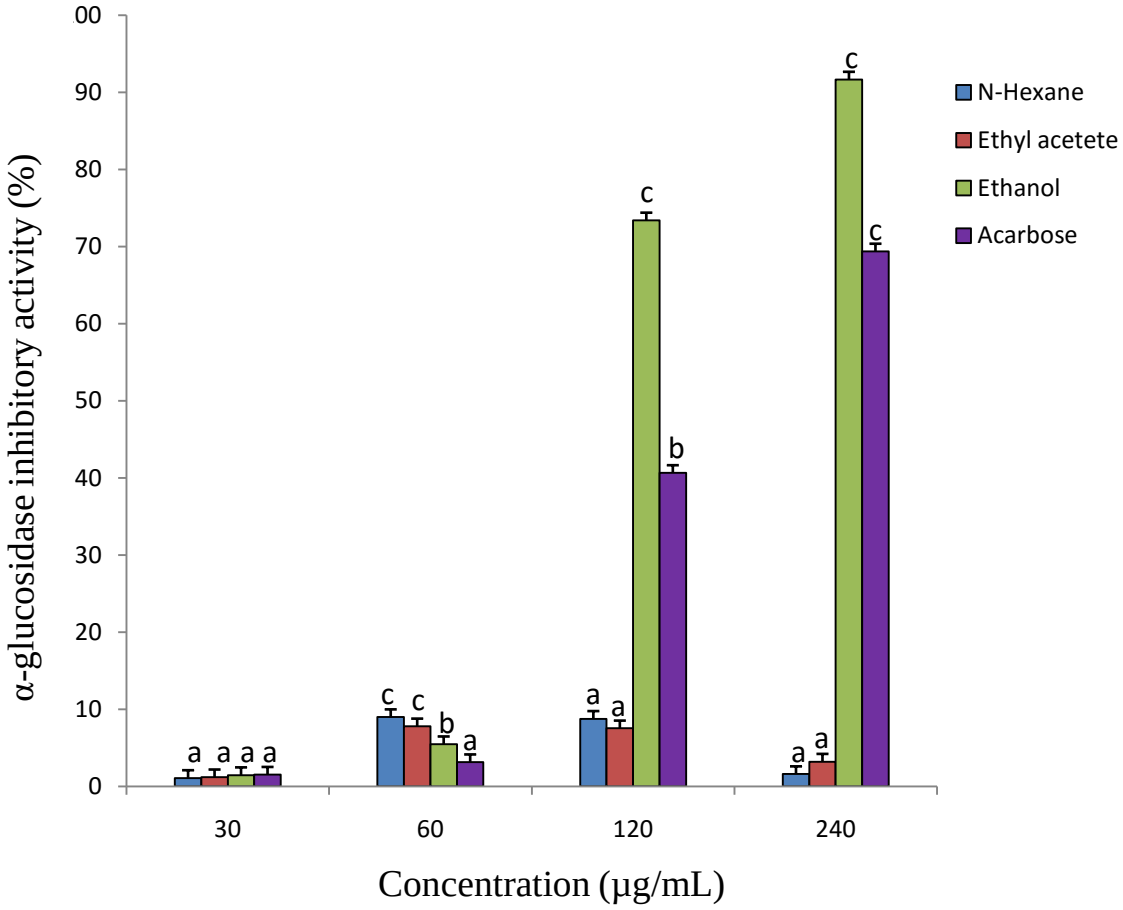


Figure4.2: The Effect of *A.senegalensis* Leaf Extracts on *In vitro* α-Glucosidase Activity

Data are presented as mean ± SD of triplicate analysis. Different letters presented above the bars for a given concentration for each extract are significantly different from each other ((Duncan's multiple range *posthoc*tests,$p < 0.05$);the same letters stand for non-significant difference

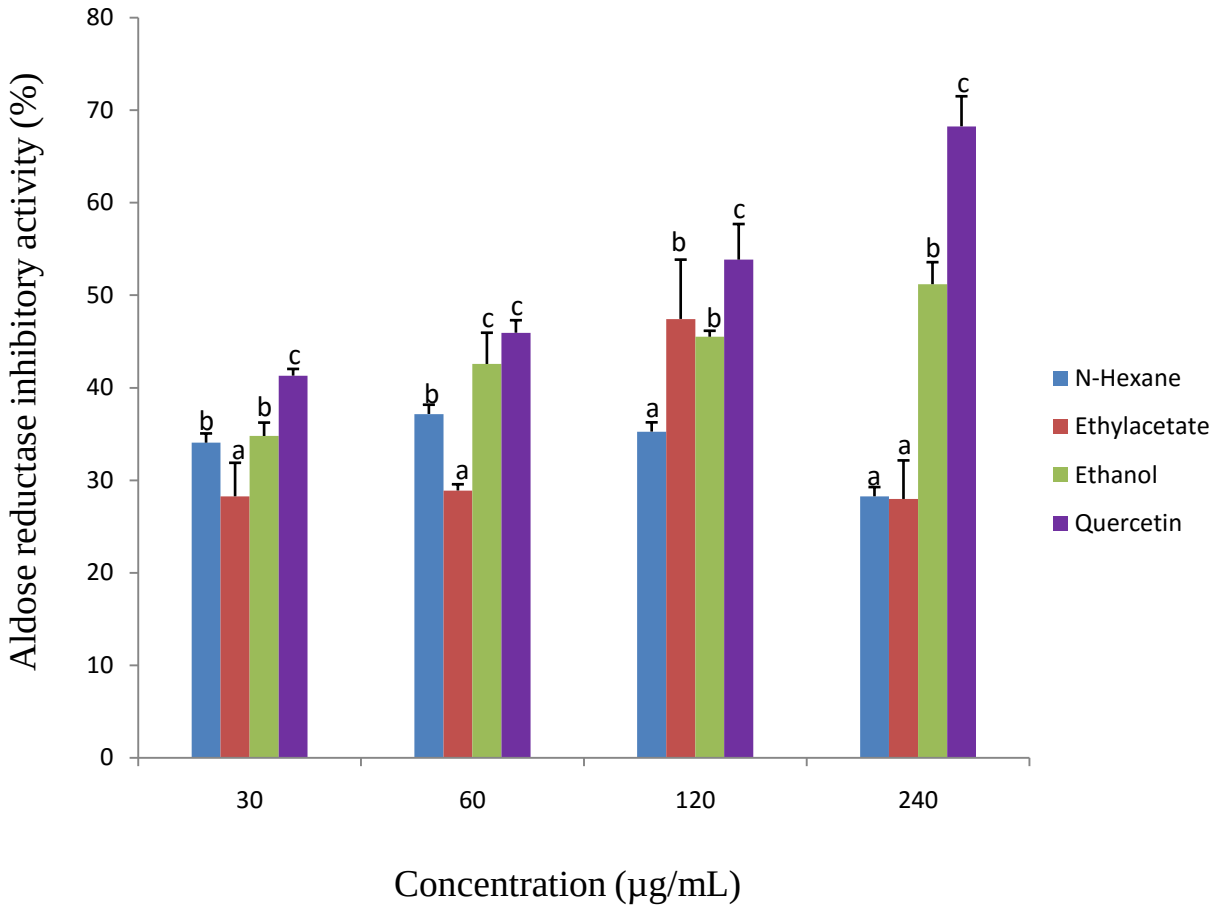


Figure4.3: The Effect of *A.senegalensis* Leaf Extracts on *In vitro* Aldose Reductase Activity

Data are presented as mean ± SD of triplicateanalysis. Different letters presented above the bars for a given concentration for each extract are significantly different from each other (Duncan's multiple range *posthoc*tests,$p < 0.05$); the same letters stand for non-significant difference

Table 4.2: IC_{50}Values for the Inhibition of α-Amylase, α-Glucosidase and Aldose Reductase Activity by the Extracts of the Leaf of *A.senegalensis*

Extract/standard	**IC_{50} (µg /mL)**		
	α-amylase	α-glucosidase	Aldose reductase
n-Hexane	359.96±14.16^{c}	4145.43±136.94^{d}	253.23±6.62^{c}
Ethyl acetate	617.72±62.93^{d}	253.57±36.30^{c}	355.22±67.84^{d}
Ethanol	204.04±6.38^{b}	97.91±2.40^{a}	119.58±12.85^{b}
Acarbose	151.31±4.01^{a}	121.29±0.10^{b}	___
Quercetin	___	___	96.41±15.67^{a}

Results are presented as mean ± SD. Values with different letters down the column are significantly different from each other(Duncan's multiple range *post hoc* tests,$p< 0.05$);the same letters stand for non-significant difference

4.3Thin Layer and Column Chromatography Results of the Ethanolic Extracts of the Leaf of *A. senegalensis*

Plate II shows the thin layer column chromatography (TLC) profiles of the EtOH extract of the leaf of *A. senegalensis* with different solvent systems. These TLC profiles paved the way in selecting Hexane (HEX), EtOAc and MeOH solvents for the mobile phase at the column chromatographic stage.

Plate III shows the TLC profiles of the fractions from fraction 1 to fractions 30 and how the fractions were pooled to fractions (A, B, C, D), TLC profiles of fractions 31 to 42 and how they were pooled to fractions E andTLC profiles of fractions 43 to 85 and how they pooled to fraction (F and G) based on their similarity profile and numbers of spots.

Plate IV shows the TLC profile of the recovered fractions A - G after pooling Fraction A had no spots while the rest of the pooled fractions were had different spotson TLC profiles.

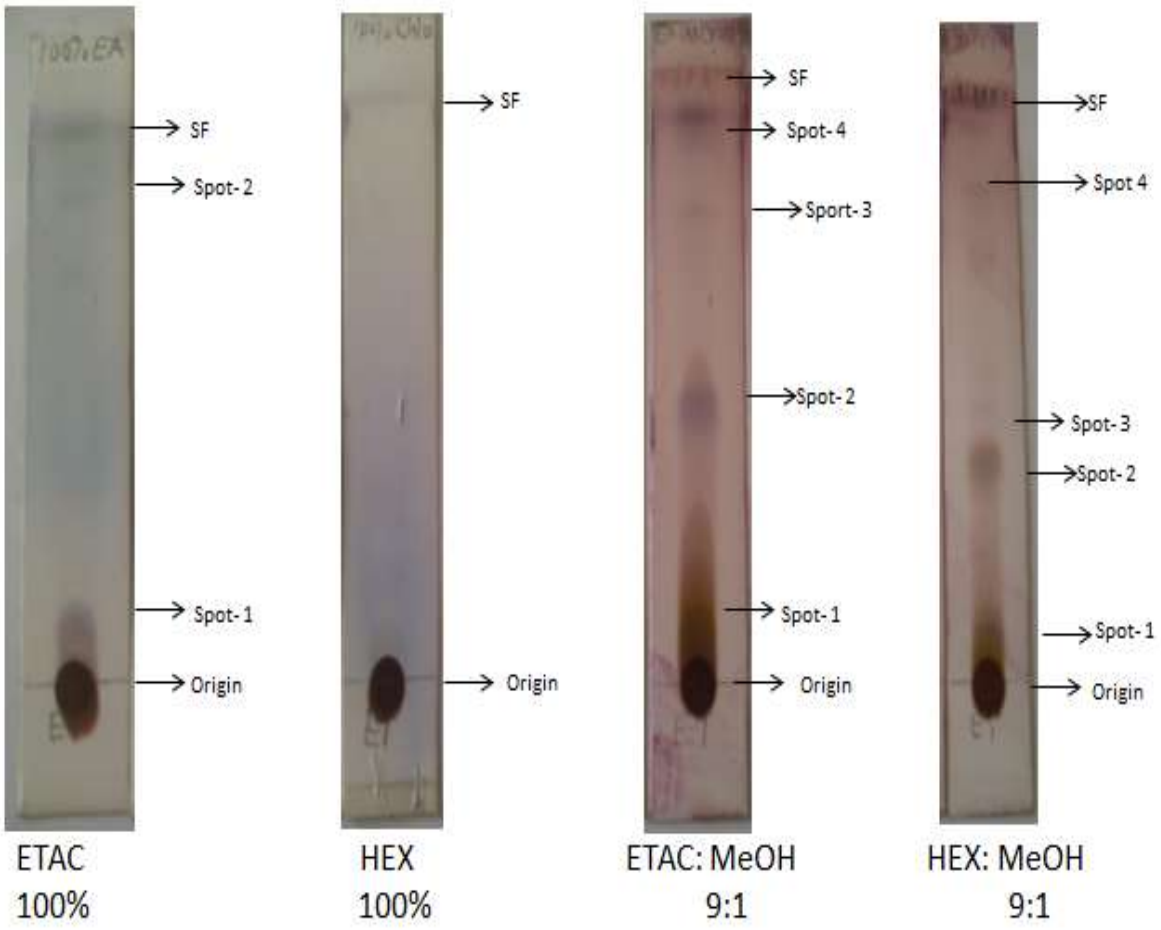
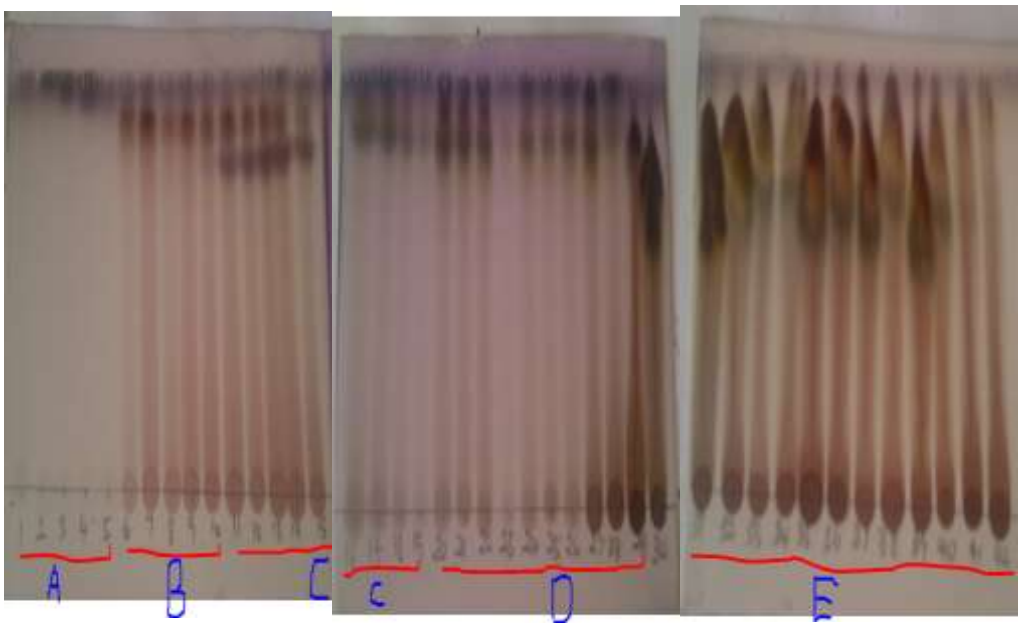
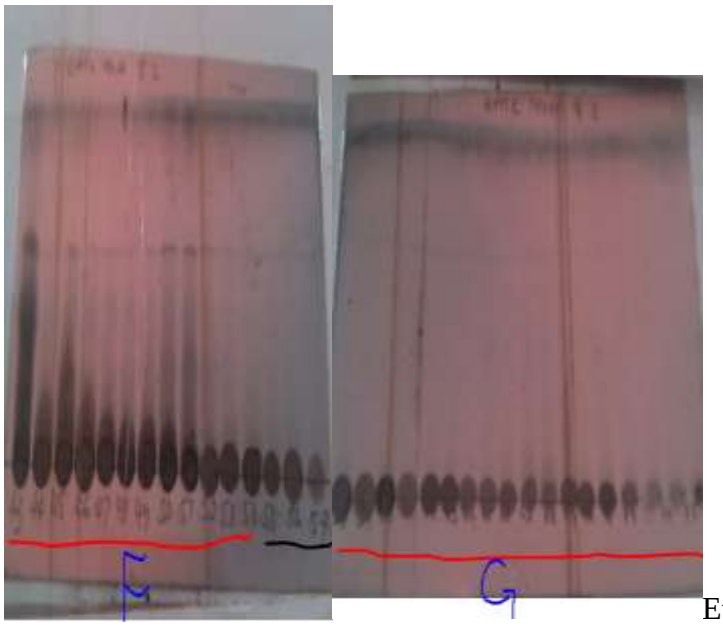


Plate II: Thin Layer Chromatogram of the *A.senegalensis* Leaf Extract using Different Solvent Systems.

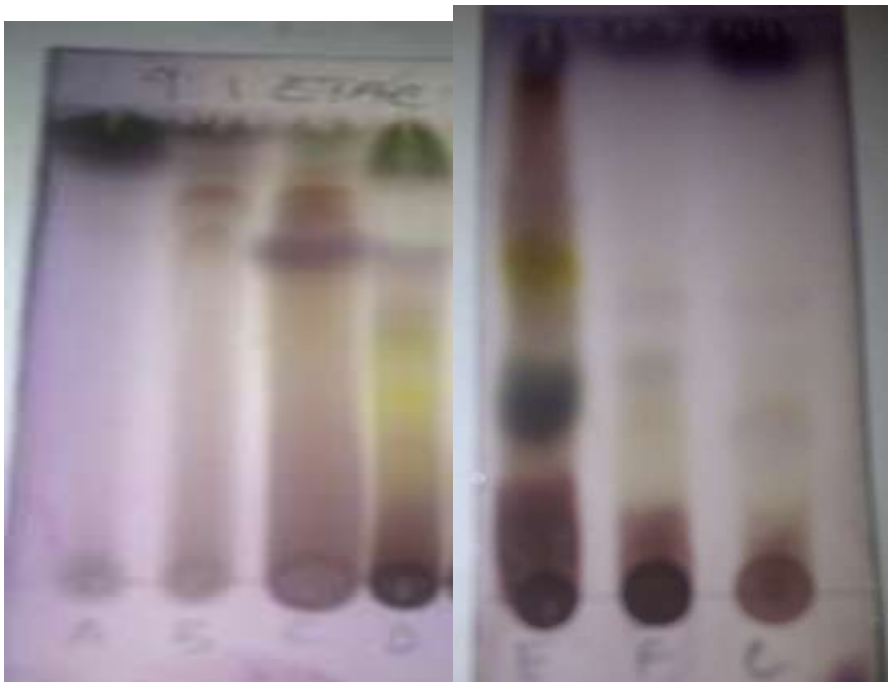


EtOAc 100% EtOAc 100% EtOAc: MeOH (8:2)



EtOAc: MeOH (8:2) EtOAc: MeOH (8:2)

Plate III: Thin Layer Chromatogram of Ethanolic Leaf Fractions of *A. senegalensis*



EtOAc: MeOH (9:1) EtOAc: MeOH: W (10:1:1)

Plate IV: Thin layer Chromatogram of pooled fractions of the Ethanolic extract of *A.senegalensis* Leaf (A-G)

4.4The Inhibition of α-Amylase, α-Glucosidase and Aldose Reductase by the Fractions of *A. senegalensis* Ethanolic Leaf Extract.

Figures 4.4, 4.5 and 4.6 shows the variation of the percentage inhibition onα-amylase, α-glucosidase and aldose reductase as the concentration of the fractions increases. It was observed that fraction-**F** hadsignificantly ($p< 0.05$) lower IC_{50} values compared to other fractions against the activities of α-amylaseand α-glucosidase while fraction-**B** hadsignificantly ($p< 0.05$) lower IC_{50} values on Aldose reductase activity (Table 4.3).

Table 4.3 revealed the effect of the different pooled fractions on α-amylase, α-glucosidase and aldose reductase. The potency of the extracts was evaluated by determining the inhibitory concentrations (IC_{50}) of all the fractions on the enzymes.The IC_{50} value recorded for the fraction-F of EtOH extract was significantly ($p< 0.05$)higher compared to the standard acarbose on α-amylase, α-glucosidase.

Similarly, the IC_{50} value exhibited by the fraction-**B** of EtOH extracton aldose reductase was found to be significantly ($p< 0.05$) lower compared to fraction-**F** and other fractions including standard quercetin (Table 4.3). However, fraction-**F** was the most active against the two enzymes having the least IC_{50} values and it was selected for the enzymes kinetics, and GC-MS analysis.

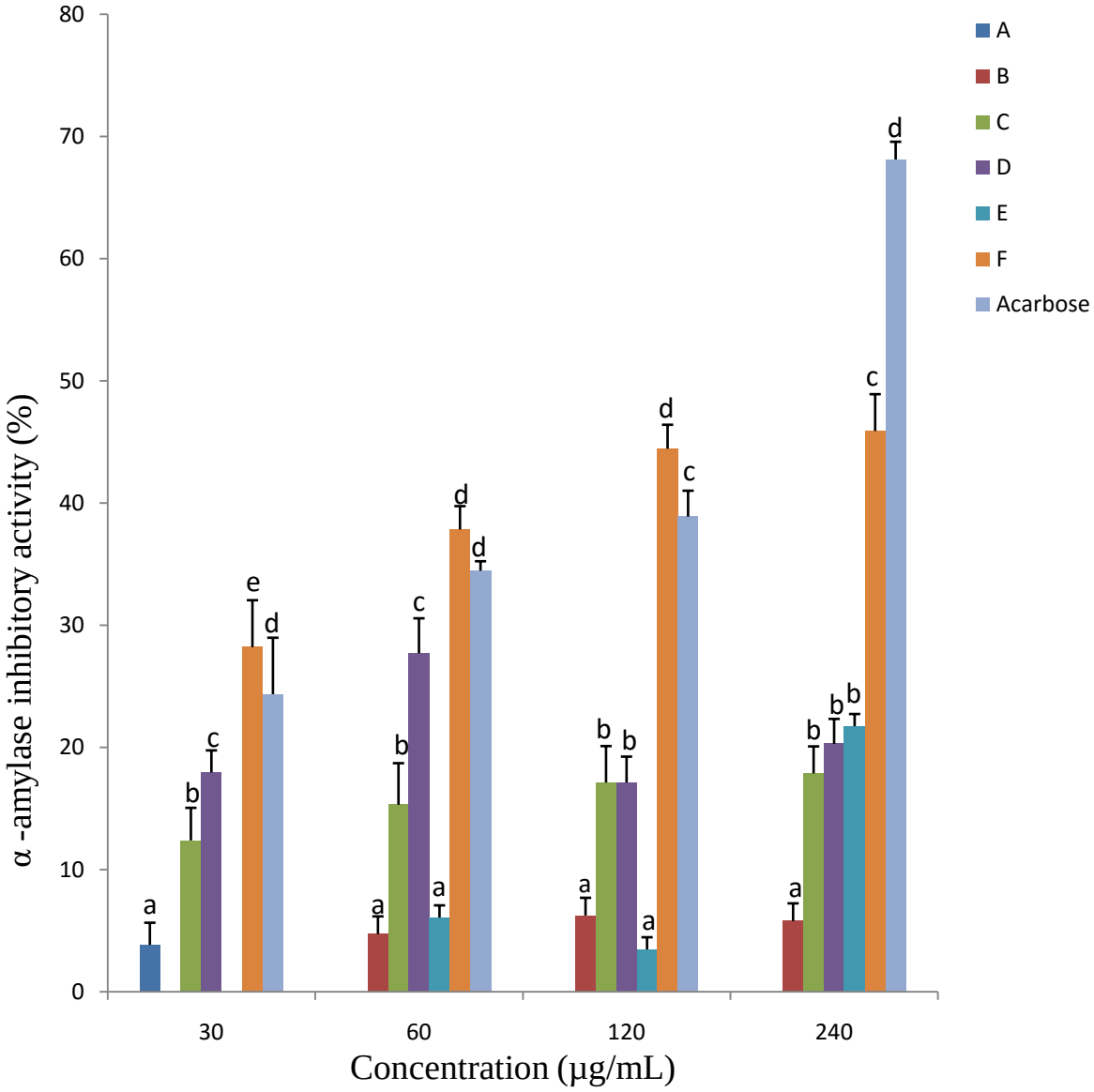


Figure4.4: The Effect of *A.senegalensis* Leaf Fractions on *In vitro* α-Amylase Activity

Data are presented as mean ± SD of triplicate analysis. Different letters presented above the bars for a given concentration for each extract are significantly different from each other (Duncan's multiple range *post hoc* tests,$p < 0.05$);the same letters stand for non-significant difference

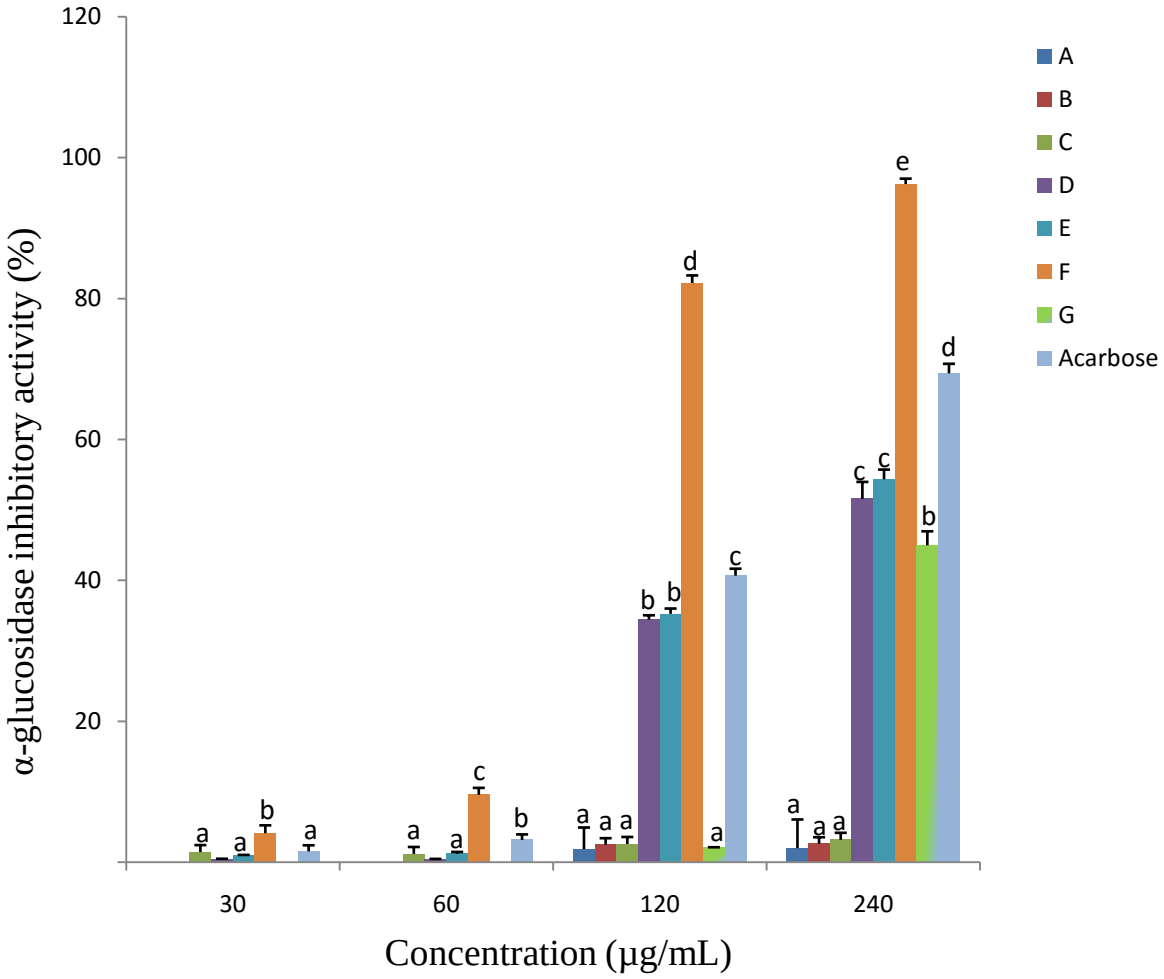


Figure4.5:The Effect of *A.senegalensis* Leaf Fractions on *In vitro* α-Glucosidase Activity

Data are presented as mean ± SD of triplicate analysis. Different letters presented above the bars for a given concentration for each extract are significantly different from each other (Duncan's multiple range *post hoc* tests,$p < 0.05$);the same letters stand for non-significant difference

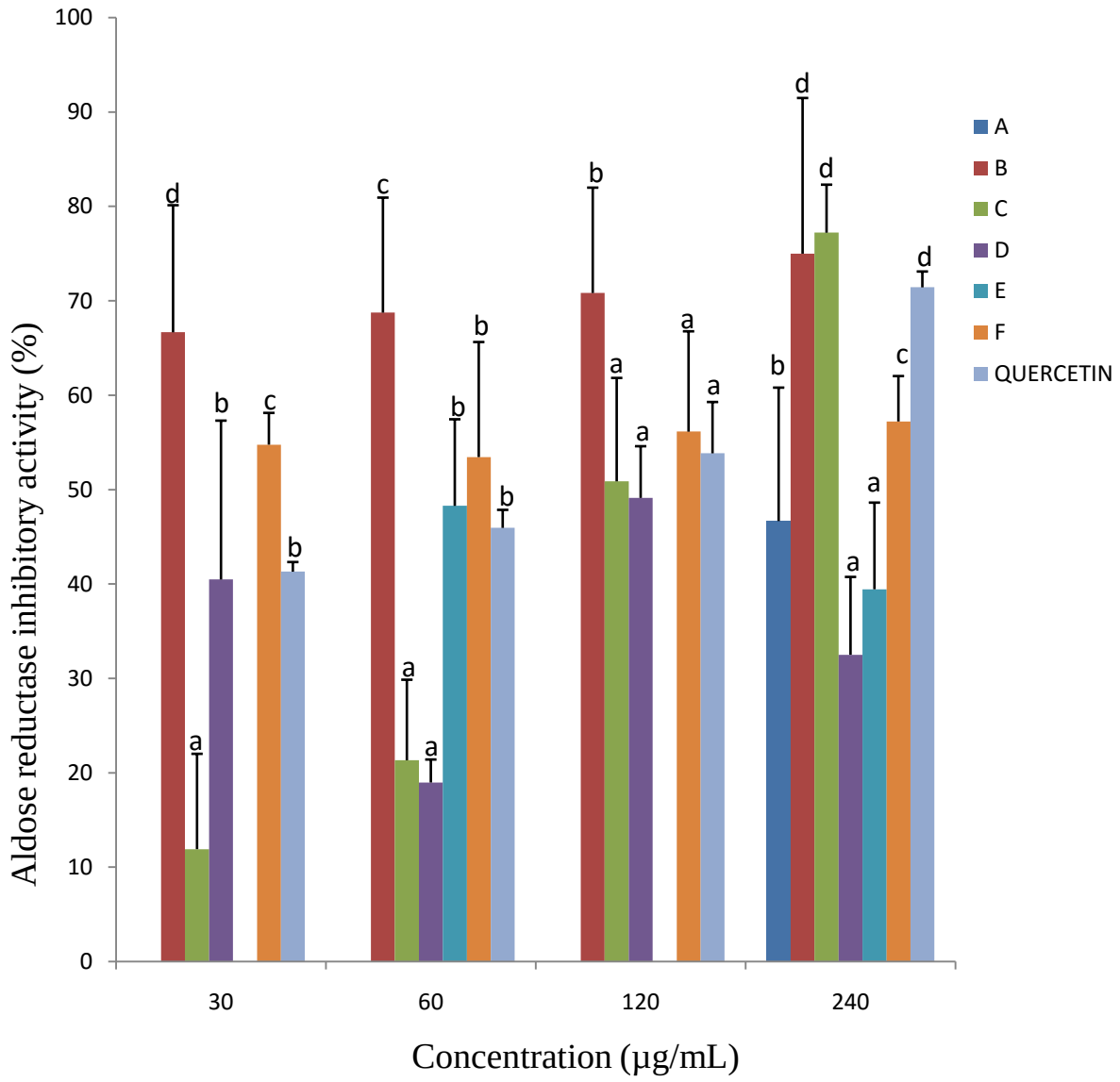


Figure4.6: The Effect of *A.senegalensis* Leaf Fractions on *In vitro* Aldose Reductase Activity

Data are presented as mean ± SD of triplicate analysis. Different letters presented above the bars for a given concentration for each extract are significantly different from each other (Duncan's multiple range *post hoc* tests,$p < 0.05$);the same letters stand for non-significant difference

Table 4.3: IC_{50}Values of the Inhibition of α-Amylase, α-Glucosidase and Aldose Reductase Activity by the Ethanolic Fractions of the Leaf of *A.senegalensis*

Fraction/standard	**IC_{50} (µg /mL)**		
	α-amylase	α-glucosidase	Aldose reductase
A	527.11±34.13[d]	4589.89±283.90[g]	265.19±21.62[d]
B	1437.36±150.08[c]	3816.37±168.60[f]	71.86±19.61[a]
C	5659.60±1390.48[f]	283.41±47.64[d]	151.66±10.72[c]
D	19779.90±10983.6[g]	224.70±0.39[c]	132.79±5.61[c]
E	870.66±16.18[e]	227.83±2.71[c]	236.75±38.32[e]
F	237.14±31.19[b]	88.25±0.59[a]	125.76±14.10[bc]
G	———	552.39±19.35[e]	___
Acarbose	151.31±4.01[a]	121.29±0.10[b]	___
Quercetin	___	___	96.41±15.67[ab]

Results are presented as mean ± SD. Values with different letters down the column are significantly different from each other (Duncan's multiple range *post hoc* tests,$p< 0.05$); the same letters stand for non-significant difference

4.5EnzymeKinetics Study of α-Amylase and α-Glucosidase Inhibition

The kinetics results on the mode of inhibition of fraction-**F** on α-amylase and α-glucosidase action is presented in Figures 4.7 and 4.8. Fraction-**F**exhibited a competitive inhibition on α-amylase and non-competitive mode of inhibition on α-glucosidase.

Table 4.4 shows that fraction-**F** had higher K_m(0.24) with unchangedV_{max}(27.03) compared to the K_m (0.11) of the substrate (starch) and computed k_i (8.46) for inhibiting α-amylase. Then, alsoV_{max} (1.10)for the fraction-**F**decreased compared to theV_{max} (2.30) of substrate (*p*NPG) withunaffectedK_m (3.70) andk_i (1.26)on α-glucosidase inhibition.

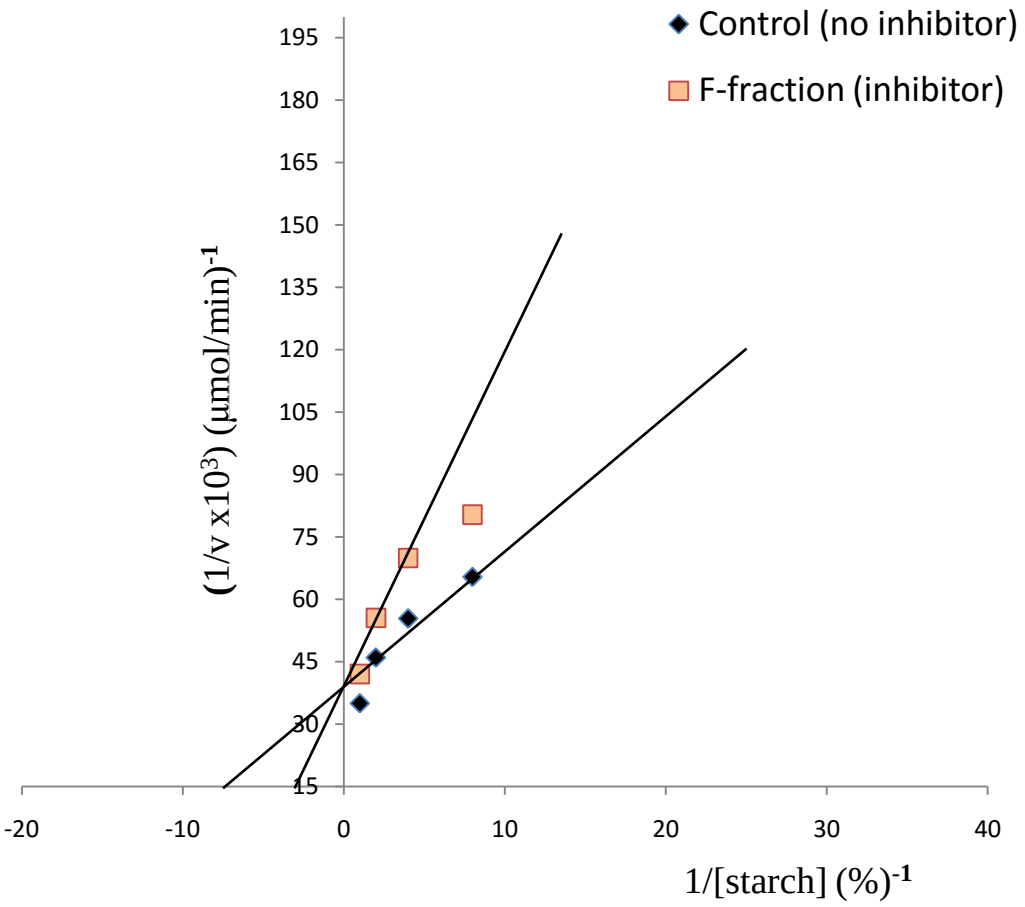


Figure 4.7: Lineweaver-Burk Plot for α-Amylase in the Absence and Presence of Inhibitor (Fraction-**F**)

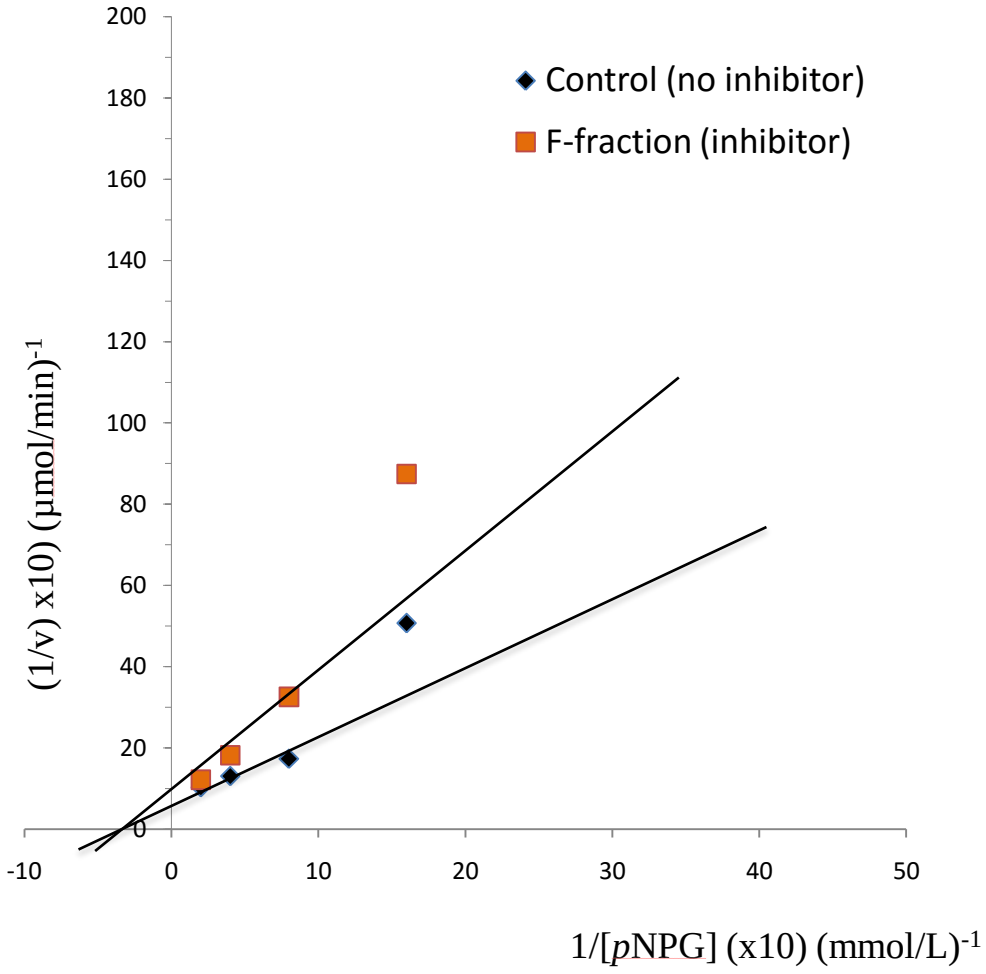


Figure 4.8: Lineweaver-Burk Plot for α-Glucosidase in the Absence and Presence of Inhibitor (fraction-**F**)

Table 4.4:Enzyme kineticsof α-Amylase and α-Glucosidase Inhibition by the Active fraction-F of *A.senegalensis* Leaf

Fraction	**α-amylase inhibition**			**α-glucosidase inhibition**		
	K_m (%)	V_{max} (µmol/min)	K_i (µg/mL)	K_m (mmol/L)	V_{max} (µmol /min)	K_i (µg /mL)
Control	0.11	27.03	___	3.70	2.30	___
Fraction-**F**	0.24	27.03	8.46	3.70	1.10	1.26

4.6 Phytochemical Screening of Fraction-F of *A.senegalensis* Leaf

The result of Phytochemical Screening offraction-**F** is presented in Table 4.5.Steroids, triterpenes and phenolic compounds were found to be present.

4.7Possible compounds present in fraction-F by Gas Chromatography-Mass Spectrometry (GC-MS) Analysis.

The GC-MS chromatogram of fraction-**F** of *A. senegalensis* leafshowed the presence of some compounds. The interpretation of mass spectrum was done by comparing with the data from National Institute standard and Technology (NIST) database. The peaks number, retention time (RT), compound name, mass to charge ratio and similarity index of the compounds of fraction-**F** of the ethanolic extract of the leaf are shown in Figure 4.9 and Table 4.6

The bioactive compounds identified from fraction-**F** were1,2-Benzenedicarboxylic acid, butyl octyl ester,1, 2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester,Hexadecanoic acid, methyl ester and Methyl 9-methyltetradecanoate.

Table 4.5: Some Phytochemicals Identified in Fraction-**F** of *A.senegalensis* Leaf Extract

	Test	Observation	Inference
1.Steroids and triterpenes	Lieberman burchards spray	Blue green and purple colouration	+ve
2.Flavanoid	Aluminum chloride spray	Yellow fluorescence under VU lamp	-ve
3.phenolic compound	Ferric chloride spray	Blue black colouration	+ve
4.Alkaloids	Dragendorff	Orange coloured	-ve
5.Anthraquenones	Bontragers spray	No yellow colored	-ve

Key
+ve present
-ve absent

Table 4.6: Compounds Identified by GC-MS in Fraction-**F** of *A.senegalensis*Leaf Extract.

S/no	Retention Time	Compound	Mass/amu	Similarity Index
1	32.581	1,2-Benzenedicarboxylic acid, buty l octyl ester	334 $[M]^+$	90
2	32.581	1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester	278 $[M]^+$	90
3	33.668	Hexadecanoic acid, methyl ester	270 $[M]^+$	81
4	37.674	Methyl 9-methyltetradecanoate	242 $[M]^+$	59

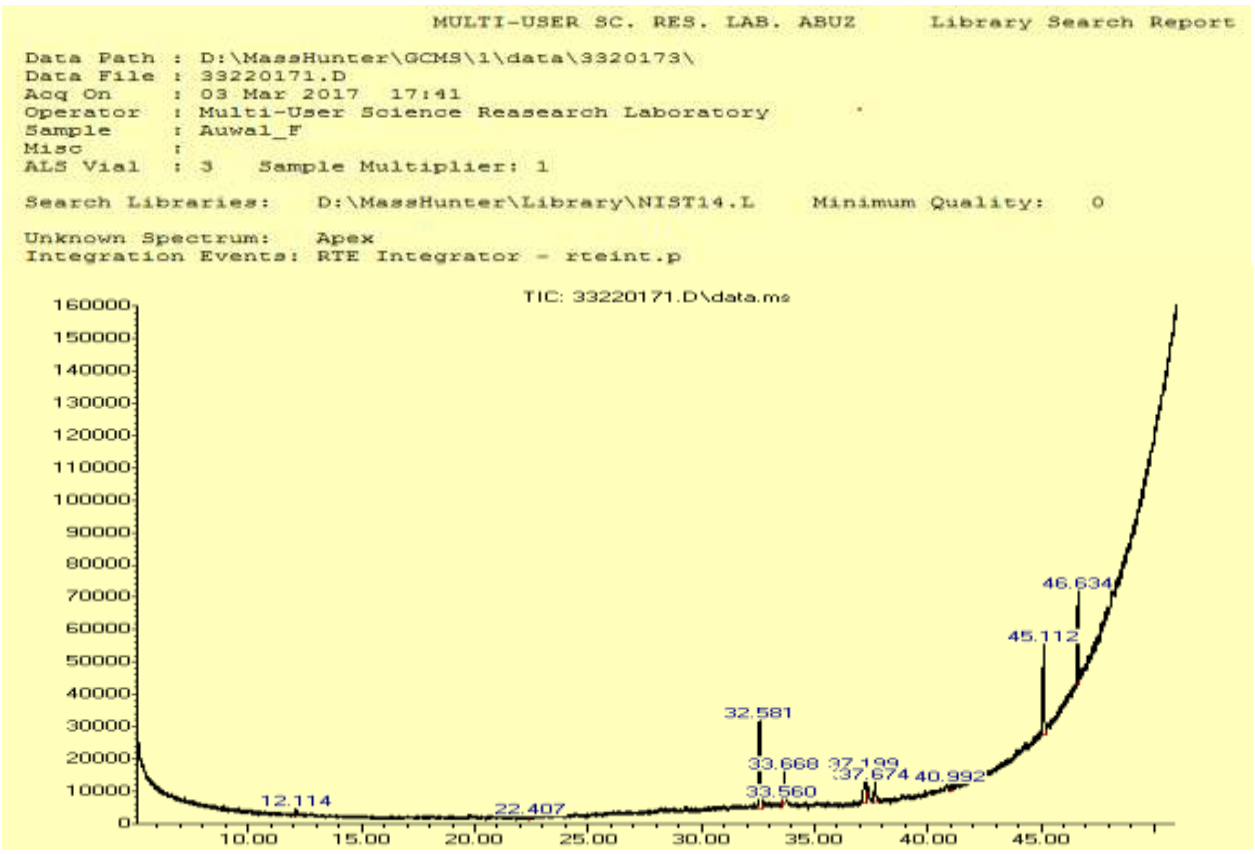


Figure 4.9: GC-MS Total Ion Chromatogram (TIC) of Fraction-**F** from *A. senegalensis* Leaf

CHAPTER FIVE

5.0 DISCUSSION

Hyperglycemia plays an important role in the progression of diabetes and diabetic complications; control of postprandial hyperglycemia has been shows to be a practical way in the management of diabetes and its complications (Jaspinder*et al.,* 2014).The plant,*A.senegalensis* have been used by folk medicine in the treatment of DM. Report from previous research has shown that the ethanol extract of the stem bark of *A.senegalensis* exerts a hypoglycemic,antihyperglycaemic, and glucose suppressive activities (Ibrahim*et al.*,2017). According to the data from our *in vitro* study, shows that the HEX, EtOAc and EtOH extract of*A. senegalensis* had differentpercentage yield. The selection of the solvents for extraction was based on the differences in their polarities. Moreover, based on our recent search *A. senegalensis* alcoholic extracts showed greater promising potentials compared to the HEX and EtOAc extract, with EtOH extract having the highest activity (More *et al.,* 2008; Yeo *et al,.* 2011; Suleiman *et al.,* 2014). This could be attributed to the nature of EtOH polarity that allows the extraction of both polar and some non-polar compounds compared to either strongly polar or non-polar solvents. Occasionally this increases extraction yield as well, leading to higher pharmacological activity and is in coherent with our present data.Theinhibitory effectiveness of varioussolvent extracts from *A. senegalensis* leaf was determined by calculating IC_{50} values. The significantly ($p<0.05$) lower IC_{50} valuesrecorded for the EtOH extract inhibition ofα-amylase, α-glucosidase and Aldose reductase activities compared to other extracts,indicated that most of the bioactive compounds are present in this extract.In addition, the lower IC_{50} value exhibited by EtOH extract on the α-glucosidaseis of pharmacological interest as greater inhibitory activity

toward α-glucosidase activity may lead to delayed hydrolysis of complex dietary carbohydrate to the absorbable monosaccharides. Consequently, this limits the intestinal glucose absorption and thus, reduced postprandial hyperglycemia.On the other hand, inhibition of aldose reductase activity has been reported to mitigate diabetes-induced oxidative complications (Akileshwari*et al.*, 2012). Interestingly, the lower IC_{50}value exhibited by the EtOH extract against Aldose reductase signify the higher availability of active ingredients with inhibitory potentials, which is in line with previous data that showed potent antioxidant and anti-inflammatory actions of *A. senegalensis*(Potchoo*et al.*, 2008; Ajboye*et al.*, 2010; Yeo *et al.*, 2011). Since the ethanolic extract of the leaf of *A. senegalensis* had the least IC_{50} against the enzymes, it was selected for the column chromatography in order to have a better insight into the part of the extract that is responsible for the inhibition of the enzymes. Hence, theethanolic extract was subjected to column chromatography using different solvents of varying polarity to separate the compounds in the extract. The solvents used as the mobile phase were hexane, ethyl acetate and ethanol. Based on the results, it can be hypothesized that fraction-F, in part, diminishes glucose absorption through the intestine by inhibition of the activity of intestinal α-amylase and α-glucosidase (the same mechanism acarbose exhibits). Moreover, fraction-B also showed potential for inhibiting the activity of aldose reductase.The inhibitory activity of fraction-F may be attributedto the presence of phenolic compounds;Phenolic compounds have an electron donor capability and are readily oxidized to form phenolate ion or quinone, which is an electron acceptor (Michalak, 2006). Thus, they have theability to block or enhance enzymes activity, due to the presence of nucleophillic and electrophilic groups at the active site of specific enzymes responsible for

digestion of carbohydrates.However, since the fraction-Fhad significant inhibitory potentials it was then, selected for thestudies of the mode of inhibition on the α-amylase and α-glucosidase activities and the possible compounds present by GC-MS analysis.

The kinetics data of the inhibitory mode of α-amylase enzyme by the fraction-F are presentedwith uncharged $V_{max}$and higher$K_m$values onα-amylase activity. This implies that certain compounds in fraction-F compete with the active site of α-amylase thereby, preventing the binding of the substrate and subsequently reducing the activity of the enzyme.Additionally, fraction-F showed a non-competitive type of inhibition for α-glucosidase activity where The K_m value is unchanged and the higher V_{max}value thanthat of the control. Hence, it is suggested that some compound(s) in fraction-F bound to other site(s), other than the active site of enzymes and induced conformational change(s) in the three-dimensional structure of the enzyme and thus inhibited its activity (Ibrahim *et al.*, 2014). The equilibrium constants for inhibitor binding (K_i) of fraction-F were lower for α-glucosidase than α-amylase, indicating greater stability of the enzymes-substrate complex for α-glucosidase compared to α-amylase.

In fraction-F, prominent compounds were identified by GC-MS and were volatile aromatics (1,2-benzenedicarboxylic acid, butyl octyl ester and 1,2-benezenedicarboxylic acid, bis (2-methylpropyl) esters) and fatty esters (hexadecanoic acid, methyl ester and methyl 9-methyltetradecanoate) which are components of essential oils. Interestingly, essential oils or some of its active ingredients such as 1,2-benzenedicarboxylic acid, butyl octyl ester and hexadecanoic acid, methyl ester were shown to reduce hyperglycemia and improve diabetes related complications (Kim *et al.*, 2014; Tahir *et al.*, 2016; Al-hajj *et al.*, 2016). Therefore, despite the limitation of our present study to specifically isolate the

active ingredient(s) responsible for the observed inhibitory effects of fraction-F, we conclude that this activity is attributed to either individual or combined action of the compounds detected.

CHAPTER SIX

6.0SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 SUMMARY

1. EtOH leaf extract of*A. senegalensis* had higher percentage yield compare to HEX, EtOAc exrtacts

2. EtOH leaf extract of*A. senegalensis* had inhibitory effectiveness on α-amylase, α-glucosidase and Aldose reductase enzymes important in the pathophysiology of diabetes mellitus.

3. The enzyme kinetics of Pooled fraction-F of *A. senegalensis* leaf extract revealedcompetitive and non-competitive mode of inhibition for α-amylase and α-glucosidase respectively, with lower inhibition binding complex (K_i) for α-glucosidase than α-amylase.

4. Compounds identified by GC-MS in Pooled fraction-F of *A. senegalensis* leaf extract were 1,2-benzenedicarboxylic acid, butyl octyl ester and 1,2-benezenedicarboxylic acid, bis (2-methylpropyl) esters, hexadecanoic acid, methyl ester and methyl 9-methyltetradecanoate.

6.2 CONCLUSION

The result of the observed Inhibitory effects, IC_{50}values of ethanol extract and that of fraction-F of *A. senegalensis* and the compounds detected that were shown to reduce hyperglycemia and improve diabetes related complicationof these finding, may partly

involved in the mechanism behind the hypoglycemic effect and thisprovide some scientific support to their traditional use.

6.3 RECOMMENDATIONS

In view of this study we recommend further study on isolation of the specific bioactive compounds anddetailed identification of the bioactive compounds using more sophisticated techniques such as nuclear magnetic resonance(NMR).

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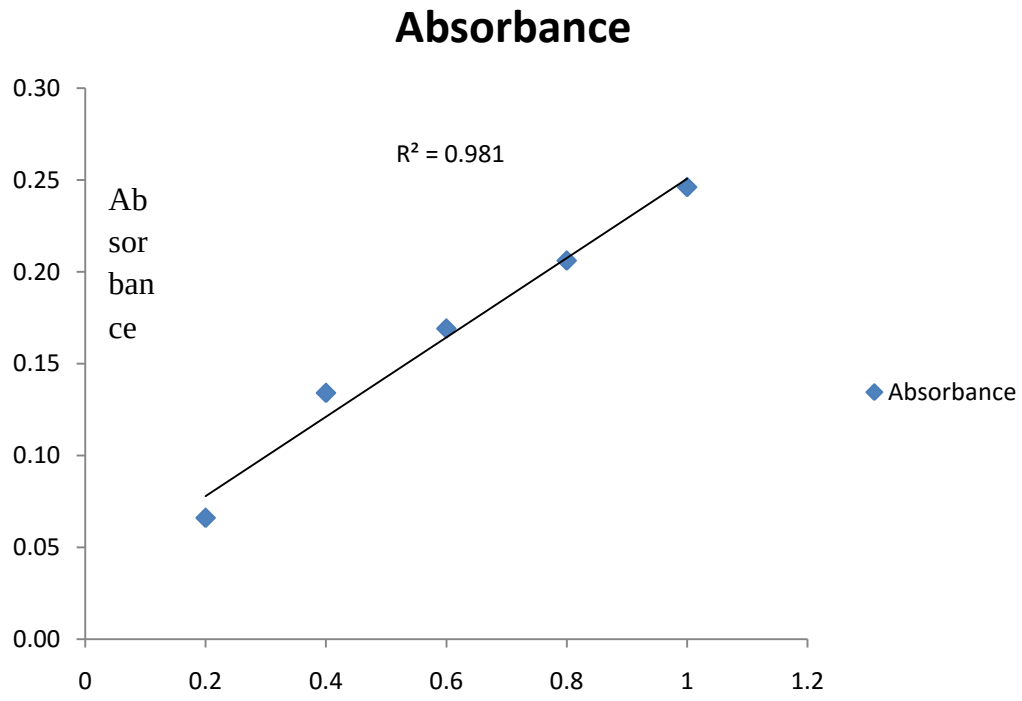
APPENDIX

Appendix A

Maltose standard curve



Concentrations

Appendix B

Paranitro phenol standard curve