EVALUATION OF FRACTIONATED METHANOL EXTRACTS OF *VERNONIA AMYGDALINA* AND *OCIMUM GRATISSIMUM* LEAVES IN TYPE 2 DIABETIC WISTAR ALBINO RATS

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

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

OCTOBER, 2017

DECLARATION

I declare that the work in this thesis entitled 'Evaluation of fractionated methanol extracts of *Vernonia amygdalina* and *Ocimum gratissimum* leaves in Type 2 diabetic wistar albino rats' has been performed by me in the Department of Biochemistry under the supervision of Prof. I. A. Umar, Prof. D. B. James and Prof. H. M. Inuwa. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree at this or any other Institution.

Stanley Irobekhian Reuben Okoduwa	…….	24th October, 2017
Name of Student	Signature	Date

CERTIFICATION

This thesis entitled "EVALUATION OF FRACTIONATED METHANOL EXTRACTS OF *VERNONIA AMYGDALINA* AND *OCIMUM GRATISSIMUM* LEAVES IN TYPE 2 DIABETIC WISTAR ALBINO RATS" by STANLEY IROBEKHIAN REUBEN OKODUWA meets the regulations governing the award of the degree of PhD (BIOCHEMISTRY) of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This research work is whole heartedly dedicated to the following: First, to God Almighty for His divine love, shield of protection, excellent provision and infinite mercy unto my life in all ramifications. Secondly, to the less privileged ones out there, striving to make all ends meet, despite all odds. Finally to all those, involved in the field of medical/scientific research working tirelessly for the betterment of humanity.

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ABSTRACT

Type 2 diabetes (T2D) accounts for 90-95 % of the present 422 million estimated cases of diabetes mellitus worldwide, yet greater part of anti-diabetic research is on Type 1 diabetes. This study evaluated the effects of fractionated methanol extracts of *Vernonia amygdalina* (VA) and *Ocimum gratissimum* (OG) leaves in Type 2 diabetic rats. In the 1st phase, the hypoglycemic efficacy of extracts obtained from three extraction methods [Soxhlet Extraction (SE), Cold Maceration (CM) and Microwave-Assisted-Extraction (MAE) was investigated. It was performed on each of 50 g of VA and OG leaves. The 2nd phase developed rat model with features of human T2D using sixty male albino rats divided into two groups and fed commercially available normal diet feed (NDF) and water or fortified diet feed (FDF) with 20 % fructose solution as drinking water. After 6 weeks of dietary regimen, both groups were each divided into 5 subgroups of 6 rats. Each subgroup received single injection (*ip*) of streptozotocin (STZ) (0, 25, 35, 45, 55 mg/kg b.w.) dissolved in citrate buffer (pH 4.5) after overnight fast. The minimum oral therapeutic dose of the crude extracts in graded dose (100, 150, 200 etc mg/kg b.w.) were evaluated in the diabetic rats over 4 h period. In the 3rd phase the activity guided fractionation of methanol extracts of VA and OG leaves using organic solvents of increasing polarity (*n*-hexane, chloroform, ethyl acetate, *n*-butanol and water) was conducted. Daily treatment of diabetic rats with the fractionated methanol extracts of VA and OG leaves (250 mg/kg b.w.) lasted for 28 days. The tolerance to oral glucose loading (2 g/kg bw) was examined after 28 days. The 4th phase isolated the antidiabetic bioactive components of the plants. The most active fractionated methanol extract of each plants were subjected to column chromatography. Column subfractions with similar Rf values were pooled together. The pooled column sub-fractions obtained were administered orally (10 mg/kg b.w) to T2D model rats. Metformin and normal saline were used as positive and negative controls respectively. The most active column sub-fractions with hypoglycemic activity were analyzed using Fourier transform infrared and Nuclear magnetic resonance spectroscopy for identification and structural elucidation of the bioactive component(s). The result showed that the CM extract gave significantly ($p<0.05$) higher hypoglycemic effect within 14 days, hence, was employed for further investigations. The FDF-fed rats exhibited significant ($p<0.05$) increase in body weight, total cholesterol, triglycerides and blood glucose compared to the NDF-fed rats. Ten days post STZ-induction, the groups treated with STZ (45 and 55 mg/kg) developed frank hyperglycemia with <46.8 % serum-insulin, a severe deficiency typical of Type 1 diabetes. The NDF groups induced with 25 and 35 mg/kg b.w STZ with 75.7 and 64.4 % serum insulin respectively had

relatively normoglycemia, whereas the FDF group induced with 35 mg/kg b.w STZ (FDF_{35}) had 85.8 % serum insulin and were notably hyperglycemia (>300 mg/dL). This FDF_{35} rats were stable over 6-weeks post diabetes confirmation and sensitive to T2D drugs (glibenclamide, metformin and pioglitazone) and were selected for further investigation. The VA and OG extracts dose of 250 mg/kg b.w significantly (p<0.05) reduced (>25%) the blood glucose of diabetic rats by 33.23 and 29.25 % respectively at 4 h post-administration, hence was used in the study. The chloroform fraction of fractionated methanol extract of VA (VACF) and *n*-butanol fraction of fractionated methanol extract of OG (OGBF) emerged as the most active in significantly (p<0.05) reducing blood glucose of diabetic rats by 66.70 ± 6.50 % and 63.52 ± 4.84 % respectively after oral treatment for 28 days as compared to the untreated diabetic control (-50.45+11.46 %). The Area under the glucose tolerance curve showed that treatment with VACF and OGBF significantly (p<0.05) reduced blood glucose of diabetic rats by 49.01 % and 48.05 % respectively, after oral glucose loading. Nine pooled column sub-fractions (C1-C9) were isolated from VACF and four (B1-B4) from OGBF. The column sub-fractions C5 and B3 were found to be the most potent hypoglycaemic in reducing blood glucose by 12.55 ± 3.55 % and 9.32 ± 0.51 % respectively at 4 h post-oral administration, when compared to the positive (18.07 ± 1.20 %) and negative (-1.99 ± 0.43 %) controls. The spectroscopic data analysis reveals that, the isolated bioactive compounds C5 and B3 were 11β,13-dihydrovernolide and 5-hydroxyhex-3-enoic acid respectively. The isolated compounds are part of the bioactive components present in VA and OG leaves which are responsible for the anti-diabetic activities. Further research is needed in the development of these compounds for pharmaceutical use.

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ABBREVIATIONS, GLOSSARIES AND SYMBOLS

ABU	-	Ahmadu Bello University
ADA	-	American Diabetes Association
ADP	-	Adenine Diphosphate
ALT	-	Alanine Transaminase
ANOVA	-	Analysis of Variance
AST	-	Aspartate Transaminase
ATP	-	Adenosine Triphosphate
^{13}C-NMR	-	Carbon 13 NMR
CHD	-	Cardiovascular Disease
DC	-	Diabetic Control
DNA	-	Deoxyribonucleic Acid
DM	-	Diabetes Mellitus
DMRT	-	Duncan Multiple Range Test
ECDCDM	-	Expert Committee on the Diagnosis and Classification of Diabetes Mellitus
EDTA	-	Ethylene-Diamine-Tetra-Acetic acid
ELISA	-	Enzyme-Linked Immunosorbent Assay
FCPD	-	Fibrocalcalous Pancreatic Diabetes
FPG	-	Fasting Plasma Glucose
FDA	-	Food and Drug Administration
FDF	-	Fortified Diet Feed
FTIR	-	Fourier Transform Infrared Spectroscopy
GAD	-	Glutamic Acid Decarboxylase
GDM	-	Gestational Diabetes Mellitus
^{1}H-NMR	-	Proton NMR
2-HPPG	-	Two Hour Post-Prandial Plasma Glucose
HOMA	-	Homeostatic Model Assessment
HDLc	-	High Density Liproprotein Cholesterol
IR	-	Insulin resistance
IDF	-	International Diabetes Federation
IFG	-	Impaired Fasting Glucose
IGT	-	Impaired Glucose Tolerance

IS	-	Insulin sensitivity
LDLc	-	Low Density Liproprotein Cholesterol
MAE	-	Microwave Assisted Extraction
MRDM	-	Malnutrition Related Diabetes Mellitus
NC	-	Normal Control
NCDs	-	Non-Communicable Diseases
NDF	-	Normal Diet Feed
NFBG	-	Non-Fasting Blood Glucose
NIDDM	-	Non-Insulin-Dependent Diabetes
NILEST	-	Nigerian Institute of Leather and Science Technology
NMR	-	Nuclear Magnetic Resonance
NPH	-	Neutral Protamine Hagedom
OG	-	*Ocimum gratissimum*
OGb	-	*n*-butanol fraction of fractionated methanol extract of *Ocimum gratissimum* leaf
OGBF	-	*n*-butanol fraction of fractionated methanol extract of *Ocimum gratissimum* leaf
OGc	-	Chloroform fraction of fractionated methanol extract of *Ocimum gratissimum* leaf
OGe	-	Ethyl-acetate fraction of fractionated methanol extract of *Ocimum gratissimum* leaf
OGh	-	*n*-Hexane fraction of fractionated methanol extract of *Ocimum gratissimum* leaf
OGLF	-	Fractionated methanol extract of *Ocimum gratissimum* leaf
OGTT	-	Oral Glucose Tolerance Test
PC	-	Positive control
PND	-	Permanent Neonatal Diabetes
PI	-	Phosphatidyl Inositol
PKB	-	Protein kinase B
PPARy	-	Peroxidase Proliferator-Activated Receptor
QUICKI	-	Quantitative Insulin Sensitivity Check Index
Rf	-	Retention factor
SPSS	-	Statistical Package for the Social Sciences
STZ	-	Streptozotocin

TMB	-	3,3’,5,5’-tetramethylbenzidine
TLC	-	Thin Layer Chromatography
T1D	-	Type 1 Diabetes
T2D	-	Type 2 Diabetes
TC	-	Total cholesterol
TG	-	Triacyglycerol
TZDs	-	Thiazolidinediones
SD	-	Standard Deviation
USCB	-	United State Census Bureau
VA	-	*Vernonia amygdalina*
VAb	-	*n*-butanol fraction of fractionated methanol extract of *Vernonia amygdalina*
VAc	-	Chloroform fraction of fractionated methanol extract of *Vernonia amygdalina*
VACF	-	Chloroform fraction of fractionated methanol extract of *Vernonia amygdalina*
VAe	-	Ethyl-acetate fraction of fractionated methanol extract of *Vernonia amygdalina*
VAh	-	*n*-Hexane fraction of fractionated methanol extract of *Vernonia amygdalina*
VALF	-	Fractionated methanol extract of *Vernonia amygdalina*
VLDL	-	Very Low Density Liproprotein Cholesterol
WHO	-	World Health Organisation.

CHAPTER ONE

INTRODUCTION

1.1 Diabetes Mellitus

Diabetes mellitus (DM) is one of the most common non-communicable diseases (NCDs) globally; first coined by a Greek physician, about two millennia ago (Laios *et al.*, 2012). Aretaeus of Cappadocia first described this devastating disease with symptoms like constant thirst (polydipsia), excessive urination (polyuria), and weight loss, which still hold true at the present time. The disease is an age long, serious heterogeneous metabolic disorder of multiple etiology characterized by hyperglycemia and glucose intolerance with disturbances of carbohydrate, fat and protein metabolism resulting from endogenous insulin deficiency, impaired effectiveness of insulin action, or both (ADA, 2014). With DM the body cannot regulate the amount of sugar in the blood. This leads to increased glucose in the body that causes deregulation of the metabolism, often accompanied by glycosuria, polydipsia, and polyuria (ADA, 2014). Diabetes is the fourth or fifth leading cause of death in most high-income countries (IDF, 2014), and the leading cause of end stage renal failure in many populations in both developed and developing countries (ADA, 2014; Seuring *et al.*, 2015). It is undoubtedly one of the most challenging health problems in the 21st century. The International Diabetes Federation estimated in 2015 that 422 million adults, aged 20–79 years, of the world's 7.4 billion population (USCB, 2016) are diagnosed with diabetes; by 2035 this will have risen to 592 million (WHO, 2016). There are basically two main types of DM. These are Type 1 diabetes (T1D) which is insulin dependence and Type 2 diabete (T2D) which is non insulin dependence.

The disease (diabetes mellitus) has become a global public health problem and its gradually getting worse particularly in developing nations where 95 % of patients are suffering from Type

2 diabetes (Wilson and Islam, 2012; ADA, 2014; WHO, 2016). This alarming rate calls for urgency to find better treatment and novel prevention strategies for the disease.

Type 2 diabetes (T2D) is a heterogeneous disorder of carbohydrate and lipid metabolism characterized by two important characteristics: one is insulin resistance (IR), which is the failure of peripheral tissues such as muscle and adipose tissue to respond to physiologic doses of insulin, and the other one is dysfunction of pancreatic beta cells to secrete sufficient insulin in response to elevated blood glucose level (Srinivasan *et al.*, 2005). Insulin resistance and β-cell failure are caused by a combination of genetic and environmental factors. Environmental factors such as lifestyle habits (i.e., physical inactivity and poor dietary intake), obesity and toxins may act as initiating factors or progression factors for T2D. The genetic factors are still poorly understood (Uusitupa, 2002; Holt, 2004). Insulin resistance always occurs in the early stage of T2D, after a long time of IR, a further decline of beta cells is induced, resulting in hyperglycemia (Hui *et al.*, 2007).

In the developing world, the diabetic epidemy is accelerating with an increasing proportion of affected people in younger age groups. The reports that T2D being diagnosed in children and adolescents (WHO, 2003), is likely to increase further the burden of chronic diabetic complications worldwide. Nigeria has 170 million people, of which 3.9 million adults between the ages of 20-79 years have been diagnosed with DM. The burden of diabetes in Nigeria may be worse by the year 2050 when the predicted population would have reach 440 million. (IDF, 2013; United Nation, 2013; ADA, 2014). Although, much progress has been made so far on basic and clinical research into getting a possible cure for diabetes, regrettably, a final cure is currently not available.

Investigation on the pathophysiology of disease in a suitable animal model is classical approach towards the development of credible therapeutic strategy for possible cure. However, the

existing therapeutic strategies for T2D are limited and involves insulin and four main classes of oral anti-diabetic agents that stimulate pancreatic insulin secretion (sulphonylureas and rapid-acting secretagogues/insulinotropics e.g., glibenclamide, glipizide, rapaglinide), reduce hepatic glucose production (biguanides e.g., metformin), delay digestion and absorption of intestinal carbohydrate (α-glucosidase inhibitors e.g., acarbose) or improve insulin action [thiazolidinediones (TZDs) e.g., pioglitazone, rosiglitazone] (Srinivasan and Ramarao, 2007). Each of the above agents suffers from generally inadequate efficacy and number of serious adverse effects (Wild *et al.,* 2004; Mane *et al.,* 2012). Hence, the shift to the use of herbal remedy, a new hopeful approach that has long been authenticated by WHO in its general assembly (WHO, 1994; Dey *et al.,* 2002; Patil *et al.,* 2012).

Management of diabetes without side effect is still a challenge for medical system, since the available oral hypoglycemic agents commonly employed in the management of diabetes are mostly synthetic drugs with undesired effects associated with their uses (Mane *et al.,* 2012). The side effects of most glucose-lowering drugs includes; severe hypoglycaemia, lactic acidosis, idiosyncratic liver cell injury, permanent neurological deficit, hematological, cutaneous, gastrointestinal reactions, disturbances in liver, kidney digestive discomfort, headache, dizziness and even death (Arif *et al.,* 2014). These undesirable side effects associated with the prolonged use of synthetic drugs with adverse effects have necessitated the demand for relatively safe and effective drugs mainly of herbal origin (Asase and Yohonu, 2016).

The advantages of medicinal plants over Synthetic drugs are relative availability, affordability, ease of preparation and absence of serious side effect. Surprisingly; Africa that includes Nigeria is blessed with enormous biodiversity of resources yet is plagued with several diseases (Atawodi, 2005). These lead researchers to continuous search and evaluation of anti-diabetic agents from plants and other natural resources used in traditional setup in accordance to WHO recommendation (WHO, 1994).

A number of medicinal plants provide a rich mine for bioactive chemicals that have been shown to be most effective, relatively non-toxic and have substantial scientific certification to attest to their efficacy in diabetes management (Singab *et al.,* 2014). These bioactive chemicals from natural resources are considered as potent candidates for drug discovery and are playing a pivotal role in drug development programs. Consequent upon the fact that plants are known to be the main sources of bioactive chemicals; the whole plant or a specific part of it such as the root, stem, leaf, flower, fruit, seed etc is usually extracted with suitable solvent. The extract thus obtained is formulated into preparations like ointment, cream, gel, lotion, pills etc. To carry out the extraction of plant material, several methods and technology are employed (Handa *et al.,* 2008). The rationale of all extraction is to separate the soluble plant metabolites, leaving behind the insoluble cellular marc (residue). The initial crude extracts using these methods contain complex mixture of many plant metabolites, such as alkaloids, glycosides, phenolics, terpenoids and flavonoids. Some of the initially obtained extracts may be ready for use as medicinal agents in the form of tinctures and fluid extracts but some may need further processing in order to isolate and characterize the specific bioactive chemical component (Azwanida, 2015).

It is believed that bioactive components isolated from natural sources such as plants provide some benefits over allopathic medicines, which allow users to feel they have control in their choice of medication (Nestor, 2004). An increasing dependence on the use of medicinal plants in the industrialized societies have been traced to the extraction and development of quite a lot of drugs and chemotherapeutics from plants (such as *Azadirachta indica*, *Dimocarpus annua* and *Curcuma longa*) as well as from traditionally used remedies (Kurian *et al.*, 2005).

In Nigeria, where about 80 % of rural people depend on herbs for treatment of various diseases, numerous plants have been screened and found to lower glycemia in model of type 1 diabetes (Fasola *et al.,* 2010; Ezuruike and Prieto, 2014; Chikezie *et al.,* 2015). Notably among them are *Vernonia amygdalina* (VA) and *Ocimum gratissimum* (OG) (Mohammed *et al.,* 2014; Ojieh *et*

al., 2016). They are well known versatile medicinal plants having a wide spectrum of biological activities both nutritionally and pharmacologically (Akpaso *et al.,* 2011; Modu *et al.,* 2013; Chikezie *et al.,* 2015). This is due to their perceived effectiveness, minimal side effect in clinical experience and relatively low cost (Muanyan 2012; Modu e*t al.,* 2013). Series of research by various researchers have attest to the efficacy of these plants (VA and OG) individually or in combination to treat ailments (Asuquo *et al.,* 2010; Agbai *et al.,* 2012; Joyce *et al.,* 2012; Muanya, 2012; Abdulazeez, *et al.,* 2013) as anti-diabetic on T1D animal model.

Vernonia amygdalina generally known as bitter leaf, is a shrub or small tree of 2 – 5 m belonging to the family *Asteraceae.* It has petiolate leaves of about 6.0 mm diameter and elliptic shape. The leaves are dark green with a characteristic odour and a bitter taste (Singha, 1996). It is a famous medicinal plant with several uses attributed to it, such as treatment of diabetes, fever, asthma, schistosomiasis, malaria, measles, diarrhea, tuberculosis, abdominal pain and a non-pharmaceutical solution to persistent headache, and joint pain associated with AIDS (IAC, 1993; Muanya, 2012; Zakaria *et al.*, 2016). The plant is known in all part of Nigeria as '*Oriwo*' in Esan, '*Ewuro*' in Yoruba, '*Etidot*' in Ibibio, '*Onugbu*' in Igbo and '*Shuwaka*' in Hausa (Akpaso *et al.*, 2011).

Ocimum gratissimum is an aromatic medicinal plant which belongs to the *Lamiaceae* family. It is popularly known as scent leaf. It is used in cooking due to its minty aromatic flavour. In Nigeria, the plant is called "*Effinrin-na*" by the Yoruba speaking tribe, "*Alumokho*" in Esan, "*Nchuanwu*" in Igbo, "*Aramogbo*" in Edo, and "*Doddoya*" in Hausa (Effraim *et al*., 2003). Traditionally, *O. gratissimum* has been used for the treatment of headache, diarrhea, wart worms, kidney infections and diabetes. The leaves of the African varieties of *O. gratissimum* are said to contain thymol oil, which is used to prevent mosquito bite.



(a)



(b)

Plate I: Pictures of the plants: *Vernonia amygdalina*[(a)] *and Ocimum gratissimum*[(b)]

On the bases that T2D is the most common form of diabetes mellitus among adult population worldwide and for the fact that *V. amygdalina* and *O. gratissimum* leaves extracts have been shown to possess antidiabetic activities in normoglycemia and T1D model, it is therefore important to investigate the antidiabetic potential of the two plants (*V. amygdalina* and *O. gratissimum*) in T2D model.

1.2 Statement of Research Problem

The report that one in every eleven adults has DM and in every six seconds a person dies from DM is a global problem that needs urgent attention (IDF, 2015). According to WHO 422 million people (aged 20-79 years) are affected with diabetes, worldwide as at December, 2015 (WHO, 2016). The number is estimated to rise above 614 million by 2040 (IDF 2015). In Nigeria alone, 3,921,500 cases of diabetes were reported as at 2013 with a prevalence rate of 4.99 % (IDF 2014) of which Type 2 diabetes accounts for 90-95 % of all the cases reported (ADA, 2014; IDF, 2014). The disease is a serious threat all over the world and it decreases the quality of

human life, yet no cure is available for it. The available anti-diabetic drugs are synthetic, limited in supply, expensive and posed several adverse effects (Mane *et al.,* 2012; Arif *et al.,* 2014; Zinman *et al.,* 2015). Hence, the need for alternative source of remedy for the management of Type 2 diabetes

1.3 Justification of the Study

Type 2 diabetes is the specific type of DM that needs urgent attention, yet greater part of anti-diabetic researches is on Type 1 diabetes (Ezuruike and Prieto 2014; Mohammed *et al.,* 2014; Chikezie *et al.,* 2015). Several investigators that have attempted to screen *V. amygdalina* and *O. gratissimum* leaves in search for alternative remedy for the management of diabetes utilized animal model of Type 1 diabetes (Chike *et al.,* 2006; Osinubi 2007; Zhang *et al.,* 2008; Fasola *et al.,* 2010; Akpaso *et al.,* 2011; Joyce *et al.,* 2012; Okon *et al.,* 2012; Onaolapo *et al.,* 2012; Modu *et al.,* 2013). More so, none of the investigators went further to isolate and characterise the bioactive components responsible for the antidiabetic effects (Mohammed *et al.,* 2014; Chikezie *et al.,* 2015). The use of animal model of Type 1 diabetes for screening a remedy intended for the management of T2D may not reliable because the Type 1 diabetes is due to absolute insulin deficiency whereas, T2D is due to insulin resistance and β-cell dysfunction. For these reasons this study was conducted.

1.4 Aim and Objectives

1.4.1 General aim

This research work is aimed at investigating the anti-diabetic efficacy of the fractionated methanol extracts of *V. amygdalina* and *O. gratissimum* leaves in T2D wistar albino rats.

1.4.2 **Specific objectives:** This study seeks to:

i. Determine the effects of three different extraction protocols of *V. amygdalina* and *O. gratissimum* leaves.

ii. Develop T2D rat model for *in vivo* bioassay screening of anti-diabetic plants.

iii. Examine the hypoglycemic effect of the fractionated methanol extracts of *V. amygdalina* and *O. gratissimum* leaves in the T2D rat model.

iv. Assess the effect of the fractionated methanol extracts of *V. amygdalina* and *O. gratissimum* leaves on some biochemical parameters in the T2D rat model.

v. Establish the effects of the fractionated methanol extracts of *V. amygdalina* and *O. gratissimum* leaves on the integrity of pancreas and liver tissues in the T2D rat model.

vi. Isolate and identify the bioactive components present in *V. amygdalina* and *O. gratissimum* leaves which are responsible for the anti-diabetic activities in the T2D rat model.

CHAPTER TWO

LITERATURE REVIEW

2.1 Historical Background of Diabetes Mellitus

Diabetes mellitus has apparently plagued man for a very long time. The writings from earliest civilizations in China, Egypt and India made mention of boils and infections, excessive thirst, loss of weight, and the passing of large quantities of honey-sweet urine which often drew ants and flies (Savona-Ventura, 2002; ADA, 2014).

The word "diabetes" was first used around 250 BC by the Greek Apollonius of Memphis. It was coined from the Greek word for "*siphon*" which literally means "to go through", to reflect the early understanding of a disease that drained patients of more fluid than they could consume (Laios *et al.*, 2012; Lakhtakia, 2013). However, the first acknowledged clinical description of the disease “diabetes” appears to have been made by Aulus Cornelius Celsus around 30 BC – 50 AD; but Aretaeus of Cappadocia first provided a comprehensive and accurate account of the disease. Aretaeus describe diabetes as a chronic disease with a slow prodrome and rapid final decompensation but makes no link with obesity or overindulgence (Gale, 2013). Aretaeus did attempt to treat the disease but could not give a good prognosis. The disease must have been rare during the time of the Roman Empire with Galen commenting that he saw only two cases during his career (Poretsky, 2009).

Nevertheless, the pathologic condition of diabetes was described by the Greek Aretaeus, Celsus and Galen. Aretaeus first defined the difference between DM and Diabetes Insipidus (a rare disease characterized by frequent urination and intense thirst). Around the first century AD, the Greeks described the disease as "a melting down of the flesh and limbs into urine." Progressively the Latin word "mellitus," or “from honey”, was attached to diabetes because of its connection with sweet urine by Thomas Willis in the late 1600s to separate the condition

from diabetes insipidus (Medvei, 1993; Poretsky, 2009). It was John Rollo who actually documented around 1798, that diabetes was a disease condition arising from excess sugar in the blood as well as the urine (Lakhtakia, 2013). The first described cases of the disease are believed to be of Type 1 diabetes. Although, the Indian physicians around the same time identified the disease and classified it as "*madhumeha"* or *honey urine* noting that the urine would attract ants (Poretsky, 2009).

The first clearly dissimilarity between Type 1 and Type 2 diabetes was made by Sir Harold Percival (Harry) Himsworth, and published in January 1936 (Himsworth, 1936). The discovery of Type 1 diabetes led to greater recognition of Type 2 diabetes as a distinct variant of diabetes (Gale, 2001). The older terms "maturity onset diabetes" and "non-insulin-dependent diabetes" (NIDDM) were abandoned in favour of the newer terminology between 1980 and the 1990s (Gale, 2013).

2.2 Pathophysiology of Diabetes Mellitus

The disease diabetes mellitus is a heterogeneous metabolic disorder characterized by hyperglycaemia and glucose intolerance, due to endogenous insulin deficiency, impaired effectiveness of insulin action, or both (ADA, 2014). It is a chronic disease that occurs when the body cannot produce enough insulin or cannot use insulin effectively even when they are produced. Insulin is a hormone produced in the pancreas that enables glucose derived from food to enter the body's cells where it is converted into energy needed by muscles and tissues to function (IDF, 2013).

Glucose is not absorbed appropriately in persons with diabetes, instead they remain circulating in the blood (a condition known as hyperglycaemia) damaging body tissues over time. This damage can lead to disabling and life-threatening health complications (IDF, 2013). DM differs

from the less common diabetes insipidus, which is caused by lack of the hormone vasopressin, which controls the amount of urine secreted (Redmond, 2009).

Persons having DM are at risk of developing a number of disabling and life-threatening health problems. Consistently hyperglycaemia can result to serious diseases affecting the heart and blood vessels, eyes, kidneys, and nerves. Complications due to diabetes are a major cause of disability, reduced quality of life, and death (ADA, 2014; IDF, 2015). Diabetes complications can affect various parts of the body, manifesting in different ways in different people. Diabetic individuals are also at increased risk of developing infections. In almost all high-income countries, diabetes is a leading cause of cardiovascular disease, blindness, kidney failure, and lower-limb amputation. As the prevalence of diabetes grows in low and middle-income countries, so also is the economic cost associated with the management of the disease complications (IDF, 2015; Seuring *et al*., 2015). Maintaining blood glucose levels, blood pressure and cholesterol close to normal can help delay or prevent diabetes complications. People with diabetes needs regular monitoring for complications.

Diabetes can be found in every country. In all its forms imposes unacceptably high human, social and economic costs on countries at all income levels. Without effective prevention and management programmes, the burden will continue to increase worldwide (WHO, 1994). In spite of the predominantly urban impact of the epidemic, Type 2 diabetes is fast becoming a major health concern in rural communities both in low- and middle-income countries (Seuring *et al*., 2015). No countries are escaping the diabetes epidemic, and in states and territories worldwide it is the poor and disadvantaged who are suffering most. Indigenous communities are among those especially vulnerable to diabetes (IDF, 2013). Diabetes imposes a large economic burden on individuals and families, national health systems, and countries. The amount spent on diabetes accounted for 10.8% of total health expenditure worldwide in 2013 (Seuring *et al*., 2015).

2.3 Aetiology of Diabetes Mellitus

Type 2 diabetes occurs as a result of reduced secretion of insulin arising from pancreatic beta cell dysfunction and decreased insulin action (insulin resistance) at a cellular level (WHO, 2003). Both environmental and genetic factors are said to be involved in the aetiology of the disease. In a reported lifetime concordance, a genetic component of between 33-90% for identical twins is suggested. According to the reports of the Diabetes UK (2004), there is a 15% lifetime risk of diabetes arising from a single diabetic parent and 75% when both parents have diabetes (Diabetes UK, 2004). Regardless of family aggregations, inheritance is not straight forward and appears to be multigenic and is as yet, incompletely understood. The implication of environmental factors specifically includes sedentary lifestyle and obesity (Zimmet *et al.*, 2001).

In about 90% of people with T2D and almost all persons in the pre-diabetic state, IR is present (Groop, 1999; WHO, 2016). This happen when the body has a reduced response to circulating insulin in its target tissues such as skeletal muscle, adipose tissue and liver (Diabetes UK, 2000). Decreased insulin sensitivity results in a compensatory hyperinsulinaemia (Groop, 1999). This cannot be sustained due to pancreatic beta cell dysfunction, resulting in increasing hyperglycaemia and the development of impaired glucose tolerance and diabetes (Weyer *et al.*, 1999). Insulin resistance is associated with a cluster of atherogenic risk factors in the insulin resistance (or metabolic) syndrome, initially described by Reaven (1988). The features include insulin resistance, hyperinsulinaemia, glucose intolerance or diabetes, dyslipidaemia (high triglycerides, low HDL cholesterol), and hypertension. Visceral obesity and a pro-coagulant state were other associations noted later and now included in the syndrome.

2.4 Epidemiology of Diabetes Mellitus

The prevalence of diabetes mellitus is increasing with ageing of the population and lifestyle changes associated with rapid urbanization and westernization. The disease is found in all parts of the world and is rapidly increasing in its coverage (IDF, 2015; WHO, 2016). Diabetes

mellitus is the third leading fatal disorder after cancer and heart disease. As at 2015 a total number of 422 million people across the globe were reported to have been diagnosed. Type 2 diabetes accounts for about 95% of the reported cases (ADA, 2014). This represents about 8.3% of the adult population (WHO, 2016). The number is estimated to rise as a result of increase population growth, increase sedentary life style, obesity, dietary habit and aging (IDF, 2014; IDF, 2015). In Nigeria, 3,921,500 cases in a population of 170 million people were reported as at 2013 with a prevalence rate of 4.99%. The number has been predicted to reach 440 million by year 2050 (United Nation, 2013; ADA, 2014; IDF, 2014). The reasons behind this projected increase in prevalence rate are due to urbanization, westernization and their associated lifestyle changes, increase in life expectancy at birth, physical inactivity and obesity and possibly a genetic predisposition.

Diabetes is common in both the developed and developing world (Melmed *et al*., 2012; Seuring *et al*., 2015). It remains uncommon, however in underdeveloped world. The scourge of diabetes has been on the increase worldwide over the years. No wonder it has been recognized by the WHO as a global epidemic (WHO, 2016). For example the global prevalence of the disease diabetes was estimated to be 30 million in 1985, 135 million in 1995, 177 million in 2000, 217 million in 2005, 285 million in 2010, 387 in 2014 and 422 in 2015 with estimated number of 642 by 2040 (Figure 2.1) (IDF, 2015; WHO, 2016). This increase is believed to be primarily due to the global population aging, a decrease in exercise and increasing rates of obesity (Smith and Heron, 2006).

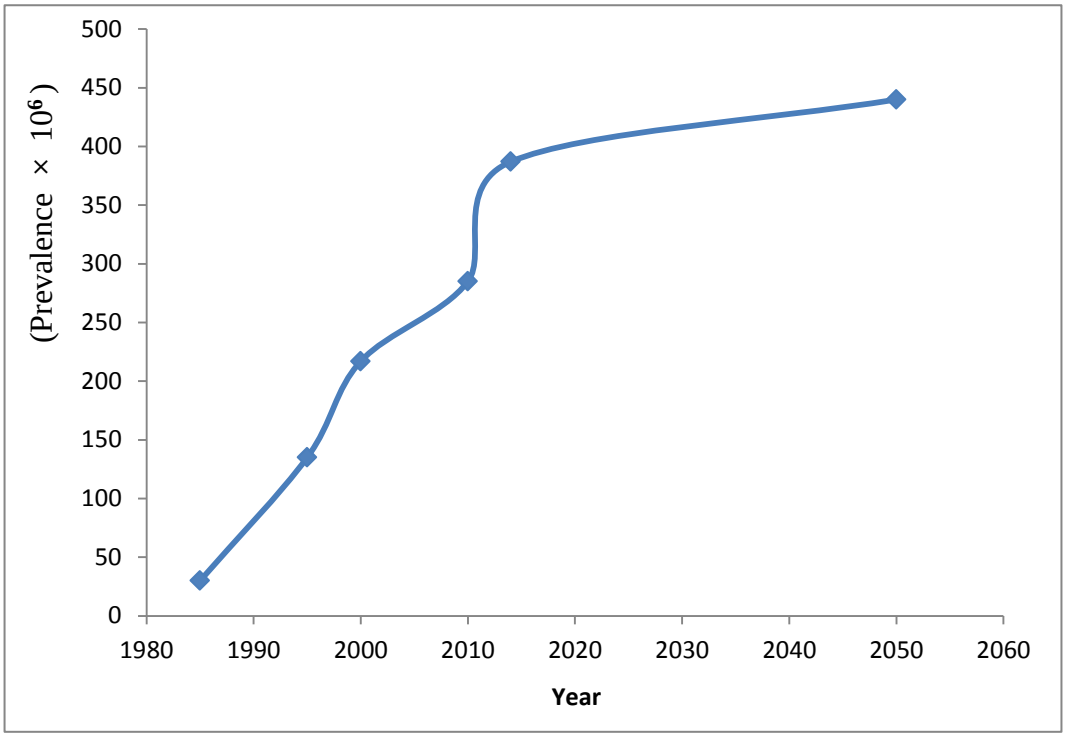


Figure 2.1: Global Prevalence of Diabetes since Year 1980 and the Projection for Year 2050 (Culled from IDF, 2015; WHO, 2016)

Between 2012 and 2014, diabetes was estimated to have resulted in 1.5 to 4.9 million deaths per year. The global economic cost for diabetes in 2014 was estimated to be $612 billion USD (IDF, 2013). There is no doubt that DM is more than a health issue and therefore requires concerted policy action across many sectors.

2.5 Pathogenesis of Diabetes Mellitus

Despite the fact that T2D account for majority of all diabetic cases, yet its pathogenesis is not certain. This could perhaps be due to the heterogeneity of the disease (Weyer *et al*., 1999; Holt, 2004). Two key features in the pathogenesis of Type 2 diabetes mellitus are a decreased ability of insulin to stimulate glucose uptake in peripheral tissues, insulin resistance, and the inability of the pancreatic β-cell to secrete insulin adequately. The major sites of insulin resistance in T2D are the liver, skeletal muscle and adipose tissue (DeFronzo, 1997).

Genetic factors and environmental influences accounts for the development of T2D. Although, the genetic factors are still poorly understood, but studies have shown that most patients have a positive family history and the risk for developing T2D is increased up to 40% by having a first-degree relative with the disease (Froguel and Velho, 2001). Environmental factors such as physical inactivity, obesity and dietary habits may interact with genetic factors and hence increase the risk of developing diabetes.

2.6 Types of Diabetes Mellitus

There are two main types of diabetes mellitus, these are: Type 1 diabetes (T1D) and Type 2 diabetes, (T2D). Other forms of diabetes mellitus include: malnutrition related diabetes mellitus (MRDM), gestational diabetes mellitus (GDM) and permanent Neonatal Diabetes (PND) (Isah, 2008).

2.6.1 Type 1 diabetes mellitus

Type 1 diabetes mellitus commonly known as insulin-dependent diabetes mellitus (IDDM), immune-mediated or juvenile-onset diabetes. This type of DM occurs as a consequence of an autoimmune-mediated destruction of pancreatic β-cells. In this case they are attacked by the body's defense system thereby leading to deficiency in insulin (IDF, 2013). It is believed that a combination of genetic and environmental factors somehow triggers the immune system to destroy these cells. However, about 20 genes that play a role in T1D have been identified, although the exact function of these genes is still under investigation (Redmond, 2009).

Environmental factors, such as certain viruses, may also contribute to the development of the disease, particularly in people who already have a genetic predisposition for the disease. T1D also can result from surgical removal of the pancreas. Markers of immune destruction of the islet-cell are present at the time of diagnosis in 90% of individuals and include antibodies to the islet cell, to glutamic acid decarboxylase (GAD), and to insulin. Individuals with T1D either

produce insufficient insulin or do not make it at all and therefore cannot control the blood glucose level (Figure 2.2). Type 1 diabetes is predominantly pronounced in persons under 30 years of age (Moussa, 2008).

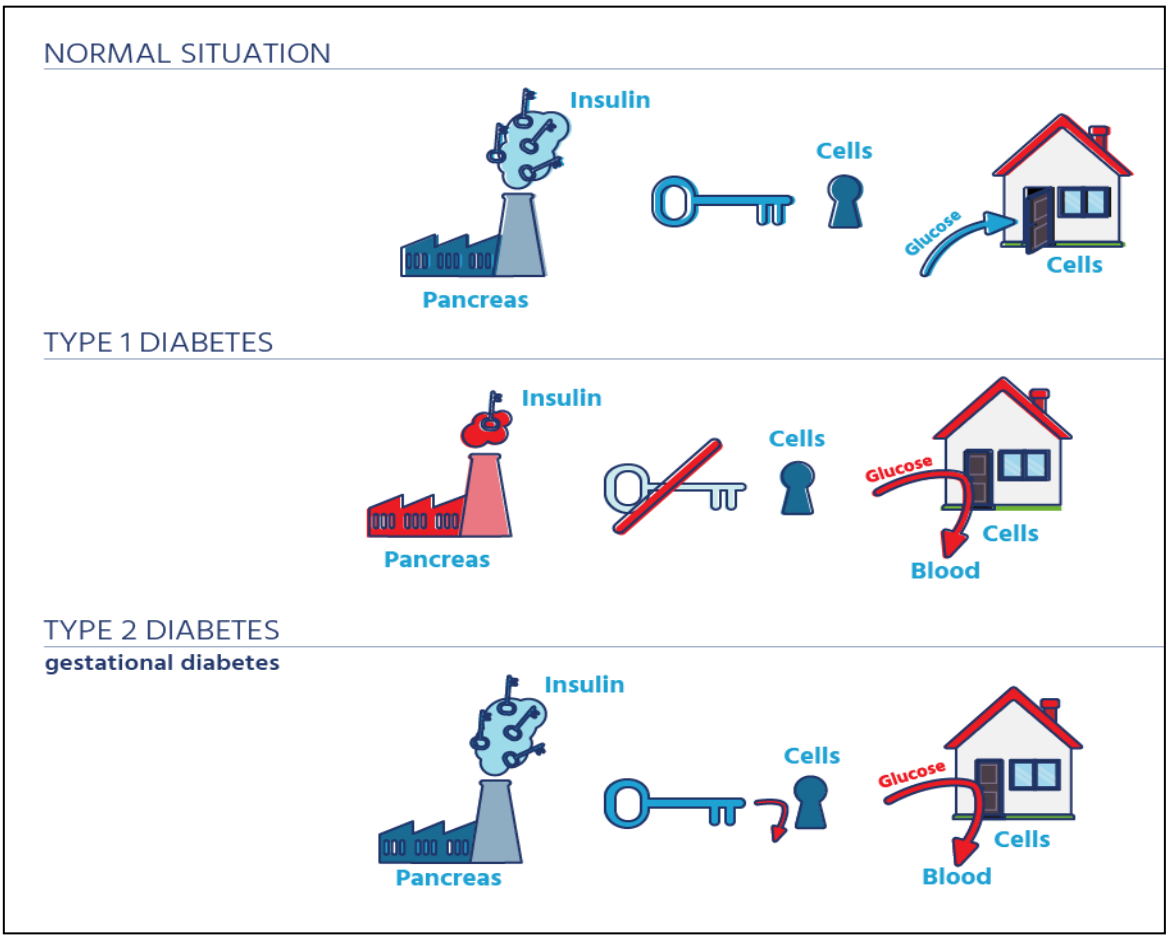


Figure 2.2: Schematic Illustration of Role of Insulin in Diabetes Mellitus (Anonymous, 2017)

Type 1 diabetes mellitus is one of the major metabolic disorders in childhood/adolescent irrespective of gender or age. Younger individuals typically have a rapid rate of β-cell destruction and ketoacidosis, while adults often maintain sufficient insulin secretion to prevent ketoacidosis for many years. Ketoacidosis is the process of breakdown of stored fat for fuel as a result of inability of the body to convert glucose into energy. The products of ketoacidosis (ketone bodies) interfere with cellular respiration. Affected individuals require the daily

administration of insulin injection in order to maintain a normoglycemia (Zimmet *et al.,* 1994; CDC, 2004; Wild *et al.,* 2004; Moussa, 2008; IDF, 2013; Zimmet *et al,* 2014).

Diabetic ketoacidosis is caused by reduced insulin levels, decreased glucose use, and increased gluconeogenesis from elevated counter regulatory hormones, including catecholamines, glucagon, and cortisol. Primarily, it affects patients with T1D, but also may occur in patients with T2D. Patients with diabetic ketoacidosis usually present with polyuria, polydypsia, polyphagia, and weakness.

2.6.2 Type 2 diabetes mellitus

Type 2 diabetes mellitus generally known as non-insulin dependent diabetes mellitus (NIDDM) occur in individuals over 40 years of age or those with a family history of diabetes. In T2D the pancreas produces insulin but the body is partially or absolutely incapable of utilizing it (Figure 2.2). In absolute terms, the concentration of plasma insulin (fasting and meal-stimulated) usually is increased, but "relative" to the severity of insulin resistance; the plasma insulin concentration is insufficient to maintain normal glucose homeostasis (DeFronzo 1997). With time, however, there is progressive beta cell failure and absolute insulin deficiency ensues. Most individuals with NIDDM exhibit intra abdominal (visceral) obesity, which is closely related to the presence of insulin resistance (Zimmet *et al.,* 1994; Zimmet *et al,* 2014). Also, hypertension, dyslipidemia (high triglyceride and low HDLc levels; postprandial hyperlipemia), and elevated PAI-1 levels often are present in these individuals. This clustering of abnormalities is what is referred to as the "insulin resistance syndrome" or the "metabolic syndrome" (Reaven, 1988). As a result of these abnormalities, those with T2D are at high risk of developing macrovascular complications such as myocardial infarction and stroke (Zimmet *et al,* 2014).

Type 2 diabetes is treated through diet changes, exercise and a desirable glycemic control. There are increasing reports of children developing T2D (Zimmet *et al,* 2014). T2D can remain

asymptomatic for many years and the diagnosis is often made from associated complications or incidentally through an abnormal blood or urine glucose test. (Zimmet *et al*., 1994; IDF, 2009). NIDDM is often, but not always, associated with obesity, which itself can cause insulin resistance and lead to elevated blood glucose levels. Several possible factors leading to the development of NIDDM include: Obesity, diet and physical inactivity, increasing age, insulin resistance, family history of diabetes, less than optimum intrauterine environment, ethnicity (Reaven, 1988; DeFronzo, 1997; CDC, 2004; IDF, 2013).

2.6.3 Malnutrition related diabetes mellitus (MRDM)

This type of DM was first recognized by the WHO in 1985 and it is further subdivided into Protein Deficient Pancreatic Diabetes (PDPD) and Fibrocalcalous Pancreatic Diabetes (FCPD). This category has been found in patients with history of childhood malnutrition, poor socioeconomic status, recurrent abdominal pain, pancreatic insufficiency and calculi. More also, these patients are often ketosis resistance requiring excess insulin (more than 60 IU/day) for stabilization of hyperglycaemia. It have been reported that areas with high consumption rate of cassava have the highest prevalence rate of the disease MRDM (Isah and Anaja, 1996).

The current hypothesis explaining this observation is that cassava contains cyanogenic glycosides, *Linamarin* and *Lotaustralin* which release cyanide when consumed. The ingested cyanide is usually detoxified to thiocyanate in a reaction requiring sulphur containing amino acid. Thiocyanate, unlike cyanide is harmless and is easily excreted by the kidney as illustrated in Figure 2.3. When protein intake is low resulting in deficiency of sulphur containing amino acid, cyanide is not converted to thiocyanate, but instead accumulate and damages the pancreas with consequent hyperglycaemia and hence MRDM (Isah and Anaja, 1996, CDC 2004, Isah, 2008).

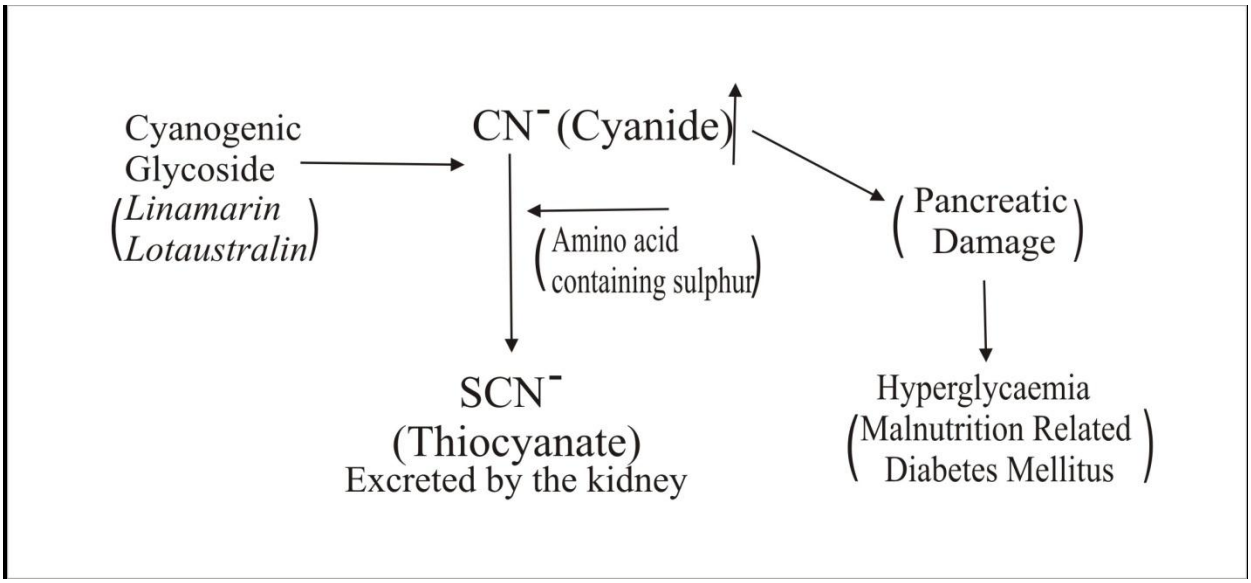


Figure 2.3 Detoxification of Cyanogenic Glycoside (Isah, 2008)

2.6.4 Gestational diabetes mellitus (GDM)

Gestational diabetes mellitus is a form of glucose intolerance that is diagnosed in some women during pregnancy. It is often common among African Americans, Hispanic/Latino Americans, and American Indians. It has also been found among obese women and women with a family history of diabetes. During pregnancy, GDM requires treatment to normalize maternal blood glucose levels to avoid complications in the infants. After pregnancy, about 5 – 10 % of women with GDM are found to have NIDDM. Women who have had GDM have a 20 – 50 % chances of developing DM in the next 5-10 years (CDC, 2004; IDF, 2009).

2.6.5. Permanent neonatal diabetes

Permanent neonatal diabetes is caused by glucokinase deficiency, and is an inborn error of the glucose-insulin signaling pathway (Njolstad *et al*., 2003).

2.7 Diagnosis of Diabetes Mellitus

The identification of glycemic cut-off point is of paramount importance in the diagnosis of DM. This helps to distinguish normal individuals from those with diabetes. The cut-points reflecting the level of glucose above which microvascular complications are likely to occur are a fasting

plasma glucose (FPG) concentration exceeding 108-116 mg/dl (6.0-6.4 mmol/l), two hour post prandial glucose (2-HPPG) level above 185 mg/dl (10.3 mmol/l), glycosylated haemoglobin (A_{1c}) greater than 5.9-6.0 %. Upon the prospective epidemiologic studies relating glycemic control to the development of diabetic retinopathy, the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus of the ADA in 1997 revised the criteria for establishing the diagnosis of diabetes (ECDCDM, 1997) (Table 2.1). To minimize the discrepancy between the fasting plasma glucose and 2-hour plasma glucose concentration measured during the oral glucose tolerance test (OGTT), cut off values of ≥ 126 mg/dl and ≥ 200 mg/dl, respectively, were chosen.

The FPG and 2-HPPG levels do not measure the same physiologic processes and so, they do not identify the same individuals as having diabetes. The FPG concentration is, in large part, determined by basal rate of hepatic glucose production (Reaven, 1988; DeFronzo, 1997) while, impaired fasting glucose (IFG) primarily reflects hepatic resistance to the action of insulin.

Table 2.1 American Diabetes Association Criteria for the Diagnosis of Diabetes Mellitus, Impaired Glucose Tolerance (IGT), and Impaired Fasting Glucose (IFG).

	Diabetes (mg/dl)	IGT (mg/dl)	IFG (mg/dl)
FPG*	≥ 126	≤ 126	≥ 110 to 125
2-Hour PG*	≥ 200	140 to 200	< 200

*FPG = fasting plasma glucose; 2-hour plasma glucose concentration during a standard oral glucose (75 g) tolerance test (ADA, 2014).

Under basal (post absorptive) conditions, the majority of glucose is taken up by insulin-independent tissues (brain and liver) although muscle tissue glucose clearance is reduced in the post-absorptive state, in absolute terms the muscle is responsible for only a small amount of glucose uptake in the basal state and is unlikely to explain the rise in FPG concentration in

individuals with IFG (DeFronzo, 1997). Also, basal insulin secretion is well preserved, even in individuals with overt NIDDM, and, therefore, cannot explain the rise in FPG concentration in individuals with IFG. In contrast, the 2-HPPG concentration primarily depends on insulin sensitivity in muscle and liver, as well as on insulin secretion by the pancreatic beta cells (DeFronzo, 1997), and defects in both tissue (muscle) sensitivity to insulin and impaired insulin secretion are responsible for IGT. Notwithstanding, both IFG and IGT predict the future development of T2D, IFG is a poor predictor of cardiovascular disease (CVD), whereas IGT is a strong predictor of myocardial infarction and stroke (DSG, 1999). This discordance most likely reflects the association of IGT with the metabolic syndrome and insulin resistance in muscle (Reaven, 1988; DeFronzo, 1997).

Although IFG and IGT are equally strong predictors of the development of future Type 2 diabetes mellitus (DSG, 1999), the prevalence of IFG in the general population is significantly less than the prevalence of IGT. In order to have similar prevalence of the two disorders (IGF and IGT) of glucose homeostasis, the cut-point for IFG would have to be reduced to approximately 103-104 mg/dl. However, the ADA recommends use of haemoglobin A_{1c} (HbA_{1c} or A_{1c}) determinations to monitor glycemic control in known diabetic patients. As there is not a "gold standard" assay and because many countries do not have ready access to the test, an A1c determination is not recommended for the diagnosis of diabetes mellitus. However, because the A1c accurately reflects the mean blood glucose concentration over a 1-3 month period and correlates well with the development of diabetic complications, it is sometimes used in diagnosis of diabetes. An A1c level above ~6.5% correlates with the present diagnostic cut-points for FPG and 2-HPPG levels (ECDCDM, 1997).

2.8 Mechanism of Diabetes Complication

Carbohydrates being the main constituent of food are broken down into simpler sugars such as glucose and then absorbed into the body. The entrance of glucose into the cell requires insulin.

Insulin facilitates the entry of glucose into the cell, where it metabolized through Kreb's cycle in the mitochondria under the control of several enzymes to release energy in the form of adenosine triphosphate (ATP). Body tissues like muscles get energy from ATP to execute mechanical functions (Forbes and Cooper, 2013).

In DM, there is a failure of the pancreas to produce sufficient insulin. In some cases the body cannot effectively utilize the insulin even when it is produced. Low insulin levels or poor response to insulin prevent cells from absorbing glucose. This affects carbohydrates, fats and proteins metabolism. Consequently, glucose builds up in the blood. When glucose overload in blood passes through the kidneys, the kidneys cannot absorb all of the excess glucose (WHO, 2016). This excess glucose spills into the urine, accompanied by water and electrolytes. This causes frequent urination to get rid of the additional water drawn into the urine; excessive thirst to trigger replacement of lost water; and hunger to replace the glucose lost in urination. Other symptoms may include blurred vision, dramatic weight loss, irritability, weakness and fatigue, and nausea and vomiting (Groop, 1999; Redmond, 2009; Forbes and Cooper, 2013).

The basic effect in lack of insulin or insulin resistance on glucose metabolism is to prevent the efficient uptake and utilization of glucose by most cells of the body, except those of the brain (Guyton and Hall, 2006). As a result of this, blood glucose concentration increases, cell utilization of glucose decreases and utilization of fats and proteins increases. Being unable to utilize glucose, resorts to other sources of energy like lipids/fats and proteins. Increase lipid/fat metabolism will eventually leads to the synthesis of cholesterol and triglycerides (Insel and Roth, 2002). The excess cholesterol produced accumulates in blood. In this disorder, plaques containing cholesterol are deposited on the walls of arteries, mainly those of small and medium size, reducing their inside diameter and the flow of blood. Clotting of blood, such as may occur in the coronary arteries to cause a heart attack, is most likely to develop at places where arterial walls are roughened by such plaques (Vorhaus, 2006).

This blockage of the blood vessels is the main cause of the complications of diabetes mellitus. This chronic condition requires careful management to avoid multi organ damage and derangements of lipid metabolism, thus preventing its complications including cardiovascular disease, nephropathy, blindness and nerve damage (Insel and Roth, 2002). Due to morbidity and mortality caused by diabetes related complications, such as increased risk of micro-vascular (retinopathy, nephropathy and neuropathy) and macro-vascular (ischemic heart disease, stroke and peripheral vascular disease) complications, which can lead to significant morbidity and diminished quality of life, there is an escalating requisite for natural therapeutic management (ADA, 2014; Arif *et al*., 2014).

2.9 Metabolic Mechanism of Action of Insulin

Metabolic actions of insulin result from its interaction with the insulin receptor in insulin responsive target cells such as liver, muscle and adipose tissue. Insulin binds to the alpha-subunit of insulin receptor and activates the intrinsic tyrosine kinase activity of the beta-subunit of the receptor. Activated insulin receptors result in the subsequent phosphorylation of intracellular substrates, including insulin receptor substrates, phosphatidyl-inositol (PI) 3-kinase, and protein kinase B (PKB). Normal insulin action leads to increased glycogen synthesis, glucose transport, and lipogenesis, and decreased gluconeogenesis, glycogenolysis, and lipolysis (Cheng and Fantus, 2005; Forbes and Cooper, 2013).

2.10 Role of Pancreas in Glucose Metabolism

The pancreas contributes a significant role in the metabolism of glucose by secreting the hormones, insulin and glucagon needed for the regulation of homeostatic level of blood glucose (Figure 2.4). Elevated postprandial blood glucose level stimulates pancreatic beta cells to produce insulin which then aid the entry of glucose into the muscle and adipose tissues, thereby clearing excess glucose from the circulation. Insulin also stimulates the processes of glycolysis and glycogenesis and inhibits both hepatic gluconeogenesis and glycogenolysis thereby

reducing the hepatic glucose output (Stanhope *et al.*, 2008)**.** The actions of insulin are opposed by glucagon, a hormone produced by the pancreatic alpha cells in hypoglycemic condition. Glucagon inhibits glycogenesis and stimulates both gluconeogenesis and glycogenolysis which releases blood glucose into the blood circulation thereby raising the blood glucose level (Stanhope *et al.*, 2008)**.**

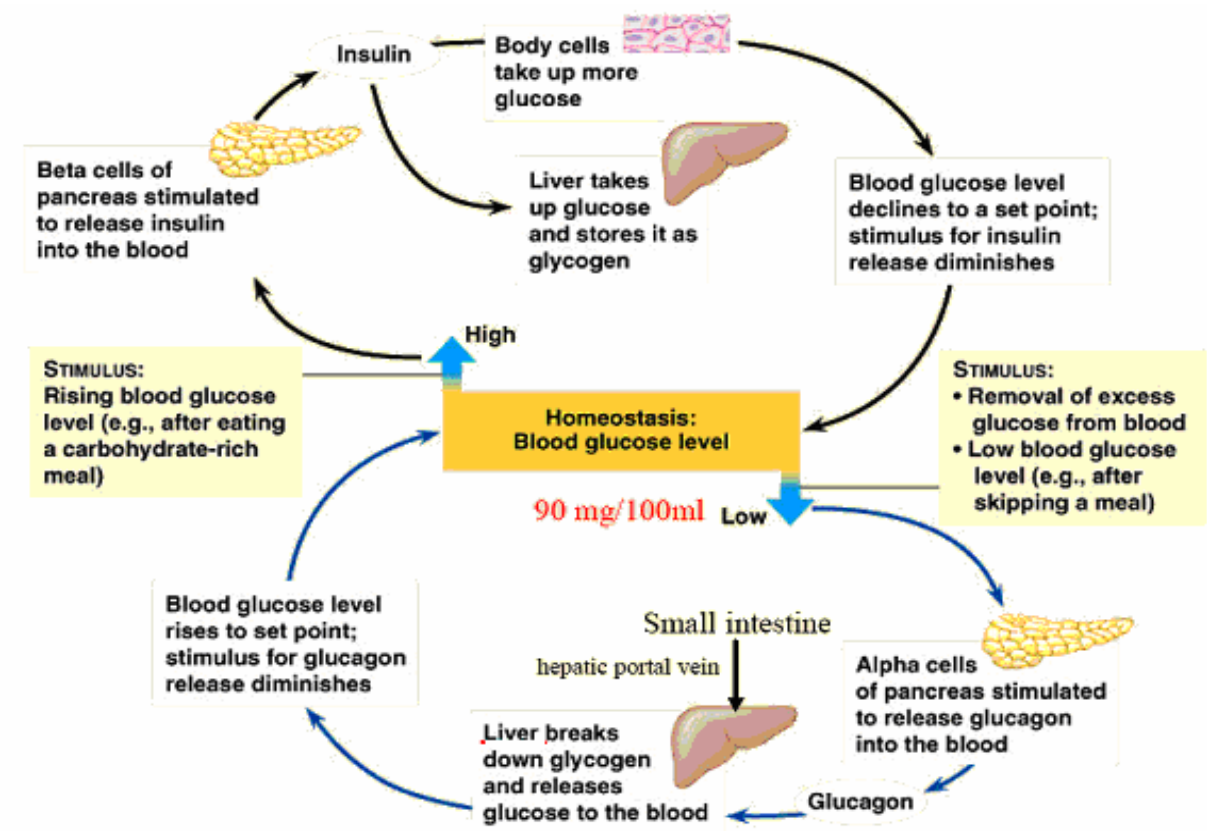


Figure 2.4: The Role of Pancreas in the Body (Cheng and Fantus, 2005)

2.11 Management of Diabetes Mellitus

The management of T1D involves the use of insulin therapy whereas in T2D, efforts are engaged to repair the fault in the line of supply of insulin or reduce the glucose load to the body, an attempt to decrease the insulin demand. The management of T2D by dietary modification entails limiting the consumption of saturated fats and replacing them with monounsaturated or polyunsaturated fats. Complex carbohydrates are substituted for simple carbohydrates and a

high fibre intake encouraged. In recent years, dietary advice has been significantly modified with greater flexibility in the proportions of energy derived from carbohydrate and monounsaturated fats (Diabetes UK, 2003). It is suggested that fats are limited to <35 % of total energy intake: saturated fats <10 %, n6 polyunsaturates <10 %, n3 polyunsaturates at least two portions per week of oily fish, and monounsaturates increased to 10-20 % of energy intake. Carbohydrates should be limited to 45-60% but sucrose can be taken up to 10 % of energy intake, provided it is taken in the context of a healthy diet (Uusitupa, 2002; Inzucchi *et al*., 2012).

Weight reduction and an increase in exercise are advocated but in practice are difficult to achieve for the majority of patients. Only 10-20% achieved good glycaemic control on diet alone. The majority require the addition of oral hypoglycaemic drugs or insulin. Insulin will be required by almost 50% of patients within 6 years of diagnosis.

The following principals of management of DM are in practiced: -

1. Administration of exogenous insulin to the body through: -

   a. Injections like Humulin or NPH insulin etc. for T1DM or uncontrolled T2DM.

   b. Inhalations (a recent development from Pfizer and Aventis pharmaceutical companies, which has been approved by Food and Drug Administration (FDA) of USA) for adults with Type 1 and Type 2 diabetes mellitus (Al-Tabakha, 2015).

2. Increasing the production of effective insulin within the body, which can be achieved through:

   a. Drugs that stimulate the production of insulin from the β-cells of islets of Langerhans in pancreas like Sulphonylureas. They act mainly by augmenting insulin secretion and consequently are effective only when some residual pancreatic beta-cell activity is present;

during long-term administration they also have an extra pancreatic action. All may cause hypoglycaemia but this is uncommon and usually indicates excessive dosage (Diabetes UK, 2003).

b. Use of herbs which increase the production of insulin by maintaining the health and integrity of insulin producing cells and modulating immune function (Al-Tabakha, 2015).

3. Increasing glucose utilization by biguanides (metformin- the only available biguanide) has a different mode of action from the sulphonylureas, and is not interchangeable with them. It exerts its effect mainly by decreasing gluconeogenesis and by increasing peripheral utilization of glucose; since it acts only in the presence of endogenous insulin and effective only if there are some residual functioning pancreatic islet cells (Inzucchi *et al*., 2012).

4. Decreasing the glucose load to the body (dietary management).

a. Restricting the carbohydrates (glucose source) in the diet.

b. Decreasing the absorption of glucose from the gut: Acarbose- an inhibitor of intestinal alpha glucosidases, delays the digestion and absorption of starch and sucrose. It has a small but significant effect in lowering blood glucose and is used either on its own or as an adjunct to metformin or to sulphonylureas when they prove inadequate. Postprandial hyperglycemia in Type 1 (insulin-dependent) diabetes can be reduced by acarbose, but it has been little used for this purpose. Flatulence deters some from using acarbose although this side-effect tends to decrease with time. Dietary fibers, like rice bran, called bowel regulators, with almost similar functions (WHO, 1994; Diabetes UK, 2003; Inzucchi *et al*., 2012).

2.12 Oral Antihyperglycemic Agents

There are four main groups of oral agents used to control hyperglycaemia. These are the insulin secretagogues, biguanides, alpha-glucosidase inhibitors and thiazolidinediones (Cheng and Fantus, 2005).

2.12.1 Insulin secretagogues/insulinotropics

Insulin secretagogues/insulinotropics (e.g., glibenclamide, glipizide, rapaglinide): There are two drug groups in this category that increases pancreatic insulin secretion. These are the sulphonylureas and the meglitinide analogues. *The sulphonylureas* stimulate pancreatic insulin secretion by binding to sulphonylurea receptors on the pancreatic beta-cell plasma membrane, causing membrane depolarisation and exocytosis of insulin granules. (Koski, 2006). They are associated with weight gain. *The meglitinide analogues* (meglitinides, repaglinide and nateglinide) bind to the sulphonylurea receptors by a different mechanism to the sulphonylureas resulting in a shorter duration of action, and are therefore used to control postprandial hyperglycaemia. Insulin secretagogues can be used alone or in conjunction with metformin (Cheng and Fantus, 2005; Koski, 2006).

2.12.2 Biguanides (e.g. metformin)

Metformin increases insulin action, although the exact mechanism is not fully understood. It decreases hepatic gluconeogenesis thus reducing glucose production. It does not cause weight gain or hypoglycaemia and in the obese is associated with a significant reduction in diabetes related events, all cause mortality, and stroke (Srinivasan and Ramarao, 2007).

2.12.3 Alpha-glucosidase inhibitors

Alpha-glucosidase inhibitors (e.g., acarbose): Alpha-glucosidase inhibitors delay digestion and absorption of intestinal carbohydrate thereby lowering postprandial blood glucose levels. They

inhibit disaccharidase enzymes and this gives a high incidence of gastrointestinal side effects. They are only effective in patients with adequate beta-cell function (Cheng and Fantus, 2005).

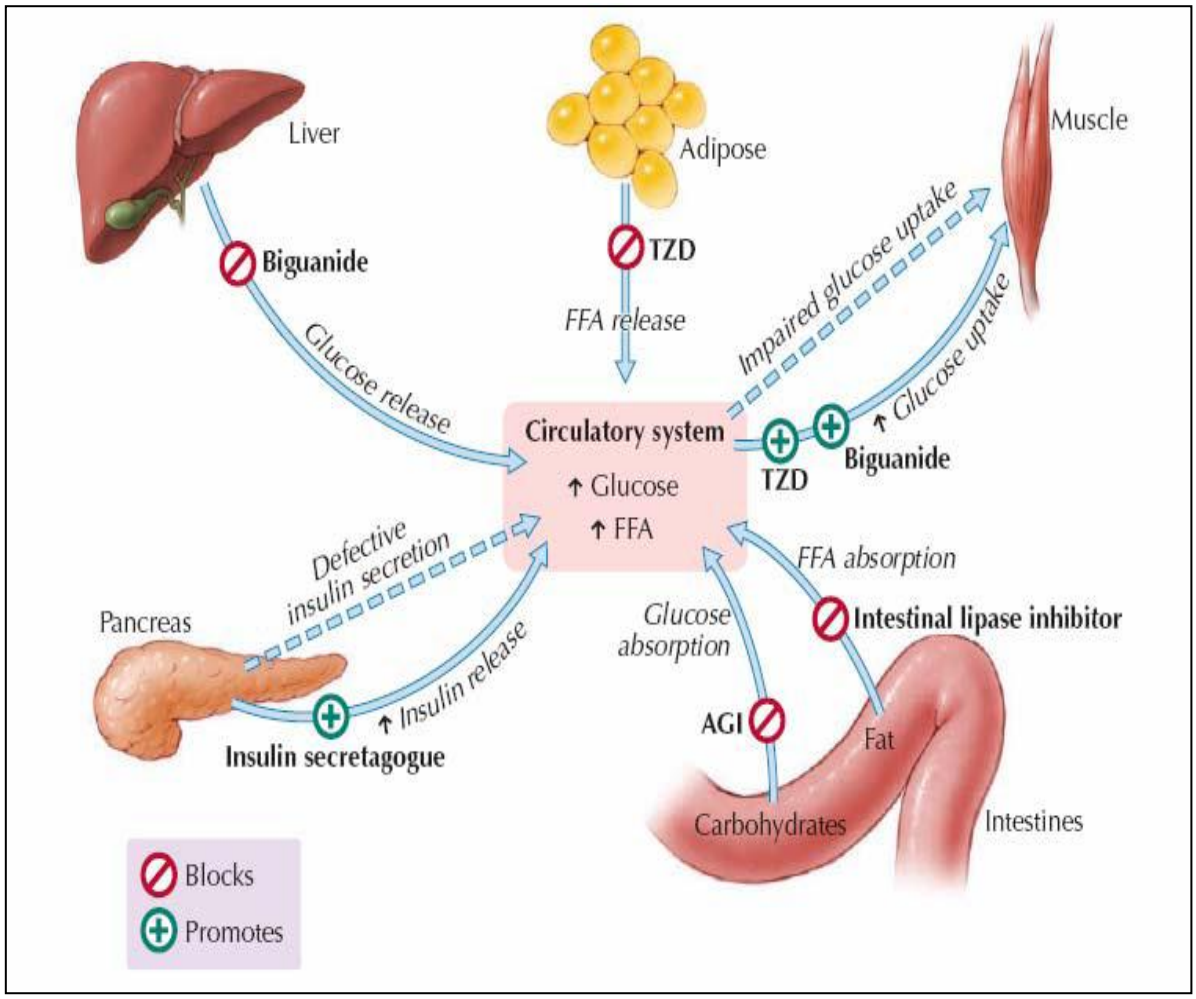


Figure 2.5: Major Target Organs and Mechanism of Actions of Orally Administered Anti-hyperglycemic Agents in T2D (Cheng and Fantus, 2005)

2.12.4 Thiazolidinediones or Glitazones

Thiazolidinediones or Glitazones (e.g., pioglitazone, rosiglitazone): This group of drugs is insulin sensitizers. They enter the cell and bind to the peroxidase proliferator-activated receptor (PPARy). This is a nuclear receptor found mainly in adipocytes, and to a lesser extent in muscle and the liver. The glitazone forms a complex in the cell that stimulates expression of insulin-sensitive genes. These increase glucose uptake, increase adipocyte lipogenesis and decrease

circulating fatty acid levels. Glitazones are associated with weight gain and fluid retention (Cheng and Fantus, 2005; Koski, 2006).

2.13 Medicinal Plants

Medicinal plants are of particular significance for the fact that they contain valuable secondary products with high potency in the management of human ailments. It is generally assumed that the active constituents contributing to the efficacy of the medicinal plants are the phytochemicals, minerals and vitamins (Singab *et al.,* 2014). Plants have continued to play a major role in primary healthcare as therapeutic remedies in developing countries. The role of plants in folklore medicine is attributed to the presence of phytochemicals; which are non nutritive plant chemicals that have disease preventing or curative properties (Arhoghro *et al.*, 2014). Medicinal plants have various effects on living systems. Some are sedatives, analgesics, antipyretics, cardioprotectives, antibacterials, antidiabetic, antivirals and antiprotozoals etc (Tanira, 1994; Singha, 1996; Asase and Yohonu, 2016).

Not fewer than 1000 plants have been described as efficacious in the treatment of diabetes mellitus. However, a lot of these descriptions are subjective accounts of traditional usage, and fewer than half of these plants or plant extracts have received a thorough medical or scientific evaluation of their purported benefits. In fact, till date there is neither any evidence nor any validation that a natural plant material can serve as a complete substitute for insulin. Though, several plant products have been reported to mimic the effects of insulin partially or enhance the effects of very low endogenous insulin concentrations, but none has sustained life in the total absence of insulin (Eddouks *et al.,* 2012; Ezuruike and Prieto, 2014).

2.13.1 Mechanism of action of anti-diabetic medicinal plants

There are several possible mechanisms of action through which medicinal plants act to regulate blood glucose level as being proposed by various investigators**.** These mechanisms of action can

be related, basically, to the ability of the plant itself or its bioactive principle to lower blood plasma glucose level by interfering with one or more of the processes involved in glucose homeostasis. The proposed mechanisms whereby herbal anti-diabetic remedies lower blood glucose levels are more or less comparable to those of the synthetic oral hypoglycemic drugs**.** These mechanisms are as follow: Alteration of the activity of some enzymes that are involved in glucose metabolism. Improvement of insulin sensitivity (enhanced glucose uptake by fat and muscle cells). Stimulation of insulin synthesis and/or secretion from pancreatic beta-cells. Slowing down the absorption of carbohydrates from the gut. Regeneration/revitalization of damaged pancreatic beta cells and mimicking the action of insulin (acting like insulin) **(**Bnouham *et al.,* 2006; Tanira, 1994**).**

2.13.2 Procedure for investigation of medicinal plant on insulin secretion *in vivo*

A description of some procedures used to investigate the *in vivo* effects of plant materials on insulin secretion, digestion and absorption of glucose, activation of the insulin receptor and the activity of some carbohydrate metabolizing enzymes is discussed.

2.13.2.1 Effect on insulin secretion: countless journal articles on the investigation of the effect of medicinal plant extract on insulin secretion *in vivo* has involved the use of streptozotocin or alloxan induced animal models of diabetes. Both streptozotocin and alloxan cause destruction of pancreatic beta cells resulting in reduced insulin secretion**.** In streptozotocin and alloxan induced animal models of diabetes, insulin is markedly depleted but not absent (Fröde and Medeiros, 2008). For this reasons these animal models have been widely used to study the effect of antidiabetic remedies on insulin secretion *in vivo.*

2.13.2.2 Intestinal digestion and absorption of carbohydrates: In order to investigate the effect of an anti-diabetic plant extract on intestinal digestion and/or absorption of carbohydrates, study animals are usually divided into experimental and control groups. Experimental animals are

given a plant extract under investigation while control animals are given a vehicle. An hour later, both groups of animals are given a fixed amount of glucose, sucrose or starch. Thereafter, blood glucose levels are measured at 0.5, 1, 1.5, 2 and 3 hrs after administration of the carbohydrate. Areas below the oral glucose tolerance curves of experimental groups are then calculated and compared with those of control groups (Hannan *et al.,* 2007). Alternatively, a glucose tolerance test can be determined in the same group of animals before and after oral administration of the plant extract. A comparison of the glucose tolerance curve before and after oral administration of the plant extract will indicate whether or not the plant extract contribute to the delay in carbohydrate digestion and subsequent lowering of the blood glucose level glucose (Hannan *et al.,* 2007).

2.13.2.3 Inhibition or activation of carbohydrate metabolizing enzymes: It has been established that some anti-diabetic remedies, such as, metformin exert its blood glucose effects by inhibiting endogenous glucose production by the liver through the process of gluconeogenesis and glycogenolysis (Bastaki, 2005). Subsequently, as part of efforts to find out the possible mode of action of anti-diabetic remedies, several researchers have investigated the effect of plant extracts on the activities of gluconeogenic enzymes: glucose 6-phosphatase, fructose 1,6-bisphosphatase; the glycogenolytic enzyme; glycogen phosphorylase and hepatic glucokinase. In order to investigate the effect of medicinal plant extract on key enzymes involved in glucose homeostasis *in vivo,* the study design used are similar to the one describe above for the study of the effect of plant extract on stimulation of insulin except that at the end of the feeding period blood and selected tissues are also collected for the measurement of the activity of selected enzymes in plasma or tissue homogenates *in vitro*.

2.14 Phytochemicals

Phytochemicals are chemical compounds or constituents that occur naturally in plants. They provide plants with characteristic colour, odour, and or flavour. They are more often than not called secondary metabolites reason being that the plants producing them may have little need for them. They are naturally synthesized in all parts of plant such as the bark, leaves, stem, root, flower, fruits, seeds, etc. in other words any part of a plant part may contain active components (Tiwari *et al*., 2011). Some examples of phytochemicals present in plants are: Phenol, flavonoids, tannins, saponins, glycosides, terpenoids etc. The quantity and quality of phytochemicals present in plant parts may differ from one part to another. In fact, there is lack of information on the distribution of the biological activity in different plant parts essentially related to the differences in distribution of active principles which are more frequent in some plant parts than in others. Some phytochemicals with physiological properties may be elements rather than complex organic molecules (Brown and Arthur, 2001).

2.14.1 Some phytochemical constituents in medicinal plants

i) Alkaloids: Alkaloids are heterogeneous group of compounds which contain one or more nitrogen atom in acyclic system. They comprise the largest single class of secondary substances of which over 2000 are known. In nature, alkaloids exist in large proportions in seeds and roots of plants and often in combination with vegetable acids (Madziga *et al.,* 2010). The compounds are derived from plants and have basic properties hence alkaline in reaction, turning red litmus paper blue. They react with acids to form crystalline salts without the production of water (Firn, 2010). Majority of alkaloids exist in solid form such as atropine, some as liquids containing carbon, hydrogen, and nitrogen. Some alkaloids are readily soluble in alcohol. Alkaloids are usually colourless and often optically active substances; but can be detected in fresh leaves by their bitter taste. They have great physiological effect on man and other animals. They are the most potent therapeutic compounds that have been manufactured as various allopathic drugs,

including the pain-killer morphine and anti-malaria quinine. In their native environment, they usually occur in the form of salts of simple organic acid, tartaric acid and citric acid and they are known to have a wide range of pharmacological activities (Kittakoop *et al.,* 2014). The investigation of bioactivity of alkaloids reveals that they are used medically as antispasmodic, mydriatic, local anesthetic drug (Harborne, 1973). They are mostly used as anti-depressant (morphine), stimulants (caffeine), anesthetics (cocaine), anti-tumour (vinblastine), anti-malaria (quinine), anti-bacteria (berberine), amoebicide (emetine) and antidiabetic (Sharma *et al.,* 2010b).

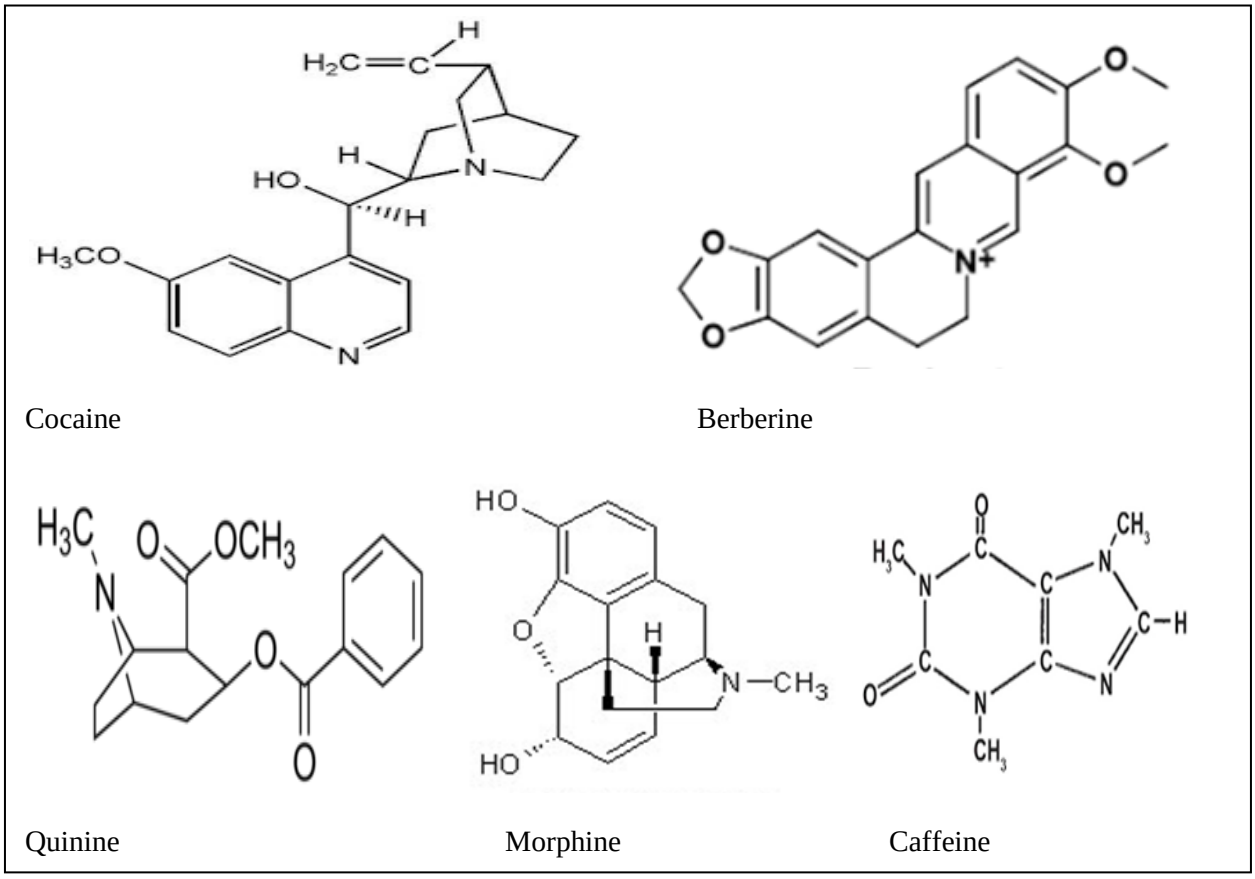


Figure 2.6: Chemical Structures of Some Alkaloids

ii) Anthraquinones: Anthraquinones also called anthraquinoids are polycyclic aromatic organic compound with the formular $C_{14}H_8O_2$. They are solely derived from anthracene giving variable oxidized derivatives such as anthrones and anthranols (Firn, 2010). Anthraquinones are used in

the manufacture of dyes in the textile industries, and as bird repellent. They have an irritant or stimulating laxative effect on the large intestine. They act directly on the intestinal mucosa, influencing several pharmacological targets and their laxative effect is largely due to increase peristalsis of the colon, reducing transit time and consequently the reabsorption of water from colon making the stool more liquid and easing bowel movements (Chien *et al.*, 2015). A large body of literature has demonstrated that naturally occurring anthraquinone possess a broad spectrum of bioactivities, such as cathrticm anticancer, anti-inflammatory, antimicrobial, diuretic, vasorelaxing and phytoestrogen activities suggesting their possible clinical application in many diseases (Chien *et al.*, 2015).

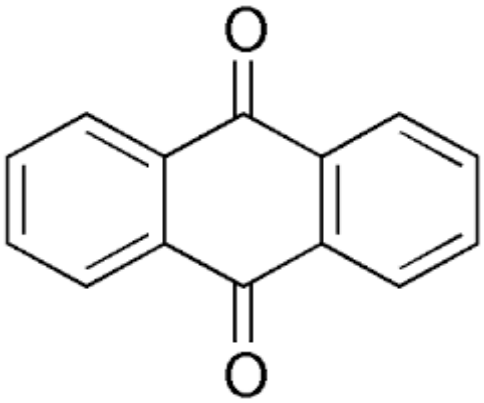


Figure 2.7: Chemical Structure of Anthraquinone

iii) Glycosides: Glycoside is a molecule in which a sugar is bound to another functional group through a glycosidic bond. Chemically, they contain a carbohydrate and a non-carbohydrate part (aglycone or genins). The word glycoside is a generic term generally used for natural product that is chemically bonded to sugar(s) e.g cardiac glycosides. In general, they are defined as the condensation products of sugars with a host of different varieties of organic hydroxyl (thiol) compounds (invariably monohydrate in character). Glycosides are colourless crystalline of carbon, hydrogen and oxygen containing water soluble phytoconstituents, found in cell sap. They are neutral in reaction and can be readily hydrolysed into its components with mineral

acids. Glycosides are classified on the basis of type of sugar component, chemical nature of aglycone or pharmacological action. They are complex groups which can be broken down to yield one or more sugar (glycones) (Firn, 2010).

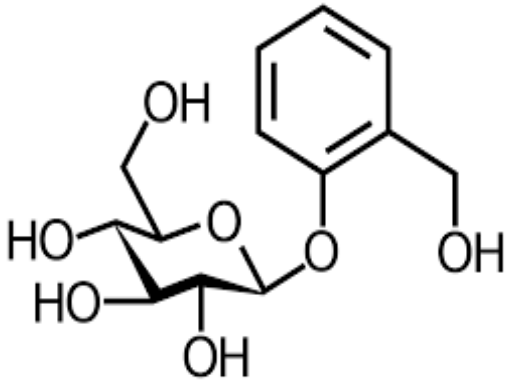


Figure 2.8: Chemical Structure of Glycoside

iv) Flavonoids and phenols: Flavonoids have the general structure of a 15-carbon skeleton, which consist of two phenyl rings and heterocyclic rings. Numerous reports attest to their use as antioxidants or free radical scavengers (Kar, 2007). Flavonoids are molecules responsible for the colour of fruits and flowers. They are class of plant and fungus secondary metabolites. They are beneficial to man as powerful anti-oxidants, anti-allergic agents, anti-viral compounds, stimulant of protein synthesis, anti-inflammatory agents, diuretic, anti-spasmodic, anti-bacterial and anti-fungal (Evans, 2005). Over four thousand flavonoids are known to exist as pigments in higher plants. Flavonoids have been referred to as nature's biological response modifiers because of strong experimental evidence of their inherent ability to modify the body's reaction to allergies, virus and carcinogens (Kasali *et al.,* 2016). They show anti-allergic, anti-inflammatory, antimicrobial, anti-cancer and antidiabetic activities (Lavle *et al.,* 2016; Testa *et al.,* 2016). Phenols are sometimes called polyphenols or phenolic compounds and they are widely found throughout the plant kingdom. They are class of chemical compounds consisting of a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon. The simplest form is phenol (Carbolic acid: C_6H_5OH). Phenolics in plants are predominantly synthesized from phenylalanine through

the action of phenylalanine ammonia lyase. They are categorized into (i) phenolic acids and (ii) flavonoid polyphenolics (flavones, flavonones, xanthones and catechins) and (iii) non- flavones polyphenolics. One of the most widespread phenolics compounds is caffeine which is distributed in the plant flora followed by chlorogenic acid known to cause allergic dermatitis among humans (Kar, 2007). It has been demonstrated that polyphenols contribute to the prevention of osteoporosis, cardiovascular diseases, cancers and anti-oxidant character with potential health benefits (Lin *et al.*, 2016). Researches have shown that phenols act as anti-tumour agents and are known to exhibit anti-viral and antimicrobial activities, hypotensive effects and anti-oxidant and antidiabetic properties (Asgar, 2013). The most significant role may be in plant defence against pathogens and herbivore predators, and consequently are applied in the control of human pathogenc infections (Vinayagam *et al*., 2016).

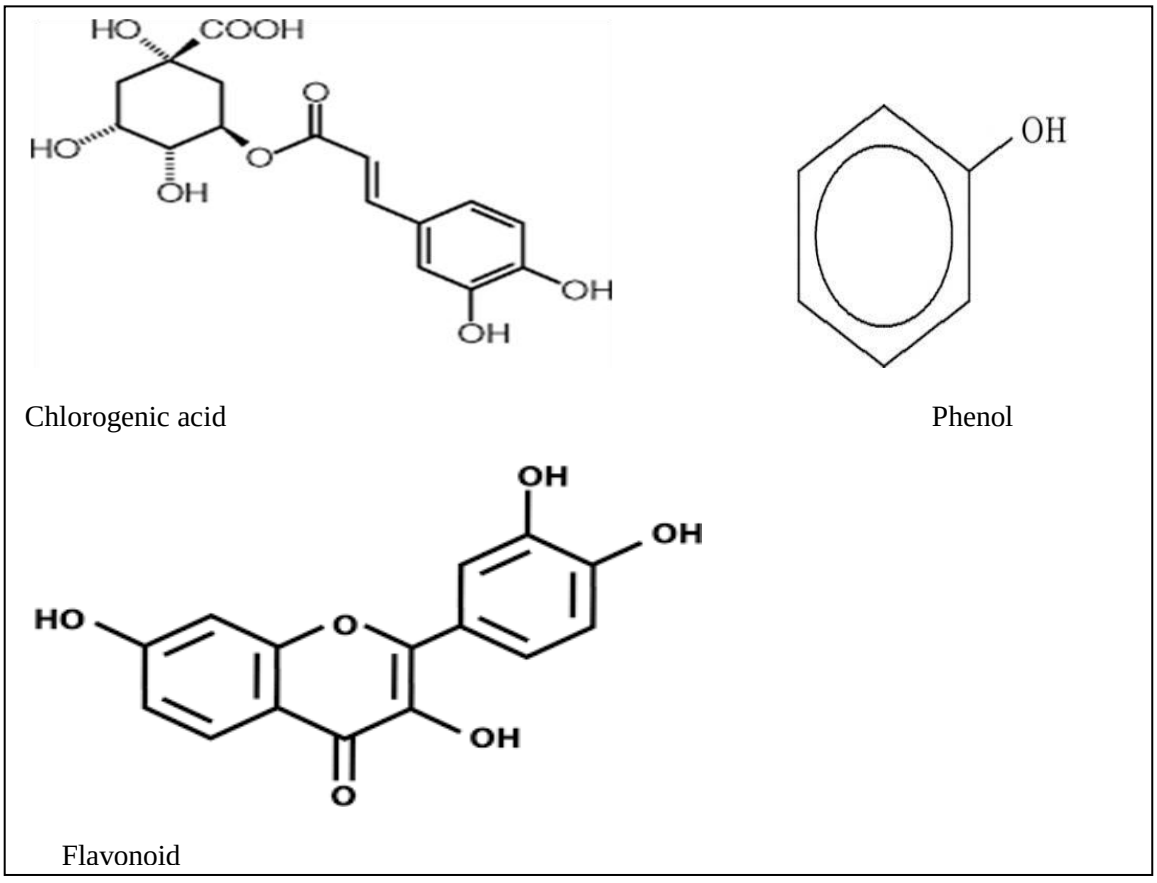


Figure 2.9: Chemical Structures of Flavonoid and Phenols

v) Saponins: Saponins are glucosides, a class of chemical compounds found abundance in various plant species. They consist of polycyclic aglycones attached to one or more sugar side chain. The aglycone part, which is also called sapogenin, is either steroid (C27) or triperpene (C30). The foaming ability of saponins is due to the combination of a hydrophobic sapogenin and hydrophilic sugar-part. Saponins possess "soaplike" behaviour in water, i.e they produce foam. On hydrolysis, an aglycone is produced, which is called sapogenin. There are two types of sapogenin: steroidal and triterpenoidal. Saponins are regarded as high molecular weight compounds in which, a sugar molecule is combined with triterpene or steroid aglycone. Saponins are extremely poisonous, as they cause haemolysis of blood and are known to cause cattle poisoning (Kar, 2007). They possess a bitter and acrid taste; besides causing irritation to mucous membranes they are mostly amorphous in nature, soluble in alcohol and water, but insoluble in non-polar organic solvents like benzene and n-hexane (Kar, 2007). They are usually detected by their ability to foam persistently and to haemolyse blood cells (Evans, 2005). It has been discovered that saponins and other flavonoid compounds at low concentrations inhibited the growth of micro-organisms and they can also act as bactericidal agents at higher concentrations by coagulating protoplasm of the organisms. Saponins are sources of sapogenins which can be converted in the laboratory to animal sterols of therapeutic importance (Sofowora, 2006). Saponin is useful in medicine and pharmaceutical industry due to its foaming ability that produces frothy effect. They are used in the manufacture of shampoos, insecticides and various drug preparations and in synthesis of steroid hormones (Okwu, 2004). Saponins are toxic, but researches have recently shown that consumption of saponins has beneficial effects on blood cholesterol levels, cancer, bone health, stimulating of the immune system (Elekofehinti, 2015; Lavle *et al.*, 2016; El-Barky *et al.*, 2017).

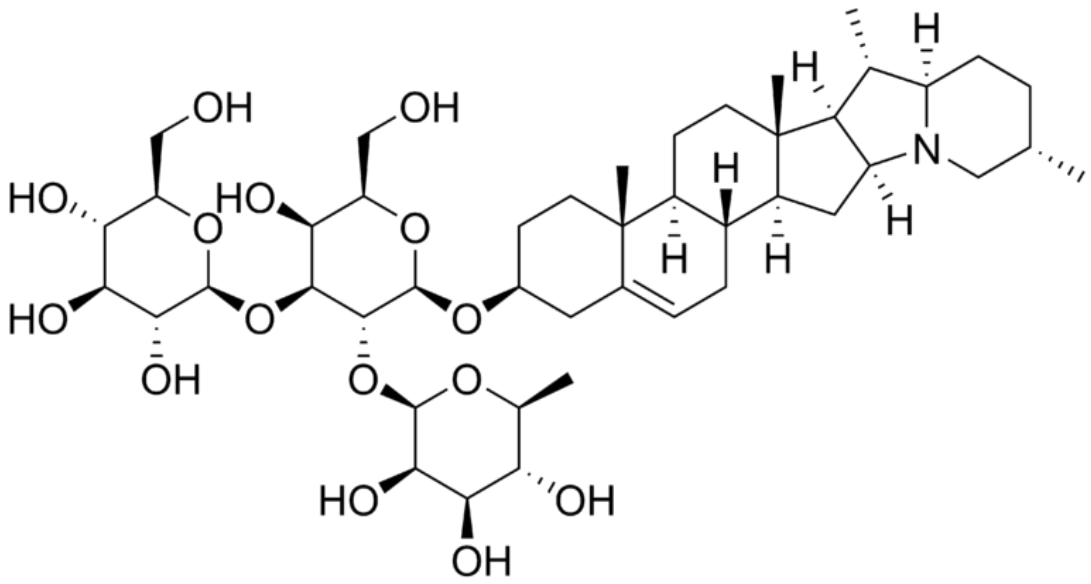


Figure 2.10: Chemical Structure of Saponin

vi) Steroids: A steroid is an organic compound with four rings arranged in a specific molecular configuration. Plant steroids also called "cardiac glycosides" are one of the most naturally occurring plant phytoconstituents that have found therapeutic applications as arrow poisons or cardiac drugs (Firn, 2010). Steroids are natural products in a class of widely distributed compounds, which develop and control the reproductive tracts in humans, and induce sexual reproduction in aquatic fungi. Therapeutically, steroids contribute in vitamin D precursors, anti inflammatory agents (corticosteroid) and anabolic agents (androgen). Steroids play critical roles in a number of disorders, including malignancies like prostate cancer, where steroid secretion within and outside the tumour promotes cancer aggressiveness (Lubik *et al*., 2017).

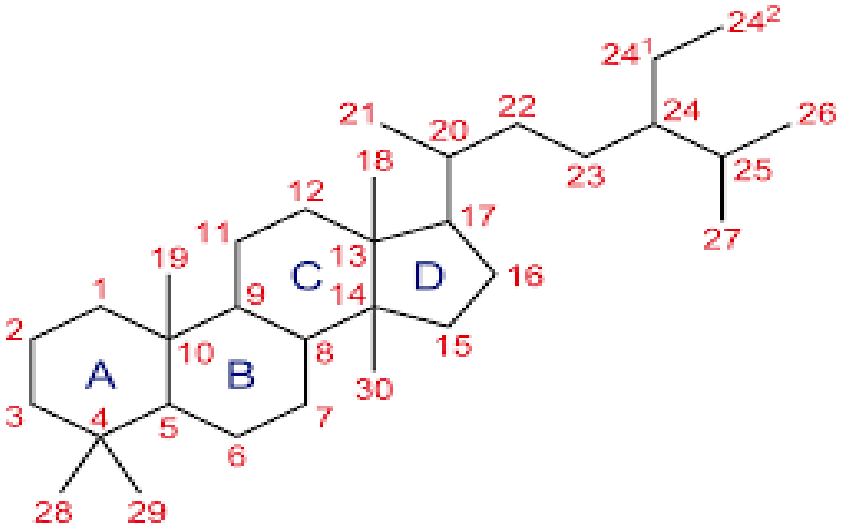
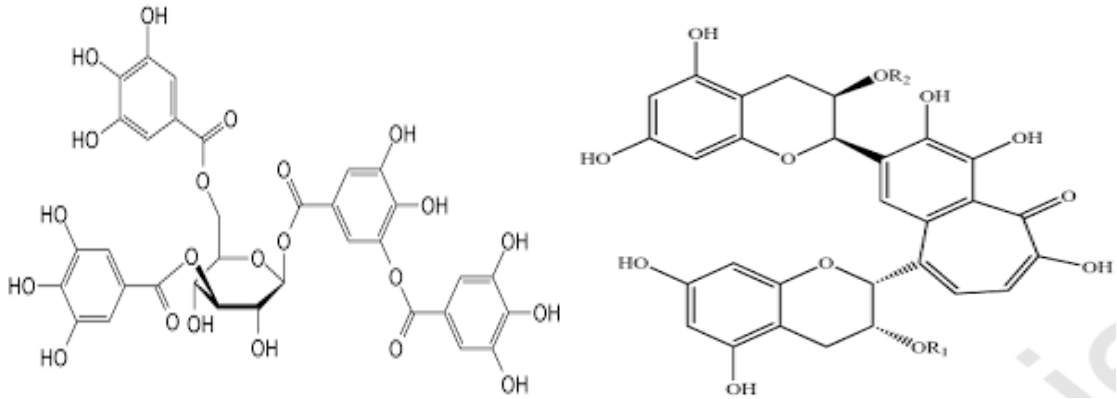


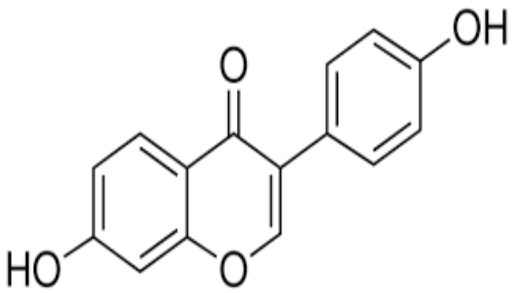
Figure 2.11: Chemical Structure of Steroid

vii) Tannins: Tannins are astringent, polyphenolic bimolecule that binds to and precipitate proteins and various organic compounds including alkaloids and amino acids. They form colloidal solutions in water and complexes with proteins, carbohydrates, gelatin and alkaloids. The word tannin is a term used to describe a group of compounds in some plants which can "tan" (convert) animal skin to produce leather. They are colourless and non crystalline compounds (Evans, 2005). Tannins are soluble in water and alcohol and are found in the root, stem, bark and outer layers of plant tissue. They are acidic in reaction, and the acidic reaction is attributed to the presence of phenolics or carboxylic group (Kar, 2007). They also serve as anti-microbial agents in plants and are used as anti-septic due to the presence of phenolic group. Two basic groups are usually recognized: hydrolysable tannins and condensed tannins. Hydrolysable tannins, upon hydrolysis, produce gallic acid and ellagic acid and these are called gallotannins or egallitannins. On heating, they form pyrogallic acid. Common examples of hydrolysable tannins include the aflavins (from tea), diadezein, genistein and glycitein (Kar, 2007). Tannins rich medicinal plants are used as healing agents in a number of diseases which includes management of Type 2 diabetes (Kumari and Jain, 2012; Kasali *et al.*, 2016).

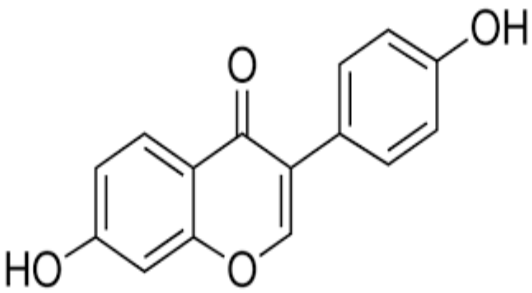


Tannic acid

Aflavins



Glycitein

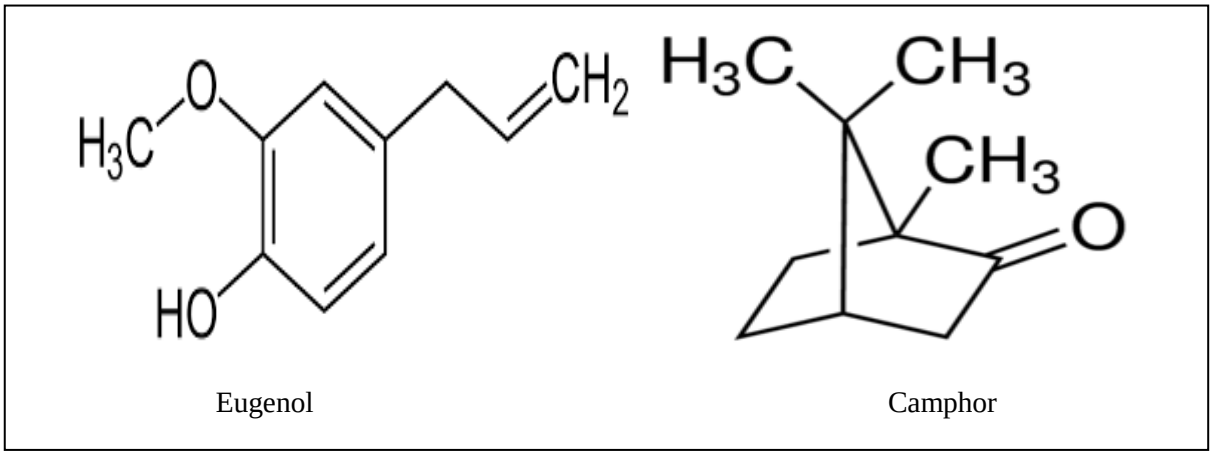


Diadzein

Figure 2.12: Chemical Structure of Some Tannins

viii) Terpenoids: Terpenoids are also called isoprenoids. They are class of naturally occurring organic chemicals derived from five-carbon isoprene units assembled and modified in thousands of ways. Most are multicyclic structure that differs from one another both in basic carbon skeleton and in their functional group. They are flammable unsaturated hydrocarbons, existing in liquid form commonly found in essential oils, resin or oleoresins (Firn, 2010). Terpenoids includes hydrocarbons of plant origin of general formula $(C_5H_8)n$ and are classified as mono-, di-, tri- and sesquiterpenoids depending on the number of carbon atoms. Examples of commonly important Monoterpenes include terpinen-4-ol, thujone, camphol, eugenol and menthol. Diterpenes are classically considered to be resins. The triterpenes include steroids, sterols and cardiac glycosides with anti- inflammatory, sedative, insecticidal or cytotoxic activity (Goto *et al.,* 2010). Sesquiterpenes like monoterpenes are major components of many essential oils. They

include camphor, beta-carotene, and digitalin, which are referred to as iso-prenoids due to the fact that all terpenoids are derived from a 5-carbon precursor isoprene. Plant terpenoids play a role in traditional herbal remedies. They contain anti-insect, anti-fungal and anti-bacterial properties (Sofowora, 2006). They are also useful for the management of metabolic disorders such as T2D, insulin resistance, hyperlipdaemia and cardiovascular diseases (Goto *et al.,* 2010; Chukwujekwu *et al.,* 2016).



Eugenol Camphor

Figure 2.13: Chemical Structure of Some Terpenoids

2.14.2 Extraction of biologically active phyto-constituents from medicinal plants

Phytochemicals have been recognized as the basis for traditional herbal medicine practiced in the past and currently in vogue in parts of the world (Lalitha and Jayanthi, 2012). In the search for phytochemicals that may be of benefit to the pharmaceutical industry, researchers sometimes follow procedures provided by local users in a specific region (Das *et al*, 2010). Following such procedures, plant parts are typically screened for phytochemicals that may be present. The presence of a phytochemical of interest may lead to its further isolation, purification and characterization. The outcome can be used as the basis for the development of a new pharmaceutical product.

Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure (Das *et al*, 2010; Tiwari, *et al.,*

2011). This therefore underscores the need to try as much solvents as possible in screening plant parts for phytochemicals. Some of the solvents that can be employed in research work for extractions of Phytochemicals are: water (aqueous), acetone, chloroform, *n*-hexane, petroleum ether, methanol and ethanol (Das *et al.*, 2010). Different solvent extracts of medicinal plants contain various phytochemicals and their medicinal values vary accordingly. In addition, the phytochemical composition of a medicinal plant may vary according to geographical location, time of harvest and to some extent the prevailing weather condition (Asante *et al*., 2016).

The quality and quantity of phytochemicals from any given plant is subject to the extraction method. Variation in extraction methods usually depend on: Length of extraction period, nature and type of solvent used, pH of the solvent used, temperature, particle size of the plant samples and finally the solvent-to-sample ratio used in the extraction. The basic principle is to grind the plant material (dry or wet) finer, which increases the surface area for extraction thereby increasing the rate of extraction. Das *et al.*, (2010) has reported ratio 10:1 (v/w) volume of solvent to dry weight of sample as ideal for maxima yield.

2.15 *Vernonia amygdalina*

2.15.1 Scientific classification of *Vernonia amygdalina*

Vernonia amygdalina belong to the Kingdom: Plantae; Division: Angiosperms; Order: Asterales; Family: Asteraceae; Genius: Vernonia and Species: *V. amygdalina*. It is a shrub or small tree of about 2-5m tall with petiolate leaf 6mm diameter and elliptic shape. The leaves are green (see plate I) with characteristic odour and a bitter taste (Singha, 1996). They do not produce seed and are basically propagated via cutting of the stem. In Nigeria it is known locally as "*onugbu*" in Ibo, "*Shuwaka*" in Hausa, "*Ewuro*" in Yoruba, "*Oriwo*" in Esan, "*Etidot*" in Efik. (Akpaso *et al.*, 2011).

2.15.2 Ecology of *Vernonia amygdalina*

Vernonia amygdalina grows under a range of ecological zones in Africa. It produces a large mass of forage and it is tolerant to drought. About 200 species of *Vernonia* has been documented in literature. The leaf of *V. amygdalina* extract is used in medicine as an antimalarial, antimicrobial, laxative, antihelmitic and antithrombotic. Both hypoglycemia and hypolidemic effects in diabetic-hyperlidemic and normoglycemic-hyperlidemic rats have been reported (Iwuji *et al*., 2010, Kaute *et al*., 2015). The leaf also exhibit anti-tumourigenic properties (Abosi and Raseroka, 2003).

2.15.3 Some medicinal uses of *Vernonia amygdalina*

Various studies conducted previously by different investigators have shown that the aqueous and various solvent extract of *V. amygdalina* has hypoglycaemic effect (Chike *et al.,* 2006, Osinubi 2007, Joyce *et al.,* 2012, Ojieh *et al.,* 2016). The leaf and root bark extract of *V. amygdalina* have been assessed for antimalarial activity (Abosi and Raseroka, 2003). The adminstration of aqueous leaf extract of *V. amygdalina* to hyperlidemic animals caused a decrease in blood plasma total cholesterol, low density liproprotein cholesterol, triacylglycerol and VLDL and an increase in blood plasma high density liproprotein cholesterol (Kaute *et al.,* 2015). The study showed that the leaf extract of *V. amygdalina* may be useful for the control of blood lipids in the prevention and treatment of coronary heart disease (Oboh and Enobhayisobo, 2009).

2.16 *Ocimum gratissimum*

2.16.1. Scientific classification of *Ocimum gratissimum*

Ocimum gratissimum is commonly known as African Basil, a herbaceous plant belonging to a genus of about thirty five species of aromatic annual and perennial herbs. It belongs to the Kingdom: Plantae, Order: Lamiales, Family: Lamiacea, Genus: Ocimum, and Species: *Ocimum*

gratissimum. It is used frequently by the different tribes in Nigeria for nutritional and medicinal purposes. *O. gratissimum,* although commonly called Scent leaf, it is known by different people of the world by different local names. It is called "*Nton*" in Ibibio/Efik, "*Efinrin*" in Yoruba, "*Daidoya*" in Hausa and "*Nchonwu*" in Igbo (Prabhu *et al.*, 2009; Effraim, 2003). *O. gratissimum* is a shrub up to 2 m, the stem is much branched and the leaves petiolate, opposite, ovate to oblong in shape, are toothed on the edges (see plate I). The inflorescences are racemes and form a set paniculé terminal. The flowers are small, irregular, whitish and grouped in spikes. The fruits are in the form of small capsules open at the apical end.

2.16.2. Ecology of *Ocimum gratissimum*

It is widely naturalized in many areas and grows predominantly in the tropics and subtropical region of the world and its greatest variability occurs in tropical Africa and India. It is widely distributed in tropical and warm temperate regions. *O. gratissimum* has been reported as a weed for pastures and prefers moist, fertile areas, but tolerates drought after flowering. It is extensively distributed all over West African Coast, Central America. It is used in Nigeria for the treatment of various ailments including diabetes mellitus (Aguiyi *et al.*, 2000; Yvette *et al.*, 2014).

2.16.3. Pharmacology of *Ocimum gratissimum*

The leaf extract of *O. gratissimum* is naturally used in the treatment of diverse disease conditions such as: diarrhea, upper respiratory tract infections, headache, conjuctunctivis, skin diseases, pneumonia, tooth and gum disorder, fever, and as mosquito repellants. *O. gratissimum* is reputed for a number of therapeutic properties including hypoglycaemic, antimutagenic activity, antibacterial activity, antihelminthic effects, antifungal properties, antidiarrhea effects and anti-convulsant activity.

According to the report of Onajobi, (1986), the thin layer chromatography analysis of the leaf fractions isolated from *O. gratissimum* indicated the presence of fairly polar compounds. Analysis of one of the components by mass spectra showed the presence of an unknown compound with a molecular mass of at least 353 daltons containing carbon, hydrogen, oxygen and possibly nitrogen. A tentative molecular formula, as deduced from computer analysis, is either $C_{21}H_{37}O_4$ or $C_{19}H_{35}N_3O$ (Prabhu *et al.*, 2009).

2.16.4. Some medicinal uses of *Ocimum gratissimum*

In the traditional system of medicine the leaves of *O. gratissimum* are said to be used by different tribes for different purposes. The leaves and flowers are used in the preparation of teas and infusion (Rabelo *et al.*, 2003). In the Coastal areas of Nigeria, the plant is used in the treatment of epilepsy, high fever and diarrhea (Effraim *et al.*, 2003). In South-eastern part of Nigeria it is used by the Ibos in the management of the baby's cord, to keep the wound surfaces sterile (Ijeh *et al.*, 2005). In the Savannah areas, the leaf decoction is used in treatment of mental illness (Akinmoladun *et al*, 2007). The Brazilian tropical forest inhabitants used a decoction of *O. gratissimum* roots as a sedative for children (Freire *et al.*, 2006). In India, the whole plant has been used for the treatment of sunstroke, headache and influenza, as a diaphoretic, antipyretic and for its anti-inflammatory activities. The leaf extract is used by some tribes in Nigeria in the treatment of diarrhea, at the same time as the cold leave infusions are used for the relief of stomach upset and haemorrhoids (Akinyemi *et al,* 2005; Ijeh *et al,* 2005). The Kenyans and Sub-Sahara Africans used *O. gratissimum* in the treatment of infections, coughs, regulation of menstruation and as a cure for the prolapsed of the rectum, abdominal pains, sore eyes, barrenness, fever, convulsions and tooth gargle. The aqueous leaf extract of *O. gratissimum* has been shown to exhibit hypoglycemic property in chemical induced diabetic rats (Aguiyi *et al.*, 2000).

It was reported that the leaf essential oil of OG (*Ocimum* oil) is effective in promoting hair growth in cyclophoshamde-induced hair loss. In the investigative report, shaved sites, 4 cm^2, were created with 30 mg/kg cyclophosphamide i.p. daily to simulate changes seen in human chemotherapy-induced hair loss. *Ocimum* oil was administered topically alone or in combination with cyclophosphamide in other groups for 14 days. Histopathology and gross morphologic observations for hair re-growth at shaved sites revealed active follicular proliferation in *Ocimum* alone and cyclophosphamide + *Ocimum* oil treated groups. The observation suggests that *Ocimum* oil may be competent in enhancing normal hair growth and promoting follicular proliferation in cyclophosphamide-induced hair loss (Orafidiya *et al.*, 2004).

Aqueous extract of *O. gratissimum* leaf has also been used to evaluate the hepatoprotective and diuretic effects (Effraim *et al.*, 2003). An investigation report of the antifungal activity of the essential oil obtained by steam-distillation (1.1% *w/w*) of the aerial parts of *O. gratissimum* and an ethanolic extract from the steam-distillation residue using the agar diffusion method revealed that the essential oil inhibited the growth of all fungi tested, including the phytopathogens, *Botryosphaeria rhodina*, *Rhizoctonia sp*. and two strains of *Alternaria* sp., while the extract from the residue was inactive (Faria *et al.*, 2006).

2.17 Animal Model of Diseases

Animal model of diseases are generally use for the study of the pathogenesis of specific disease. The investigation of *in vivo* efficacy, mode of action and side effects of anti-diabetic plants and their bioactive components involve the use of animal models (Skovso, 2014). The use of non-diabetic animals and animals with impaired glucose tolerance and insulin resistance has been used to demonstrate hypoglycemic activity and to investigate the mode of action of anti-diabetic plant constituents. It is imperative to note that agents that show a blood-glucose-lowering activity in animals are not necessarily effective in man and vice versa (Eddouks *et al.*, 2012; Suman *et al.*, 2016). This may be due at least in part to differences in hepatic metabolism, where

the metabolites are the active compounds. As a result of the heterogeneity of diabetic conditions in man, no single animal model is entirely representative of a particular type of human diabetes. Hence, several kinds of animal models have been used, each displaying a different selection of features seen in human diabetic states (Eddouks, *et al.,* 2012; Sasidharan *et al.,* 2013).

Significant variations in sensitivity to the same agent can also occur between species because of different rates of absorption, metabolism, and elimination (Serreze *et al.,* 2010). Also, a greater variety of mutations leading to diabetes observed in rodents have been characterized in more detail than those in other animal groups (Sugrue *et al.,* 2010; Eddouks *et al.,* 2012).

Considering the complex interaction among multiple susceptibility genes and between genetic and environmental factors, genetic analysis of diabetes is difficult and poorly understood in humans. Furthermore, diabetes research in humans is impeded by obvious ethical considerations, because provocation of disease is strictly impermissible in man (Srinivasan and Ramarao, 2007; Eddouks *et al.,* 2012).

Animal models of diabetes are thus greatly valuable and beneficial in biomedical studies since they offer promise of new insights into human diabetes. Inbred animal models, in which the genetic background is homogeneous and environmental factors can be controlled, are for that reason valuable in genetic dissection of multi-factorial diseases. A good number of the available models are based on rodents because of their small size, short generation interval, easy availability and economic considerations as they are less expensive to maintain than larger animals and generally show a rapid onset of their diabetic condition consistent with their short lifespan (Srinivasan and Ramarao, 2007; Sugrue *et al.,* 2010). However, non-rodent models of diabetes are urgently needed as a valuable supplement to rodents for practica reasons. The large number of different models developed for different traits and insufficient characterization of some models make it difficult to choose the right model for a given study involving

pharmacological screening and at times leading to misinterpretation of data or even to the wrong conclusions.

The choice of model will depend on the purpose of the study. For instance, in the case of pharmacological testing, the putative mechanism of the drug being tested will be instrumental in choosing an appropriate animal model. The main determinant in choosing an animal model is whether a model of autoimmunity is required. If yes, then Type 1 diabetes would be appropriate. The timing and predictability of onset is also variable in different models of Type 1 diabetes. In Type 2 diabetes, it is important to consider the mechanisms underlying the hyperglycaemia and whether this is relevant to the study. These mechanisms can include insulin resistance and/or beta cell failure. Indeed, to determine whether a drug intervention can improve symptoms in any given model may depend on whether beta cells have failed. Animal models of Type 2 diabetes can be divided into those that are obese and those that are non-obese. The majority of Type 2 diabetes models are obese, either due to genetic or dietary manipulations. However, this usually comes with a variety of associated pathologies such as dyslipidaemia and artherosclerosis (Lee *et al*., 2010; King, 2012).

Chemically induced diabetes is appropriate to use when testing drugs or therapies where the main mechanism of action is lowering blood glucose in a non-beta-cell-dependent manner; for example to test new formulations of insulin. This is also an appropriate model for testing transplantation therapies where the end point is lowering of blood glucose (Srinivasan and Ramarao, 2007). One disadvantage with chemically inducing diabetes is that the chemicals can be toxic to other organs of the body. It should also be noted that changes in P450 isozymes in the liver, kidney, lung, intestines, testis and brain have been reported after administration of STZ or alloxan, and thus, this should be considered when drugs are being tested in these models (Lee *et al*., 2010).

2.18 Streptozotocin

Streptozotocin (STZ) is an antimicrobial agent and has also been used as a chemotherapeutic alkylating agent. It is a naturally occurring chemical that is specifically toxic to the insulin-producing beta cells of the pancreas in mammals. In medical research, it is used to produce an animal model for hyperglycemia. In medicine it is used for treating certain cancers of the Islets of Langerhans. Streptozotocin is approved by the U.S. Food and Drug Administration (FDA) for treating metastatic cancer of the pancreatic islet cells. Given that it carries a substantial risk of toxicity and hardly ever cures the cancer, its use is generally restricted to patients whose cancer cannot be removed by surgery. In these patients, streptozotocin is used to reduce the tumour size and minimize symptoms like hypoglycemia due to excessive insulin secretion by insulinomas (Brentjens and Saltz, 2001). On the account of its high toxicity to pancreatic beta cells, in medical researches streptozotocin has also been used for inducing insulitis and diabetes on experimental animals (Rossini *et al.,* 1977).

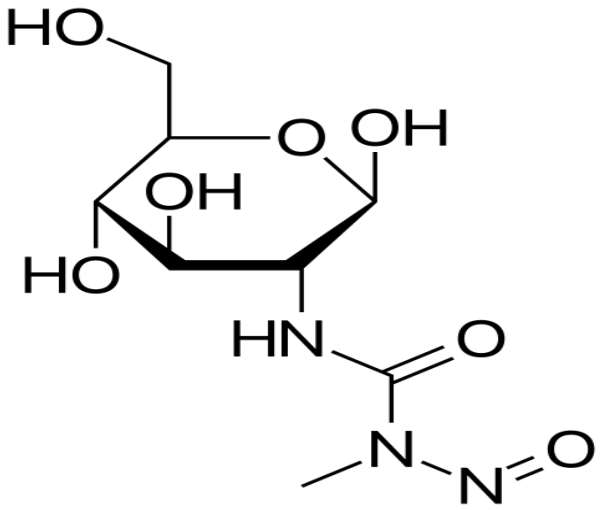


Figure 2.14: Chemical Structure of Streptozotocin

2.18.1. Mechanism of streptozotocin action

Streptozotocin is a glucosamine-nitrosourea compound. It is an alkylating agent toxic to cells by causing damage to the DNA, though other mechanisms may also contribute to its action. DNA damage induces activation of poly ADP-ribosylation, which is likely more important for

diabetes induction than DNA damage itself (Szkudelski, 2001). Streptozotocin is analogous to glucose hence transported into the cell by the glucose transport protein GLUT2, but is not recognized by the other glucose transporters. This explains its relative toxicity to beta cells, since these cells have relatively high levels of GLUT2 (Wang and Gleichmann, 1998). STZ inhibits insulin secretion and causes a state of insulin-dependent diabetes mellitus. The effect is attributed to its specific alkylating potency. Its beta cell specificity is mainly the result of selective cellular uptake and accumulation (Lenzen, 2008). In brief, Selective beta cell uptake of STZ through GLUT2 glucose transporter leads to beta cell toxicity via alkylation and the consequence is beta cell death through necrosis and ultimately ends up with Insulin dependent diabetes mellitus.

2.18.2. Beta cell selectivity of streptozotocin

Streptozotocin is a nitrosourea analogue in which the *N*-methyl-*N*-nitrosourea (MNU) moiety is linked to the carbon-2 of a hexose. The toxic action of streptozotocin and chemically related alkylating compounds requires their uptake into the cells. Nitrosoureas are usually lipophilic and tissue uptake through the plasma membrane is rapid; however, as a result of the hexose substitution, streptozotocin is less lipophilic. Streptozotocin is selectively accumulated in pancreatic beta cells via the low-affinity GLUT2 glucose transporter in the plasma membrane Thus, insulin-producing cells that do not express this glucose transporter are resistant to streptozotocin. This observation also explains the greater toxicity of streptozotocin compared with *N*-methyl-*N*-nitrosourea in cells that express GLUT2, even though both substances alkylate DNA to a similar extent. The importance of the GLUT2 glucose transporter in this process is also shown by the observation that streptozotocin damages other organs expressing this transporter, particularly kidney and liver (Wilson *et al.,* 1988; Schnedl *et al.,* 1994; Elsner *et al.,* 2000; Lenzen, 2008) .

2.19 Screening Criteria for Animal Model of Type-2 Diabetes

The age and length of exposure of rats to high fat and/or fructose diet contribute to the type of diabetes model that can be developed (Srinivasan *et al*., 2005; Zhang *et al*., 2008; Wilson and Islam, 2012). For instance, different metabolic reactions occur to similar diet at different age of rats (Willett, 2002). Han *et al.,* (1997) reported that high-fat diet altered the blood glucose level of young rats; hence it is of paramount significance to consider the age and gender of rats in experimental model of Type 2 diabetes.

The choice of gender in experimental animal is of vital significance due to hormonal differences. Sex hormone effects can be contradicting in different animal model. Male gonadectomy has been reported to protect against diabetes in some models but ineffective or increases the incidence in other models (Yoon *et al.,* 2008). It has been suggested that gender should be considered in the choice of animal model of disease (Yoon *et al.,* 2008; Zarfeshani *et al.,* 2012). Type 2 diabetes tend to occur later in life and adult rats tend to withstand the stress of STZ induction much better than the younger counterpart (Zhang *et al.,* 2008), hence, it is more reasonable to consider matured adults male rats in the development of T2D model.

The nature and duration of exposure to diet cannot be ruled out in development of T2D model. Quite a number of available animal models of T2D are either based on high-fat diet or high-fructose (Srinivasan *et al.,* 2005; Wilson and Islam 2012; Zarfeshani *et al.,* 2012; Hu *et al.,* 2013). According to Willett (2002), high fat diet model stimulates glucose intolerance with hypercholesterolemia and impaired pancreatic function, whereas, the high fructose model induces hyperinsulinemia and hypertriglyceridemia. Kuate *et al.,* (2015) demonstrated that high carbohydrate and fat diet does not induce frank diabetes in experimental rats. The blood glucose level in both diet regimens does not get to the diabetic threshold; thus, it remains unsuitable for the screening of pharmacological compounds or validation of antidiabetic drugs, like insulin secretagogues or insulin sensitizers (Basciano *et al.,* 2005; Srinivasan and Ramarao 2007).

Previously, it was reported that seven weeks old male Sprague-Dawley rats fed 40% HFD had similar blood glucose concentrations to chow-fed rats, but significantly higher insulin, free fatty acid and triglyceride concentrations indicating insulin resistance (Reed *et al.*, 2000).

Combination of high fat and high fructose in the diet of experimental animal model synergizes their metabolic outcomes. This procedure renders rat mild hyperglycemic, obese, insulin resistant, hypercholesterolemic, and hypertriglyceridemic with compensatory hyperinsulinemia (Willett, 2002). This situation is comparable to prediabetic, insulin resistant state in humans. More often than not, insulin resistance is overcomed by β-cell-induced insulin overproduction; ultimately, the conversion of prediabetes to frank hyperglycemia in patients having T2D becomes linked with a decline in the secretory ability of the overwhelmed pancreatic β-cells. For that reason, the development of frank hyperglycemia can be accomplished in insulin-resistant experimental rat using low dose of STZ.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant materials

Fresh specimen of *V. amygdalina* and *O. gratissimum* were harvested in the month of May, 2015 from local farm in Samaru (located on latitude: 11°9'55.3" longitude: 7°39'5.84"), Zaria, Kaduna State, Nigeria. The plant samples were identified and authenticated by the Herbarium unit of the Department of Botany, Ahmadu Bello University, Zaria, Nigeria and a voucher specimen number 1166 and 1285 were assigned for *V. amygdalina* and *O. gratissimum* respectively.

3.1.2 Experimental animals

Wistar albino rats weighing 150-200 g were used for the research. The animals were obtained from the animal house of the Department of Pharmacology, Ahmadu Bello University, Zaria-Nigeria. They were kept in properly ventilated cages at the Research Laboratory in the Directorate of Research and Development, Nigerian Institute of Leather and Science Technology (NILEST), Zaria, Kaduna State-Nigeria, where bedding was replaced daily, at an average room temperature and normal night/dark light cycle. They were allowed to acclimatize for two weeks prior to experimentation. During this period, they were all provided with the same commercially available rat pellets (purchased from Grand cereals Limited, Jos Nigeria and compressed into balls) and tap water *ad libitum.* Their weights were also noted. All experimental protocol was in conformity with the institutional guidelines that are in compliance with National and International Laws and Guidelines for Care and Use of Laboratory Animals in Biomedical Research.

3.1.3 Chemicals and reagents

Strepozotocin (STZ) was purchased from Adooq Bioscience, (LLC, USA). Fructose (Kem Light Laboratories PVT Ltd, India), Simas Margarine (PT Salim Ivomas Pratama Tbk, Indonesia), Normal diet feed (Grand Cereals Limited, Nigeria); kidney function and Liver enzyme kits (Randox Laboratories, UK), ultra sensitive rat insulin ELISA kit (Mercodia AB, Uppsala, Sweden), Rat C-peptide ELISA kit (WKEA Med supplies, China) and all other reagents were of analytical grade and purchased from Biorapid Diagnostics Nigeria Limited Abuja Nigeria.

3.1.4 Equipment

One-touch glucometer (Lifescan Inc 1995 Milpas, California, United States), cardiochek meter (PTS, Indianapolis, India), mission cholesterol meter (ACON Lab. Inc, USA), laboratory milling machine (Thomas-Wiley Laboratory mill Model 4, USA), rotary evaporator (Senco Rotary Evaporator, Model RE 801), rotary microtome (Heitz 150 Rotary Microtome, Cambridge model), microwave (HTMO-23EG), electronic weighing balance (JT1003B), multi plate ELISA reader (Biorad-680, BIORAD Ltd., Japan), Agilent NMR (vnmrs 400 Germany), Agilent-FTIR (Germany).

3.2 Methods

3.2.1 Extraction of the plants crude extracts

The quality of extraction of medicinal plant using conventional method such as Soxhlet extraction technique and the cold maceration were studied and compared with the microwave assisted extraction technique using leaves of *V. amygdalina* and *O. gratissimum*. The leaves of *V. amygdalina* and *O. gratissimum* were harvested, immediately sorted out to obtain only fresh samples and washed with distilled water without squeezing to remove debris and dust particles. The samples were dried under shade at room temperature to constant weights for seven days.

The dried samples were then pulverized into powder using a laboratory milling machine at the Quality Control Laboratory in NILEST, Zaria, Kaduna State, Nigeria. The powdered samples were extracted using three different extraction technologies as highlighted below:

3.2.1.1 Soxhlet extraction method

Exhaustive Soxhlet extraction was performed using classical apparatus with 50 g each of the powdered leaf samples of *V. amygdalina* and *O. gratissimum*. Extraction was performed with methanol (1:10 *w/v*) (Handa *et al*., 2008). After extraction, the methanol solvent was evaporated completely to concentrate it in vacuum by using rotary evaporator at 40 ^{0}C under reduced pressure. The solvent free methanol extract was thereafter evaluated for the study.

3.2.1.2 Cold maceration method

Maceration was carried out in a closed conical flask for 72 h at room temperature with frequent agitation (Handa *et al*., 2008), using 50 g powdered leaf samples of *V. amygdalina* and *O. gratissimum* each and methanol (1:10 *w/v*) (Azwanida, 2015). After 72 h the mixture was strained by filtration and washed with methanol (1:8 *w/v*). The suspension after maceration was centrifuged and the supernatant evaporated under reduced pressure. The solvent free methanol extracts obtained were similarly evaluated.

3.2.1.3 Microwave assisted extraction method

Exactly 50g of the homogeneous powder leaf samples *V. amygdalina* and *O. gratissimum* each was mixed with methanol (1:5 *w/v*) (Azwanida, 2015). After a preleaching time of 5 min, the suspension was irradiated with microwave set on defrost at 3 min pulse (Asghari *et al.,* 2011; Umar *et al.,* 2015). The samples were treated under microwave irradiation in an intermittent way for five times. After extraction, the samples were centrifuged at 4000 rpm (3520 × g) and the supernatant evaporated under pressure. The dried residue was evaluated accordingly.

The percentage yield (*w/w*) by the three extraction methods was calculated using the formula:

$$\text{Percentage yield} = \frac{\text{Mass of extract (g)}}{\text{Mass of plant sample (g)}} \times 100$$

3.2.2 Effect of extraction methods on the yield and hypoglycemic activity of *Vernonia amygdalina* and *Ocimum gratissimum* leaves

The extracts obtained from the three extraction methods were compared by examining the yield and phytoconstituents of the leaf extracts of *V. amygdalina* and *O. gratissimum*. The comparison was done using the same sample size (50 g) and the biological activities (hypoglycemic effects) were analyzed on rat model of Type 1 diabetes developed as follow: First, the rats were fasted overnight after which they were given a single intra-peritoneal injection (*ip*) of 55 mg/kg b.w. of streptozotocin (STZ) dissolved in 0.1 mL fresh cold citrate buffer pH 4.5. Confirmation of diabetes was done 72 h after STZ induction, using a One Touch Glucometer. Blood samples were obtained from the tail puncture of the rats. Animals with fasting blood glucose ≥ 200 mg/dL, after 10 d of STZ induction were considered diabetic and included in the study as diabetic animals. The rats were divided into eight groups of six rats each as follow:

i Group CME-VA: Diabetic rats treated with *V. amygdalina* extracts obtained from cold maceration extraction method.

ii Group SEE-VA: Diabetic rats treated with *V. amygdalina* extracts obtained from Soxhlet extraction method.

iii Group MAE-VA: Diabetic rats treated with *V. amygdalina* extracts obtained from Microwave Assisted extraction method.

iv Group CME-OG: Diabetic rats treated with *O. gratissimum* extracts obtained from cold maceration extraction method.

iv Group SEE-OG: Diabetic rats treated with *O. gratissimum* extracts obtained from Soxhlet extraction method.

v Group MAE-OG: Diabetic rats treated with *Ocimum gratissimum* extracts obtained from Microwave Assisted extraction method.

vi DC (Diabetic control): Diabetic rats treated with vehicle alone.

vii NC (Normal control): Normoglycemic rats given distilled water.

The extracts from the different extraction methods were administered at the dose of 250 mg/kg b.w for a period of 21 days and the fasting blood glucose of the experimental rats were determined weekly.

3.2.3. Further extraction of Vernonia amygdalina and Ocimum gratissimum leaves

On the bases of the preliminary evaluation of the three extraction technologies carried out as described above, the cold maceration procedure was chosen for further study. One thousand two hundred and fifty grams (1250 g) each of the powdered leaves of the two plants were respectively soaked in methanol (3 × 4.5 L) for 72 h and filtered. After filtration, the methanol solvent was evaporated completely to concentrate it in vacuum by using rotary evaporator at 40 ^{0}C under reduced pressure. The solvent free methanol extract was stored in a refrigerator at 4 °C until needed.

3.2.4 Fractionation of the methanol crude extract of Vernonia amygdalina and Ocimum gratissimum leaves

The solvent free methanol crude extracts (100 g) of each plant was suspended in 500 ml distilled water and then fractionated using organic solvents in an increasing order of polarity (*n*-hexane, chloroform, ethyl acetate, butanol and water). The crude extract was soaked in *n*-hexane in a separating funnel, shaken and allowed to stand for phase separation into two fractions. The *n*-hexane fraction was carefully decanted after partitioning. More of the *n*-hexane solvent was added and the same process repeated until no further colour change was observed with *n*-hexane. The *n*-hexane soluble fraction was obtained and allowed to dry under room temperature to obtain the *n*-hexane fraction. The same procedure was repeated for chloroform, ethyl-acetate

and butanol to obtain the respective solvent fraction of each plant. The resulting residue was dissolved in water and referred to as the aqueous fraction. Each fraction obtained was concentrated using a rotary evaporator, the remaining solvent in the extract was allowed to evaporate at room temperature to a constant weight. The fractions derived were screened for anti-diabetic activities *in vivo* using diabetic rat model.

3.2.5. Qualitative screening of phytochemicals present in *V. amygdalina* and *O. gratissimum* leaves

Chemical tests for the screening and identification of the various bioactive phytochemical constituents in the *V. amygdalina* and *O. gratissimum* was carried out on the freshly prepared crude extracts and its fractions using the standard procedures as described by Sofowora (2006), Evans (2005) and Harborne (1998). The screening involves detection of alkaloids, flavonoids, saponins, tannins, anthraquinones, glycosides, steroid compounds and phenolic compounds.

3.2.5.1 Test for alkaloids using Wagner's reagent

Two milligram of the fractionated methanol free crude extracts of the leaves of *V. amygdalina* and *O. gratissimum* were treated with dilute HCl and filtered. The filtrates were treated with 3-5 drops of Wagner's reagent (1.27 g of iodine and 2 g of potassium iodide in 100 ml of water) and observed for the formation of reddish brown precipitate (or colouration).

3.2.5.2 Test for carbohydrates using Molisch's test

Three drops of Molisch's reagent were added to 2 ml portion of the various fractionated methanol free crude extracts of the leaves of *V. amygdalina* and *O. gratissimum*. This was followed by addition of 2 ml of conc. H_2SO_4 down the side of the test tube. The mixture was then allowed to stand for two-three minutes. Formation of a red or dull violet colour at the interphase of the two layers was a positive test.

3.2.5.3 Test for cardiac glycosides using Keller Kelliani's test

Exactly 0.5 mg of each fractionated methanol free crude extracts of the leaves of *V. amygdalina* and *O. gratissimum* were dissolved in 5 ml distilled water and filtered. The filtrates were treated with 2ml of glacial acetic acid in a test tube and a drop of Ferric chloride solution was added to it. This was carefully layed with 1 ml concentrated sulphuric acid. A brown ring at the interface indicated the presence of deoxysugar characteristic of cardenolides. A violet ring may appear below the ring while in the acetic acid layer, a greenish ring may form.

3.2.5.4 Test for flavonoids using alkaline reagent test

Exactly 0.5 mg of each fractionated methanol free crude extracts of the leaves of *V. amygdalina* and *O. gratissimum* were dissolved in 5 ml distilled water and filtered. About 2 ml of the filtrates were treated with few drops of 20 % sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute hydrochloric acid, indicates the presence of flavonoids.

3.2.5.5 Test for phenols using Ferric chloride test

Exactly 0.5 mg of each fractionated methanol free crude extracts of the leaves of *V. amygdalina* and *O. gratissimum* were dissolved in 5ml distilled water and filtered. About 2 ml of the filtrates were treated with aqueous 5 % ferric chloride and observed for formation of deep blue or black colour.

3.2.5.6 Test for saponins using foam test

Exactly 0.5 mg of each fractionated methanol free crude extracts of the leaves of *V. amygdalina* and *O. gratissimum* were dissolved in 5ml distilled water and filtered. To 2 ml of filtrates were added 6ml of water in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam that confirms the presence of saponins.

3.2.2.7 Test for sterols using Liebermann-Burchard test

Exactly 0.5 mg of each fractionated methanol free crude extracts of the leaves of *V. amygdalina* and *O. gratissimum* were dissolved in 5 ml distilled water and filtered. To 1 ml of filtrates were treated with few drops of chloroform, acetic anhydride and conc. H_2SO_4 and observed for the formation of dark pink or red colour.

3.2.5.8 Test for tannins using Braymer's test:

Exactly 0.5 mg of each fractionated methanol free crude extracts of the leaves of *V. amygdalina* and *O. gratissimum* were dissolved in 5 ml distilled water and filtered. To 2 ml of filtrate was treated with 10 % alcoholic ferric chloride solution and observed for the formation of blue or greenish colour solution.

3.2.5.9 Test for terpenoids using Salkowki's test.

Exactly 0.5 mg of each fractionated methanol free crude extracts of the leaves of *V. amygdalina* and *O. gratissimum* were dissolved in 5ml distilled water and filtered. Exactly 1 ml of chloroform was added to 2 ml of each filtrate followed by a few drops of concentrated sulphuric acid. A reddish brown precipitate produced immediately indicated the presence of terpenoids.

3.2.5.10 Test for anthraquinones using Borntrager's test.

About 5 mg of the fractionated methanol free crude extracts of the leaves of *V. amygdalina* and *O. gratissimum* were boiled with 10 % HCl for few minutes in a water bath. It was filtered and allowed to cool. Equal volume of $CHCl_3$ was added to the filtrate. Few drops of 10 % NH_3 were added to the mixture and heated. Formation of pink colour indicates the presence of anthraquinones.

3.2.6. Quantitative determination of phytochemicals present in *V. amygdalina* and *O. gratissimum* leaves

The quantitative determination of the phytochemicals present in the fractionated methanol free crude extracts of the leaves of *V. amygdalina* and *O. gratissimum* were carried out using standard procedures as described below.

3.2.6.1 Determination of Alkaloids

The quantity determination of alkaloids was done using the method of Harborne (1973). Two grams each of the fractionated methanol free crude extracts of the leaves of *V. amygdalina* and *O. gratissimum* were separately put in a 250 ml beaker; 80 ml of 10 % acetic acid in ethanol was added, covered and allowed to stand for 4 h. It was then filtered and the filtrate was concentrated on a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was then added in drops to the filtrate until the precipitation was complete. The whole solution was collected and allowed to settle and then the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue which is the alkaloid was dried in an oven and weighed, using electronic weighing balance. The percentage of alkaloid is expressed mathematically as

$$Percentage\ of\ alkaloid\ = \frac{Weight\ of\ alkaloid}{Weight\ of\ sample} \times 100$$

3.2.6.2 Determination of Flavonoids

Determination of the quantity of flavonoids was done as described by Krishnaiah *et al.*, (2009). One gram each of the fractionated methanol free crude extracts of the leaves of *V. amygdalina* and *O. gratissimum* were extracted separately with 100 ml of methanol at room temperature. The whole solution was filtered using filter paper No 42 (125 mm). The filtrate was later transferred into a crucible and evaporated to dryness over a water bath and weighed to a constant weight, and the weight was calculated.

$$Percentage\ of\ flavonoid\ = \frac{Weight\ of\ flavonoid}{Weight\ of\ sample} \times 100$$

3.2.6.3 Determination of Phenolics

Determination of the quantity of Phenolic compounds by spectrophotometric method described by Ganapaty *et al.*, (2013). Exactly 100 mg each of the fractionated methanol free crude extracts of the leaves of *V. amygdalina* and *O. gratissimum* were weighed separately and dissolved in 100 ml of triple distilled water (TDW). Then 1ml of the solution was transferred to a test tube and then 0.5 ml 2 N of the Folin-Ciocalteu reagent and 1.5 ml 20 % of Na_2CO_3 solution was added and the volume was made up to 8ml with TDW, followed by vigorous shaking and allowed to stand for 2 h. Then the absorbance was taken at 750 nm. The data was used to estimate the total phenolic content using a calibration curve obtained from various diluted concentrations of gallic acid.

3.2.6.4 Determination of Saponins

Determination of the quantity of Saponins was carried out using the method reported by Obadoni and Ochuko (2001). Five grams each of the fractionated methanol free crude extracts of the leaves of *V. amygdalina* and *O. gratissimum* were put separately into a conical flask and 100 ml of 20 % aqueous ethanol was added. It was then heated over the water bath for 4 h with continuous stirring at 55 °C. This mixture was then filtered and the residue was re-extracted with another 100 ml of 20 % aqueous ethanol. This combined extracts were reduced to 40 ml over water bath at about 90 °C. These concentrates were transferred into a 250 ml separator funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and 60 ml of *n*-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5 % aqueous sodium chloride. The remaining solution was heated in the water bath. After

evaporation the samples were dried in the oven to a constant weight to give the saponin. The percentage of saponin content was then calculated.

$$Percentage\ of\ saponin\ = \frac{Weight\ of\ saponin}{Weight\ of\ sample} \times 100$$

3.2.6.5 Determination of Tannins

Determination of the quantity of tannins was carried out according to the method described by Van-Burden and Robinson (1981). Exactly 500 mg each of the fractionated methanol free crude extracts of the leaves of *V. amygdalina* and *O. gratissimum* were weighed separately into a 50 ml plastic bottle. Fifty millilitres of distilled water was added and then shaken vigorously in a mechanical shaker for one hour. This was then filtered into a 50 ml volumetric flask and made up to the mark. Five millilitres of the filtrate was pipetted out into a test tube and mixed with 2 ml of 0.1M $FeCl_3$ in 0.1N HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 minutes to get the total tannins.

3.2.7 Acute toxicity test

Acute toxicity studies of the methanol free crude extracts of the leaves of *V. amygdalina* and *O. gratissimum* were carried out separately as described by Lorke (1983) with oral administration of increasing doses of the extracts from 10 mg/kg body weight to 5000 mg/kg body weight. The animals were observed continuously for behavioural changes, palpitation, respiratory rate toxicity or mortality for 24 h.

3.2.8 Preparation of animal model for in vivo bioassay screening of the fractions

After two weeks of acclimatization, the body weight, blood glucose and lipid profile of the animals were determined.

3.2.8.1 Induction of diabetes

Two different procedures were used to induce diabetes in the rats.

Procedure 1: A set of 30 rats that were maintained on commercially available Normal Diet Feed (NDF). The rats were divided into 5 groups of 6 rats and fasted overnight. Each group was given single intra-peritoneal injection (*ip*) of 0, 25, 35, 45, 55 mg/kg b.w. of streptozotocin (STZ) dissolved in 0.1 ml fresh cold citrate buffer pH 4.5.

Procedure 2: Another set of 30 rats were treated with some modification of the combined methods described by Srinivasan *et al.,* (2005), Zhang *et al.,* (2008), Wilson and Islam (2012). The NDF was fortified with Simas Margarine at a ratio of 11:1. This was administered together with 20 % fructose solution as drinking water to the rats *ad libitum* for six weeks. After which they were fasted overnight and divided into 5 groups of 6 rats. Each group was injected (*ip*) respectively with a single dose of 0, 25, 35, 45, 55 mg/kg b.w. of STZ dissolved in a citrate buffer, pH 4.5.

3.2.8.2 Confirmation of diabetes

Confirmation of diabetes was done three days after STZ induction, using a One-touch glucometer. Blood samples were obtained exactly after ~6 h of fasting from the tail puncture of the rats.

3.2.8.3 Validation of diabetes

The mean fluid and feed intake of each animal was recorded daily. The percentage change in body weight before and after dietary regimen/ STZ induction was also recorded. Validation of diabetes was done 10 days after STZ induction. Blood sample was obtained from the tail tip of each rat by single prick. The first drop of blood was discarded in order to ensure stable hyperglycaemia. The subsequent blood obtained was analysed using glucometer. Animals with

fasting blood glucose (FBG) ≥ 300 mg/dL (16.7 mM) were considered diabetic and included in the study as diabetic animals (Srinivasan *et al.*, 2005).

3.2.8.4 Screening for T2D rat model.

After validation of diabetes, oral glucose tolerance test (OGTT) was performed after an overnight fast on the FDF-fed, NDF-fed and STZ-treated rats. The rats were orally dosed with a D-glucose solution (2.0 g/kg b.w.) and blood glucose concentrations were subsequently measured at 0 (just prior to oral glucose dosing), 30, 60, 90, and 120 min after the oral dosing of glucose using blood samples collected from the tail puncture of the rats. A one-touch glucometer was used for the measurement. The blood total cholesterol (TC) and triacyglycerol (TG) were also measured using one touch panels test strips on cardiochek meter.

At the end of the experimental phase, animals were fasted overnight and then euthanized. The terminal blood collected was used for the determination of percentage circulating C-peptide and insulin concentrations. Homeostatic Model Assessment (HOMA) score for insulin resistance and β-cell function (HOMA-IR and HOMA- β) were calculated as described by Matthew *et al.*, (1985).

$$\mathrm{HOMA-IR} = \frac{\text{Insulin (U/l)} \times \text{Blood glucose (mmol/l)}}{22.5}$$

$$HOMA-\beta = \frac{20 \times \text{Insulin (U/l)}}{\text{Blood glucose (mmol/l)}} - 3.5$$

The serum insulin and C-peptide levels were measured using ultra sensitive insulin ELISA and rat C-peptide ELISA kits respectively. Values of C-peptide equal to the normal reference range of 0.17 - 0.9 nmol/l or higher indicated T2D, while C-peptide values lower than the above reference range value indicated T1D (Vijan, 2010). The T2D rat models were selected for further studies.

3.2.8.5 Stability of experimental diabetic model and validation for pharmacological screening.

Based on the outcome of section 3.2.8.4, the FDF_{35} group was selected for further investigation. The stability of the selected FDF_{35} model of diabetes was investigated for six weeks post validation of diabetes by monitoring the fasting blood glucose. Thereafter, the suitability of the model for screening purposes and pharmacological testing was established using three confirmed anti-diabetic drugs with different mechanism of actions for T2D. The diabetic rats were treated once daily for 7 days using pioglitazone (10 mg/kg bw), metformin (500 mg/kg bw) or glibenclamide (5 mg/kg bw) orally. The drugs were dissolved in 1 % sodium carboxymethyl cellulose as vehicle. After the one week of oral drug administration, blood sample was withdrawn for determination of blood glucose, total cholesterol, triglyceride and serum insulin level.

3.2.9 Determination of minimum therapeutic dose of *Vernonia amygdalina* and *Ocimum gratissimum* in diabetic rats

The hypoglycaemic activity of the methanol crude extracts of the leaves of *V. amygdalina* and *O. gratissimum* were evaluated separately in order to determine the minimum dosage of extract to be administered. Diabetic rats were divided into 5 groups (V_{100}, $V_{150,}$ ... G_{100}, G_{150}, ... DC) of 3 rats each. The animals of group DC served as untreated control whereas the other groups were administered with the methanol crude extract of *V. amygdalina* and *O. gratissimum* at a single dose of 100, 150, 200 mg/kg etc respectively.

Blood glucose was estimated at 0, 1, 2, 3 and 4 hours calculated for each group using the following formula:

$$\%\, Variation\ of\ glycaemic = \frac{Gi - Gt}{Gi} \times 100$$

Where Gi is the initial glycaemia (0 h) and Gt are the values of glycaemia at 1, 2, 3 and 4 h respectively. The plasma glucose levels of different groups at different time intervals were also

compared. The extract dose that lowered the glucose level by 25 % at 4 h was considered the minimum hypoglycaemic dosage (Oguanobi *et al.*, 2012).

3.2.10 Effects of fractions of fractionated methanol extracts of *Vernonia amygdalina* and *Ocimum gratissimum* leaves in rat model of Type 2 diabetes

The T2D rats were divided into two sets of 48 rats each. Parts of the first set of 48 rats were treated with the fractionated methanol crude extracts of the leaves of *O. gratissimum* while some parts of the second set of 48 rats were treated with the fractionated methanol crude extracts of the leaves of *V. amygdalina*.

3.2.10.1 Groups treated with fractionated methanol extracts of Ocimum gratissimum leaf

The first set of T2D rats were divided into eight groups of six rats each.

i. *Group NC:* Normal control: normoglycemic rats given distilled water.

ii *Group DC:* Diabetic control: diabetic rats treated with vehicle alone.

iii *Group OGh:* Diabetic rats treated with hexane fraction of the fractionated methanol crude extracts of *O. gratissimum* leaf.

iv *Group OGc:* Diabetic rats treated with chloroform fraction of the fractionated methanol crude extracts of *O. gratissimum* leaf.

v *Group OGe:* Diabetic rats treated with ethyl-acetate fraction of the fractionated methanol crude extracts of *O. gratissimum* leaf.

vi *Group OGb:* Diabetic rats treated with *n*-butanol fraction of the fractionated methanol crude extracts of *O. gratissimum* leaf.

vii *Group OGa:* Diabetic rats treated with aqueous fraction of the fractionated methanol crude extracts of *O. gratissimum* leaf.

viii *Group PC:* Positive control: diabetic rats treated with metformin (500 mg/kg b.w.)

3.2.10.2 Groups treated with fractionated methanol extracts of Vernonia amygdalina leaf

The second set of T2D rats were divided into eight groups of six rats each.

i *Group NC:* Normal control: normoglycaemic rats given distilled water.

ii *Group DC:* Diabetic control: diabetic rats treated with vehicle alone

iii *Group VAh:* Diabetic rats treated with hexane fraction of the fractionated methanol crude extracts of *V. amygdalina* leaf.

iv *Group VAc:* Diabetic rats treated with chloroform fraction of the fractionated methanol crude extracts of *V. amygdalina* leaf.

v *Group VAe:* Diabetic rats treated with ethyl-acetate fraction of the fractionated methanol crude extracts of *V. amygdalina* leaf.

vi *Group VAb:* Diabetic rats treated with *n*-butanol fraction of the fractionated methanol crude extracts of *V. amygdalina* leaf.

vii *Group VAa:* Diabetic rats treated with aqueous fraction of the fractionated methanol crude extracts of *V. amygdalina* leaf.

viii *Group PC:* Positive control: diabetic rats treated with metformin (500 mg/kg b.w.)

3.2.10.3 Treatment of animals with fractionated methanol crude extracts of *V. amygdalina* and *O. gratissimum* leaves

The fractionated methanol crude extracts were administered at a dose of 250 mg/kg body weight by oral intubation to the diabetic rats daily for 4 weeks. The 250 mg/kg b.w. extract dose used in the treatment was from the study on section 3.2.9.

Daily feed, fluid intake and weekly body weight changes were measured during the entire experimental period. At the end of the experimental period, all animals were fasted overnight and then euthanized by anaesthesia. Blood was collected into plain tubes through cardiac puncture and placed immediately on ice for 3 hours then centrifuged at 3,000 rpm for 15 minutes to obtain serum, which was stored at -30^{o}C until further analysis. Liver and pancreatic tissues were collected, washed with normal saline, wiped, weighed and preserved in buffered formalin (10% formaldehyde in phosphate buffered saline) for histopathological study.

3.2.10.4 Oral glucose tolerance test (OGTT)

At the end of the 28th day of treatment, at 3 h after the last dose of treatment OGTT was performed. Rats were orally dosed with a D-glucose solution (2.0 g/kg b.w.) and blood glucose concentrations were subsequently measured at 0 (just prior to oral glucose dosing), 30, 60, 90, and 120 min after the oral dosing of glucose (Wilson and Islam, 2012). The blood sample was obtained through the tail puncture of the rats. A one-touch glucometer was used for the measurement. The Area under the glucose curve (AUC) for OGTT was calculated using the Trapezoid rule. The trapezoid rule works by approximating the region under the graph of the function $f(x)$ as a trapezoid and calculating its area for each rat as follow

$$\int_a^b f(x)\mathrm{dx} = (\mathrm{b} - \mathrm{a})[\frac{\mathrm{f(a)} + \mathrm{f(b)}}{2}]$$

Where "a"= initial blood glucose and "b" = final blood glucose; "b-a" The time interval

3.2.10.5 Biochemical analyses.

(i) Fasting plasma glucose (FPG): Blood sample collected from the sacrificed animals at the end of the experiment was used to determine the FPG using glucometer. The glucometer test strips works by the modified glucose-oxidase method described by Trinder, (1969).

Reaction Principle: The enzymatic reaction sequence of the test strips and the meter in the assay of glucose is as follows:

β-D-Glucose + $2H_2O$ + O_2 ⟶ H_2O_2 + D-Gluconic Acid

H_2O_2 + 4-aminoantipyrine + **P**-HBS ⟶ Iminoquinone + H_2O

β-D-Glucose is oxidized by glucose oxidase to produce D-gluconic acid and hydrogen peroxide. The hydrogen peroxide is then oxidatively coupled with 4-aminoantipyrine and phenol substitute, p-HBS, in the presence of peroxidase to yield a red iminoquinone dye. The intensity of red coloured complex formed on the test strips is proportional to glucose concentration and can be displayed by the glucose meter.

Test procedure: The lateral tail veins of animal were pricked using sterile needle to obtain blood sample. A drop of the blood was applied to the end tip of the glucometer test strip. A transient electrical current is formed during the reaction and the blood glucose concentration is calculated based on the electrical current detected by the meter, then the results are shown on the meter display. The meter is calibrated to display concentration of results in mg/dL or mmol/L.

(ii) Liver function enzymes: Liver function enzymes (aspartate transaminase AST and alanine transaminase ALT) were measured according to the method of Reitman and Frankel, (1957) using reagents kits (Randox kits).

Reaction principle for AST: Two substrates participate in the reaction catalyzed by AST, *L*-aspartate and Oxoglutarate. With the help of NADH coenzyme, Malate dehydrogenase (MDH) contained in the reagent catalyses the transformation of Oxaloacetate released in the first reaction. The oxido-reductive process of NADH /NAD^+ is indicated by a decrease in absorbance at 340 nm.

L-Aspartate + α-Ketoglutarate $\xrightarrow{AST}$ *L*-glutamate + Oxaloacetate

Oxaloacetate + NADH $\xrightarrow{MDH}$ *L*-Malate + NAD^+

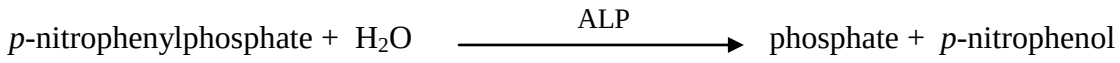
Reaction principle for ALT: Two substrates participate in the reaction catalyzed by ALT, *L*-alanine and Oxoglutarate. With the help of NADH coenzyme, Malate dehydrogenase (MDH) contained in the reagent catalyses the transformation of Oxaloacetate released in the first reaction. The oxido-reductive process of NADH /NAD^+ is indicated by a decrease in absorbance at 340 nm.

L-Alanine + α-Ketoglutarate $\xrightarrow{ALT}$ *L*-glutamate + Pyruvate

Pyruvate + NADH $\xrightarrow{MDH}$ *L*-Lactate + NAD^+

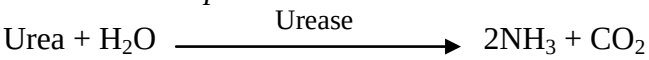
(iii) Alkaline phosphatase (ALP): Serum ALP was determined according to the method described by Gscc, (1972) using reagents kits (Randox kits).

Reaction Principle for ALP:



(iv) Serum urea: Serum urea was determined according to the method described by Patton and Crouch, (1977).

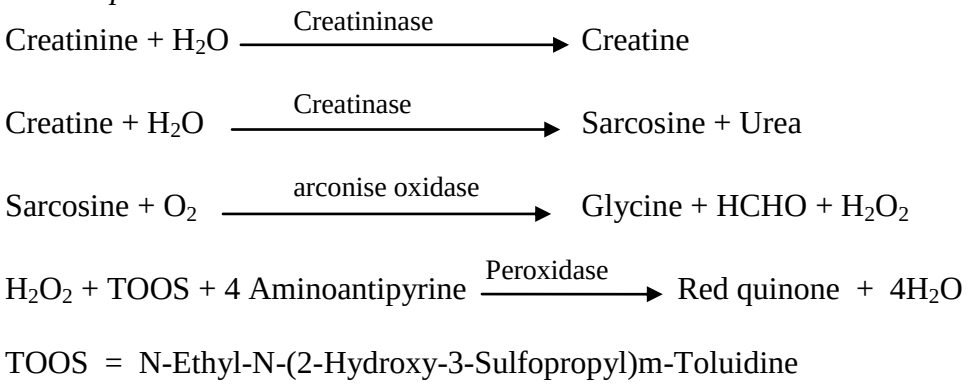
Reaction Principle:



Salicylate and hypochlorite in the reagent react with the ammonium ions to form a green complex (2,2 dicarboxylindophenol).

(v) Serum creatinine: Serum creatinine was determined according to the method described by Badiou *et al*., (2003)

Reaction Principle:



TOOS = N-Ethyl-N-(2-Hydroxy-3-Sulfopropyl)m-Toluidine

(vi) Uric acid: Uric acid was measured colorimetrically using Randox kits (Randox Laboratories Ltd, UK) (Krieg *et al*., 1986). Uric Acid is converted by uricase to allantoin and hydrogen peroxide, which under the catalytic influence of peroxidase, oxidizes 3,5-Dichloro-2-hydroxybenzenesulfonic acid and 4-aminophenazone to form a red-violet quinone-imine compound.

Reaction Principle

$$\text{Uric Acid} + O_2 + 2H_2O \xrightarrow{\text{uricase}} \text{Allantoin} + CO_2 + H_2O_2$$

$$2H_2O_2 + \text{3,5-dichloro-2-hydroxybenzenesulfonic acid} + \text{4-aminophenazone} \xrightarrow{\text{Peroxidase}} \text{N-(4-antipyryl)-3-chloro-5-sulfonate-p- benzo-quinoneimine}$$

(vii) Serum lipid profile: The lipid profile was measured using One-touch panels test strips cardiochek meter (Williams, 2002). The Test Devices uses a timed-end point method to measure the TC/HDL, HDL/TG concentrations in whole blood. The concentration of LDL is calculated by the values of TC, TG and HDL. The system monitors the change in absorbance at 635 nm at a fixed time interval. The change in absorbance is directly proportional to the concentration of lipid in the specimen. After the test strip is inserted into the analyzer and blood applied to the strip, test results are displayed in about two minutes.

General principles of the test device: When blood is applied to a test strip, the blood reacts to produce colour that is read by the analyzer using reflectance photometry. The amount of colour produced is proportional to the concentration of the lipid in the specimen. The specific enzymatic reactions that occur are highlighted below.

Specific reaction principle

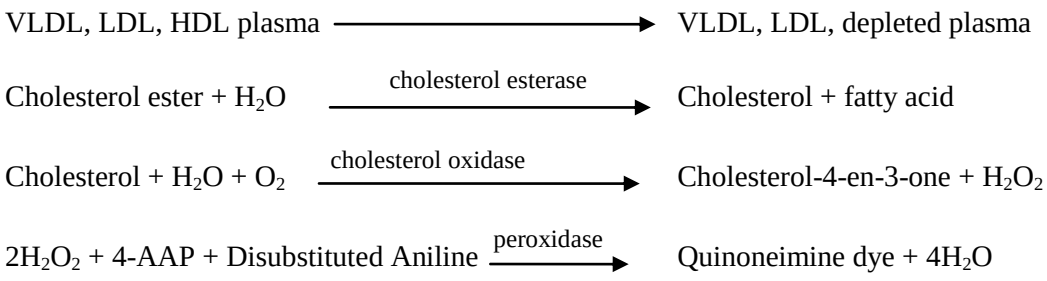
Total cholesterol: In the reaction, cholesterol esterase hydrolyses cholesterol esters to free cholesterol and fatty acids. The free cholesterol is oxidized to cholesten-3-one and hydrogen peroxide by cholesterol oxidase. Peroxidase catalyses the reaction of hydrogen peroxidase with 4-aminoantipyrine and phenol to produce a coloured quinone-imine product.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{Cholesterol} + \text{fatty acid}$$

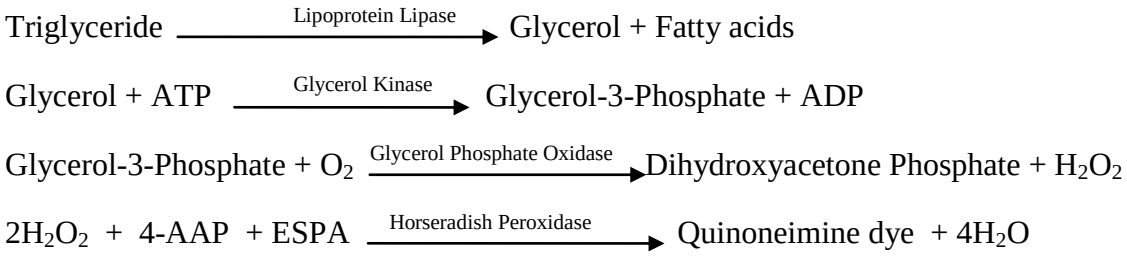
$$\text{Cholesterol} + H_2O + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{Cholesterol-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4-AAP} + \text{Disubstituted Aniline} \xrightarrow{\text{peroxidise}} \text{Quinoneimine dye} + 4H_2O$$

High density lipoprotein cholesterol: The dextran sulphate/Mg^{2+} on the test device precipitates the chylomicrons, VLDL and LDL, leaving HDL in the specimen. The cholesterol concentration of this HDL is then determined enzymatically (Williams, 2002), the same as TC.



Triglyceride: Triglycerides in the specimen are hydrolysed to glycerol and free fatty acids by action of lipase. A sequence of three coupled enzymatic steps using glycerol kinase (GK), glycerophosphate oxidase (GPO), and horseradish peroxidise (HPO) causes the oxidative coupling of 4-aminotipyrine to form a blue dye (Williams, 2002).



Low density lipoprotein cholesterol: when the concentration of TG in the specimen is equal to or lower than 400 mg/dL, LDL concentration can be calculated by the meter with the Friedeward equation (Friedewald *et al.*, 1972):

$$\text{LDLc (mM)} = (\text{Tc} - \text{HDLc}) - \text{TAG}/2.2$$
$$\text{LDLc (mg/dl)} = (\text{Tc} - \text{HDLc}) - \text{TAG}/5$$

The atherogenic ratio (Tc:HDLc) was calculated by dividing the Tc by HDLc.

Test procedure: The lateral tail veins of animal were pricked using sterile needle to obtain blood sample. The first drop of blood was wiped away and 35 µL of blood was collected using a capillary tube. The cardiocheck meter is switch on and the test device slot in. When the meter

starts flashing blood drop symbol, the blood specimen is applied on the teat area of the test device. Three dashed lines appear on the meter indicating test in progress and the results are displayed on the screen after 2 minutes. The cardiochek test device uses a timed-endpoint method to measure the total cholesterol/HDL/triglyceride concentrations in whole blood, serum or plasma. The concentration of LDL is calculated from the values of TC, TG and HDL. The system monitors the change in absorbance at 635 nm at a fixed-time interval

(viii) Serum insulin: Serum insulin was analyzed by using an ultra sensitive rat insulin ELISA kit using a multi plate ELISA reader according to the manufacturer's guideline as described by Byersdorfer *et al,* (2005).

Reaction principle: During incubation, insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies which are bound to the micro-titer wells. A sample washing step removes the unbound enzyme-labelled antibody. The bound conjugate was detected by the reaction with 3, 3', 5, 5'-tetramethylbenzidine. The reaction was stopped by adding acid to give a colorimetric end-point and optical density was measured by using a microplate auto-reader (iMark^{TM}; Bio-Rad, Hercules, CA, USA) at a wavelength of 450 nm

(ix) Rat C-peptide: Rat C-peptide ELISA which is a solid phase two-site enzyme immunoassay was used. The reaction principle is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the C-peptide molecule (Wahren *et al*., 2007).

Reaction principle: During incubation C-peptide in the sample reacts with anti-C-peptide antibodies bound to the microtitration well. After washing, peroxidise conjugated anti-C-peptide antibodies are added. After a second incubation and a simple washing step, the bound conjugate is detected by reaction with 3,3’,5,5’-tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

(x) Insulin sensitivity: Insulin sensitivity (IS) was quantified using the quantitative insulin sensitivity check index QUICKI (Matthew *et al.,* 1985). This is derived using the inverse of the sum of the logarithm of the fasting insulin and fasting glucose.

$$IS = \frac{1}{\text{Log [fasting insulin (U/l)]} \times \text{log [fasting glucose (mmol/l)]}}$$

(xi) Insulin resistance and β-cell function: Homeostatic Model Assessment (HOMA) score for insulin resistance and β-cell function (HOMA-IR and HOMA- β) were calculated as described by Matthew *et al.,* (1985) using the following formula:

$$\text{HOMA-IR} = \frac{\text{Insulin (U/l)} \times \text{Blood glucose (mmol/l)}}{22.5}$$

$$\text{HOMA-}\beta = \frac{20 \times \text{Insulin (U/l)}}{\text{Blood glucose (mmol/l)}} - 3.5$$

Conversion factor: Insulin (1U/l = 7.174 pmol/l) and blood glucose (1 mmol/l = 18 mg/dl).

(xii) Histopathological examination of liver and pancreatic tissue: The preserved Liver and pancreatic tissues were treated according to a standard laboratory protocol (Spitalnik, 2016). In brief, some of the pancreas and liver were excised and fixed in 10% formalin. The tissues were further processed with the Automatic Tissue Processor and sectioned at 5 µm using the Rotary Microtome. Sections were stained according to haematoxylin and eosin (H and E) technique for microscopical examination. The sections were examined using Swift Binocular Microscope with in-built lighting system. The sections were then photographed using a microscope-digital-camera with an Olympus photomicroscope at 100 × magnification.

3.2.10.6 Isolation of the bioactive components of the plants

After the *in vivo* assay, the content of the most active fractionated methanol crude extracts (VAc and OGb) used in the bio-assay study was subjected to further fractionation in order to isolate

the bioactive component responsible for the hypoglycemic effect of the plants. The detail procedure is described as follow.

(i) Fractionation of chloroform fraction of the fractionated methanol extract of Vernonia amygdalina leaf: The chloroform fraction of fractionated methanol extract of *Vernonia amygdalina* leaf (VAc) which was the most active fractionated extract was subjected to column chromatography to separate the component into sub-fractions. Silica gel was used in packing the column while varying solvent combinations of increasing polarity were used as the mobile phase.

Packing of column: In the packing of the column, 150 g of silica gel (G60-200 mesh size) was mixed in 200 ml of absolute hexane to make the slurry. The chromatographic column (3.5 cm diameter by 100 cm height) was packed with the slurry and was allowed free flow of the solvent into a conical flask below. The tap was locked and the column was allowed 24 h to stabilize after which, the clear solvent at the top of the silica gel was allowed to drain freely.

Elution: The chloroform fraction (20 g) was dissolved in 20 ml absolute chloroform and the solution was applied unto a chromatographic column The following solvent combinations (400 ml) were sequentially used in the elution process: Hexane : ethyl acetate 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90 and 0:100. The eluted sub-fractions were collected in aliquots of 30 ml in beaker. The sub-fractions were thereafter concentrated to dryness at room temperature.

(ii) Fractionation of butanol fraction of the fractionated methanol extract of Ocimum gratissimum methanol leaf: The butanol leaf fraction of fractionated methanol extract of *O. gratissimum* leaf which was the most active fractionated extract was subjected to column chromatography to separate the component into sub-fractions. Sephadex C-25 was used in packing the column while methanol was used as the mobile phase.

Elution: The butanol fraction (20 g) was dissolved in 20 ml absolute methanol and the solution was applied unto a chromatographic column. Elution of the extract was done with methanol. The eluted sub-fractions were collected in aliquots of 30 ml in beaker. The sub-fractions were thereafter concentrated to dryness at room temperature.

iii) Analytical thin layer chromatography (TLC) and pooling of fractions: The concentrated sub-fractions derived from column fractionation were spotted on pre-coated (silica gel F254) aluminium plates in a small chromatographic tank to separate the different components based on their relative mobilities in solvent systems and colour reactions with ultra-violet light. A strip of the precoated silica gel was cut out. With the aid of a micro pipette, a spot of the sample was applied on the plate about 1.0 cm from the edge. It was dried using hot air dryer. The strip was then lowered into a chromatographic jar containing the solvent system. The jar was covered with a glass lid. The solvent was allowed to ascend until the solvent front is about 75 % of the length of the strip. The strip was removed and dried by a hot air dryer and viewed under UV lamp at 365 and 254 nm to identify the fluorescing spot. The fluorescent spot was marked and then sprayed with spray reagent: [0.16 g vanillin in 30 ml concentrated tetraoxosulphate (IV) acid (H_2SO_4)]. The strip was placed in hot oven at 110 ^{0}C for 5 seconds for visibility of fluorescent bands. The colour reaction was recorded and the relative Retention factor (Rf) value was calculated based on the formula:

$$Rf = \frac{\text{Distance travelled by the streak from the starting point}}{\text{Distance travelled by the solvent from the starting point to the solvent front.}}$$

The column fractionated subfractions with similar profile on the TLC were combined. The combined sub-fractions were concentrated and evaporated using rotary evaporator and dried under vacuum then kept at 4 ^{0}C in the refrigerator until needed for further hypoglycemic activity examination.

3.2.11 Determination of the hypoglycaemic effects of the column fractionated subfractions

The combined sub-fraction(s) were subjected to further bioassay-guided screening and purification with preparative TLC. The resultant eluents were screened for biological activity using the T2D model rats described previously and the procedures from sections 3.2.8 to 3.2.8.3 were repeated. The dose administered was calculated accordingly; from 20 g of VACF and OGBF, 0.79 g and 0.82 g respectively were obtained after column chromatography. The dose of 250 mg/kg b.w. employed on bioassay of fractions contained 9.875 mg and 10.23 mg of column fractionated subfractions of VACF and OGBF respectively. Hence the dose for the determination of the hypoglycaemic effect of the column fractionated subfractions was approximated as 10 mg/kg each for VACF and OGBF. The most active column fractionated sub-fractions (obtained from VACF and OGBF) were sent for Nuclear magnetic resonance (NMR) and FTIR spectroscopy for structural elucidation.

3.2.12 Nuclear magnetic resonance (NMR) spectroscopy analysis

Samples of purified sub-fractions were dissolved in $CDCl_3$, the NMR data were recorded at 31ºC using Agilent-NMR-vnmrs400, Germany at the Multiuser Science Research laboratory, ABU Zaria Nigeria. Proton nuclear magnetic resonance (1H-NMR) and ^{13}C-NMR spectra were determined with TMS as internal standard reference which was added to the sample before recording, operating at 300 and 75.5 MHz respectively. The ^{1}H-NMR was recorded depicting the number of signals (which indicate the different "kinds" of protons present in the molecule), the position of the signals (signifying the electronic environment of each kind of proton), the intensities of the signals (accounting for each kind of protons present), the splitting of a signal into several peaks (signifies the environment of a proton with respect to other, nearby protons). The ^{13}C NMR Spectrum or CMR spectrum was generated in the fundamental way as the proton NMR spectrum. The determinations conducted were as follow: the number of signals (indicating the number of different carbons or different sets of equivalent carbons present in a molecule),

the splitting of a signal (signifying the number of hydrogen attached to each carbon), the chemical shift (indicating the hybridization of each carbon) and the chemical shift (which reveals the electronic environment of each carbon with respect to other nearby carbons or functional groups). Subsequent to the structure proposed, the ChemDraw (Version 8 software) was used to simulate the named structure and compared with NMR generated in parts per million (ppm).

3.2.13 FTIR analysis

Samples of purified sub-fractions were dissolved in $CDCl_3$, the FTIR data were recorded at 31 ºC using Agilent-FTIR, Germany at the Multiuser Science Research Laboratory, ABU Zaria Nigeria.

3.2.14 Statistical analysis

All statistical analyses were done using computer software, Statistical Package for the Social Sciences (SPSS Cary, NC, USA) version 20.0. The results are expressed as mean ± S.D. The data were analyzed by one way analysis of variance (ANOVA) and *post hoc* test. Differences between extracts and animal groups were compared using Duncan Multiple Range Test (DMRT). Values of $p < 0.05$ were considered significant.

CHAPTER FOUR

RESULTS

4.1 Extracts Obtained from Three Different Extraction Methods

The result showing the extracts obtained from three different extraction methods of the two medicinal plants (*V. amygdalina* and *O. gratissimum* leaves) are presented the Table 4.1. The percentage yield obtained from *V. amygdalina* via MAE for 39 minutes was higher (20.90 ± 1.05 %) than that of Soxhlet extraction method (15.75 ± 0.71 %) for 265 min and that of maceration extraction method (14.35 ± 0.28 %) for 4320 min. Similarly, the yield obtained from microwave extraction method from *O. gratissimum* (19.10 ± 1.67 %) for 39 min was higher than that of Soxhlet extraction method (11.25 ± 0.42 %) for 255 min and that of maceration extraction method (10.15 ± 0.65 %) for 4320 min. The MAE also used the least volume of solvent 250 ml when compared to the other methods, 500 ml and 900 ml for Soxhlet and cold maceration respectively (Table 4.1).

4.2 Effect of Extraction Methods on Phytoconstituents of *Vernonia amygdalina* and *Ocimum gratissimum* Leaves

The results of the qualitative and quantitative phytoconstituents of methanol extract of *V. amygdalina* and *O. gratissimum* leaves are presented in the Tables 4.2 and 4.3 respectively. There were no observable differences in the qualitative phytoconstituents obtained by the three extraction methods. However, the quantitative phytochemical constituents showed that Soxhlet extraction method had significant ($p<0.05$) higher quantity of alkaloids. Phenolics, saponins and tannins were significantly ($p<0.05$) higher in quantity from the MAE method, while flavonoids were significantly ($p<0.05$) higher quantity in the maceration extraction method. There was no significant ($p>0.05$) difference in the quantity of alkaloids obtained from the Soxhlet and MAE method. Also, there was no significant ($p>0.05$) difference in the yield of tannins obtained from *V. amygdalina* via the MAE and Soxhlet extraction method (Table 4.3).

Table 4.1: Percentage Recovery Yield, Extraction Time and Volume of Solvent Used for Different Extraction Methods of *Vernonia amygdalina* and *Ocimum gratissimum* Leaves.

	Vernonia amygdalina			*Ocimum gratissimum*		
Parameter	**MAE**	**Soxhlet**	**Maceration**	**MAE**	**Soxhlet**	**Maceration**
Sample (g)	50.00	50.00	50.00	50.00	50.00	50.00
Solvent Volume (ml)	250.00	500.00	900.00	250.00	500.00	900.00
Extraction Time (min)	39.00±0.00	265.00±3.50	4320.00±0.00	39.00±0.00	255.00±4.55	4320.00±0.00
Recovered Yield (g)	10.50 ± 0.53^{b}	7.90 ± 0.35^{a}	7.20 ± 0.14^{a}	9.60 ± 0.84^{b}	5.60 ± 0.21^{a}	5.08 ± 0.32^{a}
Percentage Recovered (%)	20.90 ± 1.05^{b}	15.75 ± 0.71^{a}	14.35 ± 0.28^{a}	19.10 ± 1.67^{b}	11.25 ± 0.24^{a}	10.15 ± 0.65^{a}

Values are mean ± SD of triplicate determinations; same superscript across the row under the same plant indicate no significant difference ($p > 0.05$)

Table 4.2: Phytochemical Constituents of Methanol Extracts of *Vernonia amygdalina* and *Ocimum gratissimum* Leaves

S/N	Constituents	Maceration		Soxhlet		Microwave	
		VA	OG	VA	OG	VA	OG
1	Carbohydrates	+	+	+	+	+	+
2	Anthraquinones	-	-	-	-	-	-
3	Phenols	+	+	+	+	+	+
4	Cardiac glycosides	+	+	+	+	+	+
5	Saponins	+	+	+	+	+	+
6	Steroids	+	+	+	+	+	+
7	Terpenoids	+	+	+	+	+	+
8	Tannins	+	+	+	+	+	+
9	Flavonoids	+	+	+	+	+	+
10	Alkaloid	+	+	+	+	+	+

(+) Present; (-) Not detected; VA: *Vernonia amygdalina;* OG: *Ocimum gratissimum*

Table 4.3: Quantitative Phytochemical Constituents of Methanol Extract of *Vernonia amygdalina* and *Ocimum gratissimum* Methanol Leaves

	Vernonia amygdalina (mg/100 g)			*Ocimum gratissimum* (mg/100 g)		
	MAE	**Soxhlet**	**Maceration**	**MAE**	**Soxhlet**	**Maceration**
Alkaloids	4.00±0.65 [a]	7.00±0.57[b]	5.00±0.46 [a]	6.50±0.75[b]	7.00±0.15[b]	5.00±0.90[a]
Flavonoids	13.00±0.35[b]	9.0±0.50[a]	15.00±0.21[c]	10.00±0.35[b]	8.00±0.18[a]	12.00±0.25[c]
Phenolics	17.50±0.25[c]	15.00±0.21[b]	12.00±0.70[a]	15.00±0.22[c]	14.00±0.21[b]	11.00±0.65[a]
Saponins	4.50±0.45 [b]	3.00±0.30[a]	2.00±0.75[a]	6.10±0.75[b]	4.00±0.92 [a]	5.00±0.20 [a]
Tannins	14.00±0.55 [b]	13.00±0.50[b]	10.00±0.25[a]	12.00±0.18[b]	9.00±0.24[a]	9.10±0.25 [a]

Values are mean ± standard deviation of triplicate determinations. Same superscript across the row under the same plant signifies no significant difference at $p>0.05$

4.3 Effect of Extraction Method on Hypoglycemic Agents of *Vernonia amygdalina* and *Ocimum gratissimum* Leaves

The effects of extraction methods on the hypoglycemic efficacy of *V. amygdalina* and *O. gratissimum* leaves are presented in Table 4.4. The cold maceration extract exhibited the highest hypoglycemic effect within the first 7 days (15.16 % for *V. amygdalina*; 10.56 % for *O. gratissimum*) and 14 days (28.71 % for *V. amygdalina*; 25.52 % for *O. gratissimum*) of treatment when compared to others extracts. But the hypoglycemic effects of extract obtained from MAE were better (*V. amygdalina*: 48.50% and *O. gratissimum*: 45.35%) after 28 days post induction (21 days treatment). The extract from MAE exhibited the least hypoglycemic effect within the first 7 days of treatment. There was no significant ($p>0.05$) difference between the hypoglycemic effects of extracts obtained from Soxhlet and the cold maceration method at the end of the 21 days (Table 4.4).

Table 4.4: Change (%) in Fasting Blood Glucose (mg/dL) in Experimental Rats Treatment with Methanol Extracts of *Vernonia amygdalina* and *Ocimum gratissimum* Leaves Obtained Using Different Extraction Methods

Group	Before Induction	7 Days after Induction	Days After Treatment		
			7Th	14Th	21St
CME-VA	73.80±04.10	315.20±8.60	267.40±12.10 (15.16%)	224.70±15.10 (28.71%)	172.40±9.60 (45.30%)
SEE-VA	77.40±02.90	328.10±11.30	299.60±11.50 (8.69%)	255.90±13.30 (22.01%)	176.50±12.00 (46.21%)
MAE-VA	79.20±03.40	323.70±14.10	289.40±13.80 (10.60%)	244.40±10.40 (24.50%)	166.70±08.20 (48.50%)
CME-OG	83.50±04.50	305.60±10.30	273.50±11.40 (10.50%)	227.60±13.70 (25.52%)	180.3±9.7 (40.94%)
SEE-OG	80.10±03.20	316.20±15.70	291.90±7.80 (7.69%)	253.00±10.80 (19.20%)	179.10±12.20 (43.36%)
MAE-OG	78.90±05.10	321.00±13.60	296.20±9.50 (7.73%)	251.80±05.90 (21.56%)	175.4±11.60 (45.35%)
DC	75.40±04.30	335.50±08.80	359.70±12.30 (-7.21%)	375.30±10.20 (-11.86)	401.80±15.10 (19.76%)
NC	81.00± 03.30	83.20±02.90	81.80±05.20 (1.68%)	83.40±04.70 (0.24%)	84.50±05.40 (1.56%)

Data are presented as mean ± standard deviation of 6 animals per group. The values in bracket are percentage blood glucose change with respect to values obtained 7 days post STZ induction. VA: *Vernonia amygdalina*; OG: *Ocimum gratissimum*; CME-VA: Extract of *V. amygdalina* leaves obtained from cold maceration extraction method; SEE-VA: Extract of *V. amygdalina* leaves obtained from Soxhlet extraction method; MAE-VA: Extract of *V. amygdalina* leaves obtained from Microwave assisted extraction method; CME-OG: Extract of *O. gratissimum* leaves obtained from cold maceration extraction method; SEE-OG: Extract of *O. gratissimum* leaves obtained from Soxhlet extraction method; MAE-OG: Extract of *O. gratissimum* leaves obtained from Microwave assisted extraction method; DC: Diabetic control; NC: Normal control.

4.4 Recovery Yield and Phytochemical Constituents of Fractionated Methanol Extracts of *Vernonia amygdalina* and *Ocimum gratissimum* Leaves

The percentage recovery yield and the general characteristics of the various fractions of the two plant extracts are presented in Table 4.5. It was observed that chloroform and hexane fractionated methanol extract from *V. amygdalina* had the highest percentage recovery yield of 28.09 % and 18.06 % respectively. The least percentage recovery yield for *V. amygdalina* leaf was recorded for the ethyl acetate fraction (1.73 %). The aqueous fractionated methanol extract of *O. gratissimum* leaf had the highest percentage recovery yield of 42.70 % and the least recovery yield was recorded for the butanol fractionated (2.21 %).

The presence of tannins and saponins was detected in chloroform and *n*-butanol fractionated methanol extracts of *Vernonia amygdalina* but absent in hexane, ethyl acetate and aqueous fractionated. Flavonoids were not detected in *n*-hexane fractionated of both plants. Anthraquinones were not detected in all the fractionated extracts of both plants. Chloroform and *n*-butanol fractions of the fractionated methanol extract of *V. amygdalina* and *O. gratissimum* leaves respectively had higher concentrations of the majority of phytochemicals tested. Alkaloids were not detected in the aqueous fractionated methanol extract of *O. gratissimum* leaf but tannins were present in both the butanol and aqueous fractionated *O. gratissimum* leaf extract (Table 4.6).

4.5 Acute Toxicity Study

Acute toxicity studies revealed the non-toxic nature of the *V. amygdalina* and *O. gratissimum* leaves extracts on normal healthy rats even up to 5000 mg/kg body weight (Table 4.7). There were no significant (p>0.05) changes in behaviours such as alertness, breathing, restlessness, diarrhea, convulsions, coma and appearance of the animals. No mortality was observed in the extract-treated rats and behaviour of all the experimental groups appeared the same and normal up to day 7.

Table 4.5: Percentage Yield of Fractionated Methanol Extract of *Vernonia amygdalina* and *Ocimum gratissimum* Leaves

Fractions	***Vernonia amygdalina***		***Ocimum gratissimum***	
	Weight of yield (g)	**Recovery Yield (%)**	**Weight of Yield (g)**	**Recovery Yield (%)**
Hexane fraction	32.40±1.60	18.10	13.20±0.70	10.40
Chloroform fraction	50.40±0.95	28.10	6.40±1.10	5.10
Ethyl acetate fraction	3.10±1.75	1.70	17.50±0.85	13.80
Butanol fraction	4.30±2.10	2.40	2.80±1.80	2.20
Aqueous fraction	24.90±0.85	13.90	42.70±1.35	33.70

Table 4.6 Phytochemical Constituents of the Fractionated Methanol Extracts of *Vernonia amygdalina* and *Ocimum gratissimum* Leaves

	Vernonia amygdalina				
Phytochemicals	**Hexane**	**Chloroform**	**Ethyl-Acetate**	**Butanol**	**Aqueous**
Alkaloids	++	++	+	+	-
Carbohydrate	-	-	++	++	+
Anthraquinones	-	-	-	-	-
Cardiac Glycoside	++	++	++	++	-
Flavonoids	-	++	++	+	-
Phenols	+	+	+	++	+
Saponins	-	++	-	+	-
Steroids	+	++	-	-	-
Tannins	-	++	-	++	-
Terpenoids	-	+	+	++	-
	Ocimum gratissimum				
Alkaloids	+	+	+	++	-
Carbohydrate	+	+	+	++	+
Anthraquinones	-	-	-	-	-
Glycoside	+	+	++	+	+
Flavonoids	-	++	+	++	+
Polyphenols	+	+	+	++	-
Saponins	-	-	-	++	+
Steroids	+	+	+	-	-
Tannins	-	-	-	+	+
Terpenoids	-	+	-	+	-

(+++): Highly present; (++): Moderately present; (+): Present; (-): Absent/Not detected

Table 4.7: Mortality (%) of Rats Administered Crude Methanol Extract of *Vernonia amygdalina*[(a)] and *Ocimum gratissimum*[(b)] Leaves (Orally) at Different Doses

Methanol Extract of *Vernonia amygdalina* Leaf						
Group	1	2	3	4	5	6
Dose (mg/kg b.w)	0	10	100	1000	2000	5000
No. Animals	3	3	3	3	3	3
No. of death	0	0	0	0	0	0
% Mortality	0	0	0	0	0	0
Methanol Extract of *Ocimum gratissimum* Leaf						
Group	1	2	3	4	5	6
Dose (mg/kg b.w)	0	10	100	1000	2000	5000
No. Animals	3	3	3	3	3	3
No. of death	0	0	0	0	0	0
% Mortality	0	0	0	0	0	0

4.6 Rat Model of Type 2 Diabetes

The effect of dietary regimen and different doses of streptozotocin induction in experimental rats and the subsequent selection of type 2 diabetic model are presented in the following sections.

4.6.1 Effects of streptozotocin induction on fluid and feed intake of experimental rats

The mean fluid and feed intake per animal per day throughout the 28 days of experimental duration are presented in Figure 4.1. The fluid and feed intake of the STZ treated rats were significantly (p<0.05) higher compared to NDF and FDF control groups.

4.6.2 Effect of dietary regimen and streptozotocin induction on body weight of experimental rats

The effect of dietary regimen on body weight of animal for six weeks is presented in Table 4.8. It was observed that the group administered combined FDF and 20 % fructose solution had increased significantly (p<0.05) in body weight as compared to the NDF-fed group. The percentage increase in body weight was about 2 times in the FDF-fed group as against the NDF-fed group. At 10 days post STZ induction there was significant (p<0.05) reduction in body weight for groups treated with high dose of STZ (45 and 55 mg/kg b.w. *i.p.*). The percentage increase in their body weight was between 4.57-11.97 % in the groups treated with higher dose of STZ (45 and 55 mg/kg b.w. *i.p.*).

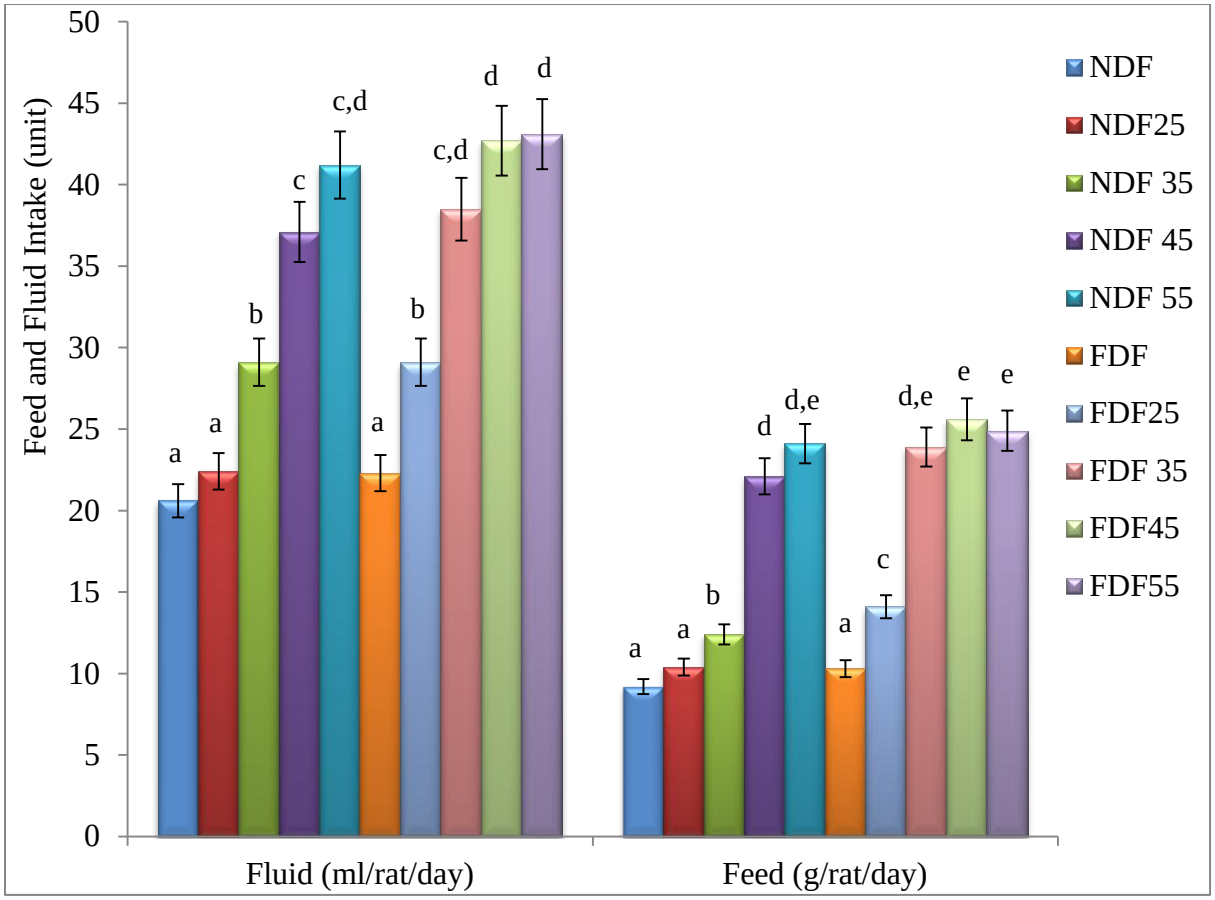


Figure 4.1: Mean Fluid and Feed Intake in Rats Fed Normal Diet-Feed or Fortified Diet-Feed and Induced with Different Doses of Streptozotocin.

Data are presented as mean ± SD of 6 animals per group. Bars with different superscript under same parameter indicate significant difference ($p<0.05$).

NDF: Group fed with Normal Diet Feed + water and induced with normal saline as control

NDF_{25}: Group fed with Normal Diet Feed + Water and induced with 25mg/kg bw, STZ.

NDF_{35}: Group fed with Normal Diet Feed + Water and induced with 35mg/kg bw, STZ.

NDF_{45}: Group fed with Normal Diet Feed + Water and induced with 45mg/kg bw, STZ

NDF_{55}: Group fed with Normal Diet Feed + Water and induced with 55mg/kg bw, STZ.

FDF: Group fed with Fortified Diet Feed + 20% fructose and induced with Normal saline

FDF_{25}: Group fed with Fortified Diet Feed + 20% fructose and induced with 25mg/kg bw STZ

FDF_{35}: Group fed with Fortified Diet Feed + 20% fructose and induced with 35mg/kg bw STZ

FDF_{45}: Group fed with Fortified Diet Feed + 20% fructose and induced with 45mg/kg bw STZ

FDF_{55}: Group fed with Fortified Diet Feed + 20% fructose and induced with 55mg/kg bw STZ

Table 4.8: Change (%) in Body Weight in Experimental Rats after Dietary Regimen and Streptozotocin Induction

S/N	Group	Body Weight (g) (Day 0)	Percentage Change in Body Weight (%)	
			Day 42 of Dietary Regimen	Day 52 of Dietary Regimen (Day 10 Post STZ Induction)
1	NDF	170.30 ± 12.30 [a]	15.00 ± 3.09 [a]	2.60 ± 0.40 [f]
2	NDF25	168.70 ± 13.90 [a]	15.10 ± 3.00 [a]	1.80 ± 0.20 [e,f]
3	NDF35	173.60 ± 19.60 [a]	14.20 ± 6.20 [a]	-1.20 ± 1.10 [d]
4	NDF45	167.80 ± 7.40 [a]	15.90 ± 3.28 [a]	-4.70 ± 0.20 [c]
5	NDF55	172.30 ± 20.90 [a]	13.10 ± 1.37 [a]	-12.00 ± 1.80 [a]
6	FDF	173.40 ± 16.30 [a]	31.10 ± 4.42 [b]	2.60 ± 1.10 [f]
7	FDF25	163.90 ± 8.70 [a]	36.70 ± 3.25 [b]	1.10 ± 0.50 [e]
8	FDF35	162.90 ± 8.30 [a]	34.50 ± 6.25 [b]	-1.20 ± 1.30 [d]
9	FDF45	170.00 ± 10.80 [a]	33.30 ± 4.74 [b]	-4.60 ± 1.10 [c]
10	FDF55	171.40 ± 9.90 [a]	33.10 ± 5.91 [b]	-9.1 ± 1.0 [b]

Data are presented as mean ± SD of 6 animals per group. Values with different superscript down the column indicate significant difference ($p<0.05$).

NDF: Group fed with Normal Diet Feed + water and induced with normal saline as control

NDF_{25}: Group fed with Normal Diet Feed + Water and induced with 25mg/kg bw, STZ.

NDF_{35}: Group fed with Normal Diet Feed + Water and induced with 35mg/kg bw, STZ.

NDF_{45}: Group fed with Normal Diet Feed + Water and induced with 45mg/kg bw, STZ

NDF_{55}: Group fed with Normal Diet Feed + Water and induced with 55mg/kg bw, STZ.

FDF: Group fed with Fortified Diet Feed + 20% fructose and induced with Normal saline

FDF_{25}: Group fed with Fortified Diet Feed + 20% fructose and induced with 25mg/kg bw STZ

FDF_{35}: Group fed with Fortified Diet Feed + 20% fructose and induced with 35mg/kg bw STZ

FDF_{45}: Group fed with Fortified Diet Feed + 20% fructose and induced with 45mg/kg bw STZ

FDF_{55}: Group fed with Fortified Diet Feed + 20% fructose and induced with 55mg/kg bw STZ

4.6.3 Effect of dietary regimen and streptozotocin induction on fasting blood glucose levels experimental rats

After 6 weeks of dietary regimen the fasting blood glucose of the FDF-fed group had increased significantly ($p < 0.05$) when compared to the NDF-fed group (Table 4.9). At day-3 post-STZ induction, there was significant increase in fasting blood glucose in the entire STZ treated groups as compared to the vehicle treated control groups. Ten days post-STZ induction, it was noticed that the blood glucose level in the NDF_{25}, NDF_{35}, NDF_{45} and FDF_{25} had significantly ($p<0.05$) reduced when compared to values obtained on day-3 post-STZ induction indicating a form of reversal effect. However, the NDF_{55}, FDF_{35}, FDF_{45} and FDF_{55} had increased in fasting glucose levels when compared to the values obtained on day-3 post-STZ induction.

4.6.4 Effect of dietary regimen and streptozotocin induction on serum total cholesterol and triglyceride levels in the experimental rats

The effect of the dietary regimen and STZ induction on total cholesterol (TC) and triglyceride (TG) levels in the experimental rats are presented in Table 4.10. It was observed that the serum TC and TG levels had increased significantly ($p>0.05$) in the FDF groups after the 6 weeks dietary regimen. Ten days post STZ induction, the serum and TG levels in all the STZ treated groups had increased significantly ($p >0.05$) when compared to the control groups for both (FDF and NDF). The increase in serum TC and TG levels were more severe in FDF_{55}, FDF_{45}, FDF_{35} and NDF_{55}.

Table 4.9: Fasting Blood Glucose (mg/dL) in Experimental Rats Before and After Dietary Regimen and Streptozotocin Induction

Group	Post Diet Feeding			
	Day 0	Day 42		
		Post STZ Induction		
		Day 0	Day 3	Day 10
NDF	85.63 ± 7.05 ^{a,1}	84.87 ± 8.18 ^{a,1}	84.61 ± 7.78 ^{a,1}	85.10 ± 7.76 ^{a,1}
NDF25	88.67 ± 5.33^{a,1}	87.80 ± 3.88 ^{a,1}	94.80 ± 3.47 ^{a,1}	88.05 ± 1.80 ^{a,b,1}
NDF35	85.90 ± 7.10 ^{a,1}	86.23 ± 4.74 ^{a,1}	120.92 ± 4.93 ^{c,3}	101.80 ± 5.61 ^{b,c,2}
NDF45	87.73 ± 3.36 ^{a,1}	89.40 ± 3.36 ^{a,1}	191.45 ± 10.85 ^{d,2}	183.62 ± 10.56 ^{d,2}
NDF55	88.17 ± 3.40 ^{a,1}	87.40 ± 3.58 ^{a,1}	340.97 ± 22.49 ^{f,2}	356.18 ± 22.16 ^{f,2}
FDF	84.82 ± 4.83 ^{a,1}	102.07 ± 3.50 ^{b,2}	102.25 ± 3.13 ^{a,b,2}	103.47 ± 5.47 ^{b,c,2}
FDF25	85.93 ± 6.60 ^{a,1}	100.68 ± 4.58 ^{b,2}	119.03 ± 3.91 ^{b,c,3}	114.93 ± 5.71 ^{c,3}
FDF35	85.83 ± 4.95 ^{a,1}	100.70 ± 4.93^{b,2}	303.48 ± 25.93 ^{e,3}	309.42 ± 19.56 ^{e,3}
FDF45	88.13 ± 3.82 ^{a,1}	100.38 ± 2.37^{b,2}	344.65 ± 22.56 ^{f,3}	371.73 ± 17.79 ^{f,3}
FDF55	81.52 ± 4.97 ^{a,1}	98.70 ± 2.37 ^{b,2}	487.57 ± 18.59 ^{g,3}	507.08 ± 14.50 ^{g,4}

Data are presented as mean ± SD of 6 animals per group. Values with different superscript down the column and different alphabet across the column indicate significant difference (p<0.05).

NDF: Group fed with Normal Diet Feed + water and induced with normal saline as control

NDF_{25}: Group fed with Normal Diet Feed + Water and induced with 25mg/kg bw, STZ.

NDF_{35}: Group fed with Normal Diet Feed + Water and induced with 35mg/kg bw, STZ.

NDF_{45}: Group fed with Normal Diet Feed + Water and induced with 45mg/kg bw, STZ

NDF_{55}: Group fed with Normal Diet Feed + Water and induced with 55mg/kg bw, STZ.

FDF: Group fed with Fortified Diet Feed + 20% fructose and induced with Normal saline

FDF_{25}: Group fed with Fortified Diet Feed + 20% fructose and induced with 25mg/kg bw STZ

FDF_{35}: Group fed with Fortified Diet Feed + 20% fructose and induced with 35mg/kg bw STZ

FDF_{45}: Group fed with Fortified Diet Feed + 20% fructose and induced with 45mg/kg bw STZ

FDF_{55}: Group fed with Fortified Diet Feed + 20% fructose and induced with 55mg/kg bw STZ

Table 4.10: Fasting Blood Total Cholesterol and Triglyceride in Experimental Rats Before and After Dietary Regimen and Streptozotocin Induction

Group	Day 0 of Dietary Regimen		Day 42 of Dietary Regimen (Day 0, STZ Induction)		Day 10, Post STZ Induction	
	Blood TC (mg/dL)	**Blood TG** (mg/dL)	**Blood TC** (mg/dL)	**Blood TG** (mg/dL)	**Blood TC** (mg/dL)	**Blood TG** (mg/dL)
NDF	78.70± 4.56^{a,1}	68.70±5.91^{a,1}	81.90± 9.84^{a,1}	70.50±5.92^{a,1}	83.60± 6.82^{a,1}	69.40± 6.80^{a,1}
NDF_{25}	85.60±7.45^{a,1}	75.40± 8.84^{a,1}	88.70±7.86^{a,1}	73.90±6.84^{a,1}	109.40±6.93^{b,2}	87.50±5.92^{b,1}
NDF_{35}	80.20±4.38^{a,1}	81.20± 8.96^{a,1}	83.50±7.55^{a,1}	80.40± 6.46^{a,1}	102.70±8.95^{b,2}	94.20± 7.85^{b,1}
NDF_{45}	74.30±7.94^{a,1}	74.60± 7.95^{a,1}	79.60± 8.38^{a,1}	77.30±5.92^{a,1}	135.40±7.97^{d,2}	276.30± 7.04^{d,2}
NDF_{55}	83.10± 6.38^{a,1}	76.80±8.87^{a,1}	87.20±5.95^{a,1}	74.50±4.85^{a,1}	141.90± 8.89^{d,2}	423.70±8.58^{f,2}
FDF	77.40±7.94^{a,1}	69.70±6.00^{a,1}	103.40±6.93^{b,2}	119.50± 8.42^{b,2}	102.20±7.92^{b,2}	224.60 ± 6.84^{c,3}
FDF_{25}	82.70±4.58^{a,1}	74.8 0± 8.82^{a,1}	114.60± 7.39^{b,2}	133.40±6.82^{b,2}	117.30± 8.72^{c,2}	338.30± 7.06^{e,3}
FDF_{35}	79.50±5.72^{a,1}	80.20±7.90^{a,1}	115.70± 8.55^{b,2}	129.20± 9.80^{b,2}	174.10± 9.57^{e,3}	420.20± 9.04^{f,3}
FDF_{45}	86.50±6.83^{a,1}	67.30±9.37^{a,1}	103.80± 8.07^{b,2}	132.50± 4.96^{b,2}	185.30± 5.85^{e,3}	431.50± 6.85^{f,3}
FDF_{55}	81.20± 8.29^{a,1}	72.60± 8.35^{a,1}	102.50± 9.35^{b,1}	127.60±7.82^{b,2}	173.40± 8.20^{e,3}	452.00± 6.92^{g,3}

Data are presented as mean ± SD of 6 animals per group. Values with different superscript down the column and different alphabets across the row (under same parameter) indicate significant difference (p<0.05). TC: Total Cholesterol; TG: Triglyceride

NDF: Group fed with Normal Diet Feed + water and induced with normal saline as control

NDF_{25}: Group fed with Normal Diet Feed + Water and induced with 25mg/kg bw, STZ.

NDF_{35}: Group fed with Normal Diet Feed + Water and induced with 35mg/kg bw, STZ.

NDF_{45}: Group fed with Normal Diet Feed + Water and induced with 45mg/kg bw, STZ

NDF_{55}: Group fed with Normal Diet Feed + Water and induced with 55mg/kg bw, STZ.

FDF: Group fed with Fortified Diet Feed + 20% fructose and induced with Normal saline

FDF_{25}: Group fed with Fortified Diet Feed + 20% fructose and induced with 25mg/kg bw STZ

FDF_{35}: Group fed with Fortified Diet Feed + 20% fructose and induced with 35mg/kg bw STZ

FDF_{45}: Group fed with Fortified Diet Feed + 20% fructose and induced with 45mg/kg bw STZ

FDF_{55}: Group fed with Fortified Diet Feed + 20% fructose and induced with 55mg/kg bw STZ

4.6.5 Oral glucose tolerance test in experimental rats

The result for OGTT showed glycaemia peak after 90 min before falling sharply in the groups treated with 45 and 55 mg/kg bw STZ. The FDF_{35} glycaemia levels were maintained elevated >300 mg/dL nearly all through the duration of 120 min glucose tolerance test with a peak at 60 min. The control groups (NDF and FDF) and the 25 mg/kg bw STZ treated groups (NDF_{25} and FDF_{25}) showed a glycaemia peak at 30 min which fell sharply to almost baseline after 2 hr (Figure 4.2).

4.6.6 Effect of normal diet-feed, fortified diet-feed and streptozotocin dose on insulin sensitivity, insulin resistance, β-cell function in experimental rats

The calculated insulin sensitivity index, HOMA-IR and HOMA-β scores are shown for all the groups of animals in Table 4.11. It was observed that pancreatic β-cell function (HOMA-β score) decreased with increase in STZ dosage. The FDF_{35} group showed highest HOMA-IR score and also had the least value for insulin sensitivity.

4.6.7 Effect of normal diet-feed, fortified diet-feed and streptozotocin dose on serum insulin and C-peptide experimental rats

The Fasting serum insulin and C-peptide in the FDF-fed groups were significantly ($p<0.05$) higher when compared to the NDF-fed groups. The induction of 45 and 55 mg/kg b.w. (*i.p.*) of STZ to the experimental rats after 6 weeks of dietary regimen resulted in significant ($p<0.05$) decrease in insulin and C-peptide both in the NDF-fed and FDF-fed groups. However, the Fasting serum C-peptide and serum insulin levels in the FDF_{25} and FDF_{35} groups where comparatively high when compared to the NDF_{25} and NDF_{35} groups but lower when compared to the NDF and FDF groups (Table 4.12).

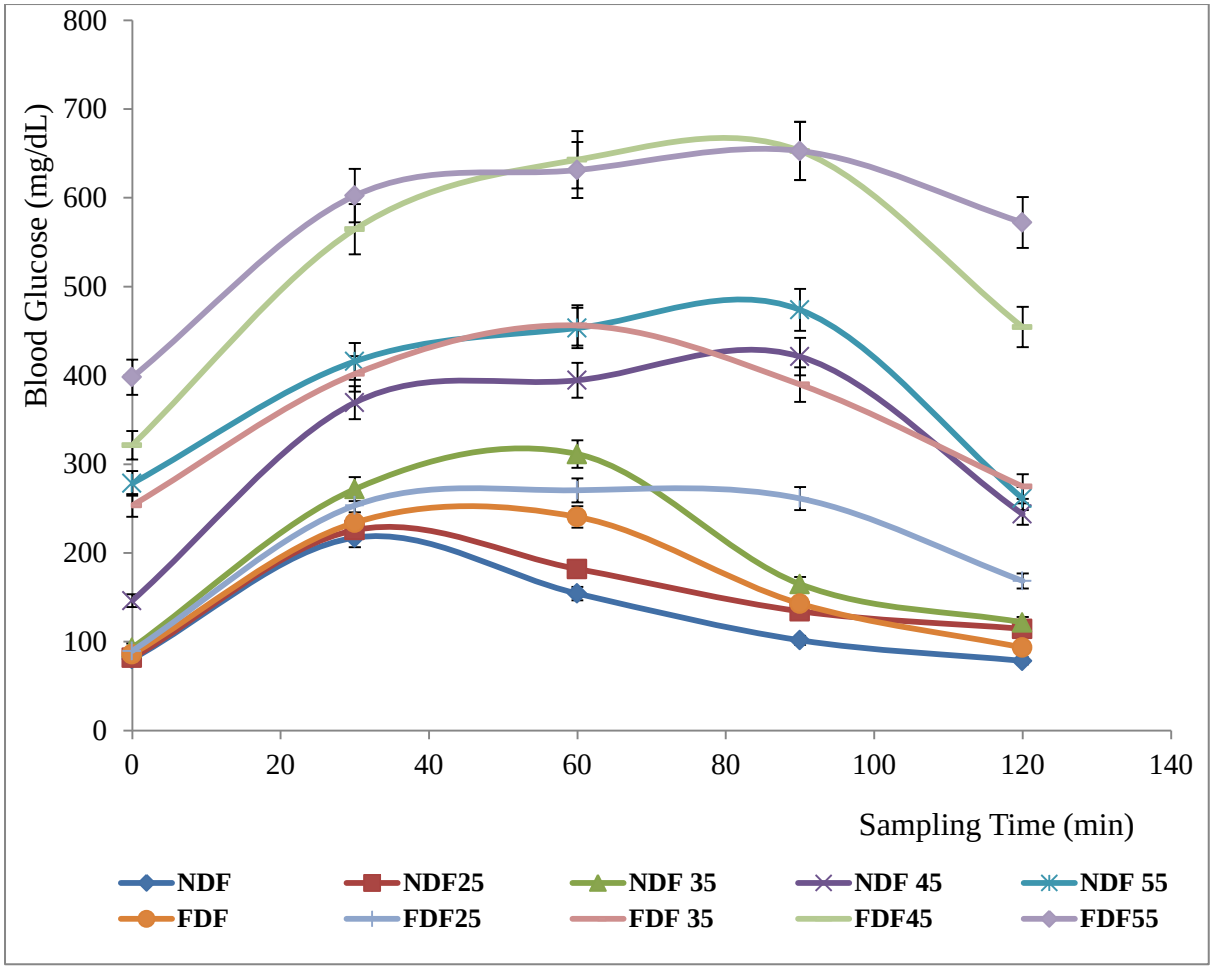


Figure 4.2: Oral Glucose Tolerance Test in Experimental Rats

Data are presented as mean ± SD of 6 animals per group.
NDF: Group fed with Normal Diet Feed + water and induced with normal saline as control
NDF_{25}: Group fed with Normal Diet Feed + Water and induced with 25mg/kg bw, STZ.
NDF_{35}: Group fed with Normal Diet Feed + Water and induced with 35mg/kg bw, STZ.
NDF_{45}: Group fed with Normal Diet Feed + Water and induced with 45mg/kg bw, STZ
NDF_{55}: Group fed with Normal Diet Feed + Water and induced with 55mg/kg bw, STZ.
FDF: Group fed with Fortified Diet Feed + 20% fructose and induced with Normal saline
FDF_{25}: Group fed with Fortified Diet Feed + 20% fructose and induced with 25mg/kg bw STZ
FDF_{35}: Group fed with Fortified Diet Feed + 20% fructose and induced with 35mg/kg bw STZ
FDF_{45}: Group fed with Fortified Diet Feed + 20% fructose and induced with 45mg/kg bw STZ
FDF_{55}: Group fed with Fortified Diet Feed + 20% fructose and induced with 55mg/kg bw STZ

Table 4.11: Beta-Cell Function, Insulin Sensitivity and Insulin Resistance in Normal Diet-Fed, Fortified Diet-Fed and Streptozotocin Induced Experimental Rats

S/N	Group	HOMA-IR	HOMA- β	Insulin Sensitivity
1	NDF	$2.83 \pm 0.33^{a,b}$	53.98 ± 8.38^{e}	$1.33 \pm 0.09^{b,c}$
2	NDF_{25}	2.22 ± 0.26^{a}	38.23 ± 5.01^{d}	$1.44 \pm 0.07^{b,c}$
3	NDF_{35}	2.18 ± 0.27^{a}	27.31 ± 5.18^{c}	$1.43 \pm 0.10^{b,c}$
4	NDF_{45}	$2.86 \pm 0.65^{a,b}$	8.85 ± 2.22^{b}	$1.27 \pm 0.15^{b,c}$
5	NDF_{55}	$3.59 \pm 1.14^{b,c}$	0.64 ± 1.36^{a}	$1.40 \pm 0.45^{b,c}$
6	FDF	4.15 ± 0.39^{c}	53.29 ± 6.77^{e}	$1.09 \pm 0.04^{a,b}$
7	FDF_{25}	3.99 ± 0.35^{c}	40.58 ± 3.79^{d}	$1.08 \pm 0.05^{a,b}$
8	FDF_{35}	8.85 ± 1.36^{d}	29.94 ± 1.22^{c}	0.77 ± 0.04^{a}
9	FDF_{45}	4.66 ± 1.01^{c}	1.48 ± 1.37^{a}	$1.11 \pm 0.16^{a,b}$
10	FDF_{55}	4.16 ± 1.53^{c}	1.17 ± 0.78^{a}	1.67 ± 0.92^{c}

Data are presented as mean ± SD of 6 animals per group. Values with different superscript down the column indicate significantly different ($p<0.05$).

NDF: Group fed with Normal Diet Feed + water and induced with normal saline as control

NDF_{25}: Group fed with Normal Diet Feed + Water and induced with 25mg/kg bw, STZ.

NDF_{35}: Group fed with Normal Diet Feed + Water and induced with 35mg/kg bw, STZ.

NDF_{45}: Group fed with Normal Diet Feed + Water and induced with 45mg/kg bw, STZ

NDF_{55}: Group fed with Normal Diet Feed + Water and induced with 55mg/kg bw, STZ.

FDF: Group fed with Fortified Diet Feed + 20% fructose and induced with Normal saline

FDF_{25}: Group fed with Fortified Diet Feed + 20% fructose and induced with 25mg/kg bw STZ

FDF_{35}: Group fed with Fortified Diet Feed + 20% fructose and induced with 35mg/kg bw STZ

FDF_{45}: Group fed with Fortified Diet Feed + 20% fructose and induced with 45mg/kg bw STZ

FDF_{55}: Group fed with Fortified Diet Feed + 20% fructose and induced with 55mg/kg bw STZ

Table 4.12**:** Effect of Different Doses of Streptozotocin on Circulating Serum Insulin and C-Peptide Levels in Experimental Rats.

Group	Circulating Serum C-Peptide (nmol/L)	Percentage Change in Serum C-Peptide (%)	Circulating Serum Insulin (mU/L)	Percentage of Available Serum Insulin (%)
NDF	0.74 ± 0.07^{f}		13.47 ± 1.31^{f}	
NDF_{25}	0.58 ± 0.06^{e}	-21.62	10.20 ± 1.20^{e}	75.72
NDF_{35}	0.51 ± 0.06^{d}	-31.08	8.67 ± 1.18^{d}	64.37
NDF_{45}	0.39 ± 0.06^{c}	-47.3	6.30 ± 1.24^{c}	46.77
NDF_{55}	$0.27 \pm 0.07^{a,b}$	-63.51	$4.08 \pm 1.29^{a,b}$	30.29
FDF	0.88 ± 0.08^{g}	18.92	15.82 ± 1.27^{g}	117.45
FDF_{25}	0.77 ± 0.52^{f}	4.05	$14.74 \pm 0.35^{f,g}$	109.43
FDF_{35}	$0.65 + 0.06^{e}$	-12.16	11.55 ± 1.28^{e}	85.75
FDF_{45}	$0.32 + 0.06^{b,c}$	-56.76	$5.10 \pm 1.24^{b,c}$	37.86
FDF_{55}	0.24 ± 0.06^{a}	-67.57	3.30 ± 1.16^{a}	24.50

Data are presented as mean ± SD of 6 animals per group. Values with different superscript down the column indicate significant difference ($p<0.05$).

NDF: Group fed with Normal Diet Feed + water and induced with normal saline as control
NDF_{25}: Group fed with Normal Diet Feed + Water and induced with 25mg/kg bw, STZ.
NDF_{35}: Group fed with Normal Diet Feed + Water and induced with 35mg/kg bw, STZ.
NDF_{45}: Group fed with Normal Diet Feed + Water and induced with 45mg/kg bw, STZ
NDF_{55}: Group fed with Normal Diet Feed + Water and induced with 55mg/kg bw, STZ.
FDF: Group fed with Fortified Diet Feed + 20% fructose and induced with Normal saline
FDF_{25}: Group fed with Fortified Diet Feed + 20% fructose and induced with 25mg/kg bw STZ
FDF_{35}: Group fed with Fortified Diet Feed + 20% fructose and induced with 35mg/kg bw STZ
FDF_{45}: Group fed with Fortified Diet Feed + 20% fructose and induced with 45mg/kg bw STZ
FDF_{55}: Group fed with Fortified Diet Feed + 20% fructose and induced with 55mg/kg bw STZ

4.6.8 Stability and validation of experimental animal model for pharmacological screening

From the data shown in Figure 4.3, it was observed that the FDF_{35} rats group had fasting hyperglycaemia stable around 300 mg/dL from week 2 althrough the 6 weeks post validation duration of the experiment. The validation and suitability of the FDF_{35} rat model was established using oral administration of three confirmed Type 2 diabetes anti-hyperglycaemic drugs. With metformin (500 mg/kg bw) once daily for one week, there was significant (p<0.05) reduction in blood glucose (46.7%), TC (65.5%) and TG (68.1%) when compared to vehicle-treated diabetic control rats. The glibenclamide (10 mg/kg bw) treated group showed significant (p<0.05) reduction in blood glucose (57.6%), TC (58.7%) and TG (74.8%). The circulating insulin levels were significantly (p<0.05) increased. The pioglitazone was observed to have also significantly (p<0.05) reduced the blood glucose by 66.1%. The TC and TG were increased significantly (p<0.05) when compared to the vehicle treated diabetic control rats (Figure 4.4).

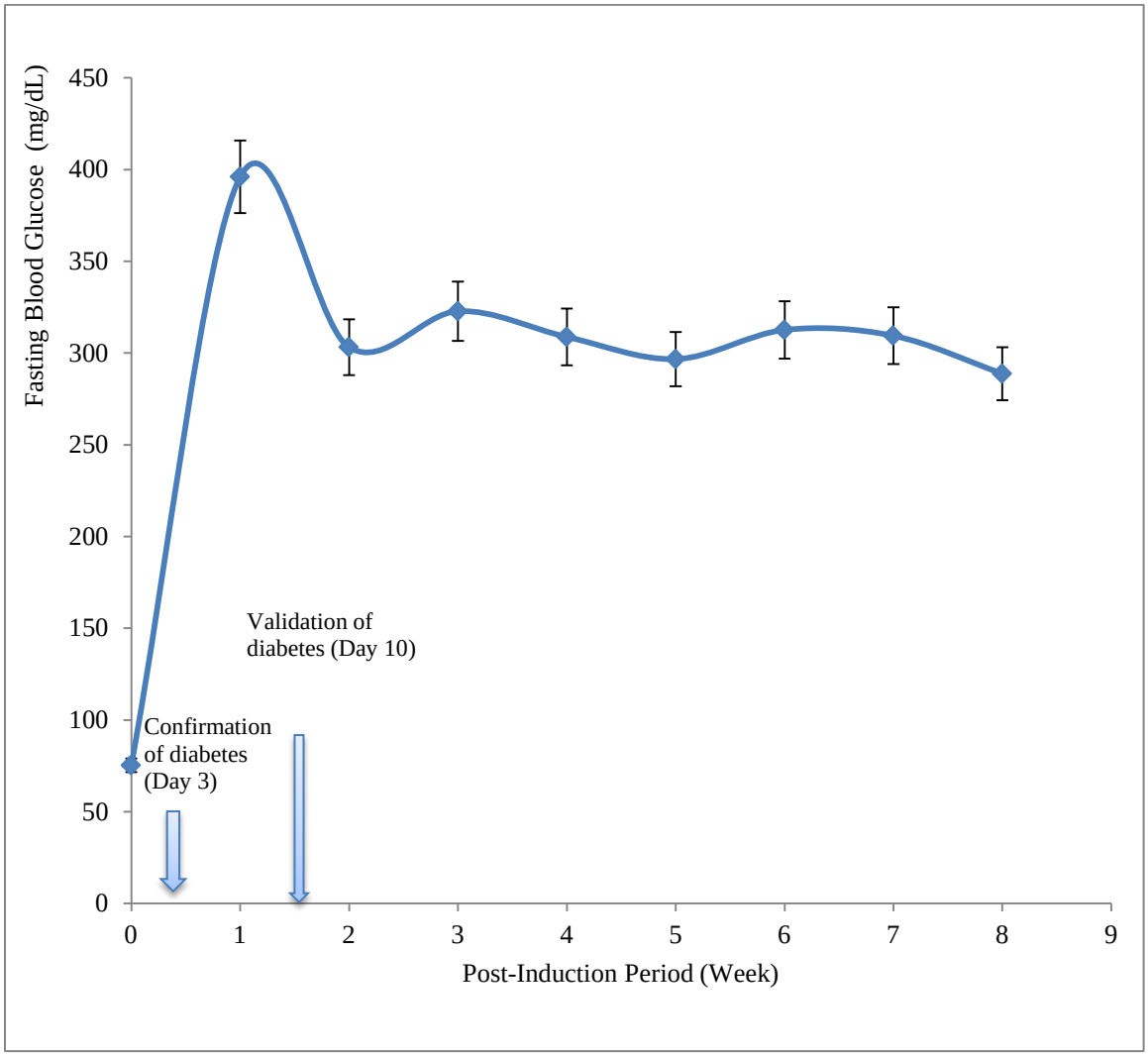


Figure 4.3: Fasting Blood Glucose Curve in the Fortified Diet-Fed and Streptozotocin Induced (35 mg/kg b.w) Rat Group for Six Weeks Post Validation of Diabetes.

Data are presented as mean ± SD of 6 animals

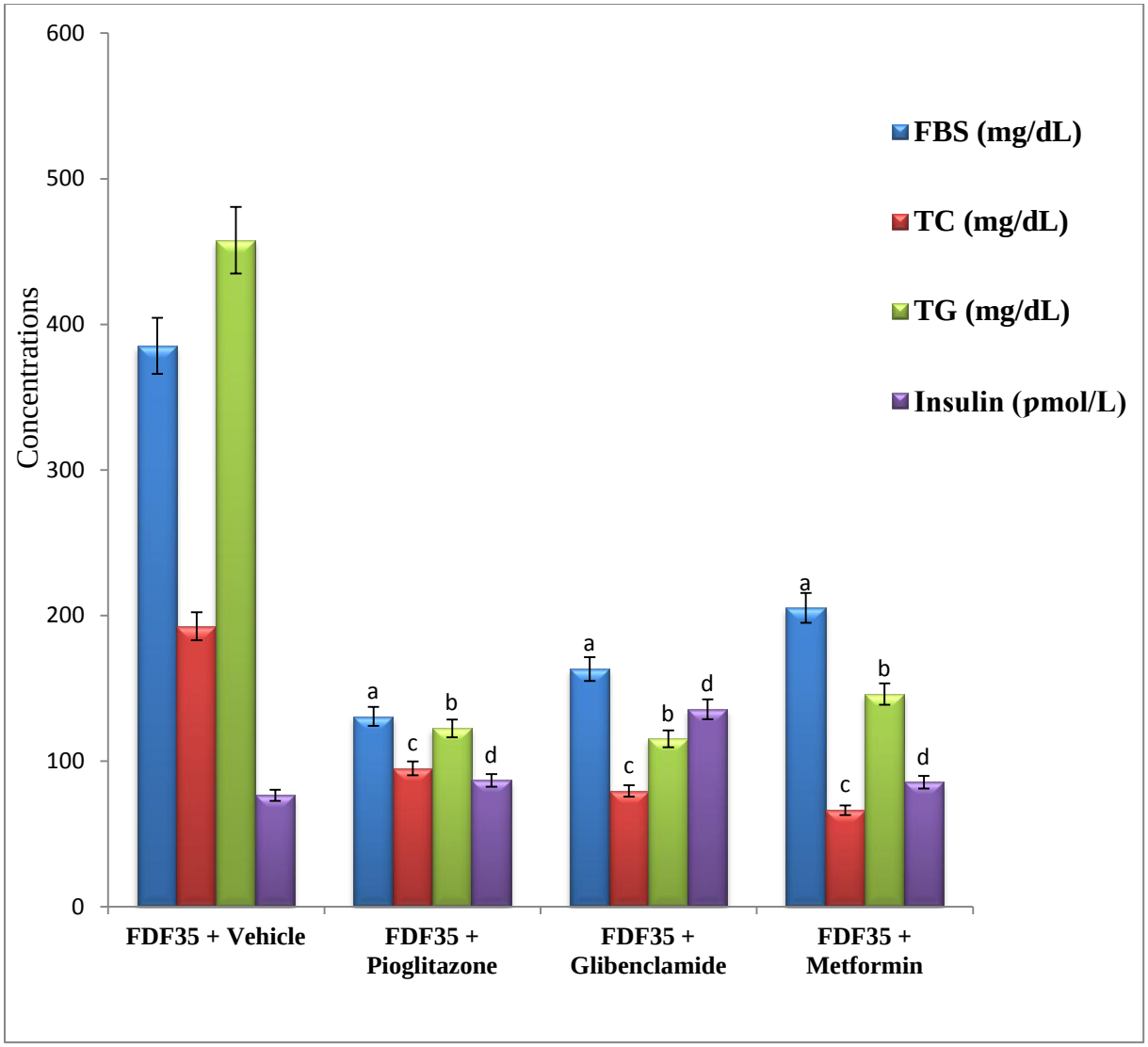


Figure 4.4: Effect of Pioglitazone, Glibenclamide and Metformin on Fasting Blood Glucose, Total Cholesterol and Triglyceride in the Fortified Diet-Fed and Streptozotocin Induced (35 mg/kg b.w) Experimental Rats.

Data are presented as mean ± SD of 6 animals per group. TC: total cholesterol, TG: triglyceride, FBS: fasting blood sugar. FDF_{35}: Group fed with Fortified Diet Feed + 20% Fructose and induced with 35 mg/kg bw STZ. Bars with alphabet indicate significant difference with respect to the "FDF_{35} + Vehicle". Conversion factor: Insulin (1U/l = 7.174 pmol/l).

4.7 Effect of Fractionated Methanol Extracts of *Vernonia amygdalina* and *Ocimum gratissimum* Leaves in Experimental Rats

The minimum oral therapeutic dose of the methanol extracts as well as the effects of the fractionated methanol extracts of *V. amygdalina* and *O. gratissimum* leaves on the feed intake fluid intake, body weight and biochemical parameters are discussed in the following sections.

4.7.1 Effective oral therapeutic dose of methanol extract of *Vernonia amygdalina* and *Ocimum gratissimum* leaves

Tables 4.13 and 4.14 shows the effective oral therapeutic dose for *V. amygdalina* and *O. gratissimum* leaves respectively as defined by the amount of extract capable of reducing the glucose level of diabetic rats by at least 25 % within 4 h of oral administration was determined. It was observed that methanol extracts of *V. amygdalina* and *O. gratissimum* leaves with a dose of 200 mg/kg b.w decreased the blood glucose of diabetic rats by 18.42 % and 20.86 % respectively, after 4 h. However, a 250 mg/kg b.w. of the same extracts were able to decrease the blood glucose of diabetic rats by 21.08 and 33.23 % after 2 and 4 h respectively for *V. amygdalina*; and 17.13 % and 29.25 % after 2 and 4 h respectively for *O. gratissimum*. The observed decrease in blood glucose of the treated diabetic rats were significant ($p<0.05$) when compared with the untreated diabetic control group (3.21 % and 4.43 % respectively after 3 and 4 h). Hence, the dose of 250 mg/kg b.w was considered as the minimum therapeutic dose for the methanol extracts of *V. amygdalina* and *O. gratissimum* leaves in this study.

Table 4.13: Minimum Oral Therapeutic Dose of Methanol Extract of *Vernonia amygdalina* Leaf in Diabetic Rats

VA Extract Dose (mg/kg b.w)	***Blood Glucose Level (mg/dL)***			
	0 h	**1 h**	**2 h**	**4 h**
V_{100}	290.50 ± 12.83	277.90 ± 15.26 (4.34%)	272.30 ± 12.93 (6.27%)	267.80 ± 11.06 (7.81%)
V_{150}	273.90 ± 11.23	256.10 ± 4.89 (6.50%)	247.30 ± 4.21 (9.71%)	235.20 ± 9.03 (14.13%)
V_{200}	302.60 ± 11.13	285.40 ± 10.94 (5.68%)	269.10 ± 10.92 (11.07%)	246.90 ± 10.22 (18.42%)
V_{250}	280.80 ± 13.47	250.10 ± 11.24 (10.93%)	221.60 ± 10.13 (21.08%)	187.50 ± 11.26 (33.23%)
DC	286.90 ± 15.48	282.30 ± 15.07 (1.60%)	277.70 ± 12.22 (3.21%)	274.20 ± 12.31 (4.43%)

The number in bracket is percentage reduction in blood glucose as compared to values obtained at 0 hr. Values are presented as mean ± SD of 3 animals per group. DC: Diabetic control group without treatment. VA: *V. amygdalina*.

Table 4.14: Minimum Oral Therapeutic Dose of Methanol Extract of *Ocimum gratissimum* Leaf in Diabetic Rats

OG Extract	***Blood Glucose Level (mg/dL)***			
Dose (mg/kg b.w)	**0 h**	**1 h**	**2 h**	**4 h**
G_{100}	261.40 ± 12.37	247.80 ± 14.31 (5.09%)	241.70 ± 11.78 (6.27%)	237.20 ± 9.14 (9.26%)
G_{150}	284.10 ± 15.79	270.60 ± 9.40 (4.75%)	259.50 ± 8.48 (8.66%)	249.30 ± 3.59 (12.25%)
G_{200}	273.70 ± 20.64	243.40 ± 14.47 (11.07%)	237.20 ± 14.31 (13.34%)	216.60 ± 17.14 (20.86%)
G_{250}	291.30 ± 14.87	260.50 ± 22.21 (10.57%)	241.40 ± 15.80 (17.13%)	206.10 ± 20.50 (29.25%)
DC	286.90 ± 15.48	282.30 ± 15.07 (1.60%)	277.70 ± 12.22 (3.21%)	274.20 ± 12.31 (4.43%)

The number in bracket is percentage reduction in blood glucose as compared to values obtained at 0 hr. Values are presented as mean ± SD of 3 animals per group. DC: Diabetic control group without treatment. OG: *O. gratissimum*.

4.7.2 Effect of fractionated methanol extracts of *Vernonia amygdalina* and *Ocimum gratissimum* leaves on feed and fluid intake of experimental rats

The mean feed and fluid intake of each experimental animal on a daily basis throughout the experimental duration are presented in Figures 4.5 to 4.8 respectively. Few days after diabetic induction with STZ, a significant (p<0.05) decrease in feed intake was observed among the STZ induced groups when compared with the normal control (NC) group. A few days after confirmation of diabetes, however, it was observed that the feed intake had significantly (p<0.05) increased among the diabetic groups when compared with the NC group. From two weeks of treatment till the end of the experiment, it was observed that the feed intake among the diabetic rats treated with OGLF and VALF were significantly (p<0.05) lower when compared with the untreated diabetic group (DC), but higher than the NC group. There were no significant (p>0.05) differences in the feed intake among the various fractions treated groups (Figures 4.5 and 4.6). The fluid intake was observed to be significantly (p<0.05) increased among the STZ induced groups when compared with the NC group. All through the duration of the experiment, it was observed that the fluid intake among the diabetic rats treated with VALF was significantly (p<0.05) higher than the NC but lower than the DC group. There was no significant difference in fluid intake between the positive control group and the VA chloroform fraction treated group at 4 week of treatment when compared with the NC group (Figures 4.7 and 4.8).

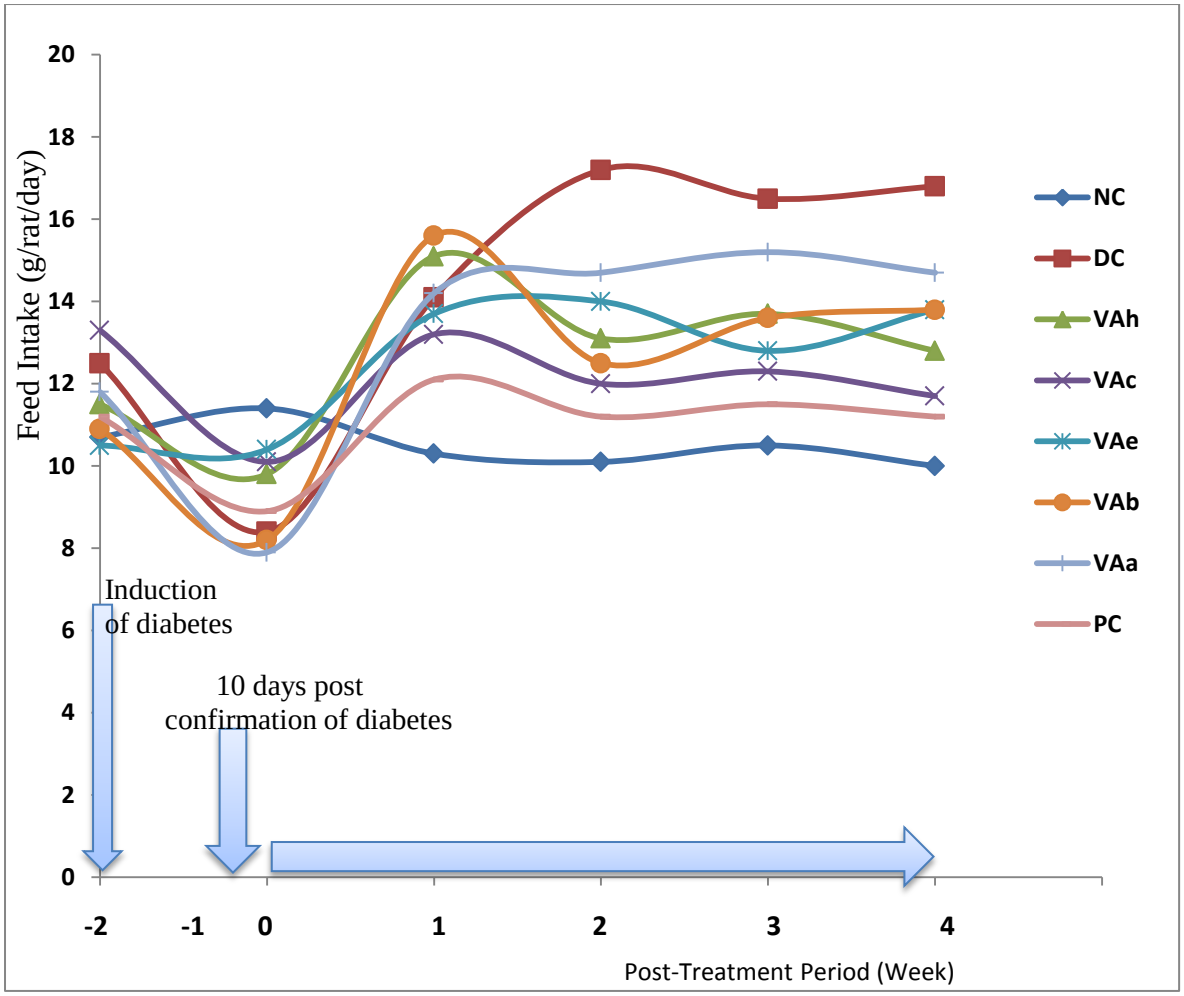


Figure 4.5: Effect of Fractionated Methanol Extract of *Vernonia amygdalina* Leaf on Feed Intake in Experimental Rats.

Data are presented as mean of 6 animals per group. NC: Normal control; DC: Diabetic control; PC: Positive control; VAh: *V. amygdalina n*-hexane fraction; VAc: *V. amygdalina* chloroform fraction; VAe: *V. amygdalina* ethyl-acetate fraction; VAb: *V. amygdalina n*-butanol fraction; VAa: *V. amygdalina* aqueous fraction. *Note: Error bars were not included for clarity.

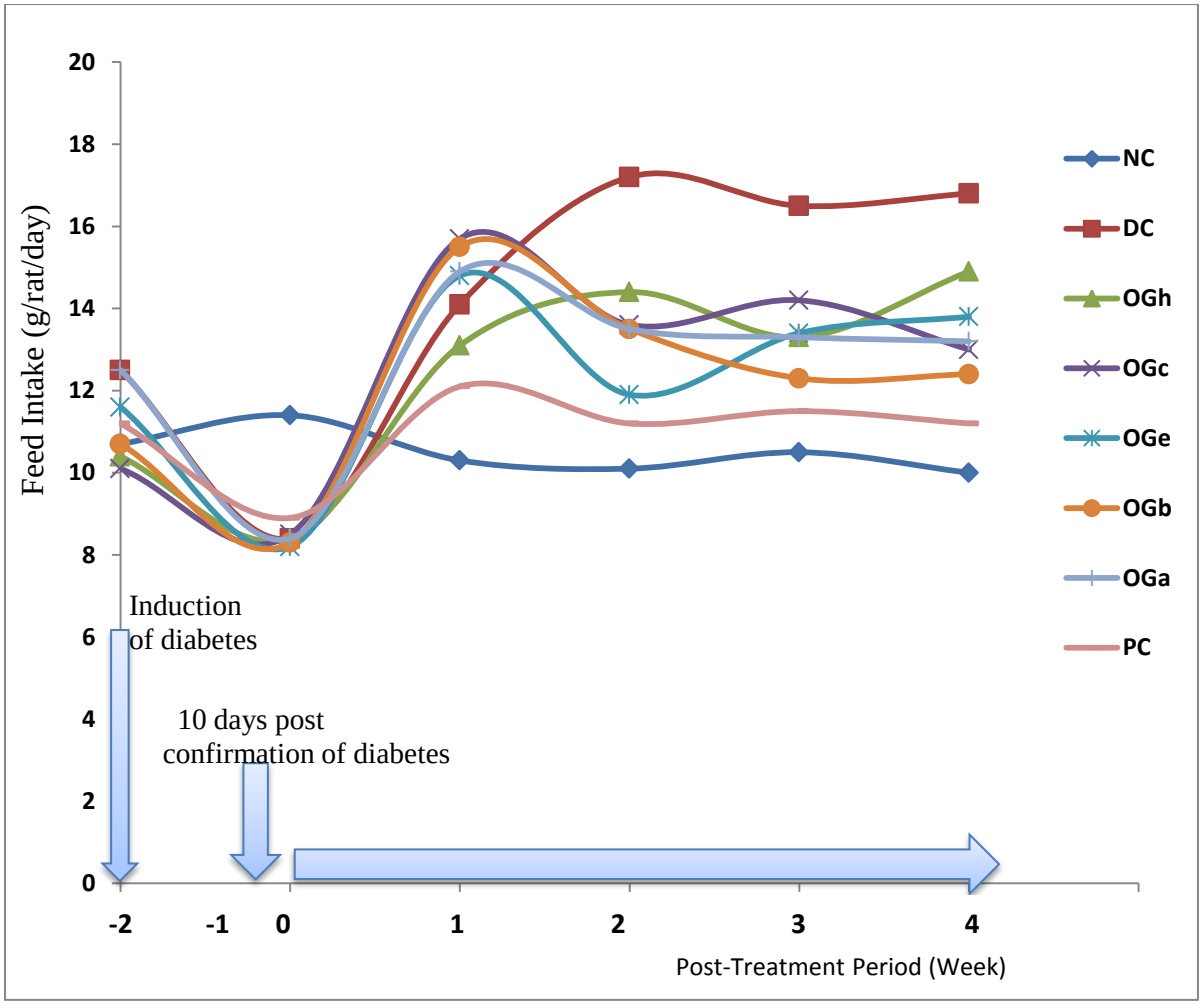


Figure 4.6: Effect of Fractionated Methanol Extract of *Ocimum gratissimum* Leaf on Feed Intake in Experimental Rats.

Data are presented as mean of 6 animals per group. NC: Normal control; DC: Diabetic control; PC: Positive control; OGh: *O. gratissimum n*-hexane fraction; OGc: *O. gratissimum* chloroform fraction; OGe: *O. gratissimum* ethyl-acetate fraction; OGb: *O. gratissimum n*-butanol fraction; OGa: *O. gratissimum* aqueous fraction. *Note: Error bars were not included for clarity.

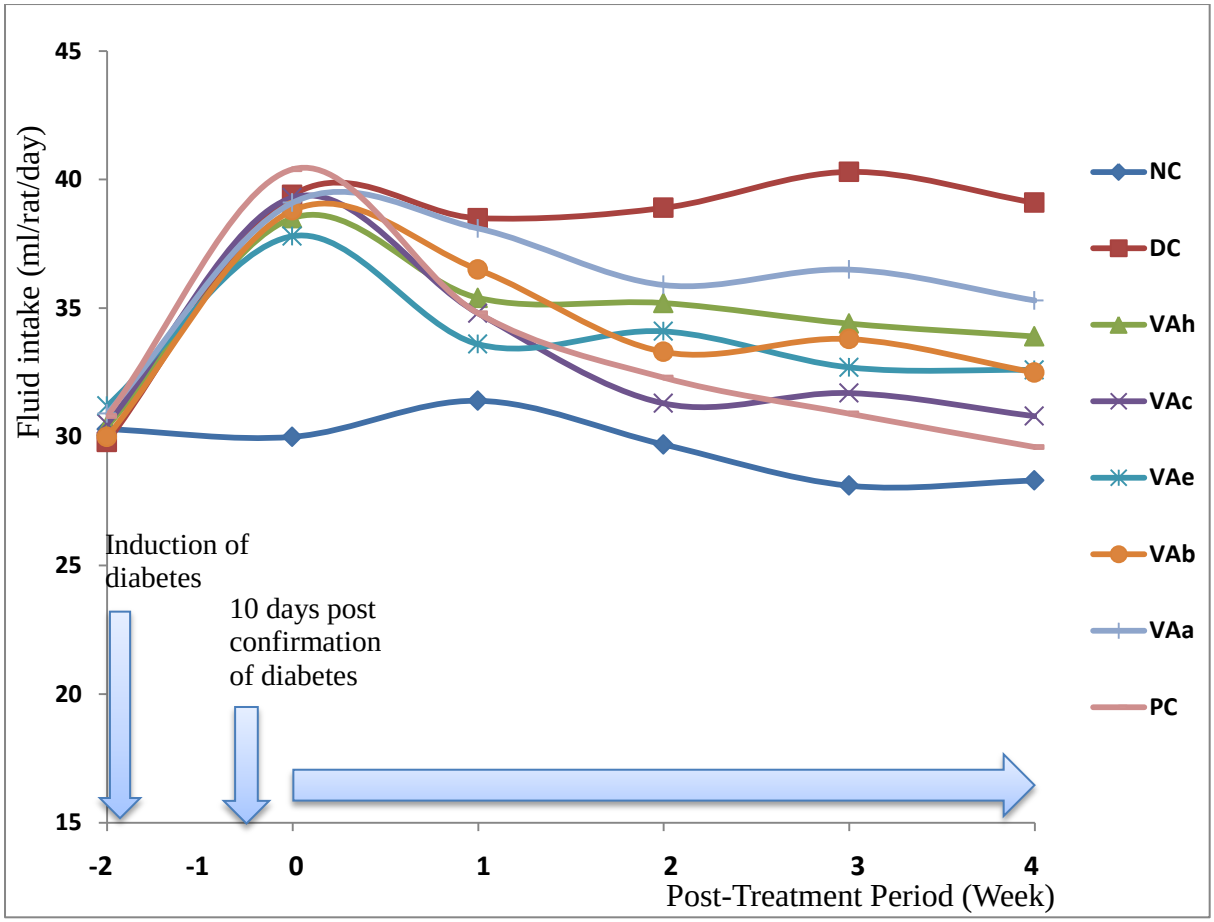


Figure 4.7: Effect of Fractionated Methanol Extract of *Vernonia amygdalina* Leaf on Fluid Intake in Experimental Rats.

Data are presented as mean of 6 animals per group. NC: Normal control; DC: Diabetic control; PC: Positive control; VAh: *V. amygdalina* hexane fraction; VAc: *V. amygdalina* chloroform fraction; VAe: *V. amygdalina* ethyl-acetate fraction; VAb: *V. amygdalina n*-butanol fraction; VAa: *V. amygdalina* aqueous fraction. *Note: Error bars were not included for clarity.

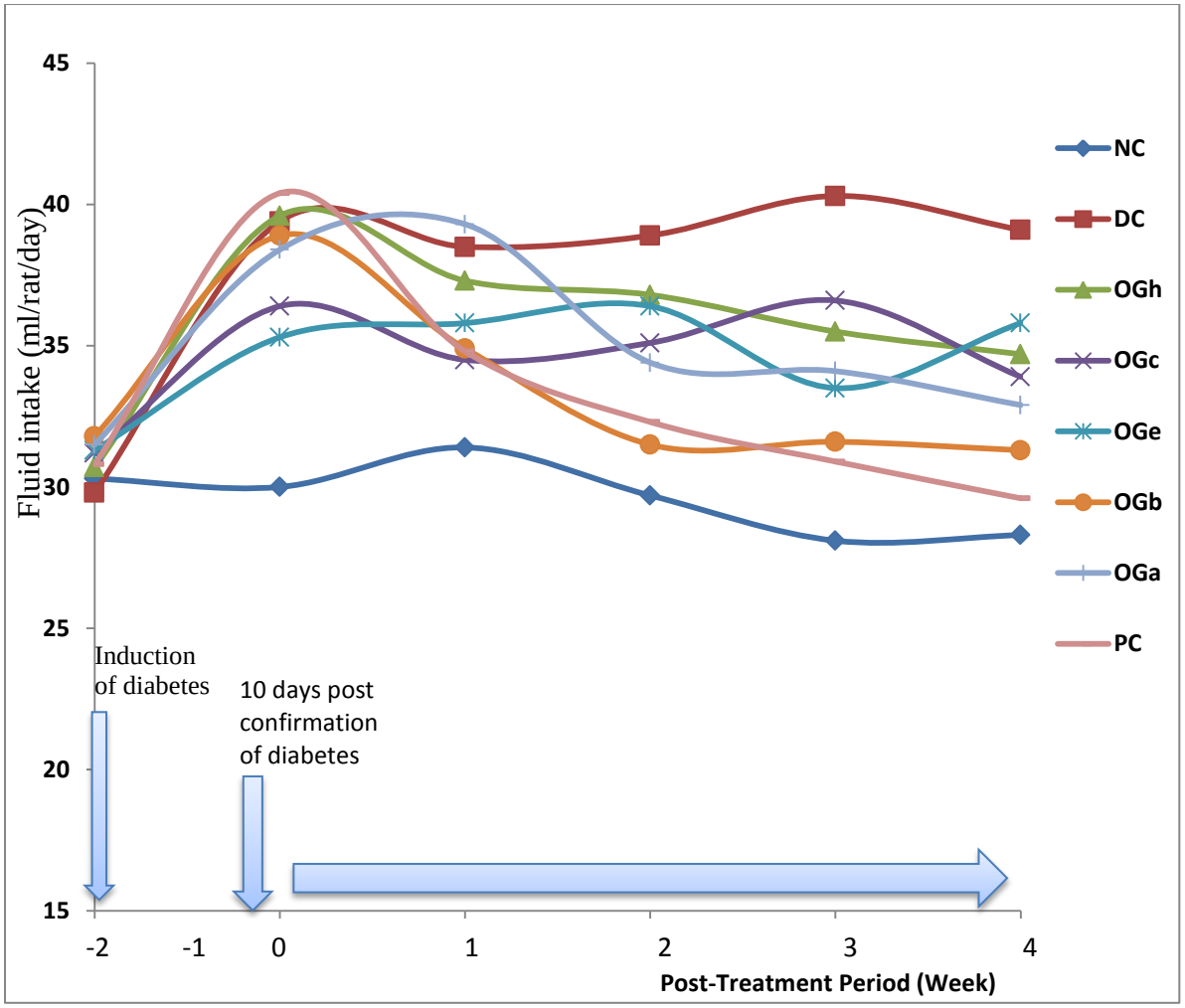


Figure 4.8: Effect of Fractionated Methanol Extract of *Ocimum gratissimum* Leaf on Fluid Intake in Experimental Rats.

Data are presented as mean of 6 animals per group. NC: Normal control; DC: Diabetic control; PC: Positive control; OGh: *O. gratissimum* hexane fraction; OGc: *O. gratissimum* chloroform fraction; VAe: *O. gratissimum* ethyl-acetate fraction; OGb: *O. gratissimum n*-butanol fraction; OGa: *O. gratissimum* aqueous fraction. *Note: Error bars were not included for clarity.

4.7.3 Effect of fractionated methanol extract of *Vernonia amygdalina* and *Ocimum gratissimum* leaves on total body weight of experimental rats

The result shows a significant (p<0.05) difference in both percentage weight increase among the treated groups and control (Figures 4.9 and 4.10). Two weeks after STZ induction, a significant (p<0.05) decrease in body weight was observed among the diabetic groups when compared with the NC group and their initial body weight just before commencement of the experiment. A significant (p<0.05) increased in weight gain was observed among the diabetic rats treated with the fractionated methanol extracts of *V. amygdalina* and *O. gratissimum* leaves and metformin groups when compared with the NC group. With respect to all the diabetic treated groups, the chloroform fraction of the fractionated methanol extract of *V. amygdalina* leaf, butanol fractions of fractionated methanol extract of *O. gratissimum* leaf treated group and the PC group had increased in body weight significantly (p<0.05) when compared to the NC group (Figures 4.9 and 4.10).

4.7.4 Effect of fractionated methanol extract of *Vernonia amygdalina* and *Ocimum gratissimum* leaves on fasting blood glucose in experimental rats

The blood glucose levels (mg/dl) of rats after 28 days of administration of fractionated methanol extracts of *V. amygdalina* and *O. gratissimum* leaves are presented in Figures 4.11 and 4.12. There was a significant (p<0.05) increase in blood glucose after diabetic induction before the commencement of treatment. During the treatment period, gradual decrease in blood glucose was observed among the VALF, OGLF treated groups and the PC group when compared with the NC and the DC groups (Tables 4.15 and 4.16). The fasting blood glucose of the DC group was significantly (p<0.05) higher than those of the treated groups. A significant difference (p<0.05) in the percentage change in fasting blood glucose level was observed among treated groups (Tables 4.15).

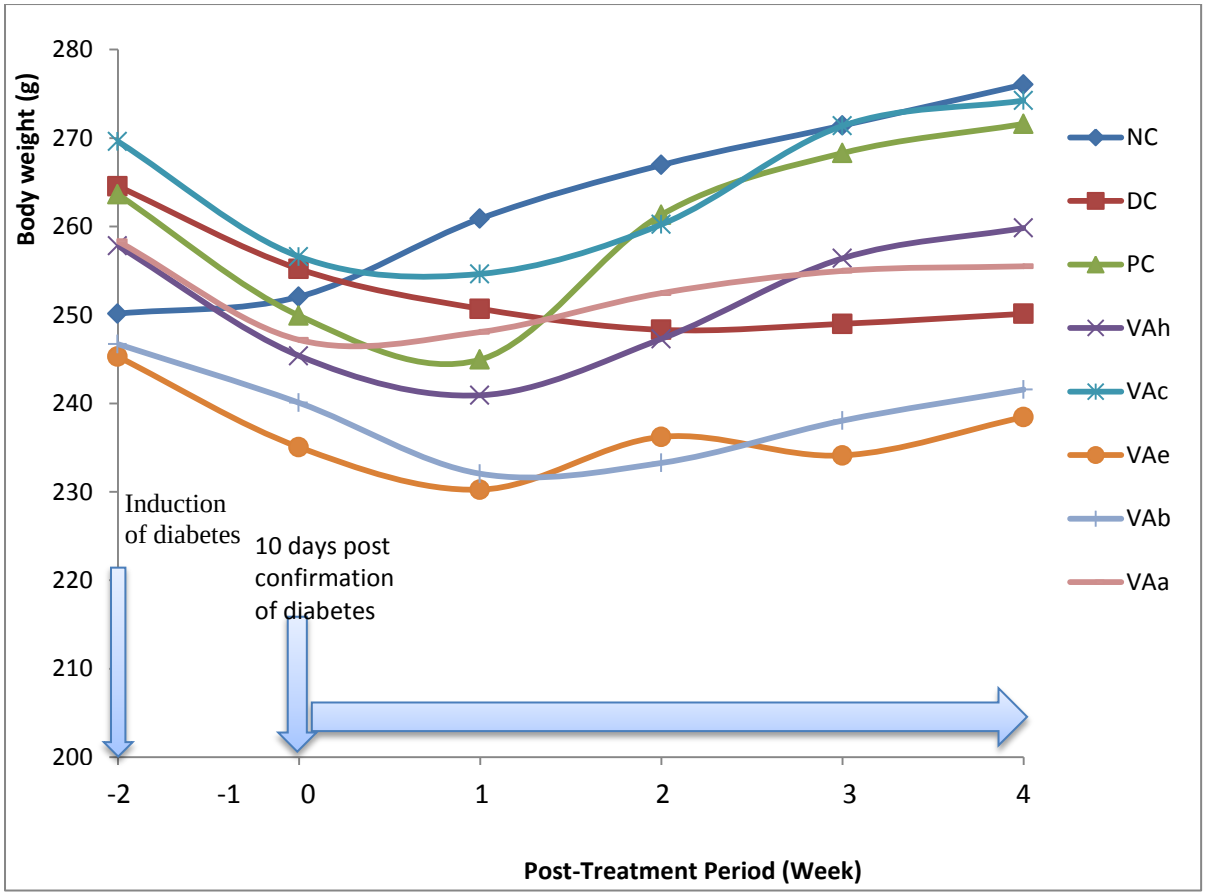


Figure 4.9: Effect of Fractionated Methanol Extract of *Vernonia amygdalina* Leaf on Body Weight in Experimental Rats

Data are presented as mean of 6 animals per group. NC: Normal control; DC: Diabetic control; PC: Positive control; VAh: *V. amygdalina n*-hexane fraction; VAc: *V. amygdalina* chloroform fraction; VAe: *V. amygdalina* ethyl-acetate fraction; VAb: *V. amygdalina n*-butanol fraction; VAa: *V. amygdalina* aqueous fraction. *Note: Error bars were not included for clarity.

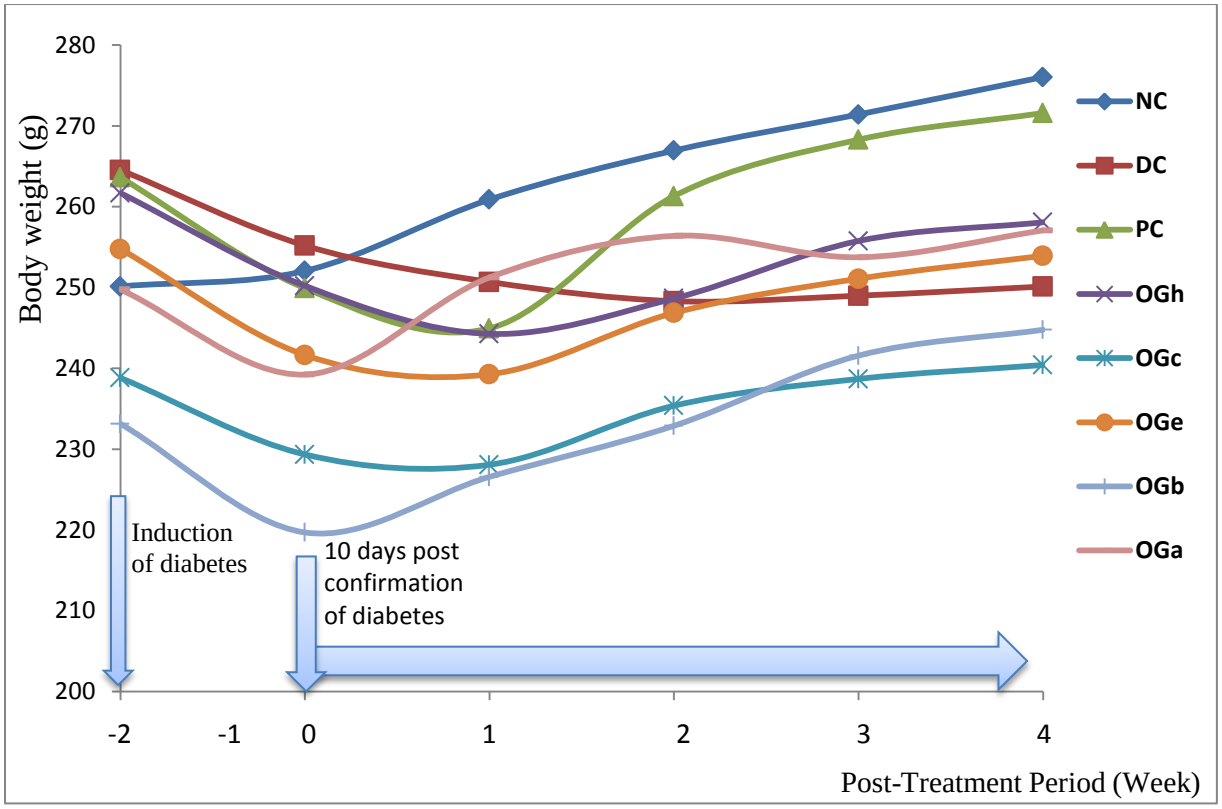


Figure 4.10: Effect of Fractionated Methanol Extract of *Ocimum gratissimum* Leaf on Body Weight in Experimental Rats

Data are presented as mean of 6 animals per group. NC: Normal Control; DC: Diabetic control; PC: Positive control; OGh: *O. gratissimum n*-hexane fraction; OGc: *O. gratissimum* chloroform fraction; OGe: *O. gratissimum* ethyl-acetate fraction; OGb: *O. gratissimum n*-butanol fraction; OGa: *O. gratissimum* aqueous fraction. *Note: Error bars were not included for clarity

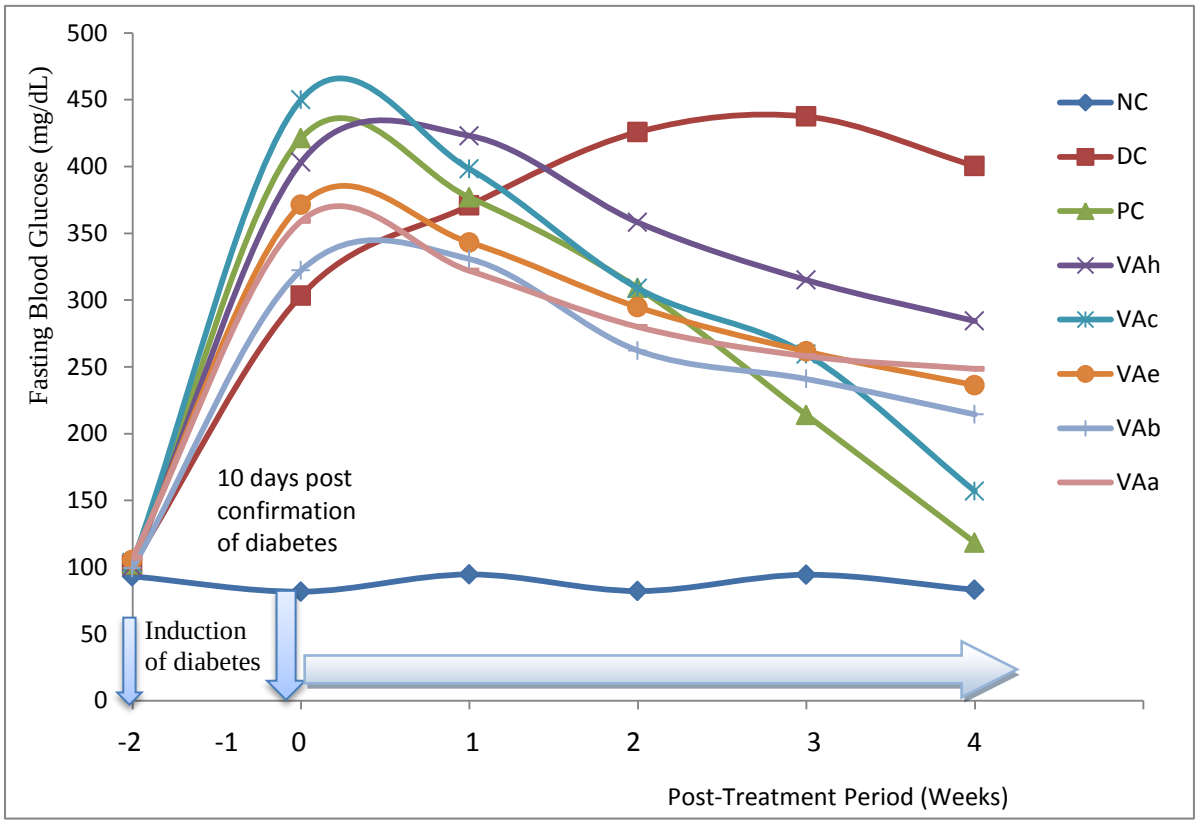


Figure 4.11: Effect of Fractionated Methanol Extract of *Vernonia amygdalina* Leaf on Fasting Blood Glucose in Experimental Rats

Data are presented as mean of 6 animals per group. NC: Normal control; DC: Diabetic control; PC: Positive control; VAh: *V. amygdalina n*-hexane fraction; VAc: *V. amygdalina* chloroform fraction; VAe: *V. amygdalina* ethyl-acetate fraction; VAb: *V. amygdalina n*-butanol fraction; VAa: *V. amygdalina* aqueous fraction. *Note: Error bars were not included for clarity.

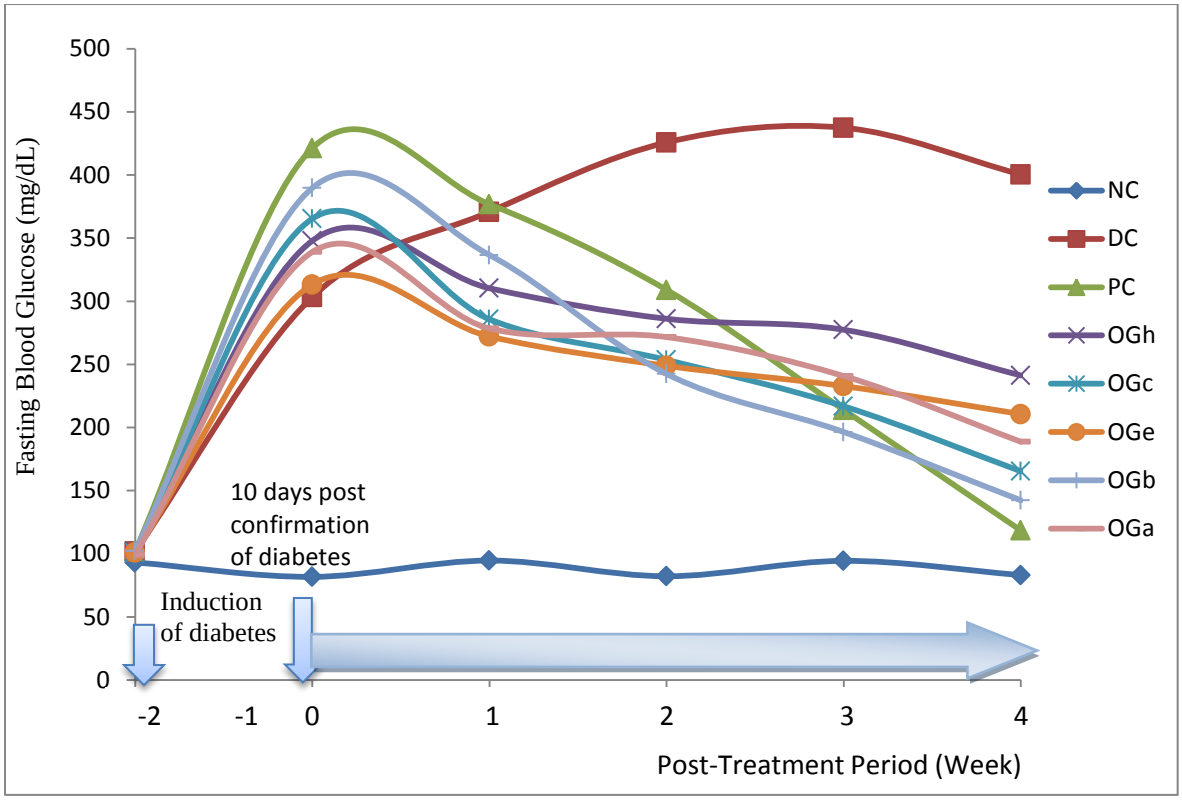


Figure 4.12: Effect of Fractionated Methanol Extract of *Ocimum gratissimum* Leaf on Fasting Blood Glucose in Experimental Rats

Data are presented as mean of 6 animals per group. NC: Normal Control; DC: Diabetic control; PC: Positive control; OGh: *O. gratissimum n*-hexane fraction; OGc: *O. gratissimum* chloroform fraction; OGe: *O. gratissimum* ethyl-acetate fraction; OGb: *O. gratissimum n*-butanol fraction; OGa: *O. gratissimum* aqueous fraction. *Note: Error bars were not included for clarity.

Table 4.15: Decrease (%) in Fasting Blood Glucose in Diabetic Rats, After Treatment with Fractionated Methanol Extract of *Vernonia amygdalina* Leaf

Groups	Decrease in Fasting Blood Glucose After Treatment (%)			
	Day 7	**Day 14**	**Day 21**	**Day 28**
NC	2.40±6.11^{b,c}	1.60±6.96^{b}	-0.90 ±5.57^{b}	0.61 ±5.55^{b}
DC	-22.30±6.96^{a}	-40.60± 7.99^{a}	-44.60 ±13.03^{a}	-50.50 ± 11.46^{a}
VAh	-5.40 ±22.09^{b}	11.02± 24.77^{b,c}	21.50 ±21.48^{c}	29.30 ± 16.12^{c}
VAc	11.50±3.47^{c}	27.10± 5.27^{d}	40.30 ±3.33^{d,e}	66.70 ± 6.50^{e}
VAe	7.70 ±4.10^{c}	20.70± 2.60^{c,d}	29.40 ±3.13^{c,d}	36.20 ± 3.88^{c,d}
VAb	10.10 ±4.57^{c}	18.90± 5.61^{c,d}	25.40± 5.31^{c}	42.90 ± 11.53^{d}
VAa	9.30 ±8.16^{c}	21.10± 8.43^{c,d}	27.00± 8.60^{c}	39.30 ± 9.41^{c,d}
PC	10.10 ±11.46^{c}	26.70±14.14^{d}	49.10 ± 7.78^{e}	71.70 ± 2.23^{e}

Data are presented as mean of 6 animals per group. Values with different superscript down the column indicate significant difference ($P<0.05$). NC: Normal Control; DC: Diabetic control; PC: Positive control; VAh: *V. amygdalina n*-hexane fraction; VAc: *V. amygdalina* chloroform fraction; VAe: *V. amygdalina* ethyl-acetate fraction; VAb: *V. amygdalina n*-butanol fraction; VAa: *V. amygdalina* aqueous fraction. Negative sign (-ve) before a figure means increase in fasting blood glucose

Table 4.16: Decrease (%) in Fasting Blood Glucose in Diabetic Rats, After Treatment with Fractionated Methanol Extract of *Ocimum gratissimum* Leaf

Groups	Decrease in Fasting Blood Glucose After Treatment (%)			
	Day 7	**Day 14**	**Day 21**	**Day 28**
NC	2.38 ± 6.11^{b}	1.60 ± 6.96^{b}	-0.93 ± 5.57 b	0.61 ± 5.55^{b}
DC	-22.27 ± 6.96^{a}	-40.57 ± 7.99^{a}	-44.57 ± 13.03^{a}	-50.45 ± 11.46^{a}
OGh	$10.82 \pm 4.36^{b,c}$	17.77 ± 3.90^{c}	20.28 ± 3.83^{c}	30.63 ± 5.02^{c}
OGc	21.75 ± 3.00^{d}	$30.60 \pm 7.50^{d,e}$	40.60 ± 6.37^{d}	54.67 ± 6.63^{e}
OGe	12.83 ± 9.42^{c}	$20.37 \pm 9.93^{c,d}$	25.63 ± 8.94^{c}	32.60 ± 5.10^{c}
OGb	$13.55 \pm 5.33^{c,d}$	37.92 ± 7.86^{e}	49.68 ± 7.29^{d}	63.52 ± 4.84^{f}
OGa	$17.85 \pm 3.98^{c,d}$	19.87 ± 4.03^{c}	28.95 ± 3.63^{c}	44.4 ± 10.87^{d}
PC	10.10 ± 11.46^{b}	26.70 ± 14.14^{d}	49.13 ± 7.78^{e}	71.70 ± 2.23^{f}

Data are presented as mean of 6 animals per group. Values with different superscript down the column indicate significant difference ($P<0.05$). NC: Normal Control; DC: Diabetic control; PC: Positive control; OGh: *O. gratissimum n*-hexane fraction; OGc: *O. gratissimum* chloroform fraction; OGe: *O. gratissimum* ethyl-acetate fraction; OGb: *O. gratissimum n*-butanol fraction; OGa: *O. gratissimum* aqueous fraction. Negative sign (-ve) before a figure means increase in fasting blood glucose

From the results, between one and two weeks of treatment, there were no significant (p>0.05) change (%) in blood glucose among the chloroform, ethyl acetate, *n*-butanol, aqueous fractions of the fractionated methanol extract of *V. amygdalina* and the PC group, except for the hexane fraction of the fractionated methanol extract of *V. amygdalina* treated group with an increase in fasting blood glucose at first week post-treatment. At three and four weeks post-treatment, it was observed that the chloroform fraction of VA, butanol fraction of OG and the PC groups recorded no significant (p>0.05) change (%) in blood glucose (Tables 4.15 and 4.16).

4.7.5 Effect of fractionated methanol extract of *Vernonia amygdalina* and *Ocimum gratissimum* leaves on oral glucose tolerance test and area under curve

The blood glucose in normal control, diabetic control and diabetic treated with fractionated methanol extracts of *V. amygdalina* and *O. gratissimum* groups demonstrated a significant (p<0.05) change after oral loading with 2 g/kg b.w glucose solution (Figures 4.13 and 4.14 and Table 4.17). The rats in the diabetic group had a significant (p<0.05) elevation in blood glucose level throughout the total measurement period (120 min) with respect to the normal control; moreover it did not return back to the initial value (0 min level) even at the end of 120 min.

Pre-treatment of diabetic rats with metformin or the fractionated methanol extract of *V. amygdalina* and *O. gratissimum* leaves induced a significant reduction in area under the curve relative to the negative control group by between 31.69-49.01 and 36.71-48.05 for VA and OG respectively (Figures 4.13 and 4.14 and Table 4. 17).

4.7.6 Effect of fractionated methanol extract of *Vernonia amygdalina* and *Ocimum gratissimum* leaves treatment on biochemical parameters in experimental rats

The levels of serum liver marker enzymes (AST, ALT and ALP) and biomarkers of kidney function (serum urea, uric acid and creatinine) were significantly elevated in the untreated diabetic control group (DC) as compared to the normal control, positive control and VALF treated groups (Table 4.18) and OGLF treated group (Table 4.19).

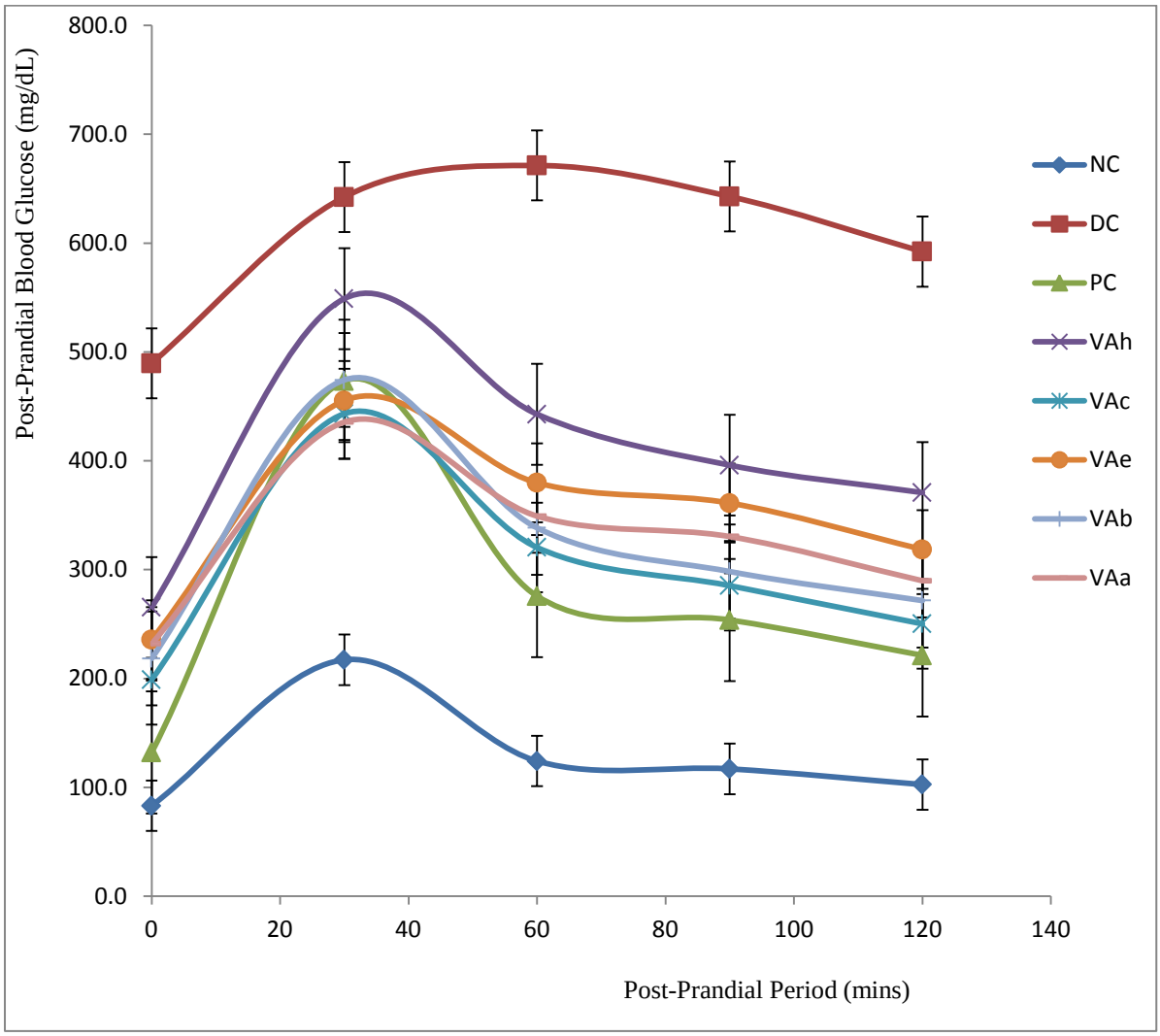


Figure 4.13: Changes in Blood Glucose Concentration during Oral Glucose Tolerance Test in Diabetic Rats Treated with Fractionated Methanol Extract of *Vernonia amygdalina* Leaf.

Data are presented as mean of 6 animals per group. NC: Normal control; DC: Diabetic control; PC: Positive control; VAh: *V. amygdalina* hexane fraction; VAc: *V. amygdalina* chloroform fraction; VAe: *V. amygdalina* ethyl-acetate fraction; VAb: *V. amygdalina* butanol fraction; VAa: *V. amygdalina* aqueous fraction.

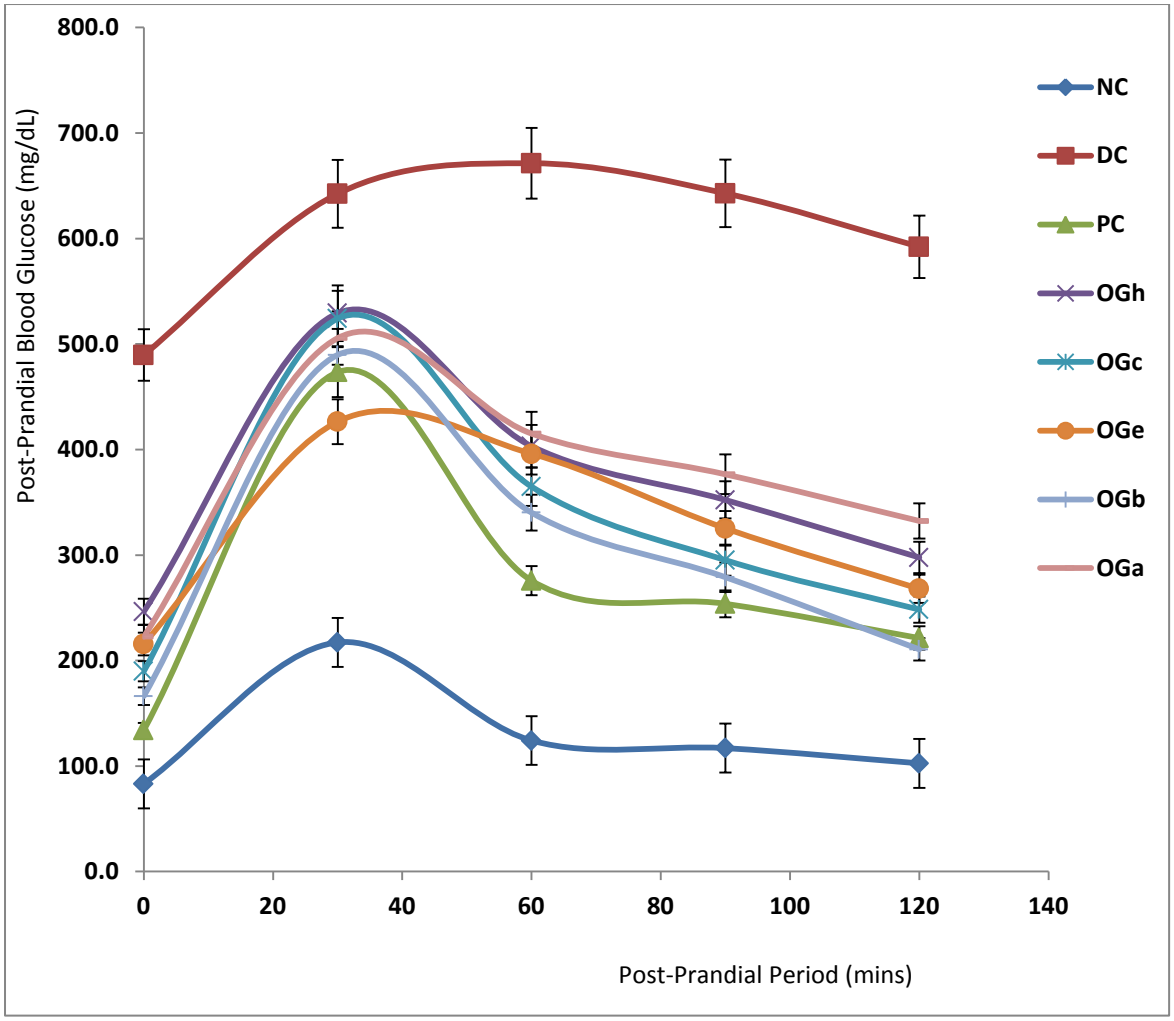


Figure 4.14: Changes in Blood Glucose Concentration During Oral Glucose Tolerance Test in Rats Treated with Fractionated Methanol Extract of *Ocimum gratissimum* Leaf.

Data are presented as mean of 6 animals per group. NC: Normal control; DC: Diabetic control; PC: Positive control; OGh: *O. gratissimum* hexane fraction; OGc: *O. gratissimum* chloroform fraction; OGe: *O. gratissimum* ethyl-acetate fraction; OGb: *O. gratissimum* butanol fraction; OGa: *O. gratissimum* aqueous fraction.

Table 4.17: Total Area Under Curve (AUC) After Oral Glucose Loading (2 g/kg bw) in Diabetic Rats Treated with Fractionated Methanol Extract of *Vernonia amygdalina and Ocimum gratissimum* Leaves (250 mg/kg/day) for 28 Days

Groups	Total AUC (mg/dL min)	Percentage Reduction (%)
NC	16538.00 ± 429.23	-
DC	74922.00 ± 1045.17	0.00
VAh	51176.00 ± 1358.06	31.69
VAc	38206.00 ± 744.11	49.01
VAe	44208.00 ± 511.37	40.99
VAb	40439.00 ± 1002.62	46.03
VAa	41282.00 ± 410.18	44.90
OGh	46697.00 ± 423.44	37.67
OGc	42095.00 ± 348.86	43.81
OGe	40606.00 ± 577.86	45.80
OGb	38921.00 ± 174.85	48.05
OGa	47241.00 ± 933.54	36.95
PC	47418.00 ± 113.52	36.71

Values are mean ± SD of 6 rats. NC: Normal control; DC: Diabetic control; PC: Positive control (Metformin); VAh: *Vernonia amygdalina* hexane fraction; VAc: *Vernonia amygdalina* chloroform fraction; VAe: *Vernonia amygdalina* ethyl-acetate fraction; VAb: *Vernonia amygdalina* butanol fraction; VAa: *Vernonia amygdalina* aqueous fraction. OGh: *Ocimum gratissimum* hexane fraction; OGc: *Ocimum gratissimum* chloroform fraction; OGe: *Ocimum gratissimum* ethyl-acetate fraction; OGb: *Ocimum gratissimum* butanol fraction; OGa: *Ocimum gratissimum* aqueous fraction.

Table 4.18: Liver and Kidney Function Biomarkers in Diabetic Rats Treated with Fractionated Methanol Extract of *Vernonia amygdalina* Leaf (250mg/kg/day) for 28 Days

Groups	AST (U/I)	ALT (U/I)	ALP (U/I)	Urea (mg/dL)	Creatinine (mg/dL)	Uric acid (mg/dL)
NC	27.69±2.93 [a]	23.40 ±4.86 [a]	32.65±7.41 [a]	25.80 ±2.41[a]	0.88 ± 0.13 [a]	2.46 ±0.34 [a]
DC	69.65± 6.40 [e]	62.44±8.41[e]	97.80±13.70 [e]	40.69± 2.50[c]	2.21 ± 0.21 [e]	4.81 ±0.65 [e]
VAh	40.62±3.04[c,d]	32.49±3.78 [b,c]	53.18± 6.56 [b]	31.91± 2.45[b]	1.56 ± 0.22[c,d]	3.33 ± 0.27 [b,c,d]
VAc	28.17±2.85 [a]	26.53±.32 [a,b]	43.51 ± 6.58 [a,b]	28.84±2.38[a,b]	1.09 ± 0.36 [a,b]	2.92 ±0.29 [b]
VAe	40.10±4.21[c,d]	35.62±6.50 [c,d]	72.36 ± 6.14 [d]	31.83± 2.42[b]	1.74 ± 0.24 [d]	3.38 ±0.26 [c,d]
VAb	35.48±3.58 [b,c]	33.78±1.18 [c,d]	55.20 ± 8.16 [b,c]	30.91± 2.49[b]	1.18 ± 0.42 [a,b]	3.21 ± 0.29 [b.c.d]
VAa	43.67±6.70 [d]	39.71±6.02[d]	65.09 ± 7.69 [c,d]	31.88± 2.22[b]	1.85 ± 0.13 [d]	3.49 ±0.26 [d]
PC	31.95±1.41[a,b]	37.78± 3.53 [c,d]	49.53 ±14.92 [b]	29.71±2.17[a,b]	1.40 ± 0.21 [b,c]	3.01± 0.27 [b,c]

Data are presented as mean ± SD of 6 animals per group. Values with different superscript down the column indicate significant difference (p<0.05). AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; NC: Normal control; DC: Diabetic control; PC: Positive control; VAh: *V. amygdalina* *n*-hexane fraction; VAc: *V. amygdalina* chloroform fraction; VAe: *V. amygdalina* ethyl-acetate fraction; VAb: *V. amygdalina* *n*-butanol fraction; VAa: *V. amygdalina* aqueous fraction.

Table 4.19: Liver and Kidney Function Biomarkers in Diabetic Rats Treated with Fractionated Methanol Extract of *Ocimum gratissimum* Leaf (250mg/kg/day) for 28 Days

Groups	AST (U/I)	ALT (U/I)	ALP (U/I)	Urea (mg/dL)	Creatinine (mg/dL)	Uric Acid (mg/dL)
NC	27.69±2.93^{a,b}	23.40 ±4.86^{a}	32.65 ± 7.41^{a}	25.80 ±2.41^{a}	0.88 ± 0.13^{a}	2.46 ± 0.34^{a}
DC	69.65±6.40^{d}	62.44 ± 8.41^{f}	97.80 ± 13.70^{e}	40.69± 2.50^{c}	2.21 ± 0.21^{e}	4.81 ± 0.65^{c}
OGh	41.38±5.97^{c}	35.86 ± 5.59^{c,d}	57.13 ± 8.62^{b,c}	30.22± 3.14^{b}	1.52 ± 0.39^{c,d}	3.24 ± 0.55^{b}
OGc	37.53±2.88^{c}	42.71 ± 2.24^{d,e}	70.28 ± 12.55^{d}	31.57± 2.54^{b}	1.39 ± 0.18^{b,c}	2.87 ± 0.27^{a,b}
OGe	30.46±4.08^{a,b}	46.73 ± 5.73^{e}	64.69 ± 7.83^{c,d}	30.12±2.25^{b}	1.71 ± 0.11^{d}	3.36 ± 0.28^{b}
OGb	26.07±2.94^{a}	25.38 ± 5.32^{a,b}	33.15 ± 7.35^{a}	29.62±2.43^{a,b}	1.14 ± 0.31^{a,b}	2.84 ± 0.54^{a,b}
OGa	42.66±5.05^{c}	32.04 ± 6.09^{b,c}	51.24 ± 6.00^{b}	32.87± 2.51^{b}	1.68 ± 0.15^{c,d}	3.16 ± 0.40^{b}
PC	31.95±1.42^{d}	29.77±10.00^{a,b,c}	49.53 ±14.92^{b}	29.71±2.17^{a,b}	1.40 ± 0.21^{b,c}	3.01 ± 0.27^{a,b}

Data are presented as mean ± SD of 6 animals per group. Values with different superscript down the column indicate significant difference ($P<0.05$). AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; NC: Normal control; DC: Diabetic control; PC: Positive control; OGh: *O. gratissimum* hexane fraction; OGc: *O. gratissimum* chloroform fraction; OGe: *O. gratissimum* ethyl-acetate fraction; OGb: *O. gratissimum* butanol fraction; OGa: *O. gratissimum* aqueous fraction.

When VALF and OGLF were administered to diabetic rats for 28 days, the serum urea, uric acid, and creatinine contents decreased significantly ($p<0.05$). The chloroform fraction of the fractionated methanol extract of *V. amygdalina* and the butanol fraction of the of fractionated methanol extract of *O. gratissimum* leaves showed better improvement. In a likewise manner, oral administration of VALF and OGLF for 28 days restored the enzyme levels significantly ($p<0.05$).

Table 4.20 shows the effect of the various fractionated methanol extract of VA and OG leaves on serum lipid profile levels in diabetic rats. The results indicated that serum total cholesterol (Tc), serum triacylglycerol (TG), serum low density lipoprotein cholesterol (LDLc) and the ratio of total cholesterol to high density lipoprotein cholesterol ratio (Tc/HDLc) were significantly increased ($p<0.05$) in the diabetic control group when compared to the normal control group, the VALF and OGLF treated groups. However, high density lipoprotein (HDLc) level was significantly lower in the DC group when compared to the NC group and the VALF/OGLF treated group (Tables 4.20 and 4.21).

The HOMA indices for insulin resistance (HOMA-IR), β-cell function (HOMA-β cell), serum C-peptide, serum insulin and insulin sensitivity for the diabetic treated (VALF and OGLF) and untreated rats are presented in Tables 4.22 and 4.23 respectively. The results indicated that HOMA-IR index was significantly ($p<0.05$) higher in the DC group when compared to the NC, PC, VALF and OGLF treated groups. There were no significant ($p>0.05$) difference in the HOMA-IR index between the various group treated with the fractionated methanol extract of *V. amygdalina* and *O. gratissimum* leaves respectively. The insulin sensitivity and HOMA- β cell functioning index were significantly ($p<0.05$) lower in the DC group when compared to the NC group, PC and the VALF treated group (Table 4.22) and OGLF treated group (Table 4.23).

Table 4.20: Serum Lipid Profile in Diabetic Rats Treated with Fractionated Methanol Extract of *Vernonia amygdalina* Leaf (250mg/kg/day) for 28 Days

Groups	TC (mg/dL)	HDLC (mg/dL)	TG (mg/dL)	LDLC (mg/dL)	TC/HDLC
NC	78.00 ± 7.61 [a]	58.60 ± 5.82 [f]	73.60 ± 7.99 a	4.70 ± 9.21 [a]	1.34 ± 0.15 [a]
DC	176.70 ± 11.82 [d]	17.80 ± 2.63 [a]	448.70 ± 11.35[e]	69.10 ± 10.26 [c]	10.07 ± 1.33 [e]
Vah	114.50 ± 14.92 [b]	31.20 ± 4.51 [b,c]	125.30 ± 7.71 [d]	58.20 ±12.64 [b,c]	3.69 ± 0.31 [c]
VAc	102.70 ± 16.37 [b]	44.70± 6.80 [e]	82.20 ± 7.09 [b]	41.60 ± 17.40 [b]	2.34 ± 0.49 [b]
VAe	109.60 ± 15.34 [b]	36.90 ± 4.22 [c,d]	121.50 ± 3.51 [d]	48.40 ± 15.22 [b]	3.00 ± 0.51 [b,c]
VAb	105.80 ± 21.07 [b]	28.20 ± 4.51 [b]	97.30 ±6.28 [c]	58.20 ± 23.27 [b,c]	3.85 ± 1.03 [c]
VAa	107.90 ±10.54 [b]	37.60 ± 4.56 [d]	118.50 ± 5.82 [d]	46.60 ± 6.40 [b]	2.88 ± 0.17 [b,c]
PC	137.50 ± 12.81[c]	29.60 ± 5.93 [b]	105.20 ± 6.33 [c]	86.9 ± 17.69 [d]	4.83 ± 1.16 [d]

Data are presented as mean ± SD of 6 animals per group. Values with different superscript down the column indicate significant difference (p<0.05). TC: Total cholesterol; HDLC: Serum High density liproprotein cholesterol; LDLC: Serum Low density liproprotein cholesterol; TG: Serum Triglyceride; NC: Normal control; DC: Diabetic control; PC: Positive control; VAh: *V. amygdalina* hexane fraction; VAc: *V. amygdalina* chloroform fraction; VAe: *V. amygdalina* ethyl-acetate fraction; VAb: *V. amygdalina* butanol fraction; VAa: *V. amygdalina* aqueous fraction.

Table 4.21 Serum Lipid Profile in Diabetic Rats Treated with Fractionated Methanol Extract of *Ocimum gratissimum* Leaf (250mg/kg/day) for 28 Days

Groups	TC (mg/dL)	HDLC (mg/dL)	TG (mg/dL)	LDLC (mg/dL)	TC/HDL
NC	78.0 ± 7.61 [a]	58.6 ± 5.82 [e]	73.6 ± 7.99 [a]	4.7 ± 9.21 [a]	1.34 ± 0.15 [a]
DC	176.7 ± 11.82 [d]	17.8 ± 2.63 [a]	448.7 ± 11.35 [e]	69.1 ± 10.26 [c,d]	10.07 ± 1.33 [f]
OGh	109.5 ±10.28 [b]	35.1 ± 3.26 [c]	119.2 ± 7.35 [d]	50.6 ± 13.24 [b,c]	3.15 ± 0.48 [b,c]
OGc	117.1 ± 10.68 [b]	32.3 ±4.68 [b,c]	99.2 ± 5.58 [c]	65.0 ± 9.70 [c]	3.67 ±0.45 [c,d]
OGe	111.2 ±7.69 [b]	27.5 ± 4.94 [b]	115.1 ± 5.73 [d]	60.7 ± 8.90 [b,c]	4.14 ± 0.73 [d,e]
OGb	102.6 ± 24.6 [b]	41.4 ± 6.59 [d]	87.0 ± 6.77 [b]	43.8 ± 24.30 [b,c]	2.51 ± 0.60 [b]
OGa	117.4 ± 18.86 [b]	28.2 ± 5.26 [b]	101.8 ± 6.81 [c]	68.8 ±18.1 [c,d]	4.26 ± 0.84 [d,e]
PC	137.5 ± 12.81 [c]	29.6±5.93 [b,c]	105.2 ± 6.33 [c]	86.9 ± 17.69 [d]	4.83 ± 1.16 [e]

Data are presented as mean ± SD of 6 animals per group. Values with different superscript down the column indicate significant difference (p<0.05). TC: Serum Total cholesterol; HDLC: Serum High density liproprotein cholesterol; LDLC: Serum Low density liproprotein cholesterol; TG: Serum Triglyceride; NC: Normal control; DC: Diabetic control; PC: Positive control; OGh: *O. gratissimum* hexane fraction; OGc: *O. gratissimum* chloroform fraction; OGe: *O. gratissimum* ethyl-acetate fraction; OGb: *O. gratissimum* butanol fraction; OGa: *O. gratissimum* aqueous fraction.

Table 4.22 Fasting Blood Glucose, Insulin Resistance, Beta-Cell Function and Insulin Sensitivity in Diabetic Rats Treated with Fractionated Methanol Extract of *Vernonia amygdalina* Leaf (250mg/kg bw) for 28 Days.

Groups	FBS (mg/dL)	HOMA-IR	HOMA-β	Insulin Sensitivity
NC	83.10 ± 6.38 ^{a}	2.65 ± 0.43 ^{a}	52.33 ± 5.62 ^{e}	1.36 ± 0.07 ^{d}
DC	489.60±53.80 ^{e}	12.44±2.79 ^{c}	4.04 ± 0.91 ^{a}	0.70 ± 0.05 ^{a}
VAh	265.32±66.16 ^{d}	7.38 ± 2.43 ^{b}	12.17 ± 3.17 ^{b}	0.87 ± 0.10 ^{b}
VAc	198.90±17.29 ^{c}	5.73 ±1.13 ^{b}	17.7 ± 3.87 ^{c}	0.87 ± 0.03 ^{b}
VAe	235.80±21.08 ^{c,d}	6.45 ± 0.98 ^{b}	13.75 ±3.91 ^{b,c}	0.86 ± 0.04 ^{b}
VAb	218.40±30.67 ^{c}	5.77 ± 1.05 ^{b}	14.39 ±2.94 ^{b,c}	0.87 ± 0.06 ^{b}
VAa	231.90±18.44 ^{c,d}	6.13 ± 1.23 ^{b}	13.08 ±2.66 ^{b,c}	0.86 ± 0.03 ^{b}
PC	134.10±12.92 ^{b}	3.86 ± 0.54 ^{a}	28.16 ±5.55 ^{d}	1.09 ±0.07 ^{c}

Data are presented as mean ± SD of 6 animals per group. Values with different superscript down the column indicate significant difference (p<0.05). NC: Normal control; DC: Diabetic control; PC: Positive control; VAh: *V. amygdalina* hexane fraction; VAc: *V. amygdalina* chloroform fraction; VAe: *V. amygdalina* ethyl-acetate fraction; VAb: *V. amygdalina* butanol fraction; VAa: *V. amygdalina* aqueous fraction.

Table 4.23 Fasting Blood Glucose, Insulin Resistance, Beta-Cell Function and Insulin Sensitivity in Diabetic Rats Treated with Fractionated Methanol Extract of *Ocimum gratissimum* Leaf (250mg/kg bw) for 28 Days

Groups	FBS (mg/dL)	HOMA-IR	HOMA-β	Insulin Sensitivity
NC	83.10 ± 6.38 ^{a}	2.65 ±0.43 ^{a}	52.33 ± 5.62 ^{e}	1.36 ± 0.07 ^{e}
DC	489.60±53.80 ^{f}	12.44±2.79 ^{d}	4.04 ± 0.91 ^{a}	0.70 ± 0.05 ^{a}
OGh	246.40 ±19.12 ^{e}	6.46±0.56 ^{c}	12.27 ± 3.04 ^{b}	0.86 ± 0.03 ^{b}
OGc	189.80±17.78 ^{c,d}	4.97 ± 0.51 ^{b,c}	16.85 ± 3.05 ^{b}	0.95 ± 0.05 ^{b,c}
OGe	215.60±20.23 ^{d,e}	5.75 ± 0.99 ^{c}	14.55 ± 2.27 ^{b}	0.92 ± 0.06 ^{b,c}
OGb	166.30±16.86 ^{b,c}	4.79 ± 1.01 ^{b,c}	21.63 ± 3.22 ^{c}	0.97 ± 0.05 ^{c}
OGa	222.70±55.43 $_{d,e}$	6.15 ± 1.96 ^{c}	15.34 ± 5.38 ^{b}	0.89 ± 0.18 ^{b,c}
PC	134.10±12.92 ^{b}	3.86 ± 0.54 ^{a,b}	28.16 ± 5.55 ^{d}	1.09 ±0.07 ^{d}

Data are presented as mean ± SD of 6 animals per group. Values with different superscript down the column indicate significant difference (P<0.05). NC: Normal control; DC: Diabetic control; PC: Positive control; OGh: *Ocimum gratissimum* hexane fraction; OGc: *Ocimum gratissimum* chloroform fraction; OGe: *Ocimum gratissimum* ethyl-acetate fraction; OGb: *Ocimum gratissimum* butanol fraction; OGa: *Ocimum gratissimum* aqueous fraction.

4.7.7 Effects of fractionated methanol extract of *Vernonia amygdalina* and *Ocimum gratissimum* leaves on the integrity of pancreas and liver tissues in Type 2 diabetes rat model

The plates II and III show the photomicrographs of liver tissues from rats treated with fractionated methanol extract of *V. amygdalina* leaf (VALF) and fractionated methanol extract of *O. gratissimum* leaf (OGLF) respectively. There were no observable changes between the photomicrographs of the various treated groups.

The photomicrographs of pancreas of experimental rats treated with VALF and OGLF are presented in the plates IV and V respectively. The photomicrographs revealed pancreatic islets with partial destruction of the β-cells in the DC when compared with the NC and PC group with apparently healthy pancreatic β-cells. Treatment with VALF and OGLF (250 mg/kg b.w/day) for 28 days reduced the damaged pancreatic islets cells and improved its microstructure and morphological structure. A considerable number of healthy pancreatic β-cells were observed in the group treated with chloroform fraction of the fractionated methanol extract of *V. amygdalina* leaf (Plate IV) and butanol fraction of of the fractionated methanol extract of *O. gratissimum* leaf (Plate V). However, the islet cells were significantly smaller in size compared to the normal control (NC) and positive control (PC) groups. The plate IV (a) NC: Normal control (non-diabetic group) shows normal histology of pancreas with increase number of ß-cells. (b) DC: present islets with highly dispersed and morphologically deformed ß-cells. The Positive control has normal arrangement of islet cells and their ß-cells are likened to that of the NC group.

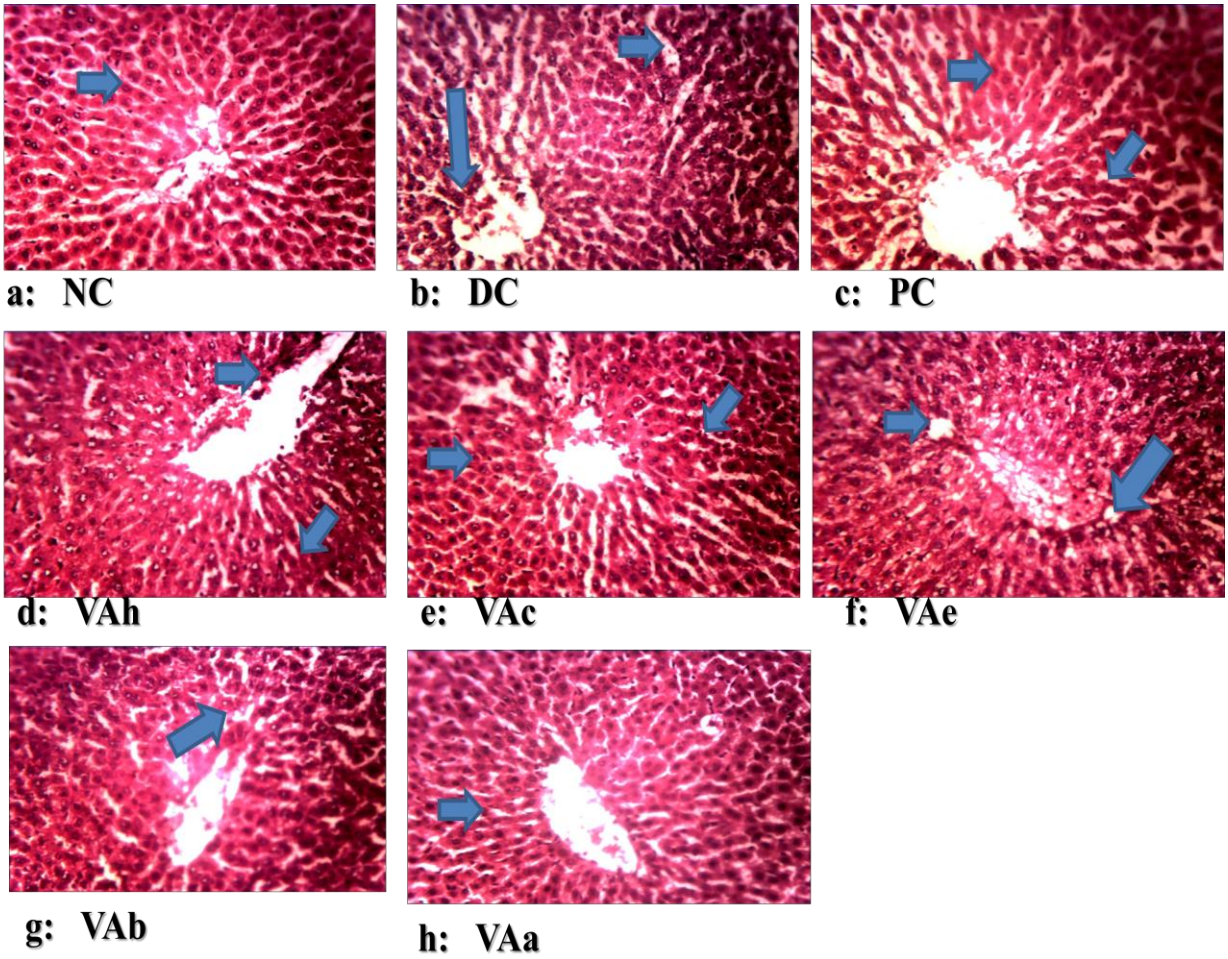


Plate II: Photomicrographs of Liver Tissues of Diabetic and Non-diabetic Rats, Pre-treated with Fractionated Methanol Extract of *Vernonia amygdalina* Leaf (250 mg/kg bw) after 28 Days (H&E stain 100x).

(a) NC: Normal control; (b) DC: Diabetic control; (c) PC: Positive control; (d) VAh: *V. amygdalina* hexane fraction; (e) VAc: *V. amygdalina* chloroform fraction; (f) VAe: *V. amygdalina* ethyl-acetate fraction; (g) VAb: *V. amygdalina* butanol fraction; (h) VAa: *V. amygdalina* aqueous fraction.

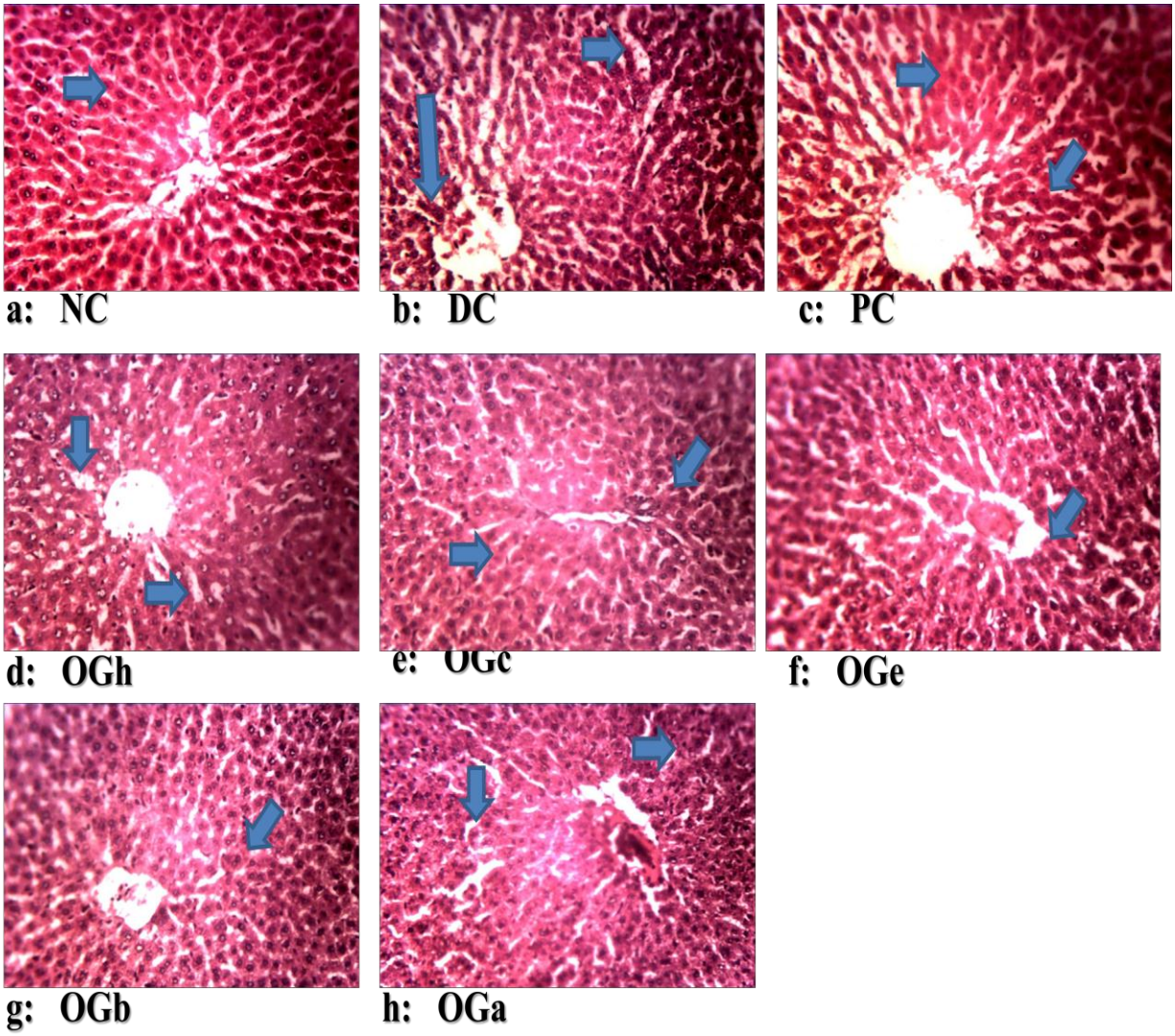


Plate III: Photomicrographs of Liver Tissues of Diabetic/Non-diabetic Rats, Pre-treated with Fractionated Methanol Extract of *Ocimum gratissimum* Leaf (250 mg/kg bw) after 28 Days (H&E stain 100x).

(a) NC: Normal control; (b) DC: Diabetic control; (c) PC: Positive control; (d) OGh: *O. gratissimum* hexane fraction; (e) OGc: *O. gratissimum* chloroform fraction; (f) OGe: *O. gratissimum* ethyl-acetate fraction; (g) OGb: *O. gratissimum* butanol fraction; (h) OGa: *O. gratissimum* aqueous fraction.

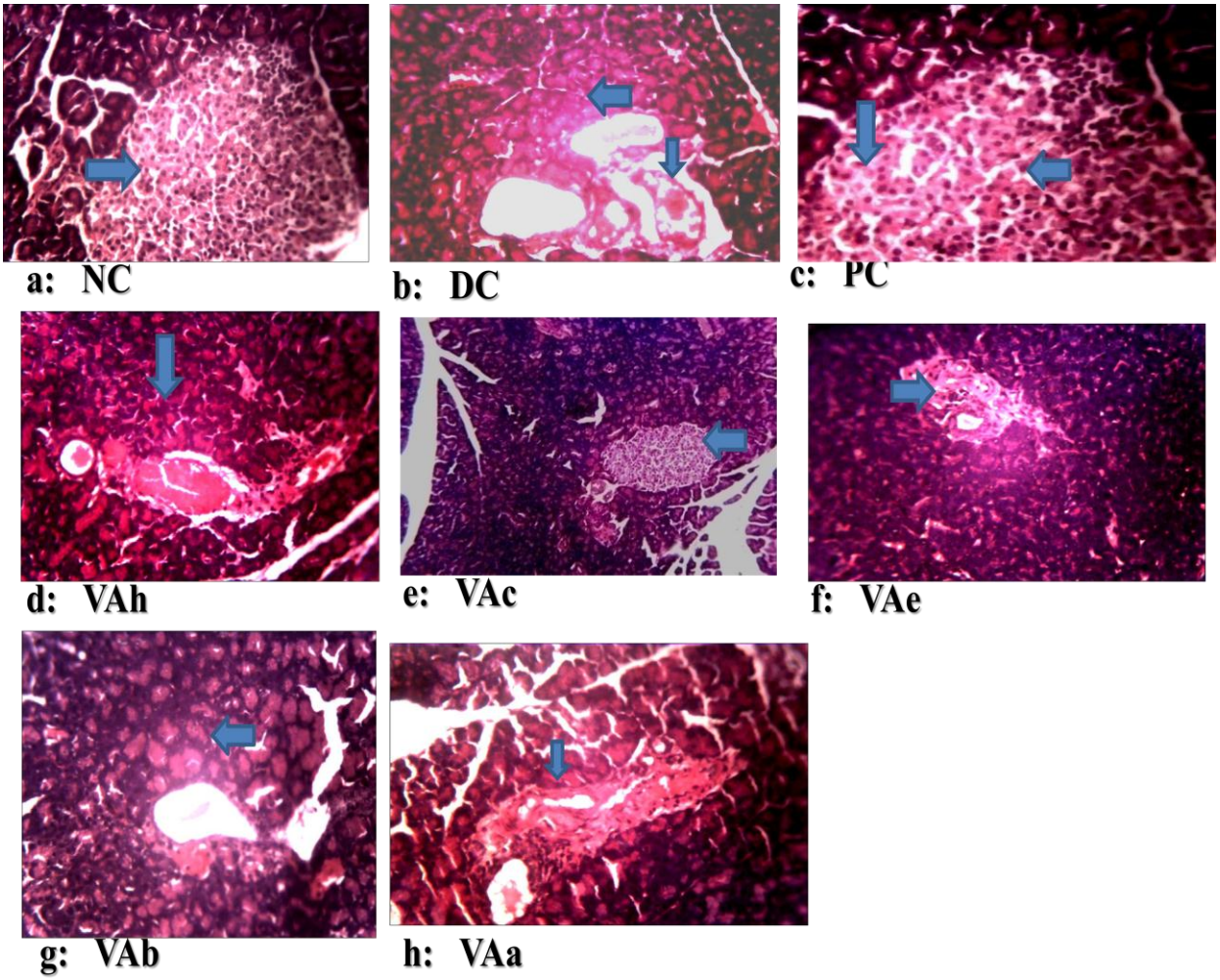


Plate IV: Photomicrographs of Pancreatic Tissues of Diabetic/Non-diabetic Rats, Pre-treated with Fractionated Methanol Extract of *Vernonia amygdalina* Leaf (250 mg/kg bw) for 28 days (H&E 100x).

(a) NC: Normal control; (b) DC: Diabetic control; (c) PC: Positive control; (d) VAh: *V. amygdalina* hexane fraction; (e) VAc: *V. amygdalina* chloroform fraction; (f) VAe: *V. amygdalina* ethyl-acetate fraction; (g) VAb: *V. amygdalina* butanol fraction; (h) VAa: *V. amygdalina* aqueous fraction.

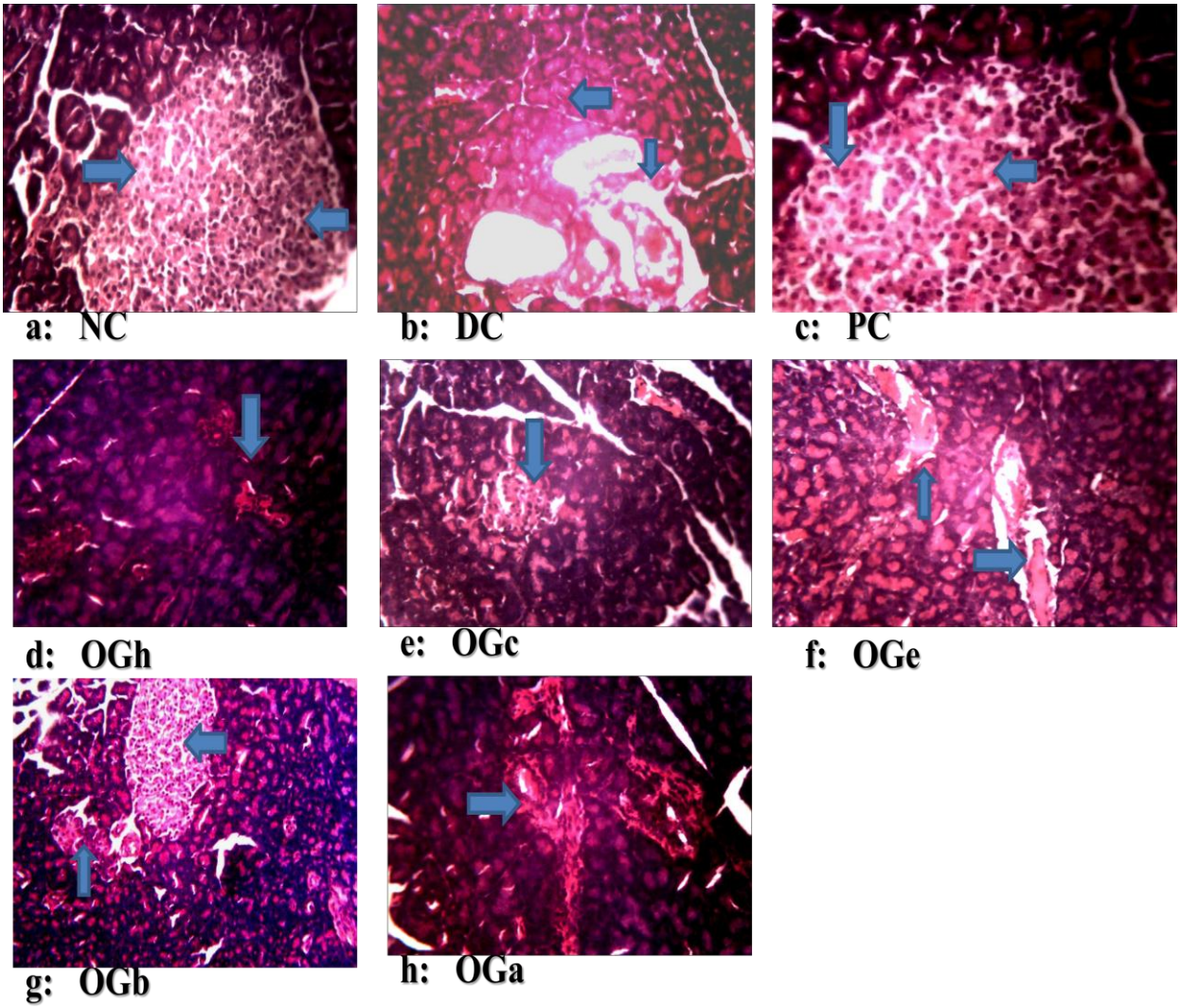


Plate V: Photomicrographs of Pancreatic Tissues of Diabetic/Non-diabetic Rats, Pre-treated with Fractionated Methanol Extract of *Ocimum gratissimum* Leaf (250 mg/kg bw) for 28 days (H&E stain 100x).

(a) NC: Normal control; (b) DC: Diabetic control; (c) PC: Positive control; (d) OGh: *O. gratissimum* hexane fraction; (e) OGc: *O. gratissimum* chloroform fraction; (f) OGe: *O. gratissimum* ethyl-acetate fraction; (g) OGb: *O. gratissimum* butanol fraction; (h) OGa: *O. gratissimum* aqueous fraction.

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4.8: Chloroform Fraction of *Vernonia amygdalina* Methanol Leaf Extract and Butanol Fraction of *Ocimum gratissimum* Methanol Leaf Extract

The percentage recovered yield upon column fractionation of the chloroform fraction of *V. amygdalina* and butanol fraction of *O. gratissimum* methanol leaves extract are presented in the subsequent section.

4.8.1 Percentage yield of pooled column sub-fractions

The yield (%) from the pooled column chromatographic fractionation of the chloroform fraction of the fractionated methanol extract of *V. amygdalina* leaf (VACF) and butanol fraction of the fractionated methanol extract of *O. gratissimum* leaf (OGBF) are presented in Table 4.24. The pooled column sub-fractions with similar Rf values from the TLC resulted in a total of nine (9) column sub-fractions (C1, C2 ... C9) obtained from the VACF, and four (4) column sub-fractions (B1, B2, B3 and B4) obtained from the OGBF (Plate VI). It was observed that a total of 0.790 g was obtained from 20 g of VACF, whereas 0.818 g was obtained from 20 g of OGBF.

4.8.2 Effects of pooled sub-fractions of column fractionated chloroform fraction of *Vernonia amygdalina* methanol leaf extract and butanol fraction of *Ocimum gratissimum* methanol leaf extract on blood glucose in diabetic rats

The effects of the pooled column sub-fractions of VACF and OGBF are presented in the Figures 4.15 and 4.16 respectively. Significant ($p<0.05$) reduction in fasting blood glucose of diabetic rats was noticed after 2 hours of oral administration of the column sub-fractions. A relative stable glycemia was noticed between 3 and 4 h after treatment of diabetic rats with the column sub-fractions. Tables 4.25 and 4.26, shows the changes (%) in fasting blood glucose after oral administration of the pooled column sub-fractions of VACF and OGBF respectively. It was observed that the pooled column sub-fractions C5 and B3 obtained (from VACF and OGBF respectively) were most active in reducing the blood glucose of diabetic rats by 12.55 % and 9.32 % respectively when compared to the untreated diabetic (DC) and metformin treated diabetic group (PC) with -1.99 % and 18.07 % respectively.

Table 4.24 Percentage Yield of Pooled Subfractions of Column Fractionated: Chloroform Fraction of *Vernonia amygdalina* Methanol Leaf Extract and Butanol Fraction of *Ocimum gratissimum* Methanol Leaf Extracts

VACF Pooled fractions	Yield (g)	Yield (%)	OGBF Pooled fractions	Yield (g)	Yield (%)
C1	0.055	0.275	B1	0.28	1.40
C2	0.095	0.475	B2	0.22	1.10
C3	0.101	0.505	B3	0.172	0.86
C4	0.105	0.525	B4	0.146	0.73
C5	0.142	0.710			
C6	0.054	0.270			
C7	0.067	0.335			
C8	0.074	0.370			
C9	0.097	0.485			

VACF: Chloroform fraction of *V. amygdalina* methanol leaf extract; OGBF: Butanol fraction of *O. gratissimu*m methanol leaf extract; B1, B2, B3, and B4: are the pooled sub-fractions obtained from column fractionation of OGBF; C1, C2 ... C9: are the pooled sub-fractions obtained from column fractionation of VACF.

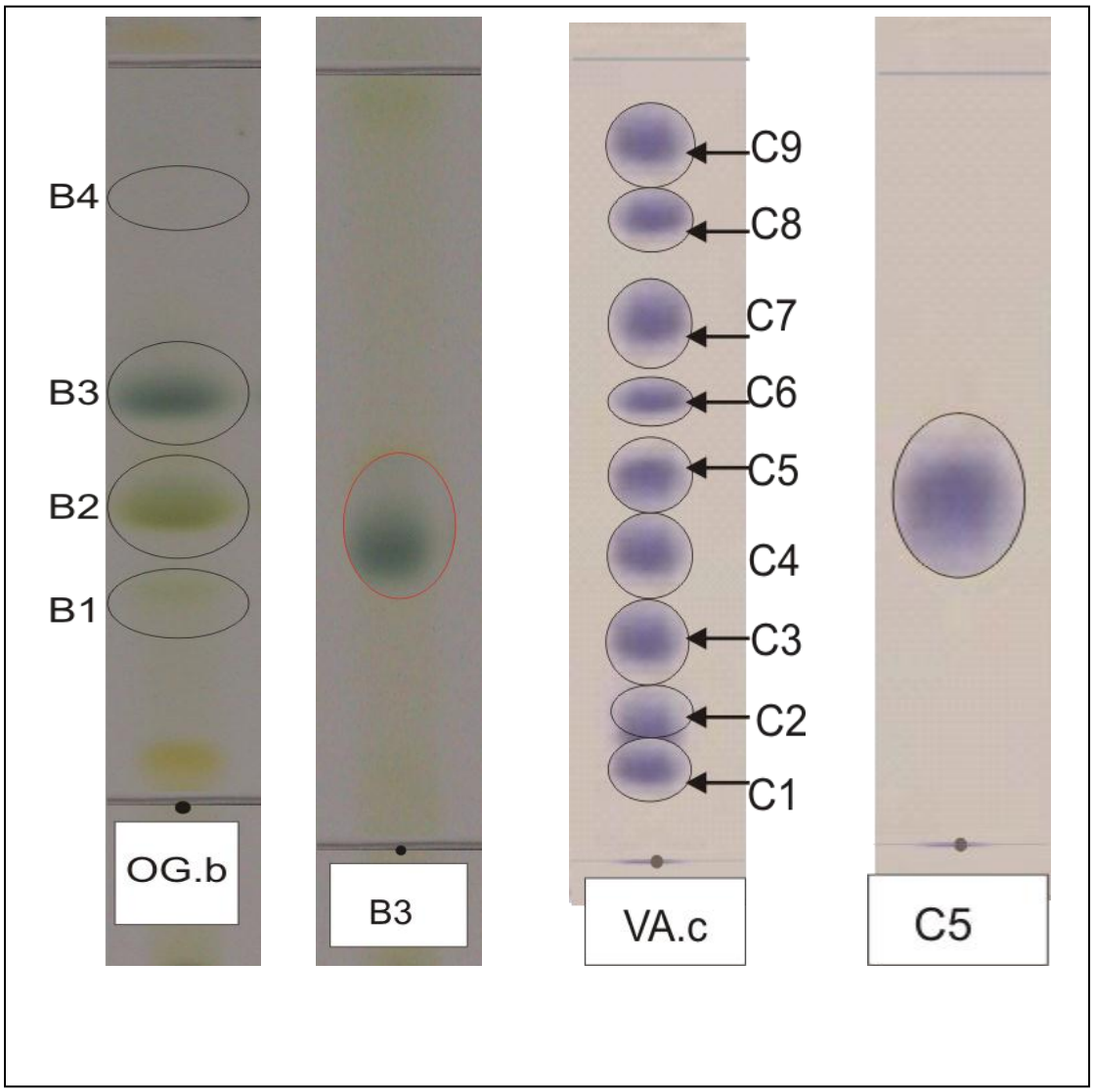


Plate VI TLC Chromatogram of Pooled Subfractions of Column Fractionated: Chloroform Fraction of *Vernonia amygdalina* Methanol Leaf Extract and Butanol Fraction of *Ocimum gratissimum* Methanol Leaf Extracts

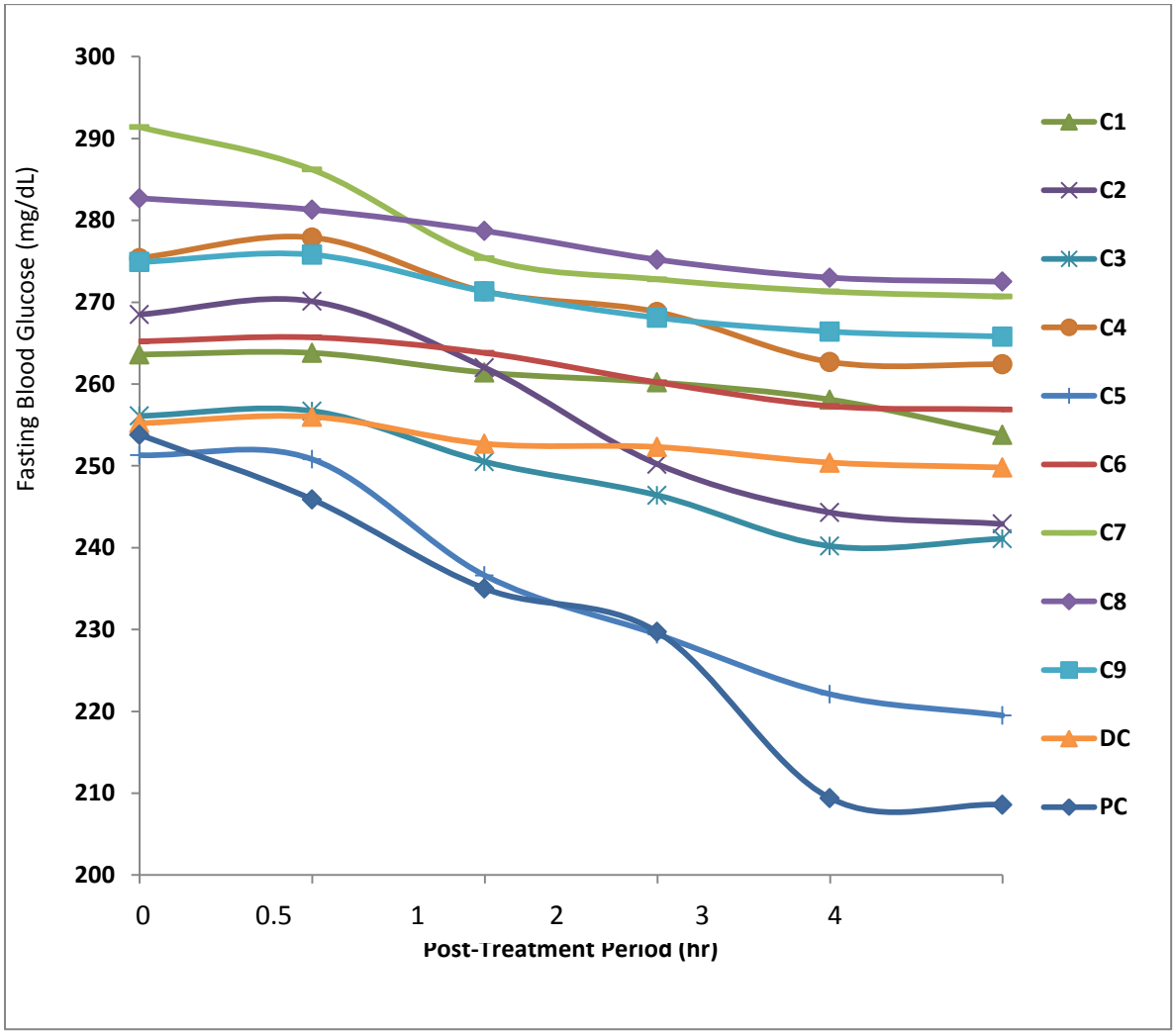


Figure 4.15: Fasting Blood Glucose in Type 2 Diabetic Rats Before and After Treatment with Pooled Sub-Fraction of Column Fractionated Chloroform Fraction of *Vernonia amygdalina* Methanol Leaf Extract (10 mg/kg bw)

Data are presented as mean ± SD of 3 animals per group. PC: Positive control; DC: Diabetic control; C1, C2,… and C9: Pooled sub-fractions of column fractionated chloroform fraction of *V. amygdalina* methanol leaf extract

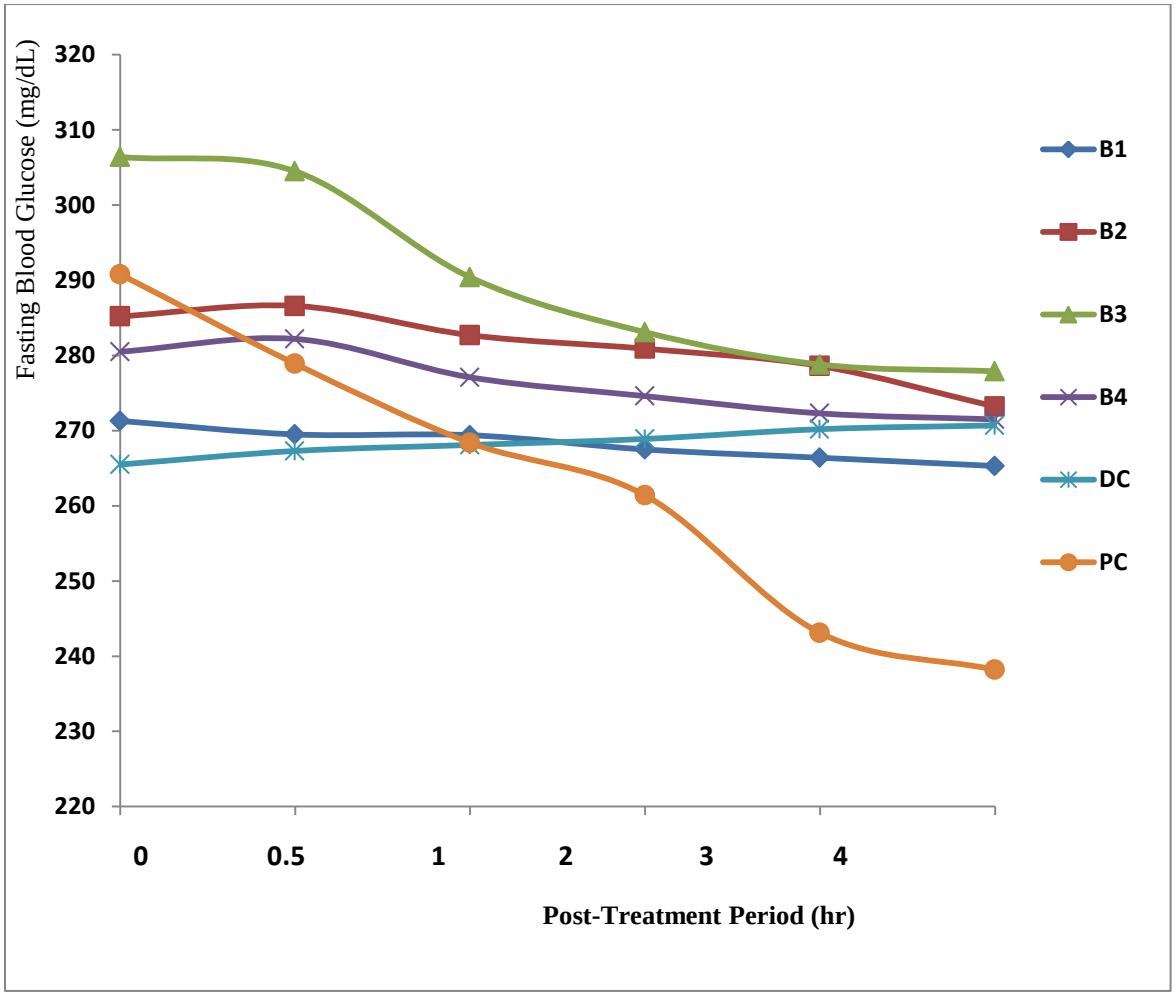


Figure 4.16: Fasting Blood Glucose in Type 2 Diabetic Rats Before and After Treatment with Pooled Sub-Fraction of Column Fractionated Butanol Fraction of *Ocimum gratissimum* Methanol Leaf Extract (10 mg/kg bw)

Data are presented as mean ± SD of 3 animals per group. PC: Positive control; DC: Diabetic control; B1, B2, B3 and B4: Pooled sub-fractions of column fractionated butanol fraction of *O. gratissimum* methanol leaf extract

Table 4.25 Decrease (%) in Fasting Blood Glucose in Type 2 Diabetic Rats After Treatment with Pooled Sub-Fraction of Column Fractionated Chloroform Fraction of *Vernonia amygdalina* Methanol Leaf Extract (10 mg/kg bw)

VACF Pooled fraction	0.5 h	1 h	2 h	3 h	4 h
C1	0.07 ± 0.42^{cd}	0.84 ± 0.48^{a}	1.30 ± 0.37^{a}	2.10 ± 0.64^{a}	3.75 ± 1.13^{ab}
C2	0.59 ± 0.23^{ab}	2.40 ± 1.97^{a}	6.79 ± 1.72^{bcd}	8.96 ± 1.96^{cd}	9.50 ± 1.75^{cd}
C3	0.22 ± 0.27^{bcd}	2.20 ± 1.78^{a}	3.77 ± 0.86^{ab}	6.15 ± 3.48^{bc}	5.79 ± 3.42^{abc}
C4	0.91 ± 0.15^{a}	1.50 ± 0.12^{a}	2.42 ± 0.38^{a}	4.63 ± 0.87^{ab}	4.74 ± 0.58^{ab}
C5	0.20 ± 0.21^{de}	5.76 ± 3.10^{b}	8.63 ± 2.67^{cd}	11.52 ± 2.98^{d}	12.55 ± 3.55^{d}
C6	0.17 ± 0.21^{bcd}	0.55 ± 0.17^{a}	1.87 ± 2.01^{a}	2.94 ± 1.71^{ab}	3.10 ± 2.55^{ab}
C7	1.79 ± 0.25^{f}	5.41 ± 2.79^{b}	6.24 ± 3.95^{bc}	6.77 ± 3.94^{bc}	6.96 ± 4.41^{bc}
C8	0.49 ± 0.30^{e}	1.42 ± 0.28^{a}	2.62 ± 1.04^{a}	3.40 ± 1.21^{ab}	3.58 ± 1.68^{ab}
C9	0.32 ± 0.18^{bc}	1.32 ± 0.55^{a}	2.48 ± 0.74^{a}	3.09 ± 0.41^{ab}	3.33 ± 0.64^{ab}
DC	0.31 ± 0.10^{bc}	1.00 ± 0.70^{a}	1.15 ± 0.73^{a}	1.90 ± 0.25^{a}	2.11 ± 0.35^{a}
PC	3.09 ± 0.32^{g}	7.41 ± 1.04^{b}	9.50 ± 0.08^{d}	17.48 ± 0.92^{e}	17.80 ± 0.96^{e}

Data are presented as mean ± SD of 3 animals per group. Values with different superscript down the column indicate significant difference ($p<0.05$). VACF: Chloroform fraction of *V. amygdalina* methanol Leaf extract; PC: Positive control; DC: Diabetic control; C1, C2, … and C9: Pooled sub-fractions of column fractionated VACF..

Table 4.26 Decrease (%) in Fasting Blood Glucose in Type 2 Diabetic Rats After Treatment with Pooled Sub-Fraction of Column Fractionated Butanol Fraction of *Ocimum gratissimum* Methanol Leaf Extract (10 mg/kg bw)

OGBF Pooled fraction	0.5 h	1 h	2 h	3 h	4 h
B1	0.66±0.36b	0.70±0.29a	1.38±0.43b	1.78±0.51b	2.21±0.76b
B2	-0.48±0.20a	0.81±0.73a	1.50±0.61b	2.32±0.77b	4.18±1.83b
B3	0.63±0.17b	5.25±1.39b	7.61±1.18c	9.01±0.45c	9.32±0.51c
B4	-0.68±0.46a	1.22±1.07a	2.11±1.24b	2.94±1.38b	3.21±1.27b
NC	-0.68±0.35a	-0.98±0.15a	-1.28±0.14a	-1.77±0.36a	-1.99±0.43a
PC	4.10±1.29c	7.72±2.30c	10.11±1.16d	16.40±1.33d	18.07±1.20d

Data are presented as mean ± SD of 3 animals per group. Values with different superscript down the column indicate significant difference ($p<0.05$). OGBF: Butanol fraction of *O. gratissimum* methanol Leaf extract; PC: Positive control; DC: Diabetic control; B1, B2, B3 and B4: Pooled sub-fractions of column fractionated OGBF; Negative sign (-ve) before a number means increase in fasting blood glucose

4.8.3 Nuclear magnetic resonance spectroscopy

The NMR spectra for pooled sub-fraction C5 of column fractionated chloroform fraction of *V. amygdalina* methanol leaf extract are presented in the 1D ^{1}H NMR (Figure 4.17), 1D ^{13}C NMR (Figure 4.18), distortionless enhancement by polarization transfer (DEPT) 1D ^{13}DEPT (Figure 4.19). The DEPT spectra indicated that C5 contained 19 carbon resonances accounting for seven methins (CH), five methilenes (CH_2), two methyls (CH_3) and five quaternary carbons (C). The HSQC visualize AB spin system attributable to H-15 which showed a correlation with carbon at 64.3 ppm, and the correlation of the methane proton in H-14 with the corresponding carbon at 99.4 ppm confirmed the presence of a gem-di-oxigenated carbon group. The chemical shift data for ^{13}C-NMR values showed exact match with literature published data of known compound (Table 4.27). From the HMBC correlation it was confirmed that the structure of the isolated compound was 11β,13-dihydrovernolide. The NMR spectra for purified sub-fraction B3 are presented in the 1D ^{1}H NMR (Figure 4.20), 1D ^{13}C NMR (Figure 4.21).

4.8.4 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) analyses of identified functional groups present in subfraction C5 of column fractionated VACF are depicted in the Figure 4.22 and Table 4.28. The FTIR revealing the functional groups for subfraction B3 of column fractionated OGBF are presented in the Figure 4.23 and Table 4.29.

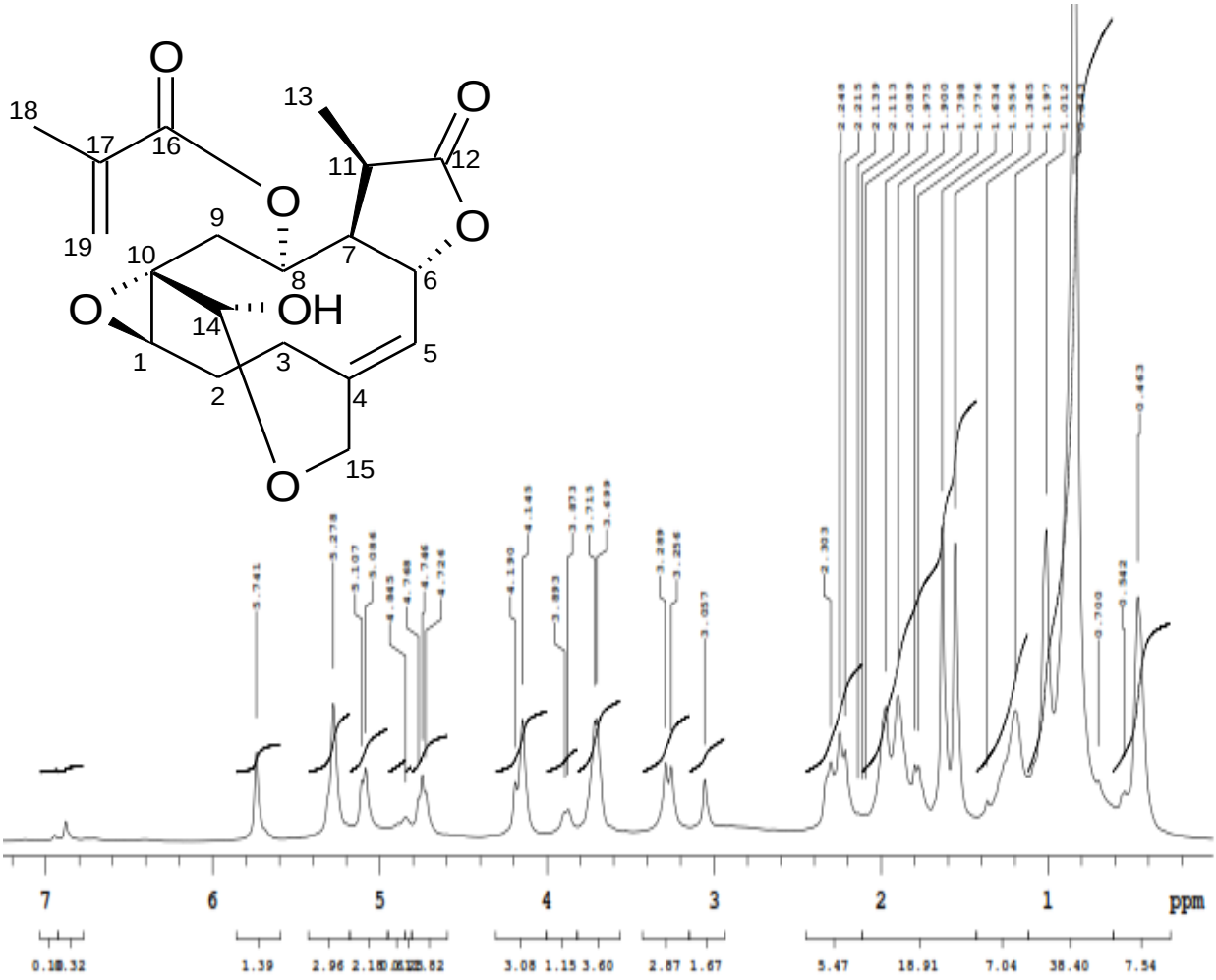


Figure 4.17: [1]H-NMR Spectra for Subfraction C-5 of Column Fractionated Chloroform Fraction of *Vernonia amygdalina* Methanol Leaf Extract

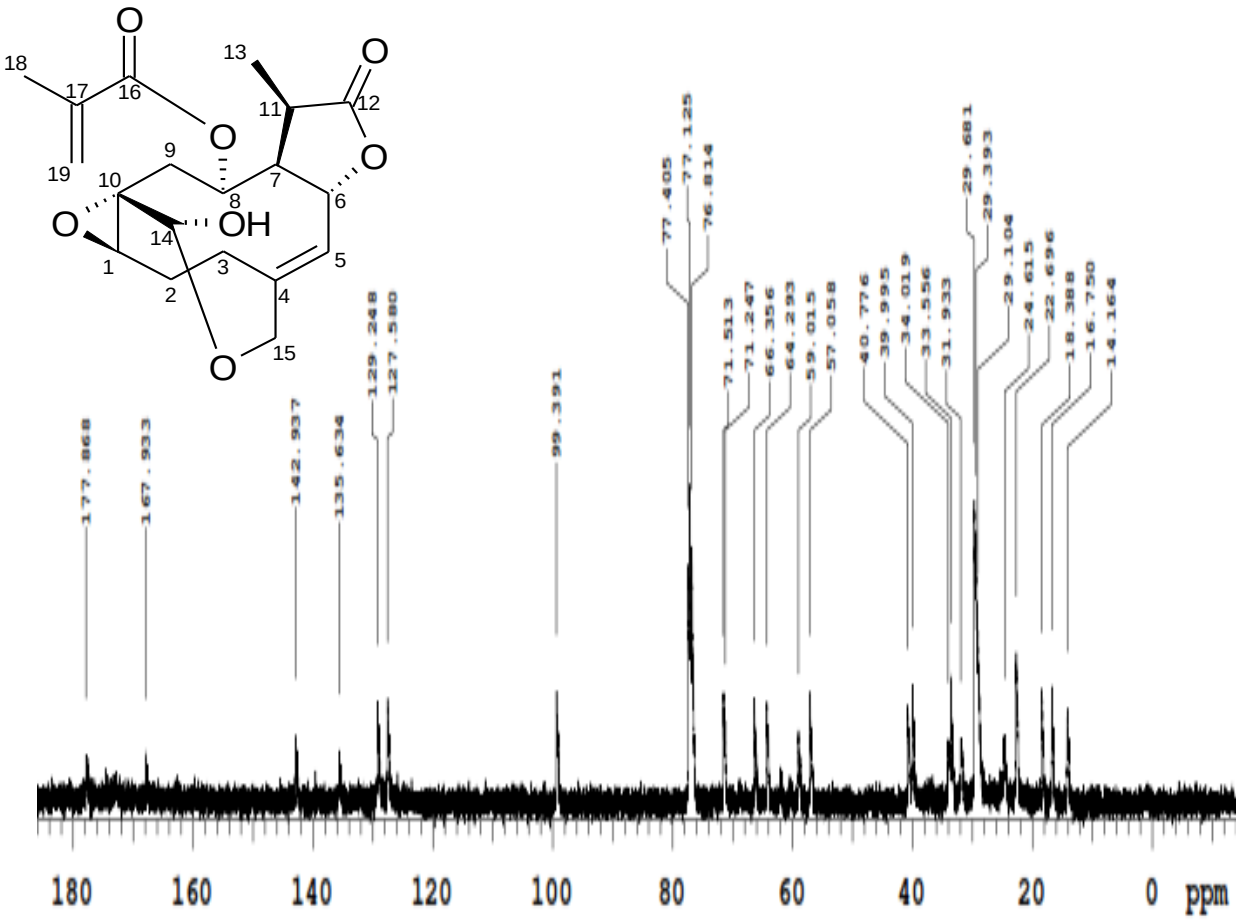


Figure 4.18: ^{13}C-NMR Spectra for Subfraction C-5 of Column Fractionated Chloroform Fraction of *Vernonia amygdalina* Methanol Leaf Extract

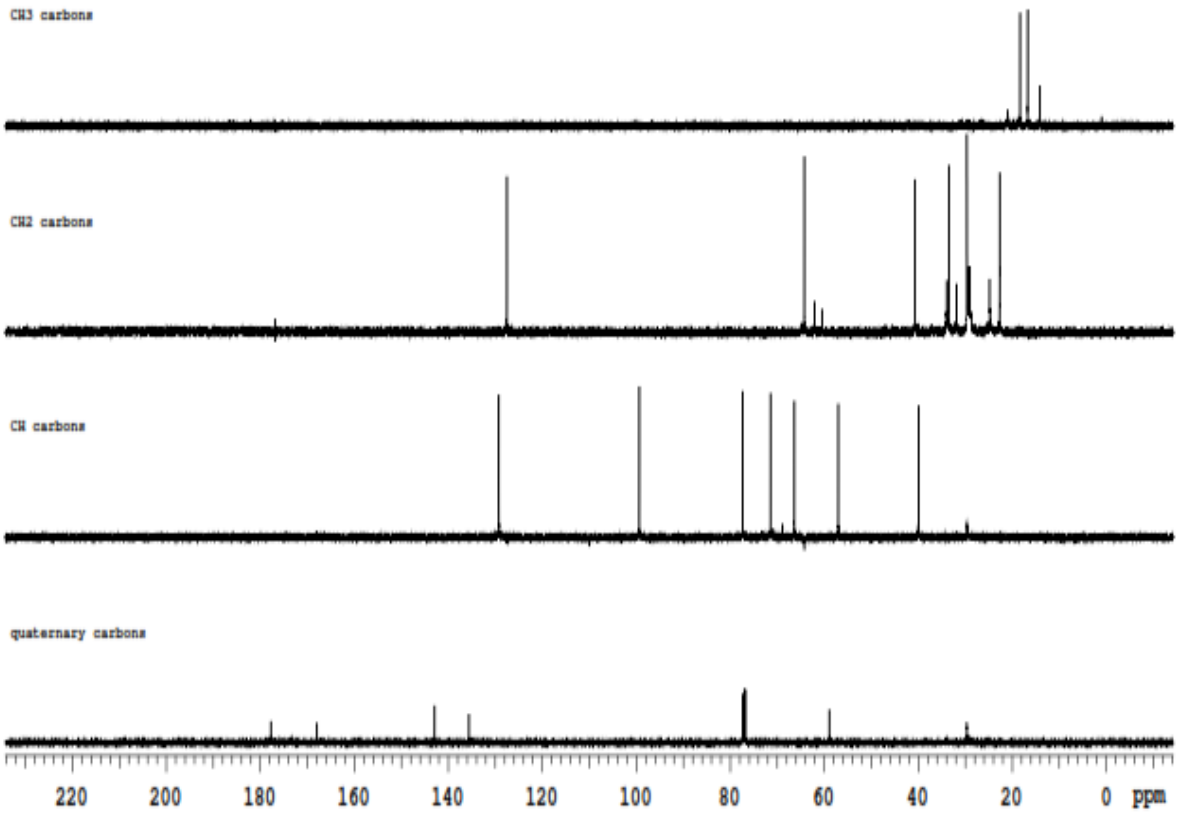


Figure 4.19: DEPT-NMR Spectra for Subfraction C-5 of Column Fractionated Chloroform Fraction of *Vernonia amygdalina* Methanol Leaf Extract

Table 4.27: Data of NMR Spectra for Subfraction C-5 of Column Fractionated Chloroform Fraction of *Vernonia amygdalina* Methanol Leaf Extract

Position	*	Experimental Data		
	^{13}C-NMR, 125 MHz, δ	^{13}C-NMR, 100 MHz, δ	DEPT	HMBC
1	66.32	66.32	CH	C2, C3, C10
2	22.70	22.74	CH_2	
3	33.56	33.56	CH_2	
4	142.94	142.85	C	
5	129.25	129.23	CH	C15
6	78.510	77.31	CH	C4, C7, C8, C11
7	57.06	57.11	CH	
8	71.51	71.50	CH	C10
9	40.78	40.79	CH_2	C14
10	59.02	59.89	C	
11	40.78	39.93	CH	C12
12	177.87	177.54	C	
13	16.75	16.72	CH_3	C11, C12
14	99.39	99.47	CH	C15
15	64.29	64.30	CH_2	
16	167.93	167.96	C	
17	135.63	135.62	C	
18	18.39	18.28	CH_3	C17, C19,
19	127.58	127.44	CH_2	C18, C16

* Rabe *et al*, 2002

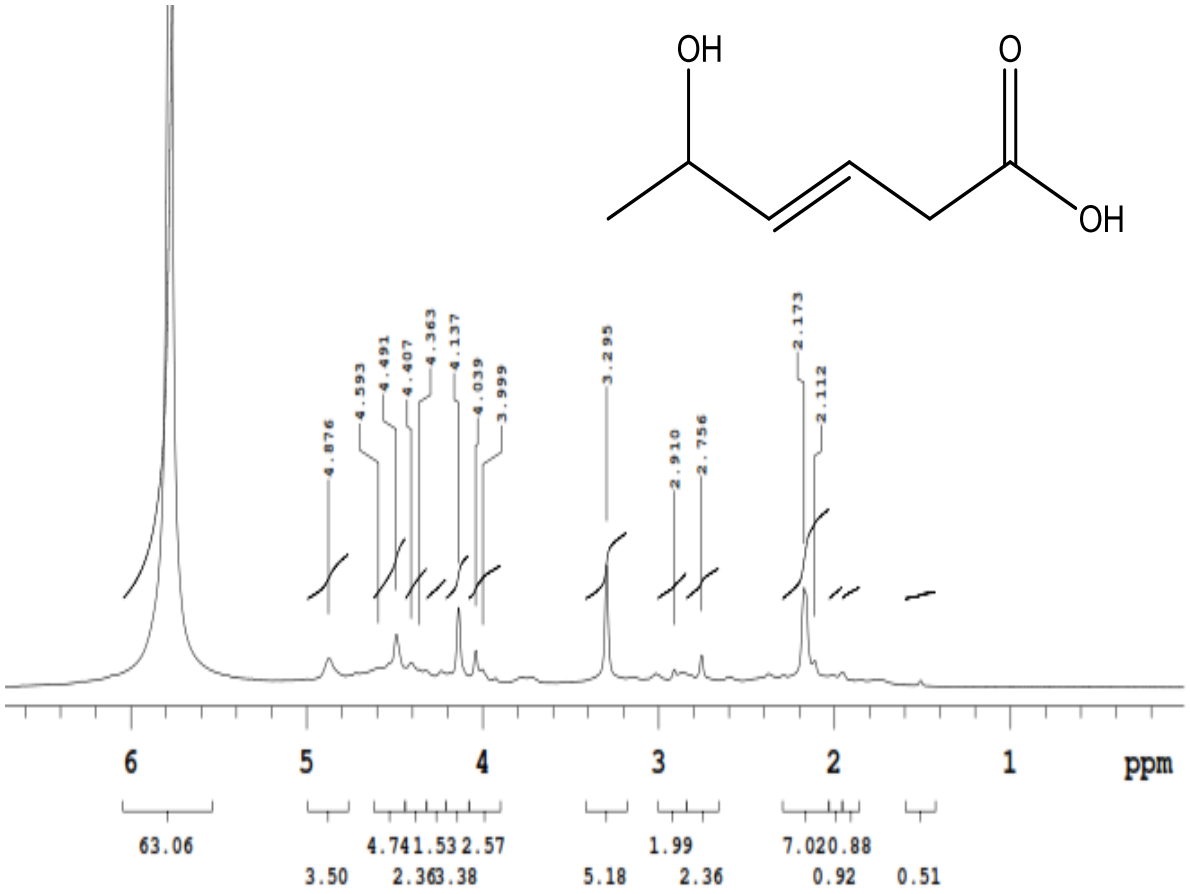


Figure 4.20: [1]H-NMR Spectra for Subfraction B-3 of Column Fractionated Butanol Fraction of *Ocimum gratissimum* Methanol Leaf Extract

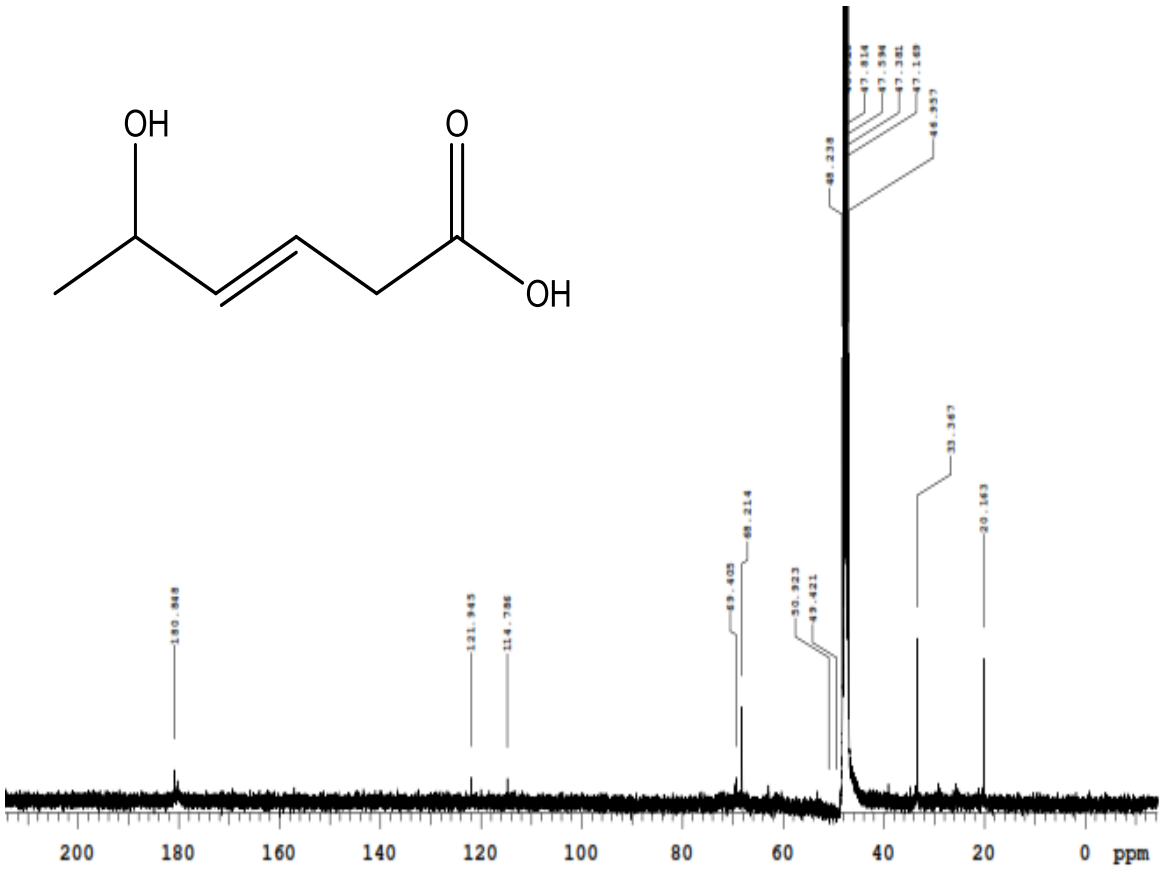


Figure 4.21: ^{13}C-NMR Spectra for Subfraction B-3 of Column Fractionated Butanol Fraction of *Ocimum gratissimum* Methanol Leaf Extract

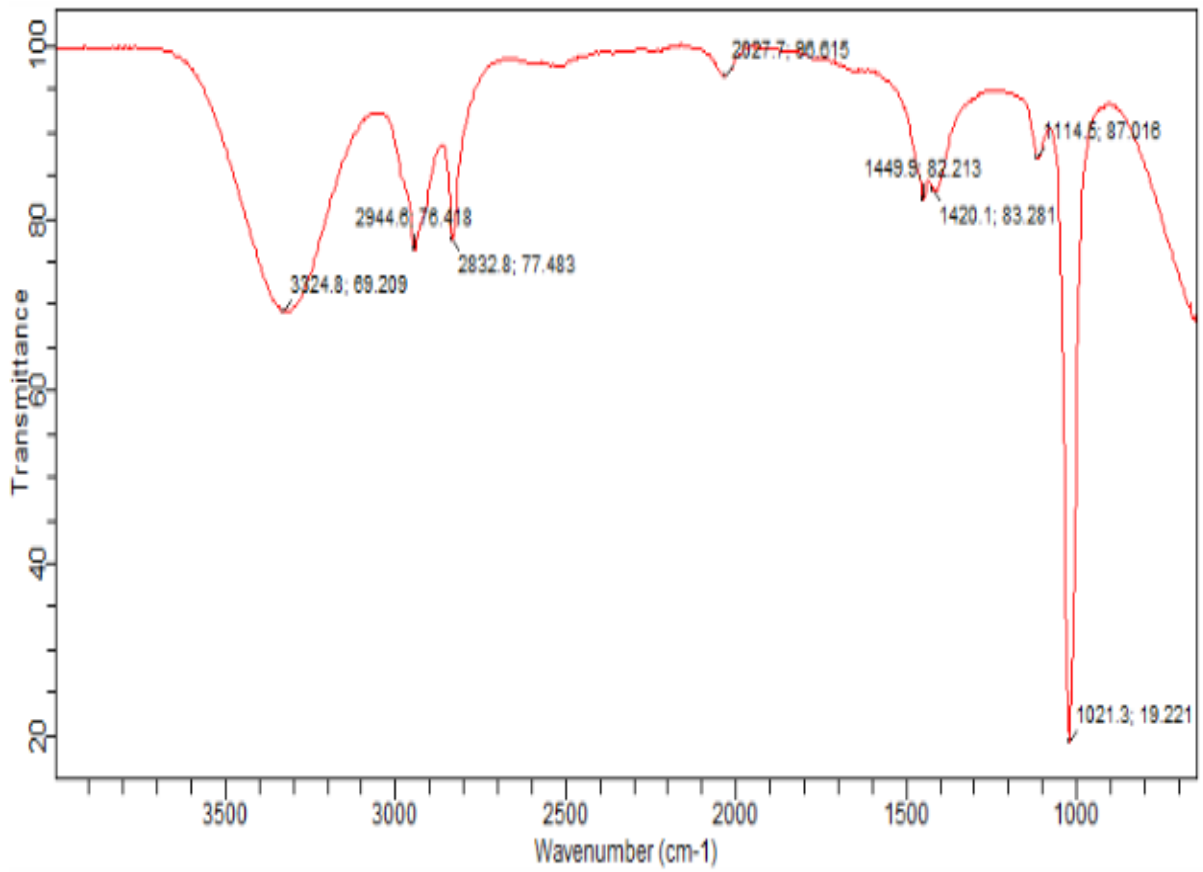


Figure 4.22: FTIR Spectra for Subfraction C-5 of Column Fractionated Chloroform Fraction of *Vernonia amygdalina* Methanol Leaf Extract

Table 4.28: Functional Groups Identified Using FTIR Spectra Analysis for Subfraction C-5 from Column Fractionated Chloroform Fraction of *Vernonia amygdalina* Methanol Leaf Extract. Wavenumbers (cm^{-1})

	Absorbance	**Absorption Ranges**	**Functional Group**
1	3324.80	3100-3500	O-H
2	2944.60	2500-3300	O-H
3	2832.80	2820-2850	=C-H
4	1449.90	1350-1480	-C-H
5	1420.10	1350-1480	-C-H
6	1114.50	1050-1150	C-O

Functional Group region: 4000 cm^{-1} - 1400 cm^{-1}

Finger print region: 1400 cm^{-1} - 400 cm^{-1}

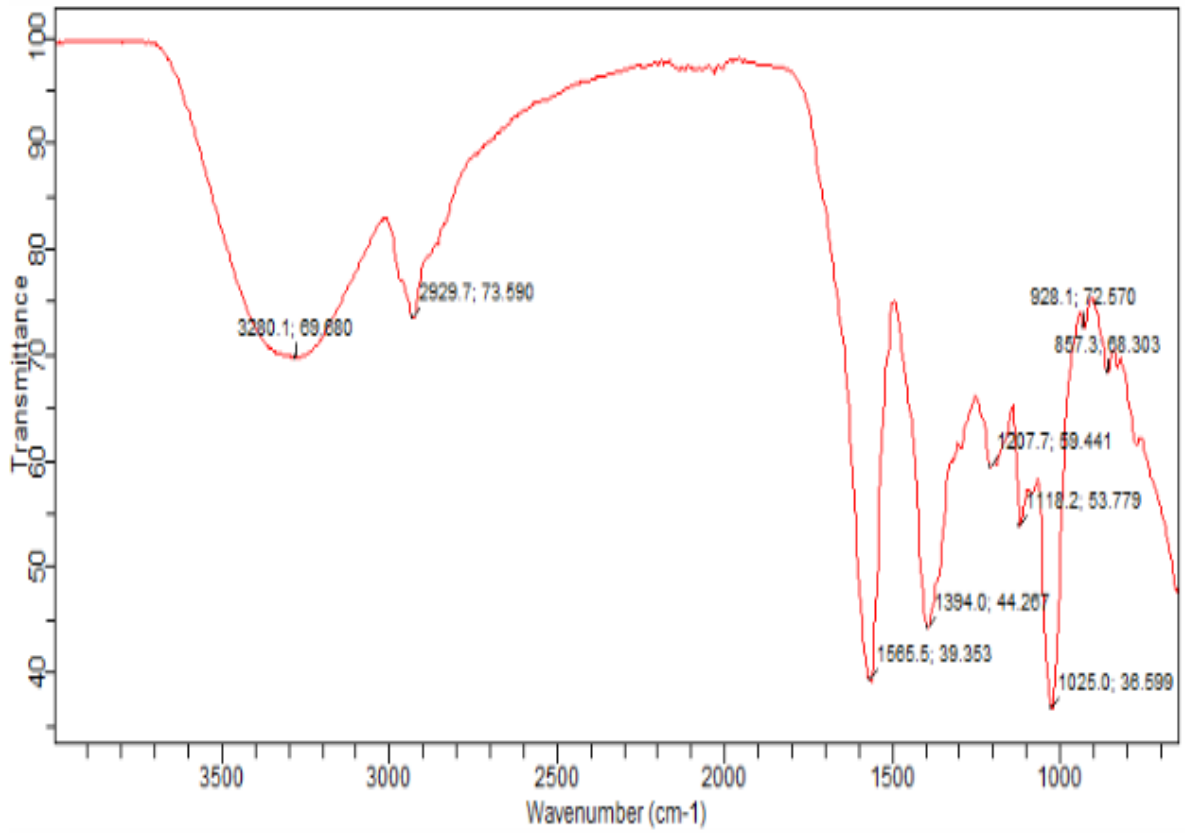


Figure 4.23: FTIR Spectra for Subfraction B-3 of Column Fractionated Butanol Fraction of *Ocimum gratissimum* Methanol Leaf Extract

Table 4.29: Functional Groups Identified Using FTIR Spectra Analysis for Subfraction B-3 from Column Fractionated Butanol Fraction of *Ocimum gratissimum* Methanol Leaf Extract

	Absorbance	**Absorption Ranges**	**Functional Group Name**
1	3280.1	3100-3500	-OH
2	2929.7	2500-3300	O-H
3	1565.5	1550-1610	$-CH_3$
4	1394.0	1380-1432	C=O

Functional Group region: 4000 cm^{-1} - 1400 cm^{-1}

Finger print region: 1400 cm^{-1} - 400 cm^{-1}

CHAPTER FIVE

DISCUSSION

5.1 Overview

Developing countries are rich in medicinal plants but, due to difficulty in accessing efficient extraction technologies; value addition to this rich bioresources are difficult. In most cases, and predominantly in developing countries, the methods used are inappropriate and not economical. The crucial setback is related to the quality of the product. Primitive extraction technologies do not guarantee a stable and high-quality product and, in some cases, unsuitable technologies and procedures result in producing contaminated product which has low market value (Fermeglia, 2008). The present study commenced by evaluating three different methods of extraction of phytoconstituents from medicinal plants for assessment of hypoglycemic effects. The reason is that medicinal plants are constantly being screened for biological activity in the quest for the discovery of new and effective therapeutic agents. Moreover, due to the high prevalence of Type 2 diabetes (T2D) worldwide, extensive research is still being performed to develop new antidiabetic agents (Soliman, 2016). This research work reports the results of investigation into the antidiabetic effect of fractionated methanol extracts of *V. amygdalina* (VALF) and *O. gratissimum* (OGLF) leaves in fortified diet-fed streptozotocin-induced rat (FDF-STZ) model of T2D.

5.2 Effect of Different Extraction Methods on Yield, Phytochemical Constituents and Hypoglycemic Agents

In the present study, it was observed that Soxhlet extraction and cold maceration methods required longer time for complete extraction of the same quantity of sample when compared to MAE (Table 4.1). The volume of the solvent used in MAE was less, confirming that, MAE is cost effective. Similar results were reported by Vongsangnak *et al* (2004) when comparing conventional extraction techniques with MAE during extraction of saponins from cultured cells

of *Panax notoginseng* and analgesic compounds from roots of *Ximenia americana* (Kenmogne *et al.,* 2014). In MAE, the cells of the plant tissues are easily broken up and this facilitate the release of its phytoconstituents. From this study, the results of the qualitative phytochemical determinations revealed that the phytoconstituents present in the studied plants (*V. amygdalina* and *O. gratissimum*) were the same in all the extracts obtained from the different extraction methods (Table 4.2), which implies that the analysed phytochemical components were not destroyed. This finding is in agreement with earlier reports by Mandal *et al* (2007) that, the plant components obtained from MAE are neither decomposed nor oxidized under optimized conditions. The yields in phytochemical constituents have been found to increase when MAE method is used compared to other conventional extraction techniques like Soxhlet and cold maceration methods. Chan *et al.,* (2011) also recorded a higher yield in the extraction of anti-diabetic agents from herbal plant. In their experiment, a yield of 1.63 mg/g was obtained using MAE from 5 g sample and 150 ml solvent in 5 min. On the contrary, with Soxhlet extraction they obtained only 0.47 mg/g in 3 hr from the same sample size and volume of solvent. In a similar study, the extraction of polyphenols and caffeine from green tea leaves gave higher yield at 4 min with MAE compared to Soxhlet and maceration extraction methods at room temperature for 20 hr (Pan *et al.,* 2003). In another study, the extraction of ginsenosides from ginseng root gave higher yield at 15 min using MAE technique compared to other conventional solvent extraction methods for 10 h (Shu *et al.,* 2003; Dhobi *et al.,* 2009).

In Soxhlet the heat transfer and the mass transfer are limiting factors compared to microwave heating, where heat transfer occurs from the centre of the samples to the outer colder environment, and volumetric heating effect leads to a faster rise in temperature. Additionally, the internal heating of *in situ* water within the plant material expands the plant cells and leads to the rupture of the glands (Tatke and Jaiswal, 2011). This might be the reason for the higher yields of phytochemicals from MAE method compared to the Soxhlet and cold maceration

methods in the present study. It is very obvious from these results, that household microwave extraction represents a promising substitute for extracting hypoglycemic phytoconstituents from natural substrate.

In the present study, there was higher yield in phenolics, saponins and tannins with MAE method from both plants: *V. amygdalina* and *O. gratissimum* leaves (Table 4.3). This implies that MAE is a better method for the extraction of these phytochemicals from *V. amygdalina* and *O. gratissimum* leaves. Although, Soxhlet extraction technique gave higher yield in alkaloids and the cold maceration technique was better for the extraction of flavonoids, the MAE is more economical as it required shorter operational time with the least volume of solvent. It has been reported that MAE possess moderately high recoveries with good reproducibility and minimal sample manipulation for extraction process (Proestos and Komaitis, 2008). Several biologically active compounds have been extracted by application of MAE, such as extraction of azadiractine related limonoids from *Azadirachta indica* seed kernels (Dai *et al.*, 1999), extraction of artemisinin from *Artemisia annua* (Hao *et al.,* 2002) and quercetin from herbal plant (Chan *et al.,* 2011). According to Pan *et al* (2008) antioxidant activity of phenolic substances extracted from the peel of *Dimocarpus Longan* using MAE was better than that of Soxhlet extraction. Also, extraction curcumin from *Curcuma longa* using MAE showed a better results and a higher yield with significant reduction in the extraction time, when compared to that of Soxhlet extraction, cold maceration and stirring extraction (Mandal *et al.,* 2008).

The hypoglycemic activity of extract from cold maceration method was better than MAE and Soxhlet within the first 14 days of treatment (Table 4.4). The higher yield of flavonoids by cold maceration extraction may account for its better efficacy within the first 14 days of treatment when compared to the soxhlet in the present study (Table 4.3). Interestingly, after 21 days of treatment, the blood glucose lowering capacity of the MAE extract was higher than extracts from Soxhlet and cold maceration techniques. This could be due to the higher concentrations of

phenols, saponins and tannins recorded in the MAE extract. Saponins, alkaloids, terpenoids and flavonoids are known to play a significant role in anti-diabetic action (Sharma *et al.,* 2010b; Chukwujekwu *et al.,* 2016; Lavle *et al.,* 2016; Testa *et al.,* 2016; El-Barky *et al.,* 2017). Differences in rate of reactions, metabolism and possibly half life of flavonoids, saponins, tannins and phenolics could account for the observed differences in the blood glucose reducing capacity of diabetic rats by these phytochemicals within the first 14 days noticed in the cold maceration and thereafter as observed in the MAE extract.

5.3 Acute Toxicity Study

The single oral administration of *V. amygdalina* and *O. gratissimum* leaves extracts at 5000 mg/kg b.w did not show any apparent toxic symptom or mortality even 7 days post-oral administration in this study. This indicated that the median lethal dose (LD_{50}) of *V. amygdalina* and *O. gratissimum* leaves extract were higher than 5000 mg/kg b.w. On the contrary Prabhu *et al* (2009) reported an LD_{50} for *O. gratissimum* to be 1264.9 mg/kg b.w in rats. However, results obtained for VA in this study were consistent with that of Adiukwu *et al.,* (2012) and Nwanjo (2005) who reported no clinical symptoms of toxicity effect at doses of 500-2000 mg/kg for 14 successive days. Interestingly, several other investigators have also reported the non–toxic nature of the *V. amygdalina* and *O. gratissimum* leaves extracts either to the kidney or to the liver (Atangwho *et al.,* 2007; Abdulazeez *et al.,* 2013; Zakaria *et al.,* 2016). The need to formally examine the safety level of plant extract when used in scientific investigation cannot be overemphasized (Fennel *et al*., 2004; Asase and Yohonu, 2016). This is especially imperative if the preparation procedure differs from the traditional method, such as using organic versus aqueous solvent (Toyang and Verpoorte, 2013). Secondly, the age of plant, geographical location, season and time of harvest affect the phytochemical compositions of the plant (Asante *et al*., 2016) and ultimately affect its toxicity. For instance, Asante and co-workers (2016)

reported that flavonoids were present in the old and not the young leaves of *V. amygdalina* ethanol extract.

5.4 Rat Model of Diabetes Mellitus

A suitable experimental model that closely mimics the pathogenesis and clinical features of T2D must adequately reflect the two prominent characteristics of human T2D which are insulin resistance and partial pancreatic beta cell dysfunction (Zhang *et al.,* 2008; Schaalan *et al.,* 2009; Sasidharan *et al.,* 2013). The experimental animals used in this study were albino Wistar rats which are predominantly available and easy to breed in this part of the country unlike the Sprague-Dawley rats used by previous investigators (Reed *et al.,* 2000; Srinivasan *et al.,* 2005; Wilson and Islam, 2012; Hu *et al.,* 2013; Khan *et al.,* 2013). Different strain and background species of rats affect the susceptibilities of replicating a specific model in different geographical terrain (Gajda *et al.,* 2007; Franconi *et al.,* 2008; Yoon *et al.,* 2008; King, 2012) hence the need for the model presented in this study. More also, since Sprague-Dawley rats are more sensitive towards T2D than Wistar rats (de-Moura *et al.,* 2008), they are less representative of the human population, where sensitivity towards T2D varies greatly from one individual to another. The albino Wistar rats are by their varied sensitivity towards T2D, closer to the human population (de-Moura *et al.,* 2008; Dupas *et al.,* 2016).

The increase in body weight observed in the FDF-fed group could be the result of consumption of diet rich in high fat (margarine) and fructose. The onset of T2D is strongly associated with weight gain, insulin resistance and pancreatic β-cell dysfunction. Lack of β-cells compensation results in impaired glucose intolerance (King, 2012). Dietary fructose stimulates less insulin secretion but enhances food intake (Bantle, 2006) Fructose metabolism cannot be controlled by insulin or leptin, which are key factors for the regulation of fat synthesis and energy intake hence, obesity will likely be the consequence (Basciano *et al.,* 2005; Mamikutty *et al.,* 2014). Obesity induced by environmental manipulation rather than gene, is thought to model the human

situation more than genetic model. The FDF-fed rats after 6 weeks significantly increased in body weight compared to the counterpart group of rats fed on NDF (Table 4.8). The consumption of high-fat diet such as the FDF leads to obesity because it facilitates the development of a positive energy balance. This observation corroborates the report presented by some previous investigators (Buettner *et al.,* 2007; Franconi *et al.,* 2008; King, 2012; Sasidharan *et al.,* 2013).

It was reported recently that 10 % fructose administration *ad libitum* results in insulin resistance after two weeks in Sprague-Dawley rats (Wilson and Islam, 2012). During the preliminary investigation in this present study, it was observed that the administration of up to 20 % fructose alone to albino Wistar rats for 2 weeks could not induce insulin resistance. This observation is in agreement with the findings of Zarfeshani *et al* (2012) in which Sprague-Dawley rat fed 21 % fructose solution for a period of 10 weeks could not develop signs of Type 2 diabetes (Zarfeshani *et al.,* 2012). In another study, Stark *et al* (2000) reported that feeding rats with high fructose (53 % *w/v*) and sucrose (10 % *w/v*) diet was not able to induce hyperglycemia, hyperinsulinemia, hypertriglyceridemia and hypercholesterolemia hence unable to develop Type 2 diabetes model among rats. These observations were contrary to that of Wilson and Islam (2012) who reported that 10 % fructose administration *ad libitum after* two weeks resulted in insulin resistance. However, the differences observed by different investigators could be due to dissimilarities in characteristics of strain/species of experimental animals and the nature of fat diet used in various studies (Franconi *et al.,* 2007; Gajda *et al.,* 2007; Yoon *et al.,* 2008; Zhang *et al.,* 2008; Sasidharan *et al.,* 2013). Different breeds are not always physiologically the same. They can show different reactions based on their sensitivity to various environmental conditions. Since Sprague-Dawley and albino Wistar rats are from diverse breeds, their colonies can present genetic variability throughout the world. The diversity of study designs could also be a contributing possible explanation (Dupas *et al.,* 2016). It is therefore acceptable that studies

conducted in different parts of the world may not demonstrate similar findings in spite of similar conditions.

The observed hyperinsulinemia in the FDF-fed rat group could be due to the high-fat present in margarine which was used in the diet fortification (Table 4.12). High-fat diet (HFD) is known to precipitate insulin resistance (Willett, 2002). Insulin resistance causes hyperinsulinemia. In insulin resistant state, there is a higher amount of circulating serum insulin when compared to normal control state. In a recent investigation, it was revealed that insulin resistance helps conserve the brain's glucose supply by preventing muscles from taking up excessive glucose (Wang, 2014). The observation of high concentration of serum insulin in the FDF-fed control group in this study was in agreement with that of the HFD-fed groups reported by Zhang *et al* (2008) and Srinivasan *et al* (2005). The induction of low dose of STZ to insulin resistant FDF-fed rats resulted in frank hyperglycaemia. Interestingly, the same low dose of STZ failed to produce obvious hyperglycaemia in the NDF-fed group (Table 4.9).

In this study, the level of β-cells destruction was observed to be directly proportional to the quantity of STZ induced in the rats as noticed from the HOMA-β (Table 4.11). When high percentages of endogenous β-cells are destroyed there will be little endogenous insulin production (Table 4.12) hence hyperglycemia and weight loss (King, 2012). The reduction in endogenous insulin production precipitates the onset of hyperglycemia (Klinke, 2008). These attributes were observed in the present study. The onset of hyperglycemia is believed to occur when 80-95 % of the β-cells mass are lost (Gale, 2002). In T1D the beta-cells are almost completely destroyed when patients are diagnosed. This was in agreement with our finding in the group administered high dose of STZ in both the NDF-fed and FDF-fed groups (Table 4.11). The level of impairment in β-cell function also contributes to the emergence of hyperglycemia. The magnitude of β-cell destruction varies with age and depends on the insulin producing capacity, which is also proportional to β-cell mass and insulin demand which is proportional to

body weight (Klinke, 2008). Subsequently, additional 3-5 % of β-cell mass may be lost yearly. Insulin secretory defect in T2D does not primarily result from insufficient β-cell mass rather from an impairment of insulin action (Nepton, 2013; Herold *et al.,* 2015). In this study, the FDF_{35} group had 55.46 % β-cell function when compared to the normal control group, unlike FDF_{45}, NDF_{55} and FDF_{55} groups with loss of >97% of their β-cell function. In addition, the histological examination of the pancreas of the FDF_{35} rats used subsequently in this study showed that their pancreatic β-cells were not completely destroyed. The UK prospective diabetes study has shown that by the time persons are diagnosed with T2D, their β-cell function is about 60% of normal. Whether the 40% reduction is the result of cells secreting less insulin or decrease in β-cell mass is unclear. However, it has been reported that the anatomical presence of β-cells do not necessarily correlate to β-cell function (Kargar and Ktorza, 2008), but the measurement of serum C-peptide and/or endogenous insulin can be used to assess β-cell function (King, 2012). In this study, serum insulin and C-peptide were measured in order to assess β-cell function. The assessment of β-cell function is highly indispensable in understanding the pathways that lead to the inability of the β-cell to secrete adequate quantity of insulin. High insulin levels can indirectly indicate insulin resistance (King, 2012).

The available serum insulin (85.8 %) and C-peptide (87.8 %) among the FDF_{35} rat group used in this study was similar to the rat model of Type 2 diabetes reported by Zhang *et al* (2008) with 83.1 %, Masiello *et al* (1998) with 88.2 % and Srinivasan *et al* (2005) with 83.0 % of circulating serum insulin respectively. This was contrary to the model of T2D presented by Suman *et al* (2016) which had 45.5 % serum insulin, Wilson and Islam (2012) which had 53.7 % serum insulin. Interestingly, Dhansh *et al* (2012) reported a model of T1D developed using 45 mg/kg b.w. STZ and they observed 28.2 %, 21.7 % and 19.8 % available serum insulin, after 15, 30 and 45 days post STZ induction respectively. These values are similar to those of human T1D (King, 2012; Skovs~~o~~, 2014). The residual circulatory insulin levels in the rat model of diabetes

reported by Suman *et al.,* (2016) (45.51 % insulin), Wilson and Islam (2012) (53.7 % insulin) and Dhansh *et al* (2012) (28.2 %, insulin) proved that the model are for Type 1 diabetes and not Type 2 diabetes. In this study we observed that in both the NDF and FDF-fed groups that were induced with 45 and 55 mg/kg STZ, obvious hyperglycemia was developed with < 46.8 % serum-insulin, a severe deficiency typical of Type 1 diabetes (King 2012; Skovsø, 2014). This value was synonymous to that reported by Suman *et al* (2016), Wilson and Islam (2012).

The blood glucose levels in the FDF rat group induced with 35 mg/kg b.w of STZ were observed to be relatively stable throughout the 6-weeks post diabetes confirmation of the study (8 weeks post STZ induction) (Figure 4.3). This implies that the model could be valuable for chronic diabetes investigations like hypertension, neuropathy and nephropathy. The suitability of the model for pharmacological screening was validated using glibenclamide, metformin and pioglitazone (Figure 4.4). The ability of these drugs to reduce the total cholesterol, triglyceride and the blood glucose level justify the model for pharmacological screening. To the best of our knowledge from available published literature, this is the first study in which three different drugs was used to validate a model. Hence, the model was used for further investigation to screen for the fractionated methanol extract of *V. amygdalina* and *O. gratissimum* leaves.

5.5 Hypoglycaemic Effect of Fractionated Methanol Extract of *Vernonia amygdalina* and *Ocimum gratissimum* Leaves in Type 2 Diabetic Rats

This study shows that the various fractions of fractionated methanol extracts of *V. amygdalina* and *O. gratissimum* leaves reduced blood glucose levels in T2D rats model after treatment for 28 days (Figures 4.11-12 and Tables 4.15-16). The highest reduction in blood glucose was observed in the positive control group (treated with metformin), followed by the treated rats that received 250 mg/kg chloroform fraction of the VA leaf and butanol fraction of OG. On the contrary, the diabetic control group manifested continuous increase in glucose levels from day 0 to the 28^{th} day. This study confirms earlier report by other investigators on the hypoglycemic

effect of the leaf extract of *V. amygdalina* and *O. gratissimum* in rat (Chike *et al.*, 2006; Osinubi, *et al.*, 2007; Fasola *et al.*, 2010; Akpaso *et al.*, 2011; Joyce *et al.*, 2012; Okon *et al.*, 2012; Onaolapo *et al.*, 2012; Modu *et al.*, 2013). These observations are in agreement with previous research reports (Goje *et al*., 2014; Asante *et al*., 2016; Ojieh *et al*., 2016), where antidiabetic medicinal plants significantly reduced the high blood glucose level in STZ induced diabetic rats.

The diabetic control rats in this study demonstrated a drastic increase in AUC of the glucose tolerance curve after oral glucose loading (Figures 4.13-14 and Table 4.17). This observation corroborates previous investigation by Elberry *et al.*, (2015). The possible explanation could be due to the reduction of glucose tissue utilization and increased hepatic glucose production as a result of decreased insulin production. Treatment with fractions of fractionated methanol extracts of *V. amygdalina* and *O. gratissimum* leaves for 28 days resulted in reduction in the AUC of diabetic rats. The implication is that fractions of fractionated methanol extracts of *V. amygdalina* and *O. gratissimum* leaves stimulate increased glucose utilization and glucose tolerance in the body tissues of the diabetic rats. In all the leaf fractions administered, the chloroform fraction of fractionated methanol extract of *V. amygdalina* leaf and butanol fraction of fractionated methanol extract of *O. gratissimum* leaf were most effective in ameliorating majority of the diabetes related complications.

The result of the daily administration of *n*-hexane, ethyl acetate, chloroform, butanol and aqueous fractions of fractionated methanol extract of *V. amygdalina* and *O. gratissimum* leaves resulted in decrease of blood glucose levels in diabetic rats. Though all the five fractions proved to be effective, the chloroform fraction of fractionated methanol extract of *V. amygdalina* leaf (VACF) and butanol fraction of fractionated methanol extract of *O. gratissimum* leaf (OGBF) were most effective as they had satisfactory capacity to restore back the blood glucose to near normal level. The VALF and OGLF stimulated insulin secretions and improved pancreatic β-

cell function and insulin sensitivity (Tables 4.22-23). Based on these observations, it can be suggested that the mechanism of anti-hyperglycemic action of VALF and OGLF were through stimulating insulin secretion, sensitivity and improving β-cell function. This hypothesis was further supported by the histopathological examinations of the pancreatic islets where the groups treated with VACF and OGBF had healthier pancreatic islets when compared with the NC and groups treated with different fractions (Plates IV and V).

5.6 Effect of Fractionated Methanol Extract of *Vernonia amygdalina* and *Ocimum gratissimum* Leaves on Biochemical Parameters in Type 2 Diabetic Rats

The abnormally high concentration of serum triacylglycerol (TG), total cholesterol (TC), low density liproprotein cholesterol (LDLc) and low concentration of serum high density liproprotein cholesterol (HDLc) observed in diabetic rats compared to control rats is in agreement with reports from previous studies (Ayinla *et al*., 2011; Abdulazeez *et al*. 2013; Elberry *et al*., 2015; Soliman, 2016) indicating that an increase in glucose level on induction of diabetes, results in high blood lipids. The liver plays a significant role in glucose and lipid homeostasis and is severely affected in diabetes (Ozkurk *et al*., 2015). Hyperlipidemia and/or dyslipidemia are known complication associated with diabetes. They are characterized by raised levels of cholesterol, phospholipids, triglycerides and other lipoproteins (Ayinla *et al*., 2011). High serum lipids in diabetes have been reported on one hand to be the effect of increased mobilization of free fatty acids from peripheral fat depots due to inhibition of the hormone sensitive lipase by insulin (Sharma *et al*., 2010a) and conversely, could be due to catecholamines, glucagon and other hormones which enhance lipolysis. The excess fatty acids produced are converted into cholesterol and phospholipids, which together with excess triacylglycerols produced at the same time in the liver, are released into the blood stream in form of lipoproteins. Hence, the significant hyperlipidemia observed in the diabetic rats may be regarded as a consequence of uninhibited actions of lipolytic hormones in fat depots (Tavasoli *et*

al., 2005). Elevated levels of TC; prominently LDLc is one of the most important coronary risk factor which is the major cause of morbidity and deaths in diabetic subjects. Treatment of diabetic rats with fractions of fractionated methanol extract of *V. amygdalina* and *O. gratissimum* leaves (VALF and OGLF) caused alleviations in all the lipid profile parameters, indicating their hypolipidemic potency. This effect may be ascribed to the presence of flavonoids, saponins, phenolics and alkaloids present in the fractions which inhibited cholesterol and/or bile acid absorption (Sharma *et al.,* 2010b; Lin *et al.*, 2016; Testa *et al.,* 2016; El-Barky *et al.,* 2017). The hypertriglyceridaemia observed among the T2D rats may be because of increased absorption and production of triglycerides in the form of chylomicrons subsequent to exogenous consumption of fructose through increased endogenous production of TG-enriched hepatic VLDL and decreased TG uptake in peripheral tissues. Hypercholesterolaemia may be attributed to the increased dietary cholesterol absorption from the small intestine following the intake of high fat (present in the FDF) in a diabetic condition. However, the levels of serum TG and TC were effectively reduced in the VACF and OGBF treated T2D rats.

One of the most common severe complications of diabetes is renal disease. Serum creatinine, uric acid and urea are used as biomarker for prediction of renal dysfunction, because they increase significantly in diabetic conditions (Almdal and Vilstrup, 1987; Tierney *et al.,* 2002). In this study, high levels of these biomarkers were observed in the untreated diabetic group as compared to the normal control group (Table 4.18). This finding is in agreement with those of previous investigators (Almdal and Vilstrup 1987; Jenson *et al.*, 1998; Akah *et al*., 2009). The significant high levels of serum urea and creatinine is an indication of diminished ability of the kidney to filter these waste products from the blood and excrete them in urine. However, treatment with VALF and OGLF for 28 days was able to reduce the serum concentration of urea and creatinine in diabetic rats to some extent. Among the various fractions administered, it was observed that the chloroform fraction of fractionated methanol extract of *V. amygdalina* leaf

(VACF) and butanol fraction of fractionated methanol fraction of *O. gratissimum* leaf (OGBF) were able to reduce the serum levels of urea and creatinine in a similar manner with the standard drug metformin. Data from this study suggest that the VACF and OGBF may have enhanced the ability of the kidneys to get rid of these waste products from the blood. It could also be explained that the VACF and OGBF have the ability to confer protective effect on the kidney or might be able to directly improve the structural and functional integrities of cells of the blood, kidney and liver.

Hepatocyte injury causes impairment in liver cell membrane permeability. Consequently, cytosolic enzymes such as transaminase enzymes (AST and ALT) escape into the circulatory system and their activities in serum increases. Alteration of membrane bound Alkaline phosphatase (ALP) affect membrane permeability and produce derangement in the transport of metabolites (Soliman 2016). In this study, the serum levels of liver biomarkers (AST, ALT, ALP) were considerably increased in the untreated diabetic group when compared to the normal control group indicating impaired liver function, which is evidently due to hepatocellular necrosis. However, treatment of diabetic rat model with VALF and OGLF caused a reduction in the activity of these enzymes (AST, ALT, ALP) (Table 4.18). This observation is in agreement with earlier report that elevated activities of serum transaminases are common signs of liver diseases observed more often in diabetic condition (Akah *et al*., 2009; Soliman, 2016). The increase in serum levels of these enzymes indicated that STZ-induced diabetes produced alteration in hepatic function, which consequently results in leakage of these hepatic enzymes from the liver cytosol into the blood stream. Ghosh and Suryawansi (2001) explained that, diabetic complications such as elevated gluconeogenesis and ketogenesis may be the result of increased transaminase activities. Thus, restoration of these biomarker enzymes towards normal level signifies decreased diabetic complications as observed in the VALF and OGLF treated

groups. Among the various fractions tested, the VACF and OGBF were the most effective in reducing the activities of the liver enzymes.

5.7 Effect of Fractionated Methanol Extract of *Vernonia amygdalina* and *Ocimum gratissimum* Leaves on Pancreatic Tissues in Type 2 Diabetic Rats

Histopathological examination in this study reveals normal pancreatic islets β-cells in the normal control group, with the diabetic negative control group reflecting evidence of deformation and not complete destruction associated with Type-1 diabetes. This includes cytoplasmic changes in the cells of the islets, particularly within the core portion of the islets where there was architectural deformation of β-cells. The normal and positive control groups had large islet cells with high number of β-cells while the untreated diabetic group had morphologically deformed islet cells with disperse β-cells (Plates IV and V). The VALF and OGLF treated groups had higher (but morphologically smaller) number of β-cells compared to untreated diabetic group, but fewer numbers compared to normal control group. The VALF and OGLF treated groups showed regeneration of the β-cells of the pancreas, and this observation was evidently supported by the significant reduction in hyperglycaemia. These changes were observed consistently in all the animals in the groups. However VACF and OGBF manifested the greatest pancreas regenerative tendency compared to the other groups. This study affirms the work done by previous investigators (Atangwho *et al*., 2007; Ekam *et al*., 2013; Goje *et al*., 2014; Elberry *et al*., 2015; Zakaria *et al*., 2016).

5.8 Identified Bioactive Components in *Vernonia amygdalina* and *Ocimum gratissimum* Leaves Responsible for the Anti-diabetic Effects in Type 2 Diabetic Rats

The isolated compound 11β,13-dihydrovernolide (Figure 4.17) from *Vernonia amygdalina* leaf was shown in this investigation to have hypoglycemic effect (Table 2.25). The compound has been isolated previously from related specie called *Vernonia colorata*, were it was reported to exhibit little antibacterial action in respect to *vernolide* and *vernodalin* (Rabe *et al*., 2002). The FTIR spectra broad band at 3324/cm was conspicuous for the hydroxyl group and 2832.8/cm for

=C-H group. The saturation of the double bond at 11-13 positions may have reduced the activity as an antibacterial observed by Rabe *et al* (2002). Although, the activity of a molecule may be related to its 3D-conformation, in the structure of 11β,13-dihydrovernolide is present an epoxide (oxirane) function which is an extremely reactive group. This functionality may be responsible for some of its activity as observed in this study. In addition, a biologically active compound may have more than one specific activity. Only few compounds show specific activity and this statement is valid also for drugs currently used in therapeutic purposes. For instance, the side effect of a drug is due to the drug itself which is administered to cure or treat a specific disease. The same is applicable for natural products which may have more than a single activity. A typical example is the case of daphnetin, a simple coumarin contained in daphne. Dapnetin resulted to be active anti-inflammatory (Li *et al.*, 2017), anti-cancer (Kumar *et al.*, 2016) and anti-diabetic (Vinayagam and Xu, 2017). Another example is syringing which showed a dose-dependent effect on sleep induction (Cui *et al*., 2015) and anti-feedant properties (Cis *et al*., 2006). The available spectra for the column sub-fraction B3 (obtained from *O. gratissimum* leaf) suggest that the compound is 5-hydroxy-hex-3-enoic acid (Figure 4.21). The hypoglycemic activity of the compound was lower than that of the *n*-butanol fraction of fractionated methanol extract of *O. gratissimum* leaf (OGBF). This implies that 5-hydroxy-hex-3-enoic acid is not the only component present in *O. gratissimum* leaf responsible for the anti-diabetic activity. The 5-hydroxy-hex-3-enoic acid may work in synergy with other components present in *O. gratissimum* leaf. This finding is in agreement with the reports from previous investigation where chicoric acid (isolated from *n*-butanol fraction of fractionated extract of *O. gratissimum* leaf by Casanova *et al* (2014)) was observed to have less activity when compared with the *n*-butanol extract in diabetic mice. The synergetic effects of these isolated compounds could account for the higher activity observed in the *n*-butanol fraction of the fractionated methanol extract of *O. gratissimum* leaf (OGBF).

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Different extraction techniques gave different yields on specific phytochemical. The use of MAE resulted in very fast extraction rate with high quantity of phytoconstituents compared to Soxhlet and cold maceration techniques. Extracts from cold maceration gave better hypoglycaemic effects in diabetic rats. A combination of margarine and fructose fortified diet-fed and single low dose of STZ induction in rats is a suitable method to develop rat model of T2D. The NDF rats induced with 25 mg/kg b.w and 35 mg/kg b.w of STZ had 75.7 % and 64.4 % serum insulin respectively and also presented relative normoglycemia, whereas the FDF rats induced with 35 mg/kg b.w of STZ had 85.8 % serum insulin and were notably hyperglycaemia. The chloroform fraction of fractionated methanol extract of *V. amygdalina* leaf and butanol fraction of fractionated methanol extract of *O. gratissmum* leaf were observed to be the most active hypoglycemic fractions of the studied plants. The fractionated methanol extracts of *V. amygdalina* and *O. gratissimum* leaves effectively ameliorates most of the alterations caused in the studied parameters in diabetic rats. The daily oral administration of fractionated methanol extracts of *V. amygdalina* and *O. gratissimum* leaves for 28 days significantly lowered blood glucose, HOMA-IR and improved insulin sensitivity. Part of the bioactive compounds responsible for the hypoglycemic activities of *V. amygdalina* and *O. gratissimum* leaves were identified.

6.2 Conclusion

The findings obtained from the present research showed that the choice of extraction techniques should be based primarily on the phytochemical entity of interest. Nevertheless, since extraction efficiency differs from efficacy, no single method can be rated as best for extracting all forms of phyto-components. The experimental rat model of T2D presented in this study is a classical

replica of the westernized unhealthy lifestyle seen in human T2D subjects, in which fructose (20%) in drinking water is co-administered with high fat diet (Margarine fortified diet feed) to produce insulin resistance. The subsequent induction of sub-diabetogenic dose of STZ (35 mg/kg b.w) elevated the blood glucose to the diabetic level without destroying much of the β-cells. The present study has established a benchmark of residual insulin levels in selecting animal model for T2D studies. The results obtained from this research suggests that selection of animal model for T2D studies should be based on circulating endogenous serum insulin of not less than 85.7% of normal control groups in a hyperglycemic condition of fasting blood glucose $\geq$ 300 mg/dL. In addition the serum C-peptide level should in relative term be insignificantly different from the normal control group. The model mimics the natural history and metabolic features of human T2D. In addition, it is cheap, easy to develop and useful for evaluating and screening of therapeutic compounds intended for treatment of T2D. The chloroform fraction and butanol fraction of fractionated methanol extracts of *V. amygdalina* and *O. gratissimum* leaves respectively, significantly (p<0.05) reduced the hyperglycemic state of diabetic rats. The compounds: 11β,13-dihrovernolide and 5-hydroxyhex-3-enoic acid isolated from *V. amygdalina* and *O. gratissimum* leaves respectively, were characterized to have hypoglycemic activity. All the set objectives for this study have been accomplished.

6.3 Recommendations

1. The details molecular mechanism of action by which 11β,13-dihydrovernolide and 5-hydroxy-hex-3-enoic acid (present in *V. amygdalina* and *O. gratissimum* leaves respectively) lowered blood glucose should be investigated.
2. The safe dose of the isolated compounds should also be investigated further.
3. Further research is required for detailed structure-activity relationships of the isolated compounds towards development of these compounds for pharmaceutical use.

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APPENDICES

Appendix I

Composition of Normal Diet Feed (NDF), Fortified Diet Feed (FDF) and Simas Margarine

Ingredient	Quantity (NDF)	Quantity (FDF)
Crude Protein	13.00%	11.82%
Fat	8.00%	16.36%
Crude fibre	15.00%	13.64%
Calcium	0.90%	0.82%
Available Phosphorus	0.35%	0.32%
Methionine	0.37%	0.34%
Lysine	0.70%	0.64%
Metabolizable energy	2600.00kcal/kg	3020.00kcal/kg

(Source: Manufacturer's pack label: Grand Cereals Limited, Nigeria)

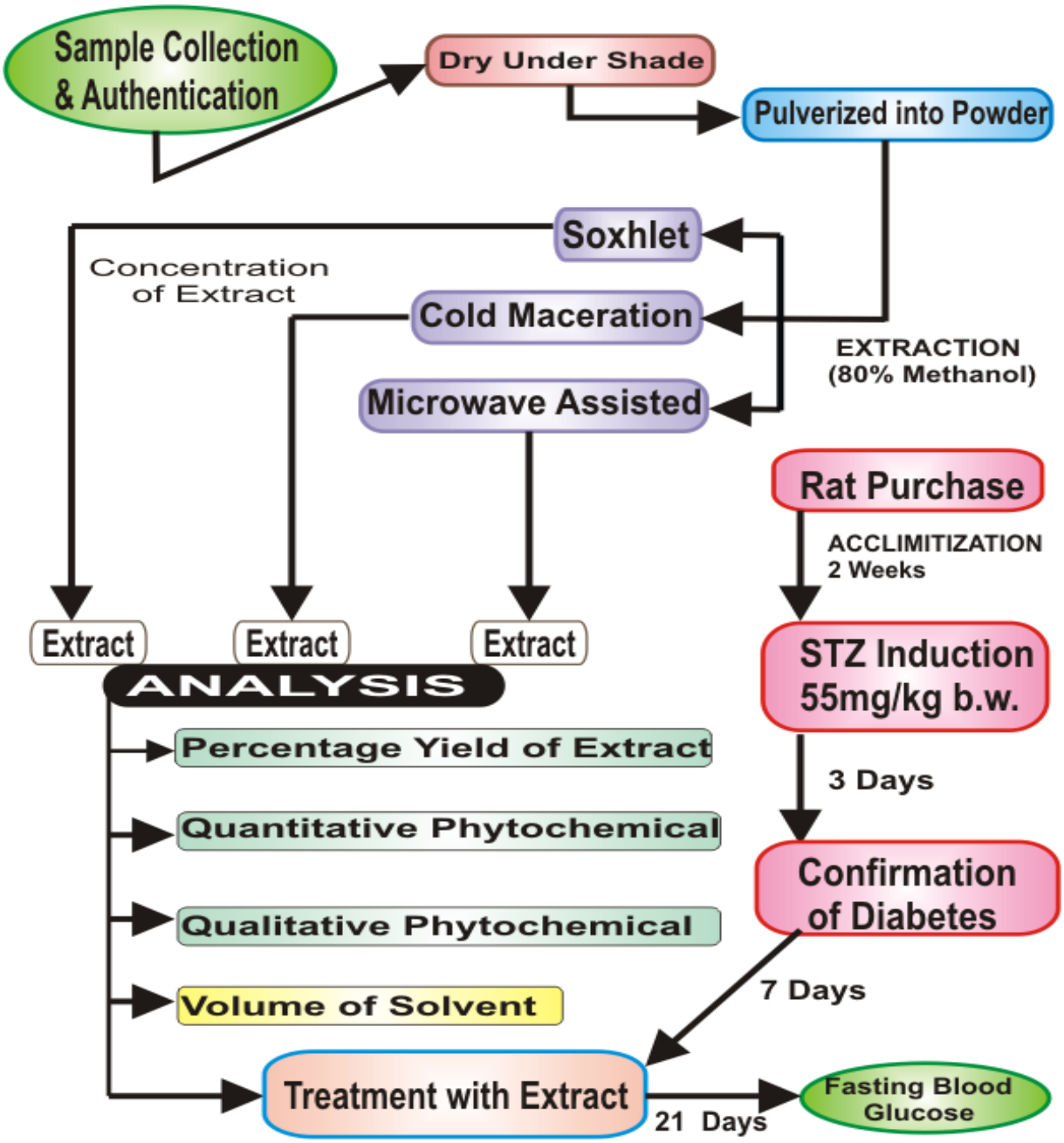
Composition of Simas Margarine

Ingredient	Quantity
Emulsifiant	E471, E322
Antioxidant	E304, E306
Beta Carotene	C175130
Vitamin A	30,000 IU/kg
Vitamin D	30,000 IU/kg

(Source: Manufacturer's pack label: PT Salim Ivomas Pratama Tbk, Indonesia)

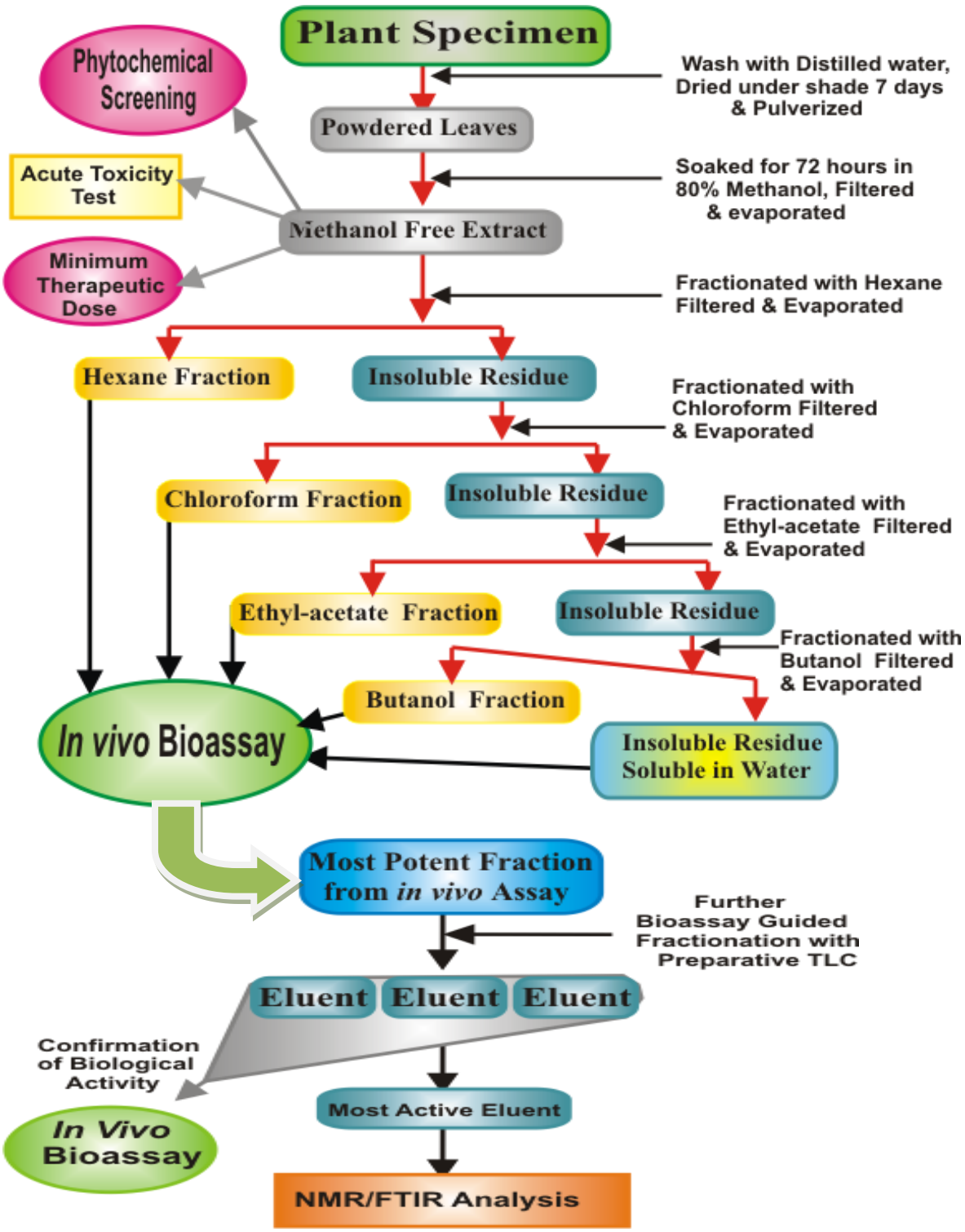
APPENDIX II

Experimental Design for the Evaluation of Extracts Obtained from Three Different Extraction Methods



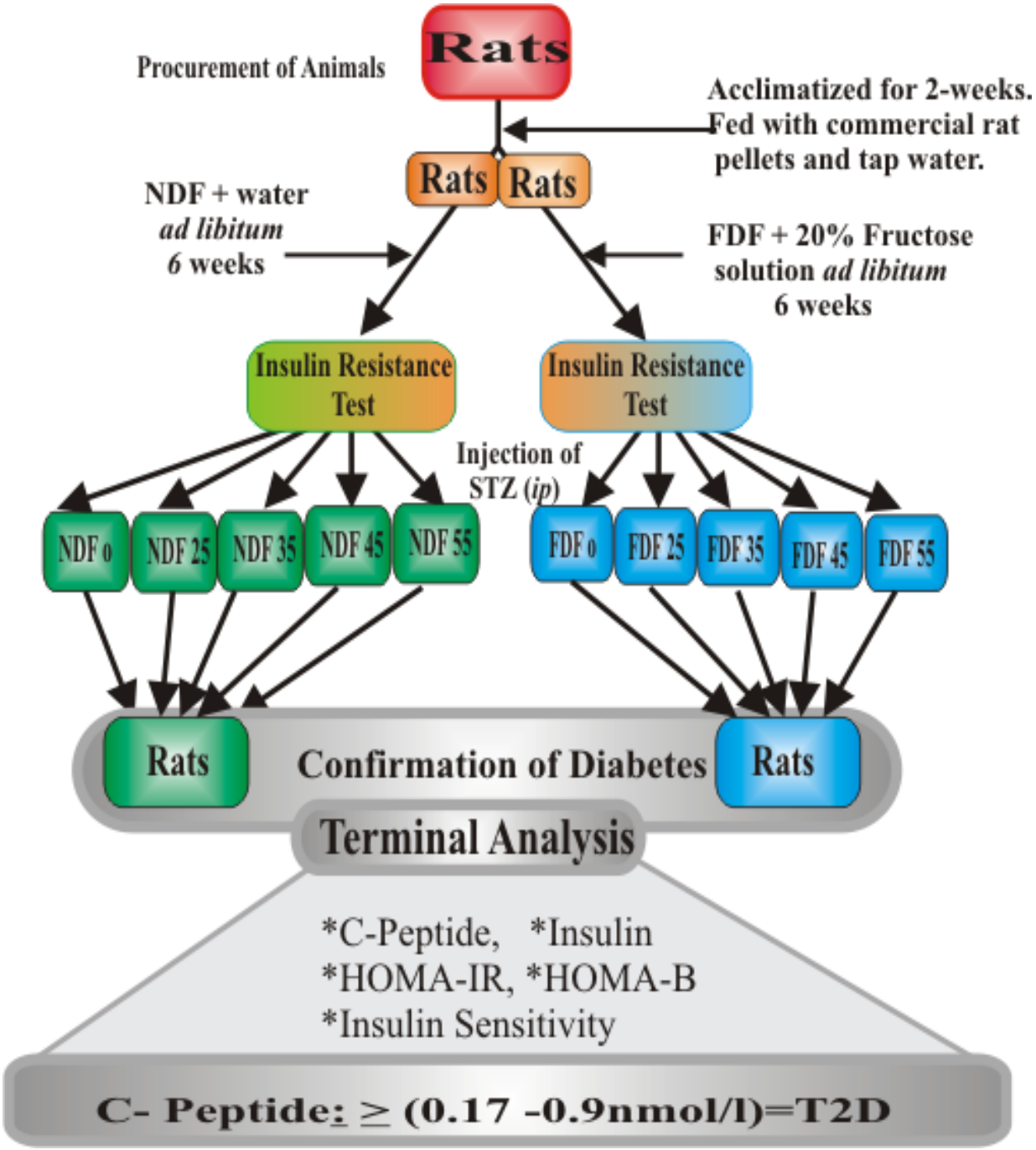
APPENDIX III

Experimental Design for the Evaluation of Fractionated Methanol Extracts of *Vernonia amygdalina* and *Ocimum gratissimum* Leaves



APPENDIX IV

Experimental Design for the Development of Animal Model of Diabetes Mellitus



APPENDIX V

Published Abstract of the 1st Conference Paper derived from this Study

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11th Asia Pacific Diabetes Conference and Expo

July 11-12, 2016 Brisbane, Australia

Evaluation of extraction protocols for anti-diabetic bioactive molecules from medicinal plants

S I R Okoduwa[1-2], I A Umar[1], D B James[1], H M Inuwa[1] and J D Habila[3]
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The successful isolation of bioactive constituents from therapeutic plants relies mostly on the use of appropriate extraction technology. This study examines the efficacy of three extraction techniques: Soxhlet-Extraction (SE), Cold-Maceration-(CM) and Microwave-Assisted-Extraction (MAE) using 80% methanol. The study was performed on each of 50 g of *Vernonia-amygdalina* (VA) and *Occimum-gratissimum* (OG) leaves respectively. The biological activities were analyzed using rat model of diabetes with a combined dose of the two plant extract (250 mg/kg-b.w.) from the three methods. The result showed that the percentage VA extract yield from MAE was highest (20.9+1.05%), when compared to the CM (14.35+0.28%) and SE (15.75+0.71%). The percentage differences in OG extract yield between: MAE versus SE was 41.05%; MAE versus CM was 46.81% and SE versus CM was 9.77%. The chemical analysis of the two plants showed no difference in the various phytoconstituents detected, but differs in the concentrations of the individual phytochemicals, as MAE had better yield on phenolics, saponins and tannins. SE technique gave the better yield on alkaloid, while CM gave the better yield on flavonoids. The CM exhibited a better percentage hypoglycemic activity within the first 14-days of treatment (43.3+3.62%) when compared to MAE (36.5+0.08%) and SE method (33.3+1.60%). However, the percentage hypoglycemic activity, 21 days post-treatment with 250 mg/kg-b.w MAE was 72.6+1.03% and it was more comparable to 10 mg/kg b.w. glibenclamide treated group (75.0+0.73%) than the SE (69.5+0.71%) and CM (69.1+1.03%). The study reveals that MAE emerges as a better option for rapid extraction procedure of phytoconstituents due to its shorter time and less solvent utilization, however, the qualitative choice of an extraction technique should depend on a specific bioactive component of interest.

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APPENDIX VI

Published Abstract of the 2nd Conference Paper derived from this Study

12th World Congress on
DIABETES & PALLIATIVE CARE
September 29-30, 2016 Toronto, Canada

Appropriate insulin level in selecting fortified diet-fed, streptozotocin-treated rat model of type 2 diabetes for anti-diabetic studies

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Pathophysiological investigation of disease in a suitable animal model is a classical approach towards development of a credible therapeutic strategy. This study examined appropriate insulin level in selecting an animal model for type 2 diabetes (T2D) studies. Albino Wistar rats (150-200g) were divided into two groups fed with commercially available normal-diet-feed (NDF) and water or fortified diet feed (FDF) (10g NDF per gram of margarine) with 20% fructose solution as drinking water. After 6 weeks of dietary regimen both groups were divided into 5 sub-groups and injected intraperitoneally with a graded dose of streptozotocin (STZ) (0, 25, 35, 45 & 55-mg/kg b.w.). The result showed that the FDF-fed rats increased significantly in body weight, basal serum insulin, total cholesterol, triglycerides and blood glucose levels as compared to NDF-fed rats. Ten days post STZ induction, the groups treated with STZ (45 & 55 mg/kg) developed frank hyperglycemia with < 46.8% serum insulin, a severe deficiency typical of diabetes type 1. The NDF_{25} and NDF_{35} groups with 75.7% and 64.4% serum insulin respectively presented relatively normoglycemia, whereas the FDF_{35} (85.8% serum insulin) were notably hyperglycemia (>300 mg/dL) throughout the 6weeks post diabetes confirmation. These FDF_{35} rats were sensitive to glibenclamide, metformin and pioglitazone in lowering hyperglycemia, hypertriglyceridemia and hypercholesterolemia, thus proved to be a suitable non-genetic stable model for T2D studies. The study suggests that circulating serum insulin $\geq$ 85.8% with overt hyperglycemia may be utilized as the benchmark in selecting animal-model for T2D studies.

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APPENDIX VII

First Page Showing the 1st Published Article Derived from this Study

World Journal of Diabetes

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DOI: 10.4239/wjd.v7.i20.605

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Evaluation of extraction protocols for anti-diabetic phytochemical substances from medicinal plants

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Author contributions: Okoduwa SIR and Umar IA got the concept and design of the study; Okoduwa SIR and Habila JD perform the study and data analysis; James DB, Inuwa HM and Habila JD participated in the interpretation of data; Okoduwa SIR wrote the first version of the manuscript; Umar IA, James DB, Inuwa HM and Habila JD critically revised the manuscript for important intellectual content; all the authors read and gave a final approval of the revised version of the manuscript to be published.

Institutional review board statement: Ethics of the study involving the use of animals were reviewed and approved by Institutional Animal Care and Use Committee of the Ahmadu Bello University and the Research Ethics Committee of Nigerian Institute of Leather and Science Technology, Zaria-Nigeria where the research was conducted.

Institutional animal care and use committee statement: The experimental protocol was review and approved by the Research Ethics Committee of the Institute. All experimental protocol was in conformity with the Institutional guidelines that are in compliance with National and International Laws and Guidelines for Care and Use of Laboratory Animals in Biomedical Research.

Conflict-of-interest statement: The authors declare no conflict of interest related to this study and publication.

Data sharing statement: No additional data are available.

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Abstract

AIM

To examine the efficacy of three extraction techniques: Soxhlet-extraction (SE), cold-maceration (CM) and microwave-assisted-extraction (MAE) using 80% methanol as solvent.

METHODS

The study was performed on each of 50 g of *Vernonia amygdalina* (VA) and *Occimum gratissimum* (OG) leaves respectively. The percentage yield, duration of extraction, volume of solvent used, qualitative and quantitative phytoconstituents present was compared. The biological activities (hypoglycemic effect) were investigated using albino wistar rat model of diabetes mellitus (n = 36) with a combined dose (1:1) of the two plants leaf extracts (250 mg/kg b.w.) from the three methods. The

APPENDIX VIII

First Page Showing the 2nd Published Article Derived from this Study

PLOS ONE

RESEARCH ARTICLE

Appropriate Insulin Level in Selecting Fortified Diet-Fed, Streptozotocin-Treated Rat Model of Type 2 Diabetes for Anti-Diabetic Studies

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Data Availability Statement: All relevant data are within the paper.

Funding: The authors received no specific funding for this work.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Background

Pathophysiological investigation of disease in a suitable animal model is a classical approach towards development of a credible therapeutic strategy. This study examined appropriate insulin level in selecting animal model for type 2 diabetes (T2D) studies.

Method

Albino Wistar rats (150-200g) were divided into two groups fed with commercially available normal-diet-feed (NDF) and water or fortified diet feed (FDF) (10g NDF per gram of margarine) with 20% fructose solution as drinking water. After 6 weeks of dietary regimen both groups were divided into 5 sub-groups and injected intraperitoneally with a graded dose of streptozotocin (STZ) (0, 25, 35, 45 & 55mg/kg bw.).

Result

The result showed that the FDF-fed rats increased significantly in body weight, basal serum insulin, total cholesterol, triglycerides and blood glucose levels as compared to NDF-fed rats. Ten days post STZ induction, the groups treated with STZ (45 & 55 mg/kg) developed frank hyperglycaemia with < 46.8% serum insulin, a severe deficiency typical of diabetes type 1. The NDF_{25} and NDF_{35} groups with 75.7% and 64.4% serum insulin respectively presented relative normoglycemia, whereas the FDF_{35} (85.8% serum insulin) were notably hyperglycaemia (>300 mg/dL) throughout the 6weeks post diabetes confirmation. These FDF_{35} rats were sensitive to glibenclamide, metformin and pioglitazone in lowering hyperglycaemia, hypertriglyceridemia and hypercholesterolemia

Conclusion

The hyperglycaemia stability of the FDF_{35} rats (85.5% insulin) together with their sensitivity to 3 different hypoglycaemic drugs strongly suggests their suitability as a non-genetic model

APPENDIX IX

First Page Showing the 3rd Published Article Derived from this Study

medicines

MDPI

Article

Anti-Diabetic Potential of *Ocimum gratissimum* Leaf Fractions in Fortified Diet-Fed Streptozotocin Treated Rat Model of Type-2 Diabetes

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Abstract: Background: *Ocimum gratissimum* (OG) is used in the traditional management of diabetes in Nigeria. This study investigated the anti-diabetic potential of OG leaf fractions (OGLF) in a rat model of Type-2 diabetes (T2D). **Methods**: Methanol crude extract of OG leaf was fractionated with solvents of increasing order of polarity (*n*-hexane, chloroform, ethyl-acetate, *n*-butanol and water). The anti-diabetic potential of the fractions was evaluated in vivo. T2D was induced in Albino Wistar rats and treated with OGLF. **Results**: The T2D rats showed significant elevation in serum levels of fasting blood glucose (FBG), liver and kidney function biomarkers. At 4-week of intervention with OGLF, the untreated diabetic control group maintained severe hyperglycaemia in the presence of 61.7% serum insulin, 17.3% pancreatic β-cell function (HOMA-β) and 51.5% Insulin sensitivity. The glucose tolerance ability was enhanced in the *n*-butanol-fraction (OGb) treated group. With 74.8% available serum insulin and 38.6% improvement in insulin sensitivity, the OGb treated group had a 63.5% reduction in FBG and it was found to be most effective as it ameliorates a majority of the changes caused in the studied parameters in diabetic rats. **Conclusions**: The data from this study suggest that OGb fraction is a potential candidate for the development of an effective drug for the management of T2D.

Keywords: Type-2 diabetes; *Ocimum gratissimum*; hyperglycaemia; hyperlipidaemia; rats

1. Introduction

Diabetes mellitus is an important chronic metabolic disorder of public health concern. It occurs either as a result of pancreatic defects in insulin secretion or the failure of the receptor cells to effectively utilise secreted insulin or both [1–3]. Hyperglycaemia is a common consequence of uncontrolled diabetes, which may over time lead to serious damage to vascular tissue, heart, eyes, nerves and kidneys [1,3,4]. Diabetes is no longer a disease of predominantly rich nations; its prevalence is steadily increasing across the globe—age [3,5], gender, socioeconomic status and ethnicity of the individual [6] notwithstanding. Globally, an estimated 422 million adults were reported to be living with diabetes in 2014, compared to 108 million in 1980; a dramatic rise in prevalence rate from 4.7% to 8.5% in the adult population [3]. In 2015 in Nigeria alone, 1.56 million cases were reported including 105,091 deaths [2]. Every six seconds a person dies from diabetes in the world. In 2012 there were 1.5 million deaths worldwide directly caused by diabetes and about 95% of all cases reported were attributed to Type-2 diabetes mellitus (T2DM), commonly called Type-2 diabetes (T2D) [2,3].

APPENDIX X

First Page Showing the 4th Published Article Derived from this Study

Original Article

Validation of the Antidiabetic Effects of *Vernonia amygdalina* Delile Leaf Fractions in Fortified Diet-Fed Streptozotocin-Treated Rat Model of Type-2 Diabetes

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Abstract

Background: *Vernonia amygdalina* (VA) is used in the traditional management of diabetes in Nigeria. Previous scientific verification of VA is on Type-1 diabetes model, in spite of the continuous increase in Type-2 diabetes (T2D) among adults. This study aimed to validate the antidiabetic effects of VA leaf fraction (VALF) in a unique T2D rat model. **Materials and Methods:** Methanol crude extract of VA leaf was fractionated with solvents of increasing order of polarity (*n*-hexane, chloroform, ethyl-acetate, *n*-butanol and water). The antidiabetic activities of the fractions were evaluated *in vivo* in T2D model rats. Albino Wistar rats were induced with T2D and treated with the VALF. Several T2D-related parameters were measured. **Results:** T2D rats showed significant increase in serum levels of fasting blood glucose (FBG), liver and kidney biomarkers. At 28-day post-oral treatment with the VALF, FBG levels were significantly ($P < 0.05$) reduced (*n*-hexane [29.3%], chloroform [66.7%], ethyl acetate [36.2%], *n*-butanol [45.59%] and aqueous [39.3%]). The glucose tolerance ability was significantly improved in the chloroform fraction (*Vernonia amygdalina* chloroform fraction [VAc])-treated groups compared to the other fractions-treated group and diabetic control group. Furthermore, the VAc was found to be most effective as it ameliorates most of the alterations caused in the studied parameters in diabetic rats when compared with *n*-hexane, ethyl acetate, *n*-butanol and aqueous fractions. **Conclusion:** The study validates the anti-diabetic effects of VALF in fortified diet-fed streptozotocin-treated rat model of T2D, and suggests that the VAc is a potential candidate for development of a more effective drug for the management of T2D.

Keywords: Anti-hyperglycaemia, hyperlipidaemia, rats, type-2 diabetes, *Vernonia amygdalina*

Introduction

Diabetes mellitus is a group of chronic metabolic disorders characterised by hyperglycaemia as a result of defects in insulin secretion, action or both.[1] The ever-increasing incidence of diabetes mellitus worldwide among all age groups,[2,3] irrespective of gender, race, socioeconomic status[4] or ethnicity, constitutes a global public health burden.[1,2] The global report on diabetes by the World Health Organization affirmed that, with a prevalence rate of 8.5%, the number of adults living with diabetes has increased by almost four times since 1980 – 422 million adults in 2015.[1,2] Moreover, the International Diabetes Federation reported that, on an average, a person dies from diabetes every 6 s.[1] In Nigeria, about 5 million people are still living with diabetes, while 1.56 million cases were recorded in 2015 with 105,091 deaths documented as of 2014.[1] This dramatic rise is largely due to increase in Type-2 diabetes (T2D), which accounts for 95% of all reported cases.[1,2]

Notwithstanding the high incidence of T2D which has become a serious threat all over the world and the advancement made thus far on basic and clinical investigations into diabetes, (particularly in this region where healthcare resources are scarce), an ultimate therapy does not exist. The existing therapeutic strategies are limited and involve insulin and four main classes of oral antihyperglycaemic agents

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