CHAPTER ONE

1.1 INTRODUCTION

Diabetes mellitus (DM) is positioned seventh among the various leading causes of death and considered third when its fatal complications are attentively regarded (Ekeocha *et al*., 2012). Diabetes is one of the major disease that causes death in most countries. It is a chronic disease due primarily to a disorder of carbohydrate metabolism with a deficiency or diminished effectiveness of insulin resulting in chronic hyperglycaemia and glycosuria. (Kumar *et al.,* 2015). Diabetes is also known to be involved in lipid metabolism and oxidative stress(Adewole *et al.,* 2009). Hyperglycaemia causes an increase in oxidative stress, causing inflammation, activation of the polyol pathway, and damage to various organs in the body (Zeinab *et al.,* 2018). Hyperglycemia increases the level of diacylglycerol and activates protein kinase C activity in the aorta of streptozotocin (stz.) induced diabetic rats (Chattopadhyay *et al.,* 2005). In Nigeria there has been active development of phyto pharmaceuticals with proven efficacy in a variety of medicinal problems (Ekeocha et al. 2012).Research has shown that a number of anti diabetic plants consists of useful properties such as antihypertensive, nephro protective and retino protective activities apart from their hypoglycaemic activity which may be useful against the most common complications of DM. Thus, consumption of these vegetable plants and local herbs are believed to contribute positively to the improvement of human health, because plants have long served as a useful and natural source of the therapeutic agents in terms of prevention and, or cure of diseases useful in the control of DM and its complications by man. The World Health Organization has approved medical centres to use herbal drugs to treat DM in addition to its complications although, there are no ultimate and specific method available to prevent and treat DM, but methods are needed to reduce the complications brought about by the disease (Zeinab *et al.,* 2018 and Arhoghro *et al*, 2009).

Medicinal plants have great impact in reduction of blood glucose level as well as other DM- related compounds (Raks *et al.,* 2017). A medicinal plant is any plant that contains active ingredient which is used for therapeutic purposes and contain compounds that can be used for synthesis of useful drugs. (Kutama *et al.,* 2018). A lot of plants are rich source of antioxidants such as flavonoids, tannins and lignin as example of phenolic compounds, vitamins A, C, E are all found in plants (Ammar *et al.,* 2017) Leaves, roots, rhizomes, stems, barks, flowers, fruits, grains, or seeds are part of plants been used and they all contain chemical components which are used for control and treatment of a diseases (Kutama *et al.,* 2018). Therefore, medicinal plant are being preferred these days with less or no side effects (Kumar *et al.,* 2015).

Vernonia amygdalina locally known as 'bitter leaf' is an example of medicinal plants widely used in Nigeria for both therapeutic and nutritional purposes for the treatment and management of diabetes, skin disease, hypertention and other metabolic diseases associated with the liver (Arhoghro et al., 2009). *V.amygdalina* is been assessed in the control of lipid complications and oxidative stress usually associated with chronic diabetes.

1.1 STATEMENT OF THE PROBLEM

DM has been identified as the most common endocrine disorder that currently affects 200 million people of the world's population and assumed to rise to over 366 million in the year 2030 (Abubakar *et al.,* 2017). The national health interview survey found that 22% of people with diabetes used some type of herbal therapy for treatment. Also, many drugs have been identified and recommended in the world for the treatment of various disease. (Omodanisi *et al.,* 2017), thousands of plants such as *V. Amygdalina* have been identified to contain medicinal qualities and are used in the cure of many disease, inflammation and ailments Other plants include*; Brassicaoleracea, Carica papaya, Citrus medica* (Abubakar *et*

al., 2017), *Cocciniagrandis, Trichosanthesdioica, Simmondsiachinensis, Garciniaindica, Momordicadioica, Celastrus paniculatus* (Kumar *et al.*,2015). Hence, this study was designed to examine the methanolic effects of *V. Amygdalina* for the treatment and management of diabetes.

1.2 AIMS AND OBJECTIVES OF THE STUDY

1.3.1 AIM

The research investigated the phytochemical analysis and effect of V. amygdalina methanolic extract on lipid profile in streptozotocin- induced diabetic rats. The study further examined specific diabetic, lipid profile in the blood sample of rats. The overall aim, therefore, is to explore the potential benefits of V. amygdalina which can be used in the prevention and management of diabetes and as a component for the pharmaceutical industry in treating diabetes in the near future especially in developing countries.

1.3.2 OBJECTIVE

1. To carry out phytochemical screening on *V. amygdalina* leaves.
2. To determine the bioactive compounds in the *V.amygdalina* (Bitter leaf) with GC-MS
3. To administer the methanolic extract and determine the effects of *V. amygdalina* on lipid profile in diabetes induced laboratory rats.
4. To evaluate the effect of oral administration of the extract on male Wister rat.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 DIABETES MELLITUS (DM)

The term diabetes was described by Aretaeus of Cappadocia as a polyuric wasting disease (Ahmed *et al*., 2002). It was proceeded from the Greek verb 'diabainein' derived from the prefix 'dia', 'across, apart' and the verb 'bainein'- 'to walk, stand'. Diabainein meaning 'to stride, walk or stand with legs as under', hence its derivatives 'diabetes' meant 'one that straddles' or precisely 'a compass, siphon'. The word 'siphon' brought up the use of diabetes as the name of a disease causing the discharge of excessive amount of urine. Diabetes has been noticed since the last 3500years. Aretaeus (130-200CE) used the word diabetes (from the Greek siphon) five hundred years later. In 1675, Dr Thomas Willis added the Latin word 'mellitus' meaning 'honey' (Pathak *et al.,* 2013). The term 'sweet urine disease' was coined from the observation of ants attracted to a person's urine due to glucose in the urine (Omodanisi *et al*., 2017).

DM is a disease caused by lack of insulin secretion, decreased sensitivity of tissues to insulin in which glucose metabolism is impaired (Ibegbu *et al.,* 2018) causes alteration in glucose, protein and lipid metabolism.DM is a diverse and chronic metabolic disease of the endocrine system (Ochalefu *et al.,* 2018).Changes in lipid concentration and consequent disorders of lipid metabolism have been observed in DM (Nwanjo *et al.,* 2015) and when the body is ineffectively use insulin that is produced (Kumar et al., 2015). In the near future, it is likely that there will be∼50% increase in diabetes in some parts of the world like Asia, the Middle East, and Africa by 2030 (Adeoye *et al.,* 2018). DM is ranked seventh among the leading causes of death and is considered third when its fatal complications are taken into consideration (Ekeocha *et al.,* 2012). There is high level of blood glucose (Hyperglycaemia)

which may project the kidneys capacity to reabsorb, with spill over of the excess glucose into the urine (Glucosuria). Chronic hyperglycaemia in DM is associated with malfunctioning, failure and tearing down of the various organs in the body such as kidney, eye, nerve, heart and blood vessels (Ochalefu *et al.,* 2018). Hyperglycemia is a condition of abnormal rise in plasma glucose level, and in type-2 diabetes is a result of insulin resistance which may be due to a number of defects in signal transduction ranging from abnormal insulin to defects in glucose transporters (Shodehinde *et al.,* 2012).

DM is one of the most common endocrine diseases worldwide, and the number of people with DM was recorded as 422 million in 2014 (Zeinab *et al.,* 2018). In 2013, DM caused 5.1 million deaths and 548 billion US dollars were expenditure globally for diabetes treatment (Kumar *et al.* 2015). In 2014, 4.9 million individuals died due to this disease worldwide, in 2015, 30.3 million Americans were regarded to have DM, 7.2 million of which were thought to be undiagnosed. In the same year, an estimated 1.6 million deaths were directly attributed to DM and a further 2.2 million deaths were ascribed to hyperglycemia (Zeinab *et al.,* 2018). A study in 2017 estimated that about 422–425 million adults were living with the disease and it was projected that this number would rise to 629 million adults by the year 2045 (Odeyemi *et al.,* 2018), 300 million in the year 2025 (Moradi *et al.,* 2018), 366 million by 2030 (Abubakar *et al.,* 2017), if necessary and adequate actions are not taken.

Diabetes mellitus is associated with reduced quality of life and increased risk factors for mortality and morbidity (Abubakar *et al.,* 2017). In the absence of proper treatment, cardiac, vascular, neurological, and renal damage and neuropathy may occur (Moradi *et al.,* 2018).

2.2 TYPES OF DIABETES

There are three main types of DM;

1. Type 1 diabetes mellitus (T1DM)
2. Type 2 diabetes mellitus (T2DM)
3. Gestational diabetes mellitus (GDM)

2.2.1 Type 1 DM: is known as insulin dependent diabetes mellitus (IDMM). It is characterized by loss of the insulin producing beta cells of the islets of Langerhans in the pancreas leading to insulin deficiency (Kumar *et al.,* 2015). It was concluded that patients with type 2 diabetes, recently started on insulin intake, would have lower hypoglycaemia risk than people with type 1 diabetes, and comparable with those taking sulfonylurea (UK hypoglycaemia study group 2007). It can also be said that type 1 diabetes is caused due to insulin insufficiency (Uma *et al.,* 2011).

2.2.2 Type 2 diabetes mellitus: is known as non-insulin dependent diabetes mellitus (NIDDM). It is insulin independent. It is due to the immunological demolition of pancreatic B cells leading to insulin deficiency (Uma *et al.,* 2011).It is characterized by insulin resistance and inadequate insulin production leading to high level of glucose (Kumar *et al.,* 2015). About 90% of all diabetes cases are type 2 and it is the second highest peril factor for developing Alzheimer's disease (Omadanisi *et al.,* 2017).

In type 2 diabetes, where insulin secretion deficiency is incomplete and progressive, the duration of insulin therapy may further influence hypoglycaemia (UK hypoglycaemia study group 2007)**.** The use of insulin in patients with type 2 diabetes is steadily rising, but relatively little is known about the frequency of hypoglycaemia in type 2 diabetic patients, treated either with sulfonylurea or insulin. It has been presumed that hypoglycaemia is rare in people with insulin-treated type 2 diabetes, but retrospective and small short-term prospective

studies suggest that severe hypoglycaemia may be relatively common. Type 2 diabetes is directly linked to obesity and accounts for 80% of diabetes cases (Tekou *et al.,* 2018).

2.2.3 Gestational DM: It is characterized by a rise in glucose level and insufficient insulin which reduces glucose level. Gestational diabetes is characterized by a slight resistance to insulin (Omodanisi *et al.,* 2017). In GDM, insulin receptors dysfunction leading to high blood glucose levels during pregnancy (Kumar *et al.,* 2015).

2.3 SYMPTOMS OF DM

Common symptoms of diabetes mellitus include; frequent urination, dehydration, intense hunger, fatigue irritability, blurred vision, wounds that does takes long before getting healed, sexual dysfunction in men, gum infections (Odeyemi *et al.,* 2018) high levels of sugar in the blood; extreme hunger; weight loss; nausea and vomiting; blurred vision; frequent tiredness, irritation etc (Uma *et al.,* 2011). An effective strategy for type 2 diabetes management has been through the inhibition of pancreatic α-amylase and limiting the absorption of glucose by inhibiting intestinal α-glucosidase enzyme (Shodehinde *et al.,* 2012).

2.4 TREATMENT OF DM

Common treatments to the symptoms of DM include regular injection of insulin or oral administration of antidiabetic drugs, such as sulfonylurea, α-glycosidase inhibitors, biguanides, thiazolidinediones, dipeptidyl peptidase-4 (DPP4) inhibitors, sodium-glucose co-transporter-2 (SGLT2) inhibitors, glaives, and parenteral administration of glucagon-like peptide-1 (GLP-1)agonists, which can cause moderate to severe side effects (Zeinab *et al.,* 2018).

2.5 AETIOLOGY OF DM

To date, there is no apparent cure for diabetes mellitus type I, and therefore those with type I diabetes need to take insulin for life to control blood glucose levels. Diabetes mellitus type II is associated with the ingestion of high-calorie foods, family history of the disease, obesity, race, genetic disorders, smoking, inactivity, viral infections and drugs or chemicals, and can be managed with drugs and/or changed diet plans (Odeyemi *et al.,* 2018).

2.6 OVERVIEW OF AVAILABLE TESTS

Converse tests for diabetes screening are many and vary from history- and anthropometric-based questionnaires to proteomics-based risk assessment. Although some of these tests might prove to be useful, the current preferred tests are limited to two groups: serum glucose–based tests and glycated proteins. Serum glucose–based tests include fasting blood glucose (FBG), random blood glucose (RBG), and the oral glucose tolerance test (OGTT) (Cox *et al.,* 2009).

2.7 STAGES OF DM

'Overt' diabetes is a type of diabetes that is not seen until the age above 40, therefore; there must be a stage of 'pre-diabetes' which starts from the time of conception.

Table 1: Stages Of Diabetic Mellitus (DM),

STAGES	GLUCOSE TOLERANCE TEST	FASTING BLOOD GLUCOSE	PLASMA INSULIN	SYMPTOMS	References
Pre-diabetes	Normal	Normal	Normal	None	Chatterjea and Ranashinde 2007.
Suspected diabetes	Abnormal	May be normal	Normal	Symptoms after stress	
Chemical/latent diabetes	bnormal	Normal or raised	Normal or raised	Unusual	
Overt diabetes	Abnormal	Raised	Normal or low	Usual	

2.8 STREPTOZOTOCIN (STZ) AND DIABETIC INDUCTION

STZ is a naturally occurring nitrosourea with molecular weight of 265 and empirical formula of $C_{14}H_{27}N_5O_{12}$ (Khaleel *et al.,* 2015). An antibiotic and anticancer agent, it has been widely used for inducing type I diabetes in a variety of animals by affecting degeneration and necrosis of pancreatic b-cells (Eidi *et al.,* 2006). This causes low insulin with subsequent increase in the blood glucose level known as hyperglycemia. Streptozotocin action on the pancreas causes β - cells destruction by necrosis. The mechanism of action of STZ causing elevated blood glucose is thought to be through its entry into the β-cells via a glucose transporter (GLUT 2) where it leads to alkylation of DNA molecule and its eventual destruction. (Ochalefu *et al*.,2018). Streptozotocin sterile powders are provided and prepared as a chemotherapy agent (Akbarzadeh *et al.,* 2007).It is a drug of choice in islet cell carcinoma and malignant carcinoid tumors. It is diabetogenic, hepatotoxic, nephrotoxic and also causes gastric ulceration (Khaleel *et al.,* 2015).

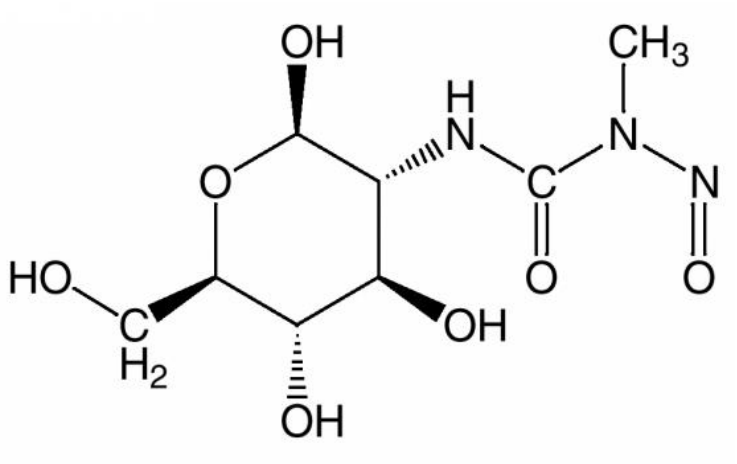

Figure 1: Structure of Streptozotocin (STZ) (National institute of health 2006)

2.8.1 STZ STABILITY

Streptozotocin should be stored at −20°C to avoid drying up. After weighing, the tube or beaker containing the sample of STZ must be covered with aluminium foil to protect it from light. STZ, as an unstable solution, even at an acidic pH, must not be mixed into citrate buffer until immediate preceding time to injection. The STZ solution should be prepared fresh and injected within 5min of being dissolved into the citric buffer because it undergoes dissolution in citrate buffer within 15 to 20 min (Furman *et al.*, 2015). The dose can vary greatly between gender and strain (Deeds *et al.*, 2011).

2.8.2 STZ ADMINISTRATION

STZ is commonly injected either intraperitoneally (IP) or intravenously S(IV), although other methods including subcutaneous, intracardiac, and intramuscular have been used in rodents (Deeds *et al*., 2011).

2.9 MEDICINAL PLANT

Medicinal plants from ancient time, have been used for preventive and curative measures for different ailments and diseases due to their readily availability and low cost of preparation. Large population of humans still rely on plants as a source of medicine

(Ogunrinola *et al.,* 2019). Medicinal plants have great impact in reduction of blood glucose level as well as other DM- related compounds (Raks *et al.,* 2017). A lot of plants are rich source of antioxidants. Antioxidants such as flavonoids, tannins and lignin as example of phenolic compounds, vitamins A, C, E are all found in plants (Ammar *et al.,* 2017).

Medicinal plants possessing antioxidant activity may reduce oxidative stress and improve the functions of various organs affected by hyperglycemia. *Vernonia amygdalina* contain natural antioxidants which have the potential to act as antioxidants against aqueous radicals and reactive species ions (Adeoye *et al.,*2018). However, population increase, inadequate drug supply, excessive cost of treatment and side-effects of several conventional drugs have increased the dependence on medicinal plant as source of medicine for a variety of ailments.

Today, medicinal plants are increasingly being used in most parts of the world as: hypolipidemic, contraceptive, or cytotoxic, antihypertensive, treatment for skin diseases, wound healers and hypoglycaemic. Hypoglycamia agents have been used in the management of diabetes mellitus (Mustafa *et al.,* 2011) that ascribe to its pharmacological functions, as anti-diabetic, antimalarial, anti-helminth, antibiotic, treatment of diarrhoea, dysentery, fertility inducer, kidney problems, stomach discomfort, hypolipidaemic, and other several uses (Ogunrinola *et al.,* 2019). Approximately 20% of known plants have been used in pharmaceutical areas positively in treating cancer and harmful disease (Ammar *et al.,* 2017) example of such plants is *Vernonia amygdalina* (VAM) known as bitter leaf.

2.10 VERNONIA AMYGDALINA (VAM)

V.amygdalina also called bitter leaf because of its bitter taste, is a shrub thatgrows predominantly in Tropical Africa (Ugwu *et al.,* 2010).VAM is a widely used local plant in Nigeria for both therapeutic and nutritional purposes. Bitter leaf is an important medicinal plant which has hypoglycemic, anti-diabetic and anti cholesterol properties (Achuba *et al.,*

2018). The bitter taste is due to anti-nutritional factors such as alkaloids, saponins, tannins and glycosides (Arhoghro *et al.,* 2009). As well as the flavonoids, oxalates, phytates, terpenes, steroids, coumarins, phenolic acids, lignans, xanthones, essential oil and sesquiterpenes (Ogunrinola *et al.*, 2019).

In Africa it is known by several local names such as Ewuro (Yoruba), Onugbu (Igbo), Oriwo (Bini/Edo), Ityuna (Tiv), Chusardoki or fatefate (Hausa) and Etidot (Ibiobio) (Egharevba et al., 2014)**,** Grawa (Amharic),Mululuza(Luganda), Labwori (Acholi), Olusia (Luo) and Andndoleh (Cameroon) (Enemali *et al.*, 2018) in English, it is referred to as bitter leaf. The plant grows throughout tropical Africa. It is drought-resistant and thrives in humid environment. It is grown commonly in Benin, Nigeria, Cameroun, Gabon and DR Congo, and to a lesser extent in their neighboring countries. (Egharevba *et al.,* 2014 and Eyo *et al.,* 2013).

Figure 2: Diagram and picture of *V. amygdalina*. (Udochukwu *et al.,* 2015).

TAXONOMICAL CLASSIFICATION

Kingdom:	Plantae
Phylum:	Spermatophyta
Subphylum:	Angiospermae
Division:	Magnoliophyta
Class:	Magnoliosida
Order:	Asterales
Family:	Asteraceae
Genus:	Vernonia
Species:	Amygdalina

Table 2: Medicinal Properties

PART	MEDICINAL USE	REFERENCES
Leaf	Flavouring and spicing of various types of food, malaria fever, diarrhoea, dysentery, hepatitis, cure for wounds as a substitute for iodine	(Enemali *et al.*, 2018, Eyo *et al.*, 2013)
Whole plant	Snakebite and insect sting treatment, dyspepsia, influenza, dysentery, malaria and respiratory infections.	(Egharevba *et al.*, 2014)
Root	Treatment of sexually transmitted disease, amenorrhoea. gastro-intestinal problems, malaria, gingivitis, toothache and fertility problems	(Eyo *et al.*, 2013, Egharevba *et al.*, 2014, Imaga *et al.*, 2013).

10.1 PHYTOCHEMISTRY OF VAM

Photochemical is a scientific word coined out from the Greek word Phyto meaning plant. Phytochemicals are defined as biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for human further than those attributed to macronutrients and micronutrients. They are chemical compounds produced by

plants which helps in their ability to thrive among competitors, predators or any pathogens. (Mamta *et al.,* 2013).

Phytochemicals are chemicals derived from plants and the term is often used to describe the large number of secondary metabolic compounds found in plants (Sasidharan *et al.,* 2011). In plants the naturally occurring chemical compounds are phytochemicals. Phytochemicals are beneficial to boost up immunolatory responses and also provide immunity against many diseases (Khalid *et al.,* 2018). They are chemical compounds formed during the Plants identification and metabolic process (Kutama *et al.,* 2018).

In the time past, some phytochemicals have been used for poisons and as traditional medicines. Recently, it was made known that more than 4,000 different phytochemicals have been catalogued and about 150 phytochemicals have been studied in detail. Phytochemicals can be found in several parts of the plants including the roots, stems, flowers, leaves, fruits and seeds but the pigment molecules are usually concentrated in the outer layers of the various plants tissues (Wingsem *et al.,* 2015).

These chemicals are referred to as secondary metabolites which comprises of several classes and these includes; alkaloids, flavonoids, phenols, tannins, coumarins, glycosides, gums, polysaccharides, terpenes, terpenoids (Uma *et al.,* 2011).

2.10.2 HYPOLIPIDEMIA EFFECT OF VAM

Regular consumption of vegetables such as VAM and *Telfaria occidentalis* (Ugu) can help to regulate blood cholesterol level, a risk factor for heart attack and stroke and lead to a significant increase in serum good cholesterol (HDL-C) showing their protective roles and function in condition that affects the heart and blood vessels such as heart attack. (Oguwike *et al.,* 2013). Low triacylglycerol and low-density lipoprotein cholesterol (LDL-C) levels and

high density lipoprotein cholesterol (HDL-C) levels are desirable health outcomes known to have resulted from the use of some plant materials (Owen *et al.,* 2011).

Methanolic extract of VAM leaves also exhibit thypolipidemic effect through influence with the lipid metabolic pathway which successfully reduced the plasma and post-mitochondrial fraction cholesterol, low density lipoprotein (LDL), triglyceride and lipid peroxidation (LPO) level and increased the plasma HDL level. Ethanol extract of the plant was also able to reduce body weight by decreasing triglyceride level (Yeap *et al.,* 2010). Also VAM leaf possesses hypoglycaemic properties and is capable of stabilizing other biochemical and hematological abnormalities associated with DM and thus could be prescribed as main therapy for DM. This investigation was aimed at determining the anti diabetic and anti lipidemic effect of a combination of the extract from the leaves of VAM with an oral hypoglycemic agent, metformin, which belongs to the class of biguanides for hyperglycemic control (Adikwe *et al.,* 2010).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Plant Material

Freshbitter leaf (*V. amygdalina*) plant was used for this study.

3.2 Collection And Preparation Of Plant Material

The bitter leaf sample (*V. amygdalina*) was collected from an individual farm at Imushin, Ijebu-Ode, Ogun state in February 2019.

The leaves were separated from the stem, air-dried for two weeks, oven dried for 30 minutes at 100^{O}C and blended to powdery form with the use of a mechanical blender. The blended sample was weighed and stored in air-tight jar. Eighty four grams (84 g) of the blended powder was weighed into three (3) jars and was macerated in methanol with occasional shaking at room temperature for 72hours twice. Filtration was done using muslin cloth. The filtrate was concentrated in a vacuum at 60°C to about one-tenth the original volume using a rotary evaporator. The concentrates were kept in the oven (40°C) for complete dryness of the methanol extracts and were then stored in a refrigerator at −4°C.

3.3 Chemicals

Streptozotocin, citric buffer, Hydrochloride, Mayer's reagent, Molisch reagent, concentrated sulphuric (H_2SO_4) acid, chloroform, ammonia solution, Ferric chloride ($FeCl_3$), sodium hydroxide (NaOH), ninhydrin reagent, potassium ferricyanide,

3.4 Qualitative Phytochemical Analysis

The methanolic extract was tested for the presence of bioactive compounds using standard methods as described by Sofowora (1993), Trease and Evans (1989) and Harbone (1973) with slight modification.

3.4.1 Test for alkaloids (Mayer's test)

0.5g of the crude extract was dissolved in 5mls of distilled water. 2ml of 1% hydrochloride (HCl) was added and heated gently. 3mlof Mayer's reagent was added to the mixture. Turbidity of the resulting precipitate indicated the presence of alkaloids.

3.4.2 Test for carbohydrates (Molisch's test)

0.5 g of extract was dissolved in 5ml of distilled water. 2ml of Molisch reagent was added and the mixture was shaken properly. 2ml of conc. sulphuric (H_2SO_4) was poured carefully along the side of the test tube. Appearance of a violet ring at the interphase indicated the presence of carbohydrate

3.4.3 Test for glycosides (Borntrager's test)

0.5g of extract was dissolved in 5ml of distilled water. 3ml of chloroform was added and the mixture was shaken. The chloroform layer was separated and 2ml 0f 10% ammonia solution was added. The appearance of pink colour indicated the presence of glycosides.

3.4.4 Test for saponin (Froth test)

0.5g of extract was diluted with distilled water to 20ml and was shaken in a graduated cylinder for 5mins. Formation of foam indicated the presence of saponin.

3.4.5 Test for terpenoids (Salkowski's test)

0.5g of extract was dissolved in 5ml of distilled water. 2ml of chloroform was added and 3ml of conc. H_2SO_4 was carefully added to form a layer. The appearance of reddish brown coloration at the interphase indicated the presence of terpernoids.

3.4.6 Test for phenol (Ferric chloride test)

0.5g of extract was dissolved in 5ml of distilled water and 4 drops of ferric chloride ($FeCl_3$) solution was added. The formation of bluish black colour indicated the presence of phenol.

3.4.7 Test for flavonoid (Alkaline test)

0.5g of extract was dissolved in 5ml of distilled water and few drops of 10% sodium hydroxide(NaOH) solution were added. The formation of intense yellow colour indicated the presence of flavonoid.

3.4.8 Test for tannin

0.5g of extract was dissolved in 5ml of distilled water and 2ml of 2% $FeCl_3$ solution was added. The formation of blue-green coloration indicated the presence of tannin.

3.4.9 Test for protein (Ninhydrin test)

0.5g of extract was dissolved in 5ml of distilled water. 2ml of 0.2% ninhydrin reagent was added and the mixture was boiled for 5minutes. The formation of violet/blue colour indicated the presence of amino acids

3.4.10 Test for phytosterol (Libermann-Burchard's test)

0.5g of extract was dissolved in 5ml of distilled water. 2 drops of conc. $H_2SO_4$was added slowly along the side of the test tube. Change in colour (violet to blue) indicated the presence of steroids.

3.4. 11 Test for polyphenol

0.5g of extract was dissolved in 5ml of distilled water. 1ml of 2% $FeCl_3$ solution and 1ml of 1% potassium ferricyanide solution were added. The formation of green-blue colour indicated the presence of polyphenol

3.4.12 Test for fat and oil (Spot test)

Small quantity of the extract was pressed between two filter papers. The appearance of oil stain on the paper indicated the presence of fixed oil.

3.4.13 Test for anthraquinone

0.5g of extract was boiled with 10ml of H_2SO_4 and filtered hot. The filtrate was shaken with 5ml of chloroform. The chloroform layer was pipetted into another test tube and 1ml of ammonia was added. The formation of a violet colour indicated the presence of anthraquinone.

3.5 Animal Study Design

3.5.1 Experimental Animals

Twenty-five (25) albino male wistar rats were used for this study and kept in a well ventilated animal cages in the animal house, Department of Biological sciences, College of Basic and Applied Sciences, Mountain Top University, Nigeria. The animals were 5 per cage

and allowed free access to feed and water ad libitum and were allowed to acclimatize for 7 days before the commencement of the experiment. Conducive temperature was maintained.

3.5.2 Experimental design

The twenty-five (25) rats were randomly distributed into five (5) groups (I-V) of 5 animals each:

Group I: Normal control; water and feed

Group II: positive control; STZ (45mg/kg) induced diabetic rats

Group III: STZ(45mg/kg) induced diabetic rat treated with Metformin

Group IV: STZ(45mg/kg)and 150mg of *V. amygdalina* plant extract

Group V: STZ(45mg/kg)and 300mg of *V. amygdalina* plant extract

3.5.3 Induction Of Diabetes

Diabetes was induced in the rats by injecting intraperitoneally freshly prepared streptozotocin STZ dissolved in citrate buffer (0.1 M pH 4.5) to overnight fasted rats at a dose based on their body weight. After 72hours of STZ administration, blood was obtained from the rat's tail to confirm the fasting blood glucose (FBG) using a glucometer (Accu-check). Hyperglycemia was confirmed four days after injection and animals with blood glucose > 200 mg/dl were classified as diabetic.

3.5.4 Drug Administration

Metformin was administered to experimental rats of group 3 and *V. amygdalina* methanolic extract was suspended in distilled water and administered via oral gavage at doses based on body weight to the experimental rats of group 4 and 5. Drug administration was done for

7days. Groups 1 and 2 were not administered any drugs because they acted as control measures, with group 1 being the normal control and group 2 being the negative control.

3.5.5 Specimen Collection and Preparation

At the end of the treatment, rats were fasted overnight to confirm the FBG. Whole blood was collected using a capillary tube to puncture the eyes and blood was introduced to a lithium heparin sample tubes and ready for centrifugation.

Blood samples were centrifuged at 4000 rpm for 10minutes at $37^{O}C$ and the plasma was obtained.

3.6 Experimental Analysis

3.6.1 Determination Of Lipid Profile Levels

Lipid profile such as high density lipoprotein (HDL), total cholesterol (CHOL) and triglyceride (TG) were analysed with plasma collected from blood using Randox kit from Randox laboratories limited UK. Protocols were followed according to the manufacturer manure procedures.

3.6.2 Cholesterol (CHOL)

Reagents

1. Reagent (R1)

Pipes buffer	pH=6.90 50 mmol/l
Phenol	24 mmol/l
Sodium cholate	0.5 mmol/l
4-Aminoantipyrine	0.5 mmol/l
Cholesterol esterase	180 U/l

Cholesterol oxidase	200 U/l
Peroxidase	1000 U/l

PROCEDURE

	Blank	**Standard**	**Sample**
Reagent	1 ml	1 ml	1 ml
Distilled water	10µl		
Standard		10µl	
Sample			10µl

The solution was incubated for 5 minutes at 37°C. The absorbance of the sample (Asample) and standard (Astandard) was read at 500nm against the reagent blank within 60 minutes.

3.6.3 Triglyceride (TRIG)

Reagent Composition

Contents Initial Concentrations

R1a buffer

R1b . Enzyme Reagent

Reconstitute one vial of enzyme reagent R1b with 15ml of buffer R1a. Stable for 21days at +2 to +8ºC or 3days at +15 to +25ºC stored protected from light.

Pipes buffer	38.7 mmol/L, pH 7.5
4-chloro-phenol	3.4 mmol/L
Magnesium ions	16.9 mmol/L
4-aminophenazone	0.25 mmol/L
ATP	1.2 mmol/L

Lipases	≥ 10 u/mL
Glycerol kinase	≥ 0.4 u/mL
Glycerol-3-phosphate oxidase	≥ 1.5 u/mL
Peroxidase	≥ 0.5 u/mL
Sodium azide	0.05

Procedures

Using fresh distilled water in test tube to carry out a water blank.

	Reagent blank(µl)	standard(µl)	sample(µl)
Sample	-	-	10
Standard	-	10	-
Reagent	1000	1000	1000

Solution was incubated for 5mins at 37^{O}C. Absorbance of the sample and standard against the reagent blank was read at 500nm within 60minutes.

3.6.4 High density lipoprotein (HDL)

REAGENT COMPOSITION

Contents	Initial Concentrations of Solution
R1. Phosphotungstic Acid	0.55 mmol/l
Magnesium Chloride	25 mmol/l

STABILITY AND PREPARATION OF REAGENTS

R1. Macro assays: Contents ready for use undiluted. Stable up to the expiry date specified when stored at +15 to +25°C.

R1. Semi-micro assays: Predilute the precipitating reagent in the ratio 4 + 1 with redistilled water (dilute the contents of **80 ml** bottle with **20 ml** redistilled water). Stable up to the expiry date specified when stored at +15 to +25°C

PROCEDURE

1. Precipitation

Pipette into centrifuge tubes:

	Semi Micro(µl)
Sample/Standard	200
Precipitant (R1)	**--**
Diluted Precipitant (R1)	500

Solution was allowed to stand for 10 minutes at room temperature. Then centrifuged for 10 minutes at 4,000 rpm, the clear supernatant was separated within thirty minutes and the cholesterol content by the CHOD-PAP method was determined.

2. Cholesterol CHOD- PAP Assay

	Reagent Blank (µl)	Standard (µl)	Sample(µl)
Distilled Water	100	--	--
Supernatant	--	--	100
Standard Supernatant	--	100	--
Reagent	1000	1000	1000

3.6.5 Low density lipoprotein (LDL)

LDL was calculated in mg/dl as:

LDL Cholesterol = Total Cholesterol - $\frac{\text{Triglycerides}}{5}$ - HDL Cholesterol

3.6.6 Very low density lipoprotein (VLDL)

VLDL was calculated in mg/dl as:

$$\text{VLDL} = \frac{\text{Triglyceride}}{5}$$

3.6.7 Statistical analysis

The data obtained were expressed as means±S.D, and analyzed. The differences between the means were analyzed statistically with one-way analysis of variance ANOVA, using Graph Pad Prism version 8.2. Values of $p<0.05$ were taken to imply statistical significance.

3.7 GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

The GC-MS analysis was carried out using a Hewlett Packard Gas Chromatograph (Model 6890 series) equipped with a flame ionization detector and Hewlett Packard 7683 series injector, MS transfer line temperature of 250°C. The GC was equipped with a fused silica capillary column- HP-5MS (30 x 0.25 mm), film thickness 1.0 μm. The oven temperature was held at 50°C for 5 min holding time and raised from 50 to 250°C at a rate of 2°C /min, employing helium gas (99.999%) as a carrier gas at a constant flow rate of 22 cm/s. 1.0 micron of extract (1 mg dissolved in 1 ml absolute alcohol), at a split ratio of 1:30 was injected. MS analysis was carried out on Agilent Technology Network Mass Spectrometer (Model 5973 series) coupled to Hewlett Packard Gas Chromatograph (Model 6890 series) equipped with NIST08 Library software database. Mass spectra were taken at 70 eV/200°C, scanning rate of 1 scan/s. Identification of compounds was conducted using the database of

NIST08 Library. Mass spectrum of individual unknown compound was compared with the known compounds stored in the software database Library (Ajayi *et al.*, 2011).

CHAPTER FOUR

4.0 RESULT

4.1 PHYTOCHEMICAL SCREENING

The result of the phytochemical analysis carried out on the methanolic leaf extract of *V. amygdalina* revealed the presence of some important bioactive components which is shown in Table 3.

Table 3; Result of phytochemical screening of *V. amygdalina* methanolic leaf extract

NO.	PHYTOCHEMICALS	RESULTS
1.	Alkaloid	Positive
2.	Carbohydrate	Positive
3.	Protein	Positive
4.	Fat and oil	Positive
5.	Terpenoids	Positive
6.	Phenol	Positive
7.	Flavonoid	Positive
8.	Tannin	Positive
9.	Glycoside	Positive
10.	Phytosterol	Positive
11.	Polyphenol	Positive
12.	Saponin	Positive
13.	Anthraquinone	Negative

4.2 GAS CHROMATOGRAPHY-MASS SPECTROSCOPY (GC-MS) ANALYSIS

4.2.1 GC-MS chromatogram of hydro-methanolic extract of VAM

Figure 3-12 shows the GC-MS chromatogram of methanolic leaf extract of V. amygdalina. Peak, retention time, library ID, %of total, and chemical formular were identified.

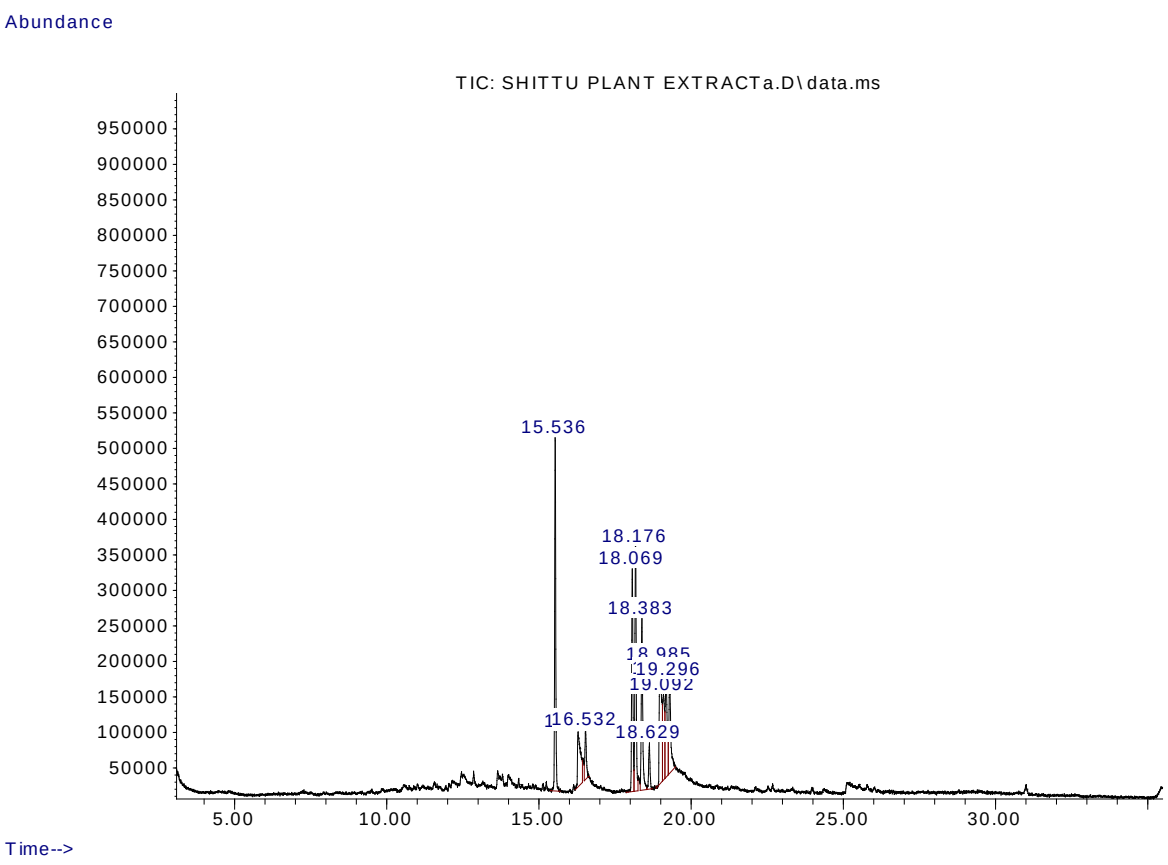

Figure 3; chromatogram of hydro-methanolic extract of VAM

Table 4; Phytochemical components identified by GC-MS in methanolic leaf extract of *V. amygdalina* showing the peak, retention time, library ID, % of total and chemical formula,

Peak	Retention time	Library ID	% of total	Chemical formula
1	15.536	Hexadecanoic acid, methyl ester	16.262%	$C_{17}H_{34}O_2$
2	16.288	n-Hexadecanoic acid	6.841%	$C_{16}H_{32}O_2$
3	16.532	Hexadecanoic acid, ethyl ester	3.197%	$C_{18}H_{36}O_2$
4	18.069	9, 12-Octadecadienoic acid (Z, Z)-, methyl ester	11.832%	$C_{19}H_{34}O_2$
5	18.176	Hexadecanoic acid, methyl ester	14.075%	$C_{19}H_{36}O_2$
6	18.383	Phytol	10.528%	$C_{20}H_{40}O$
7	18.629	heptadecanoic acid, 16-methyl-, methyl ester	2.611%	$C_{19}H_{38}O_2$
8	18.985	9, 12-Octadecadienoic acid	11.968%	$C_{18}H_{32}O_2$
9	19.180	Linoleic acid ethyl ester	8.203%	$C_{20}H_{36}O_2$
10	19.296	9, 12, 15-Octadecatrienoic acid, ethyl ester (Z, Z, Z)	7.828%	$C_{20}H_{34}O_2$

4.2.2 Chromatogram of each components of VAM

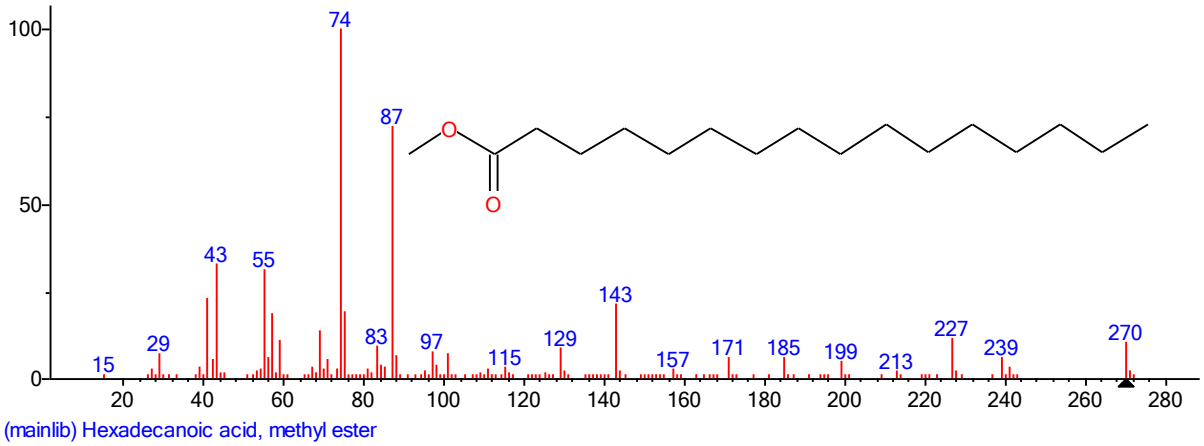

Figure 4; Chromatogram of hexadecanoic acid, methyl ester

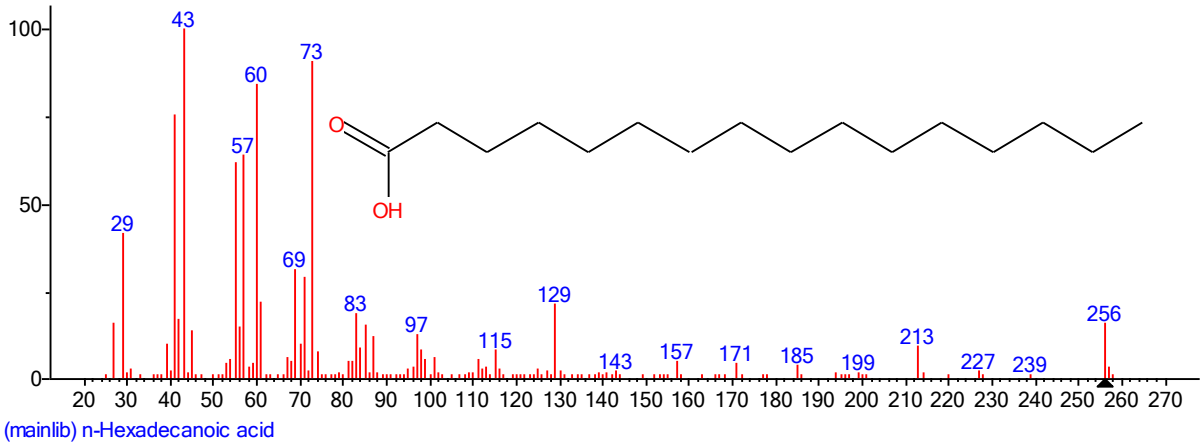

Figure 5; Chromatogram of n-Hexadecanoic acid

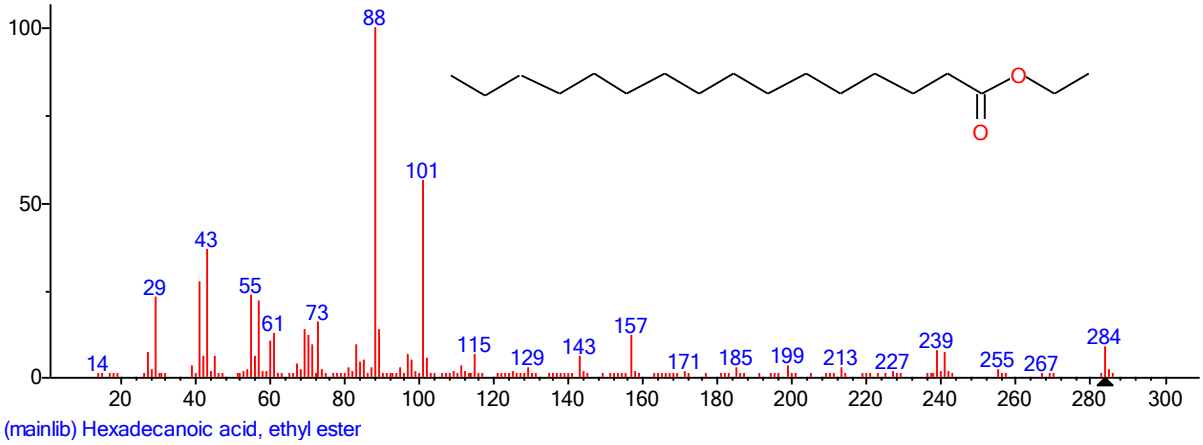

Figure 6; Chromatogram of hexadecanoic acid, ethyl ester

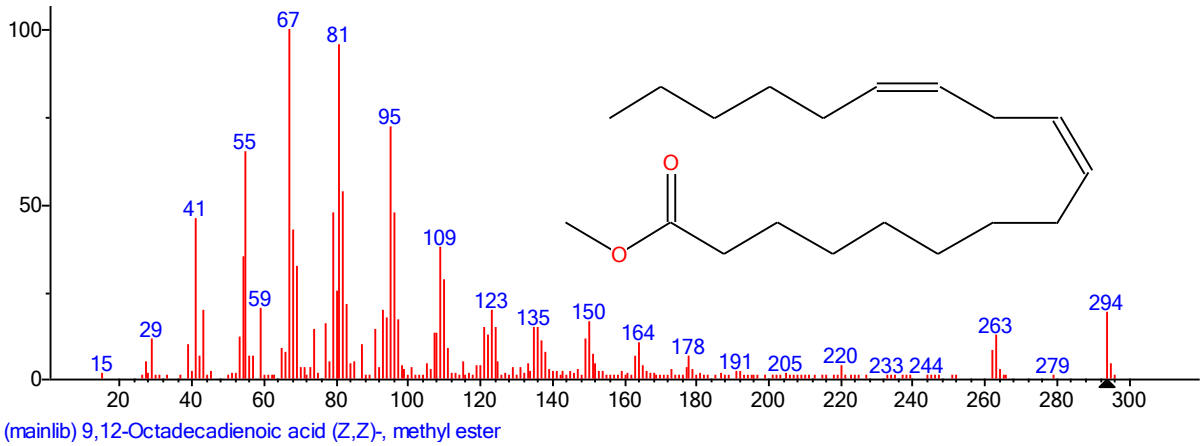

Figure 7; Chromatogram of 9, 12-Octadecadienoic acid (Z, Z)-, methyl ester

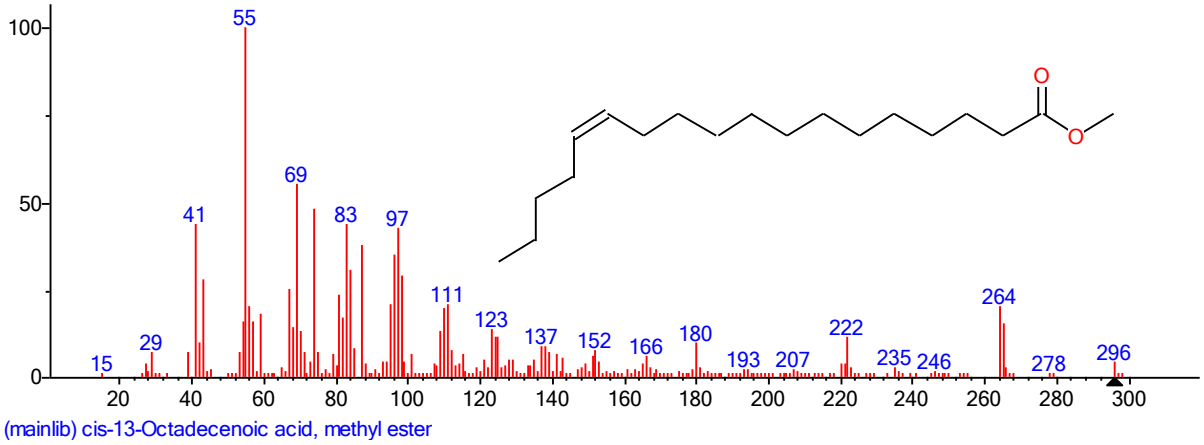

Figure 4.6; Chromatogram of cis-13-Octadecenoic acid, methyl ester

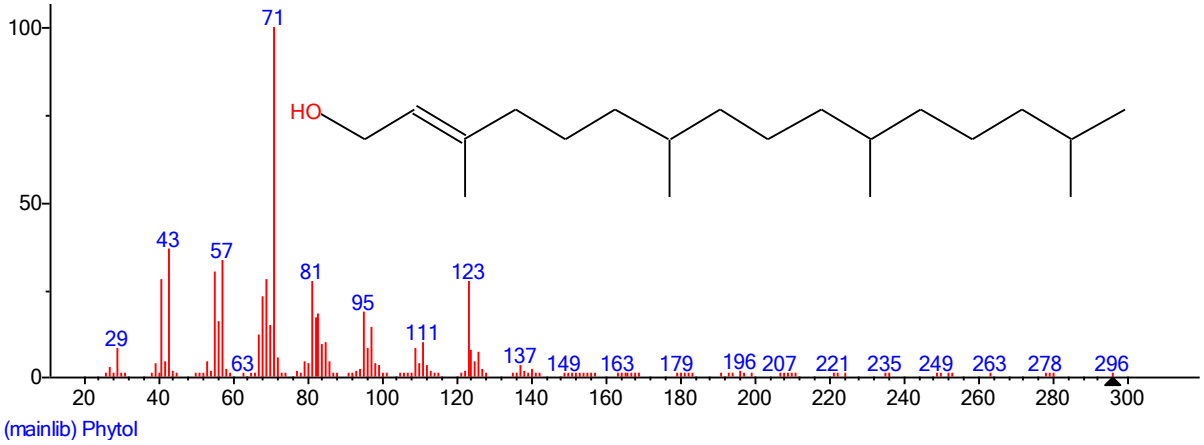

Figure 8; Chromatogram of phytol

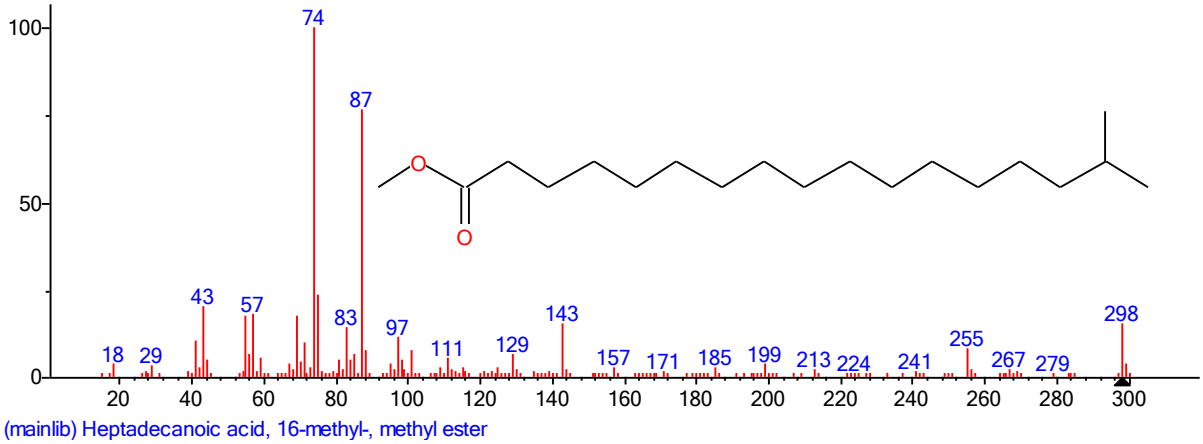

Figure 9; Chromatogram of heptadecanoic acid, 16-methyl-, methyl ester

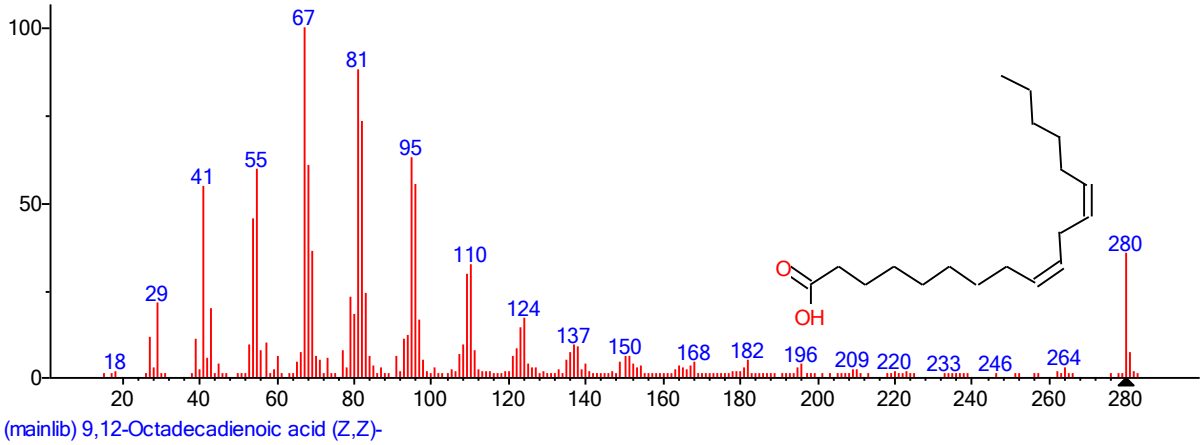

Figure 10; Chromatogram of 9, 12-Octadecadienoic acid

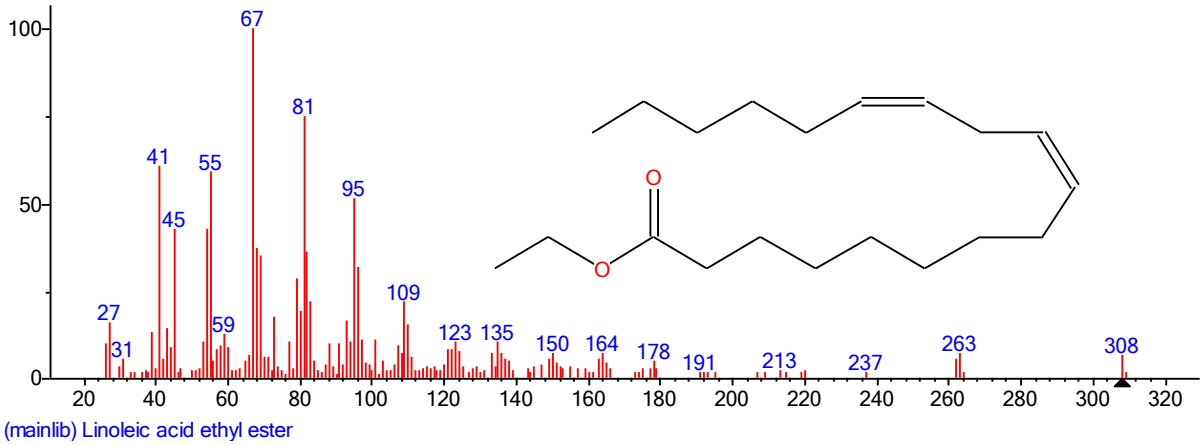

Figure 11; Chromatogram of Linoleic acid ethyl ester

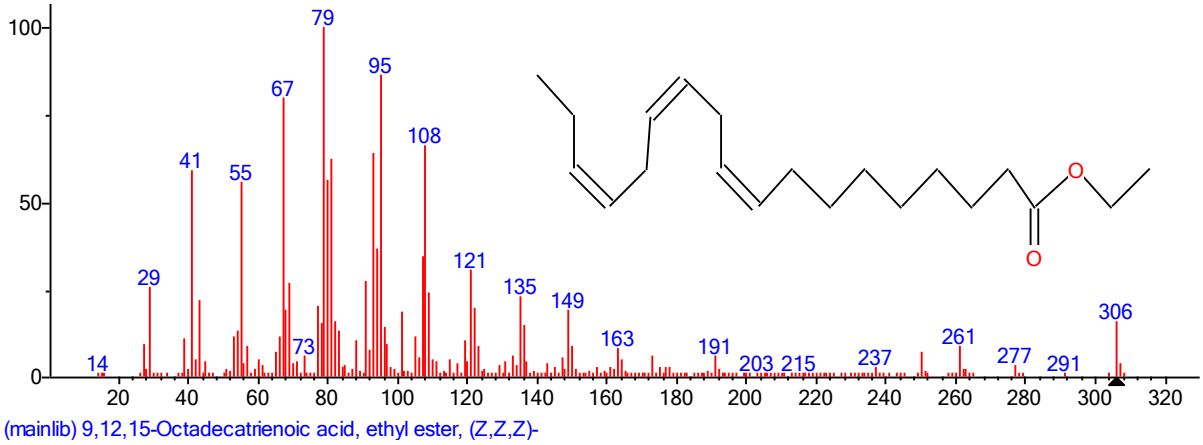

Figure 12: 9, 12, 15-Octadecatrienoic acid, ethyl ester (Z, Z, Z)

4.3 WEIGHT OF ANIMALS

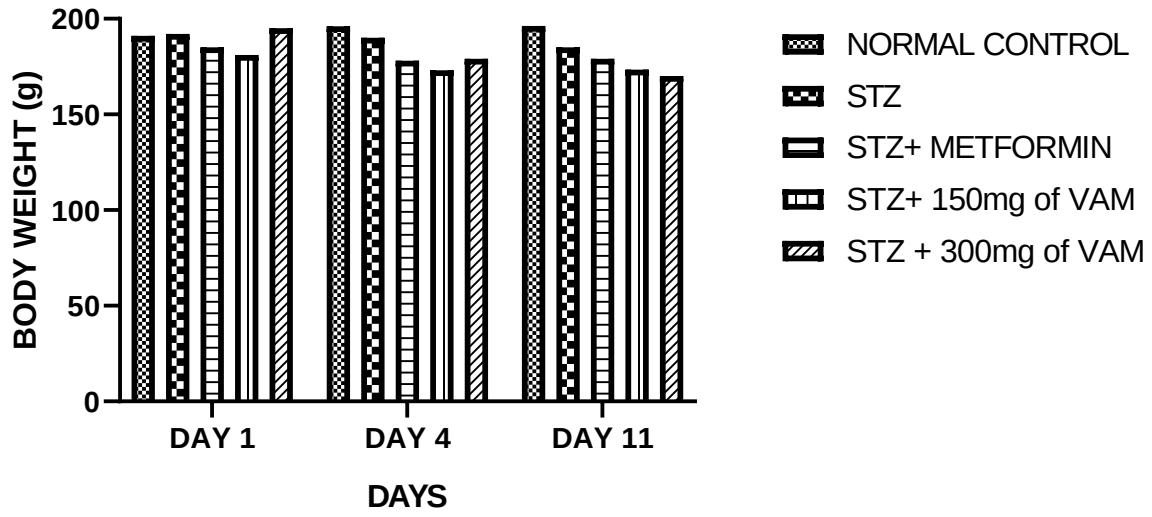

Figure 13: Values represent mean±S.E.M of 5 animals per group, significant at (P<0.05)

4.5 GLUCOSE CONCENTRATION

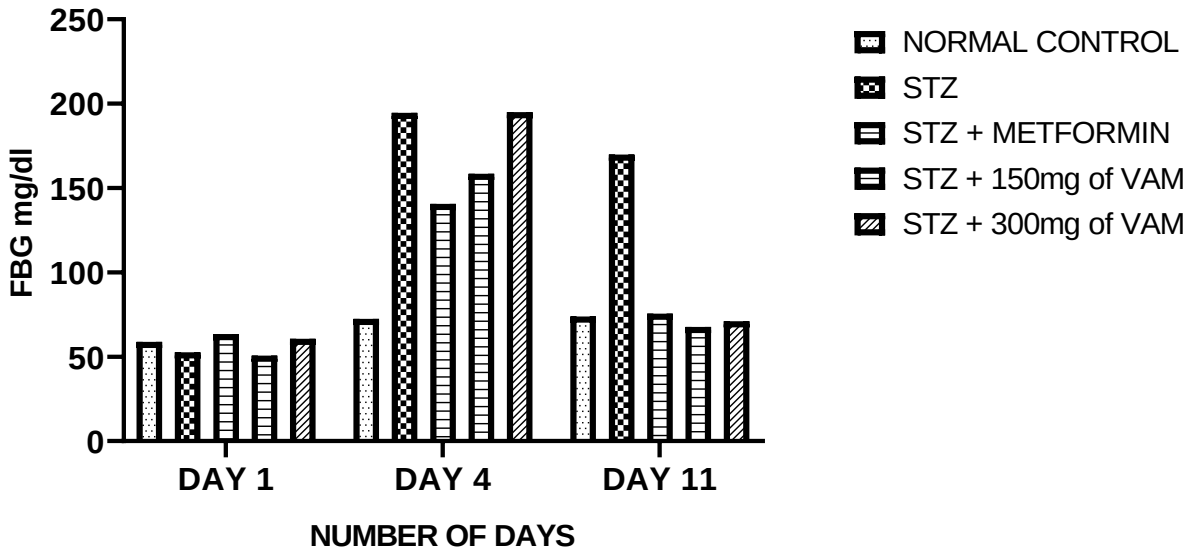

Figure 14: Values represent mean±S.E.M of 5 animals per group. There was a significant difference of (P<0.05).

4.6 LIPID PROFILE CONCENTRATION

Table 5: Lipid profile concentration in the plasma of rats

Parameters (mg/dl)	NC	STZ	STZ+MET	STZ+150mg of VAM	STZ+300mg of VAM
CHOL	44.58±4.13	46.25±4.96	40.53±11.19	53.54±6.36	49.0±5.45
TRIG	94.6±16.29	57.37±23.02	91.75±3.86	119.3±15.64	94.6±16.29
HDL	164.1±39.70	85.35±34.15	24.58±8.08	110.9±24.87	109.1±11.34
LDL	138.7±37.43	50.58±34.42	2.45±6.04	81.52±22.61	82.16±9.12
VLDL	19.06±3.26	11.47±4.59	18.4±0.80	24.12±3.19	22.02±2.27

Values represent mean±SD of 5 animals per group. Values for CHOL, TRIG and VLDL are not significantly different ($P>0.05$). there was a significant difference among the parameters for HDL and LDL ($P<0.05$).

CHAPTER 5

5.0 DISCUSSION

The effects of methanolic *V. Amygdalina* (VAM) leaf extract on lipid profiles in plasma of diabetic rat was investigated in this study. Diabetic-induced condition led to hyperglycemia. Twenty-four hours after STZ administration, all the rats treated with STZ displayed hyperglycemia, weakness, hyperlipidemia (Adewole *et al.*, 2009) and significant ($p<0.05$) loss of body weight. At the beginning of this study, the baseline weights were similar and glucose level of all the rats were normal in all groups. At day four after the administration of STZ the glucose level of each rat in the groups was high compared to the normal control group. After the administration of VAM for 7days the glucose level of the experimental rats dropped drastically to the normal glucose level. However, VAM treatment significantly reduced the blood glucose concentration of the VAM treated groups D and E. HDL and LDL was significantly ($P<0.05$) reduced in metformin and STZ treated groups compared to the normal control groups. CHOL, TRIG and VLDL improved toward normal level after treatment with VAM except the group treated with STZ+ 150mg of VAM that was high compared to the control group. Further investigation will be worked on this.

The lipid profile such as total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL) and very-low density lipoprotein (VLDL)) serves as diagnostic indices in conditions such as coronary heart disease, jaundice, hepatitis, and atherosclerosis. Hyperlipidaemia is one of the risk factors for coronary heart disease while cholesterol is the major lipid constituent of atherosclerotic plague (Imaga *et al.*, 2013).

In this study, phytochemicals such as Alkaloids, carbohydrate, protein, fats and oil, terpenoids, phenol, flavonoid, tannin, glycoside, phytosterol, polyphenol and saponin were present in V. amygdalina leaf extract while anthraquinone was absent. While major bioactive

compounds from methanolic extract as revealed by gas chromatography-mass spectrometry (GC-MS) were Hexadecanoic acid, methyl ester (16.3%), Hexadecanoic acid, methyl ester (14.1%), 9, 12-Octadecadienoic acid (Z, Z)-, methyl ester (11.8%), 9, 12-Octadecadienoic acid (11.9%), Phytol (10.5%).

CONCLUSION

Based on this study, it is concluded that *V. amygdalina* leaf extract possesses hypoglycaemic properties and is capable of stabilizing other biochemical and haematological abnormalities associated with DM and thus could be prescribed as main therapy for DM.

RECOMMENDATION.

Further investigation should be done on the effect of various extract of *V. amygdalina* (ethanolic, methanolic extract) on lipid profile.

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APPENDIX

1. FASTING BLOOD GLUCOSE PARAMETERS

Parameters (mg/dl)	NC	STZ	STZ+MET	STZ+150mg of VAM	STZ+150mg of VAM
DAY 1	58.8±2.80	52.6±1.72	63.4±5.55	50.6±1.69	60.6±4.1
DAY 2	72.40±2.85	194.50±57.45	140.60±38.28	158.40±35.33	195.00±39.39
DAY 3	196.34±4.21	184.88±60.01	179.00±4.92	173.40±18.80	169.63±9.07

2. WEIGHT PARAMETERS

Parameters (mg/dl)	NC	STZ	STZ+MET	STZ+150mg of VAM	STZ+150mg of VAM
DAY 1	190.87±6.80	191.56±4.85	185.07±7.74	181.04±14.91	194.83±9.29
DAY 2	196.04±5.82	189.93±4.48	177.79±7.05	172.68±15.71	178.67±7.66
DAY 3	196.34±5.55	184.88±5.9	179.00±7.31	173.40±13.88	169.63±7.51