AMELIORATION OF METABOLIC DERANGEMENT BY BIOACTIVE COMPOUNDS FROM *Vernonia amygdalina* IN STREPTOZOTOCIN-INDUCED DIABETES

BY

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CERTIFICATION

This thesis titled "Amelioration of Metabolic Derangement by Bioactive Compounds from *Vernonia amygdalina* in Streptozotocin-induced Diabetes"by OJIEH, Anthony Emeka, meets the regulation governing the award of the degree of Doctor of Philosophy of Delta State University and is approved for its contribution to scientific knowledge and literary presentation.

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DECLARATION

I, OJIEH, Anthony Emeka declares that "Amelioration of Metabolic Derangement by Bioactive Compounds from *Vernonia amygdalina* in Streptozotocin-induced Diabetes", is my research work and all the literature used or quoted therein have been acknowledged by complete referencing. This thesis is an original work and has not been submitted, and will not be submitted to any university or institution other than Delta State University, Abraka for the award of a Ph.D. degree.

..……..
OJIEH, Anthony Emeka

DEDICATION

This research work is dedicated to God almighty, the supreme fountain of all knowledge.

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My sincere gratitude goes to God almighty for His mercy, love and grace, love all through the period of this project work. To my wife Felicity and lovely children Valerie, Sophia and Olivia, I say I love you and thanks for your patience and understanding.

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LIST OF ABBREVIATIONS

1. 4-HNE: 4-Hydroxynonenal (58, 59)
2. ADA: American Diabetes Association (16)
3. ADH: Antidiuretic Hormone (50)
4. ADP: Adenosine Di Phosphate (31, 59, 78)
5. ALP: Alkaline Phosphatase (114, 132, 139)
6. ALT: Alanine Transaminase (42, 83, 84, 113, 114, 132)
7. AMP: Adenosine Mono Phosphate (28, 38, 136)
8. AMPK: AMP-activated Protein Kinase (136)
9. AST: Aspatate Transaminase (42, 82, 113, 132)
10. ATP: Adenosine Tri Phosphate (31, 37, 59, 75, 79, 127)
11. B-cell: Beta Cell (13)
12. BEN: Benzene (114)
13. BUN/SUN: Blood/Serum Urea Nitrogen (47, 48, 52, 53, 54)
14. BUT: Butanol (114)
15. CAT: Catalase (134)
16. cCr: Creatinine Clearance (52)
17. CHL: Chloroform (114)
18. CPK: Creatine Phosphokinase (51)
19. Cr: Creatinine (52)
20. DAN: Diabetes Association of Nigeria (4, 9)
21. DKA: Diabetes Keto-Acidosis (10)
22. DM: Diabetes Mellitus (11)
23. DNA: Deoxyribonucleic Acid (20, 31, 32, 60, 62)
24. DPP: Dipeptidyl Peptidase (137)
25. ETY: Ethyl Acetate (114)
26. F1, 6-P2: Fructose 1,6- Bisphosphate (37)
27. F6P: Fructose 6-Phosphate (37)
28. GAA: Guanido Acetic Acid (51)
29. GAD: Glutamic Acid Decarboxylase (11, 16)
30. GC-MS: Gas Chromatography – Mass Spectra (136)
31. GFR: Glomerular Filtration Rate (47, 52, 53)
32. GK: Gluckokinsae (38)
33. GLP: Glucagon-Like Peptides (137)
34. GLUT: Glucose Transporter (6, 7, 31, 32, 37, 136)
35. GSH: Glutathione (56, 57,58, 62, 92)
36. GSIS: Glucose Stimulated Insulin Secretion (34, 59)
37. GSSG: Glutathione Disulphide (57)
38. HbA1c: Glycated Hemoglobin (16, 17)
39. HDL: High Density Lipoprotein (11,112, 131)
40. HGP: Hepatic Glucose Production (127)
41. IAA: Insulin Antibodies (11)
42. ICA: Islet Cell Antibodies (11, 16)
43. IDDM: Insulin Dependent Diabetes Mellitus (10)
44. IDF: International Diabetes Foundation (8)
45. IRS: Insulin Receptor Substrates (7)
46. LDH: Lactate Dehydrogenase (132)
47. LDL: Low Density Lipoprotein (111, 112, 131, 139)

48. MDA: Malondialdehyde (58. 59, 60, 61, 62, 134)
49. MET: Metformin (114)
50. MI: Myocardial Infarction (18)
51. mRNA: Messenger Ribonucleic Acid (5)
52. NADH /NAD+: Nicotinamide Adenine Di-nucleotide (31, 42, 76, 77, 79, 80, 81)
53. NADPH: Nicotinamide Adenine Dinucleotide Phosphate (36, 40, 73, 74, 129)
54. NIDDM: Non-Insulin Dependent Diabetes Mellitus (10, 12)
55. NIH: National Institutes of Health (3)
56. OGTT: Oral Glucose Tolerance Test (16)
57. PEP: Phosphoenol Pyruvate (38, 75, 78)
58. PFK: Phosphofructokinase (38, 75)
59. PPAR: Peroxisome Proliferator-Activity Receptor (136, 138)
60. RBC: Red Blood Cells (43)
61. ROS: Reactive Oxygen Species (27)
62. RXR: Retinoid X Receptor (136)
63. SAM: S-Adenosyl Methionine (51)
64. sCr: Serum Creatinine (47, 51, 53, 54)
65. SGOT: Serum Glutamic Oxaloacetate Transaminase (41)
66. SGPT: Serum Glutamic Pyruvic Transaminase (42)
67. SOD: Superoxide Dismutase (54, 91, 123, 124, 134)
68. T-CHOL: Total Cholesterol (111, 112)
69. TG: Triglyceride (138)
70. TPVR: Total Peripheral Vascular Resistance (18)
71. TRIG: Triglycerides (111, 112)
72. UNA: Urea Nitrogen Appearance (54)
73. WHO: World Health Organization (11, 13, 16)

ABSTRACT

The search for herbal preparations with anti-diabetic properties continues to demand significant attention as the incidence of diabetes mellitus worldwide is on the increase despite all well-established treatment modalities. In recent times, herbal preparations are being recognized as a good source of medicinal formulations for diabetes mellitus management and in some instance as compliment therapy to existing anti-diabetic drugs. However, most of these herbal preparations are yet to be investigated fully with the sole aim of understanding how they the metabolism of carbohydrate, also to the extent of identifying the bioactive compounds in the herbal preparation with hypoglycemic properties. This study investigates the influence of bioactive components of Vernonia amygdalina extract on the management of diabetes mellitus. Fresh samples of bitter leaves having being air-dried, were crushed and soaked in ethanol for 48hours. The resultant mixture was sieved and allowed to stand for the ethanol to evaporate; the ethanol-free solution was subsequently subjected to liquid-liquid fractionation. Some fresh Vernonia amydgdalina leaves were also macerated and sieved to get a liquid crude extract. Adult male Wistar rats (150-200gm) were randomly selected, 5 rats per group with a total of 12 groups (n=5). The grouping is as follows; 1a and 2a were control groups,1b, 1c, 1d, 1e and 1f (normoglycemic groups), and 2b, 2c, 2d, 2e, 2f served as the diabetic groups. Diabetes was induced in the rats by injecting streptozotocin at 60mg/kg as a single dose, intraperitoneally. The treatment groups were put on Vernonia amgydalina extract at 300mg/kg/day. Group 3, a comparative diabetic group was placed on metformin at 50mg/kg/day. Fasting glucose level and body weight of the rats were monitored weekly. At the expiration the treatment period (28days), the animals were sacrificed; blood samples were collected and centrifuged and the resulting serum obtained for biochemical analysis. The live were also harvested and part (0.5gm) homogenized for biochemical analysis. Data were analyzed using SPSS package and expressed as mean ± SEM. Results showed an upsurge glycolytic enzyme activity in rats treated with the crude extract (hexokinase 422.92±13.42µU/mg.liver), compared to untreated diabetic rats (328.56±38.82 µU/mg.liver) and increased pyruvate kinase activity (treated 668.30±11.95U/g.liver; untreated 304.98±20.76U/g.liver). There were also increased the pentose phosphate pathway enzymes activity observed with the plant treatment. The bitter leave demonstrated significant reduction in fasting glucose level (diabetic rats 307.40±12.18; VA 105.67±17.68; Metformin 204.67±152.11mg/dl), showed increase serum HDL level (Diabetic rats 38.06±2.08; VA 55.58±6.01; Metformin 43.65±7.64mg/dl), also showed reduction in liver enzyme ALP (VA 19.01±3.21; Diabetic rat 90.24±6.09; Metformin 18.97±8.50) and ALT (VA 17.99±6.35; Diabetic rats 133.12±5.43; Metformin 37.32±3.61U/L). Analysis of the plant with GC-MS, elucidated bioactive compounds with hypoglycaemic properties such as Phytol, Palmitic acid, stearic acid and oxirane. Bitter leaf in this study also promoted glycolytic and the pentose phosphate pathways which are alternate glucose metabolic pathways in a hyperglycaemic state. The plant may also ameliorate renal and liver complications associated with diabetes while improving lipid metabolism.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND TO THE STUDY

For decades, there has been intense research into herbal treatments and alternative medicine for the cure of several chronic and terminal diseases such as cancers, obesity and diabetes (Jung et al., 2006). Over time, numerous drugs have been employed in the management diabetes, with burdens of cost and unpleasant effects on the part of these drugs which reduce patient's compliance, therefore the renewed curiosity in the use of traditional medicine (Zaidi, 1998). This renewed and more scientifically directed work in the field of ethno-medicine is born out of the need to alleviate the adverse effects common to orthodox and allopathic treatments, the expensive nature of such medications and to a large extent, the failure of the sole use of orthodox medications (Arias et al., 2004).

One medicinal plant that has continued to receive a lot of attention due to the numerous curative potentials that it has demonstrated is Vernonia amygdalina commonly referred to as bitter leaf which is purported to possess antioxidant activity from radical scavenging (Ayoola et al., 2008).Ingestion of crude Vernonia amygdalina and raw chewing by healthy human subjects were found to control post-prandial blood glucose without inducing severe hypoglycaemia (Okolie et al., 2008). It also showed anti-malaria activity against Plasmodium falciparum in vitro (Tona et al.,2004) and is now recommended locally for the management of hyperglycaemia related diseases, like diabetes mellitus (Cefalu et al., 2011).

Diabetes mellitus is a metabolic disorder characterized by an abnormal and chronic elevation in plasma sugar levels (hyperglycaemia), consistent polyuria, visual impairment, blindness, kidney disease, nerve damage, heart disease, and the loss of glucose homeostasis due to loss of insulin production or the effective utility of insulin or both (Ozougwu et al., 2013).

It is one of the world's commonest diseases affecting both the rich and poor with 347 million people reported worldwide to be suffering from diabetes (Danaei et al., 2013).

1.2 RESEARCH QUESTIONS

To guide the focus in this study, the following questions heve been raised;

1) DoesVernonia amygdalina have hypoglycaemic property, as has been stated by some

authors?

2) What active chemical compounds can be found in the plant?

3) DoesVernonia amygdalina exhibit dose dependent toxicity?

4) Is the plant capable of ameliorating assault to tissues resulting from injection of diabetogenic agent?

5) Is the plant able to affect lipid and protein metabolism in the body? Answers to the questions have been provided by the study, thereby providing clearer understanding and empirical basis for recommending the Vernonia amygdalina for use in management and treatment of diabetes mellitus.

1.3 OBJECTIVES OF THE STUDY

General Objective: To investigate the hypoglycemic properties of Vernonia amygdalina.

Specific Objectives:

To;

1) Ascertain the active phytochemical compounds in Vernonia amygdalina.

2) Ascertain of the hypoglycemic property of the crude and semi-purified leaf fractions of the plant.

3) Investigate the mechanism of action of the plant.

4) Assess the property of Vernonia amygdalina to heal tissues damaged by complication of Diabetes, using renal and liver function tests.

1.4 SCOPE OF THE STUDY

The research is an experimental study; it lasted 10 weeks (2 weeks for pilot study and 8 weeks for the treatment period).Only male Wistar rats were used, to eliminate possible variations due to gender related factors such as menstrual cycle. "Ethical conditions governing the use of animals for research was adhered to in accordance with Ward and Elsea (1997) and all experiments, were conducted in compliance with NIH Guidelines stated animal care and its use for experimental purposes, revised in 1985".

1.5 SIGNIFICANCE OF STUDY

Alternative medicine and herbal therapy seems to be the direction for most part of the

world today in the management of chronic diseases; results from this study may provide an insight and a basis for informed and guided use of Vernonia amygdalina as a good alternative drug for management of diabetes especially on long term.

1.6 JUSTIFICATION OF STUDY

The expensive nature of orthodox treatment and medications for diabetes has impoverished many and reduced patient's compliance to treatment. The use of injectable (such as insulin) and possible complications that can follow (such as lipodystrophy, secondary infections, and hypoglycaemia) may also affect compliance, given the fact that the management of diabetes in most cases is long-term. Ogberaet. al (2005), reported that Diabetic Association of Nigeria (DAN) estimate of diabetics in Nigeria is put at about 10 million.

Following urbanization and its effects on the life style of individuals, this figure would have certainly increased a great deal since then (Ogbera et al., 2005). Having an alternative medication which is readily available in the environment and otherwise cheap would serve as an alternative management for diabetes and its anttendant complications.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 DIABETES MELLITUS

The term diabetes mellitus according to World Health Organization (1999), describes a metabolic disease with multiple etiologies characterized by chronic and sustained hyperglycaemia manifesting as derangement in the metabolism of fat, carbohydrate and protein that result from deficiency in insulin secretion, defective insulin action, or both (Ozougwu). It arises from an inability of the body to metabolize blood glucose either due to the absence of insulin or the insufficiency of insulin in the body. Manifestations of uncontrolled diabetes mellitus ranges from long-term damage, dysfunction and failure of various organs (Kumar and Clark, 2002). Diabetes mellitus is a rather common metabolic disorder, affecting individuals both of developed and developing countries (Erasto et al., 2005).

Insulin is the key hormone involved in the storage and controlled release of the chemical energy available from food within the body. It is synthesized in the beta cells of the pancreatic islets (Kumar and Clark, 2002).Within the beta cell, ribosomes manufacture pre-proinsulin from insulin mRNA. The hydrophobic 'pre' portion of the pre-proinsulin allows it to be transferred to the Golgi apparatus, and is subsequently enzymatically cleaved off.

Proinsulin is parceled into secretory granules in the Golgi apparatus, where they mature and pass towards the cell membrane for storage prior to being released. The proinsulin molecule folds back andits stabilized by disulphide bonds. Biochemically, fragments of the inert peptide called connecting peptide (C-peptide) splits off from the proinsulin during the secretory process, leaving insulin as a complex linked by two peptide chains. Portions of insulin and C-peptide are released into the circulation. A small amount of insulin may also be secreted by the beta cells directly via the constitutive pathway, which bypasses the secretory granules (Kumar and Clark, 2002).Within plasma, insulin travels through the portal circulation to the liver. 50% or there about of the secreted insulin is degraded in the liver; and the residue degraded by the kidneys.C-peptide is only partially extracted in the liver (and hence provides a useful index of the rate of insulin secretion), but is mainly excreted by the kidneys (Nishi et al., 1990).

Insulin is the major regulator of intermediary metabolism, although its actions are modified in many aspects by other hormones, such as glucagon, epinephrine, cortisol and growth hormone; these cause greater production of glucose from the liver and less utilization of glucose in fat and muscles for a given level of insulin.

The actions of insulin in the fasting and post-prandial states differ. In the fasting state, its concentration is low and it acts mainly as a hepatic hormone, modulating glucose production (via glycogenesis and gluconeogenesis) from the liver.

In the post-prandial state, the insulin concentrations are high and it then suppresses glucose production from the liver and promotes the entry of glucose into peripheral tissues. Permeability of cell membrane to glucose requires a carrier mechanism. A family of specialized glucose transporter (GLUT) proteins carries glucose through the cell membrane into the cells (Shepherd and Kahn, 1999). These carrier proteins consists of GLUT-1, which enables basal non-insulin stimulated glucose uptake into many cells. GLUT-2 facilitates glucose uptake by beta cell, which initiates glucose sensing. GLUT-3 enables non-insulin mediated glucose uptake into the brain neurons and placenta and GLUT-4 enables much of the peripheral actions of insulin.

It is the channel through which glucose is taken up into muscles and adipose tissue cells following stimulation of the insulin receptors (Shepherd and Kahn, 1999). Insulin receptors are glycoproteins, coded in chromosome 19, the short arm which can be found on the cell membrane of many cells (Lee and Pilch, 1994). It consists of a dimer with two Alpha-subunits, which include the binding site for insulin, and two beta-subunits, which traverse the cell membrane. When insulin binds to the Alpha-subunits, it induces a conformational change in the beta-subunits, resulting in the activation of tyrosine kinase via autophosphorylation and initiation of a cascade response involving a host of intracellular substrates. These intracellular substrates include a group of enzymes referred to as insulin-receptor substrates (IRS). Varieties of IRS such as IRS-1, IRS-2, IRS-3) are usually expressed in different tissues. The net effect is to activate some of these enzymes, while inactivating others (Guyton and Hall, 2011). A resultant effect of the migration of the GLUT-4 glucose transporter to the surface cell increases transport of glucose into the cell.

The insulin-receptor complex is then internalized by the cell, insulin is degraded, and the receptor is resent to the cell surface (Seabright and Smith, 1996).

2.1.1 Epidemiology and Prevalence of Diabetes Mellitus:

The disease has gained worldwide attention because of it vast distribution among individuals of various nationality and works of life, and its socio-economic impact on nations' and the world's economy, making it one of the diseases of high world burden that raises keen interest and concern of the international community (Das and Samarasekera, 2012).

According to the International Diabetic Federation (IDF), 2010 estimate, the prevalence of diabetes was put at 285 million, equivalent to 6.4 % of the world`s adult population. With a projected estimate by 2030, put around 438 million, corresponding to 7.8 % of the adult population (IDF, 2011). Currently, with a worldwide incident of 5% in the general population, diabetes mellitus is considered as an epidemic.

The number of adult with diabetes in the world is projected to raise from 135 million in 1995 to 300 million in the year 2025 worldwide (Torben, 2002). And consequent upon this galloping rise in the prevalence of disease, it is projected that by 2030, diabetes mellitus is likely to be the seventh leading cause of death worldwide, accounting for 3.3% of total deaths in the world (WHO, 2014). A projection has been made that by 2025, countries like China, India and America would have the largest number of diabetics (Ramachandran et al., 2002). India has been acclaimed the “Diabetic capital of the world”. In 2007, there were about 23.6 million children and adults in the United States, about 7.8 % of the population, suffering from diabetes. The national diabetes information clearing house put the average cost of management of diabetes mellitus at $132 billion in the United States alone, with the management of associated complications exceeding $ 100 billion yearly. (Edwin et al., 2006). Also, there are many patients in communities worldwide with undiagnosed diabetes. Stress and unhealthy eating habits have been implicated as the possible cause of the increasing prevalence in the past two decades. According to the Diabetes Association of Nigeria (DAN, 2010), about six million Nigerians are estimated to be living with diabetes. A breakdown of the figure from DAN (2010), shows that about 3,336,000 sufferers are females and 40 % of them are elderly; 2,400,000 sufferers are male and 30% of them are elderly and about 240,000 are children. The association also stated that the disease has no particular geographic spread but is seen throughout the country with about 70% amongst urban dweller. It also pointed out that the disease is seen in all ethnic groups including minorities. Survey shows that in Nigeria,

crude prevalence in males is 1.6% and 1.9% in females below the age of 45years. After the ages of 45year, it rises to 5.4% in males and 5.6% in female. Kinnear et al., (1963), in a hospital based study in the University College Hospital, Ibadan, and reported prevalence of 0.38%. Ohwovoriole et al., (1989), during a diabetic screening exercise for the world diabetes day, "November 14th", found a prevalence of undiagnosed diabetes of 1.6% in males and 2.2% in females within Lagos and environ.

2.1.2 Classification of Diabetes Mellitus:

There are various types of diabetes discovered over the years ranging from congenital diabetes, gestational diabetes. However, considering their etiologies in 1997, the American Diabetes Association revised the nomenclature for the major types of diabetes. Insulin dependent diabetes mellitus and non-insulin-dependent diabetes mellitus, the old classification was reviewed as they were considered misleading. These terms had been confusing and had frequently resulted in classifying the patient based on treatment rather than etiology (Pittas and Greenberg, 2003). The new classification of diabetes based on etiology is shown below:

- Type 1 diabetes: pancreatic beta islet cell destruction resulting in absolute insulin deficiency
- Type 1b, a sub-type 1, presents with DKA then behaves like type 2
- Type 2 diabetes: results from varying degrees of insulin resistance and insulin deficiency
- Gestational diabetes

2.1.3 Type I Diabetes Mellitus (IDDM):

In type 1 diabetes, there is hypoinsulinaemia resulting to a constant rise in the blood glucose level (hyperglycaemia). Type I diabetes results from an autoimmune attack on the ?-cells of the pancreas (Pamela et al., 2008).

There are two basic etiologies according to WHO (1999) for type I diabetes; autoimmunity and idiopathic etiologies. Autoimmunity theory explains an autoimmune attack on the islet cells by the pancreatic antibodies. Immune dysregulation, due to genetic susceptibility and environmental modifiers, cause autoantibodies to develop against variety

islet cell components, like decarboxylase antibodies (GAD-65), glutamic acid, insulin antibodies (IAA) and islet cell antibodies (ICA512/IA-2). These antibodies serve as markers for Type I DM (Frisk and Diderholm, 2000). It is a partly inherited disorder but does not manifest at first until it is triggered by an environmental factor such as certain infections, food, toxins with some evidence pointing at Coxsackie B4 virus but its onset is unrelated to lifestyle (Frisk and Diderholm, 2000). The idiopathic theory explains that some subjects with clinical manifestations of Type I diabetespossess no marker for autoimmunity and no evidence of an autoimmune disorder is demonstrable (McLarty et al., 1990).Type 1 diabetes also can result from surgical removal of the pancreas (Muniraj and Chari, 2012).

The disease is hereditary, predisposing an individual to a possible manifestation on trigger. On exposure to the trigger there is an activation of autoimmune response on the pancreas leading to the production of pancreatic antibodies which destroy the cells of the pancreas (usually 80-90% of pancreatic?-cells population) resulting in manifestation of diabetic symptoms (Pamela et al., 2008). At this point, the pancreas is non-responsive to the presence of glucose in the blood stream and ultimately fails in the production of insulin. Type I diabetes may develop very abruptly, over a period of a few days or weeks, with three principal sequelae: (1) increased blood glucose, (2) increased utilization of fats for energy and formation of cholesterol by the liver, and (3) depletion of the body's proteins (Guyton and Hall, 2011).

2.1.4 Type II Diabetes Mellitus (NIDDM):

In type II Diabetes Mellitus, the pancreatic cells are normal; hence there is production of insulin. However, there is inability of the body to utilize the insulin produced, a phenomenon that has been referred to as, "insulin resistance". Amongst the diabetes types, Type II is the commonest, affecting approximately 90% of the diabetic population in the United States (Pamela et al., 2008).

This type of Diabetes results mainly due to resistance to insulin in the liver and skeletal muscle, increased glucose production in the liver, excessive fatty acid production by fat cells as well as relative insulin deficiency. According to Loghmani (2005), the following factors contribute to the development of Type II; obesity, Age (onset of puberty is associated with increased insulin resistance), physical inactivity, Genetic predisposition, Conditions associated

with insulin resistance, (for example, polycystic ovary syndrome) and Dietary factors such as sweetened drinks in excess, saturated fats, increases the risk of polyunsaturated and monounsaturated fat.

2.1.5 Pathophysiology of Type II Diabetes Mellitus.

In Type II diabetes, early stage clear features is reduced insulin sensitivity, after which insulin secretion decreases due to down regulation of the receptors leading to insulin resistance (Loghmani, 2005). Insulin resistance reduces ability of tissues to respond to normal circulating concentration of insulin. Insulin resistance (which increases with weight gain) alone, does not lead to Type II diabetes. Rather, Type II diabetes develops insulin-resistant in individuals who also show impaired?-cell function (Harvey and Ferrier, 2011). Insulin resistance and subsequent risk for the developing this type of diabetes is commonly observed in the elderly, and in individuals who are obese, physically inactive, or in the 3-5% of pregnant women who develop gestational diabetes. This class of individual is unable to sufficiently compensate for insulin resistance with increased insulin released.

2.1.6 Other forms of Diabetes Mellitus Gestational diabetes:

Gestational diabetes is glucose disturbance resulting in hyperglycaemia usually recognition for the first time during pregnancy. Those most at risk for developing this type of diabetes include those with previous glucose intolerance history, older women, and those with a history of large for gestational age babies, women from certain high-risk ethnic groups, and any pregnant woman who has elevated fasting, or casual, blood glucose levels (Garrison, 2015).

2.1.7 Clinical Signs and Symptoms of Diabetes:

Mellitus Regardless of the diabetes type, common presentations includes polyuria to skin manifestations such as tags and vitiligo. However, classical symptoms of diabetes are listed below and represented in the diagram that follows; Polyuria, polydipsia, fatigue, blurring of vision, weight loss, are in association with glycosuria and ketonuria (Vijan, 2010).

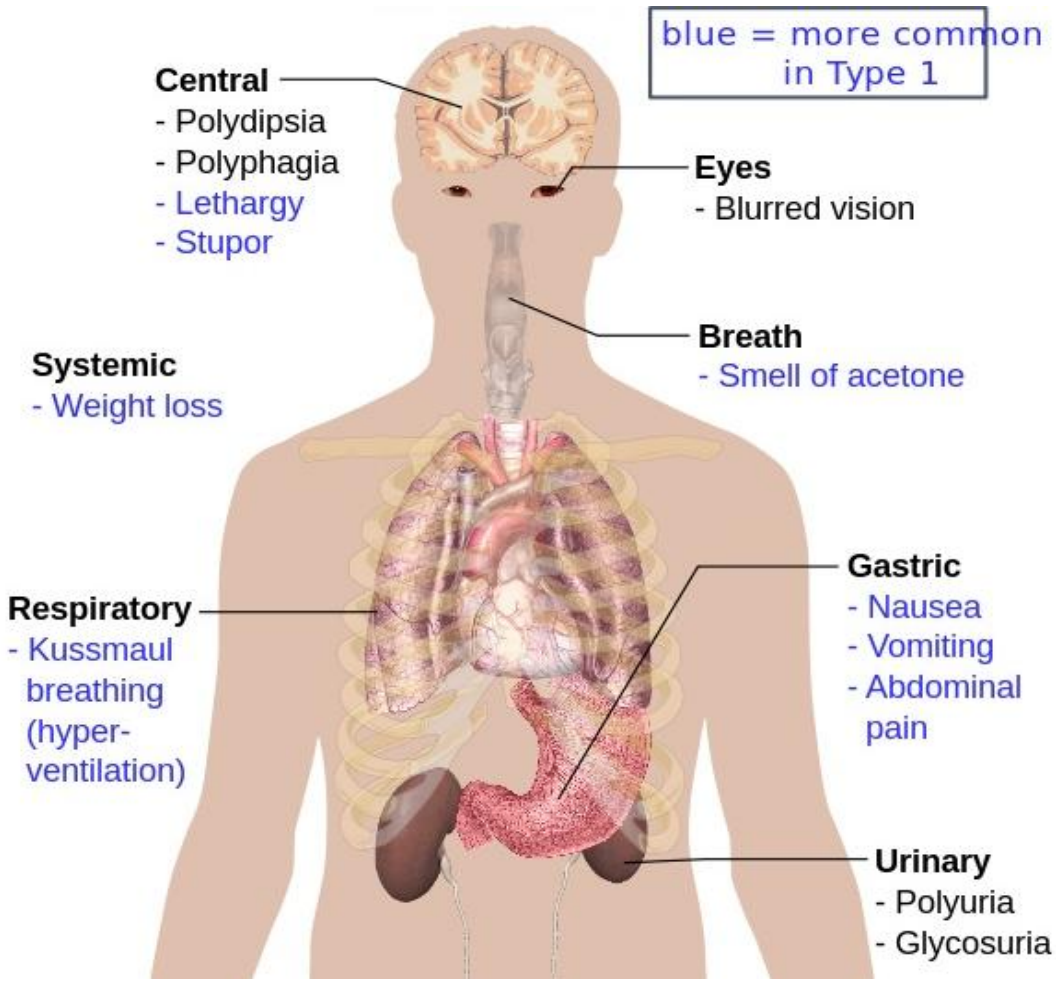


Fig 1: Diagram showing an overview of the most significant symptoms of diabetes, (Source; Vijan, 2010)

As diabetes progresses without medication and care, diabetic skin manifestations such as begin to manifest (Vijan, 2010).

2.1.8 Diagnosis of Diabetes Mellitus: Diabetes mellitus can be diagnosed in the laboratory by monitoring metabolic changes in the metabolites and constituents of the body. A very important marker is blood glucose level. Diabetes has over the years been diagnosed by measurement of blood glucose level since diabetes is marked by an elevated glucose level. However, according to WHO and ADA, the diabetic verdict should not be given on the basis of a rise in blood glucose level since various factors (postprandial absorption, stress induced gluconeogenesis) can raise the blood glucose level of a healthy individual. Hence, diagnosis should be based on the level of;

1. Blood glucose level: measurement of blood sugar level.

2. Impaired fasting blood glucose: measurement of fasting 2 hours postprandial blood glucose level.

3. Oral glucose tolerance test (OGTT): It is a further test where fasting blood sugar fails. It is used to detect or test for impaired glucose tolerance.

4. Glycatedhaemoglobin (HbA1c): demonstrates an estimate ofglucose in plasma within the previous 2-3 months in a single measure. It's usually measure without the need to fast and can be done at any time of the day. Hence is usually most dependable in assessing the severity of the glucose disturbance and mostly reliable in making a diagnoses (Rohlfing et al., 2002).

5. Test for specific autoantibody markers such as islet cell antibody (ICA), Glutamic Acid Decarboxylase (GAD) (Maclaren et al., 1999).

6. Measurement of fasting insulin and C-peptide level can also be useful in the diagnosis of type 2 diabetes in children (Eppenset al., 2009). The basic criteria for ascertaining a diabetes condition is as shown below;

- Fasting plasma glucose level ≥ 7.0 mmol/l (126 mg/dl)
- Plasma glucose ≥ 11.1 mmol/l (200 mg/dL) two hours after a 75 g oral glucose load following an oral glucose tolerance test.
- Symptoms of hyperglycaemia and casual plasma glucose ≥ 11.1 mmol/l (200 mg/dl)
- Glycated haemoglobin (Hb A1C) ≥ 6.5%.

2.1.9 Complications of diabetes mellitus

Glucose is the metabolic fuel of the body as such it is required by all tissues and at a particular quantity at which the body can carry out its metabolic functions effectively. When there is an imbalance in glucose level either high or low, every single tissue that utilizes glucose will be affected. Hence Diabetes, poses a metabolic complication in the function of most of the body's organ and tissue. However, there are specific organs that it affects primarily (liver, kidney) because they directly control and metabolize body glucose. Some of such complications are;

2.1.9.1 Cardiovascular complications:

The Cardiovascular system is one of the most affected systems (Magdalene, 2014). There are morphologic and pathologic changes in this system resulting in atherosclerosis and microangiopathy (Brown et al., 2011), large vessel arterial disease (arise as a result of atherosclerotic lipid depositions which cause degenerative changes in the vessel leading to ulcerations, infarctions, fibrosis and thrombo-embolic disorder. According to Magdalene (2014), the vessels become stiff and reduced distensibility; there is reduced vascularity and increased blood pressure). Atherosclerotic coronary heart disease is viewed as the commonest cause of death in Diabetes Mellitus, accounting for about 53% of diabetic deaths. The commonest factor is myocardial infarction (MI) with women being more affected than men.

Diabetic peripheral vascular disease is especially characterized by the involvement of arterioles (especially those below 0.3mm in diameter). These vessels are critical for the regulation of total peripheral vascular resistance - TPVR- and of blood flow through the capillary bed. There is significant thickening of capillary basement membrane of all tissues including skin, skeletal muscles, adipose tissue, kidney, pancreas and peripheral nervous system. This alters tissue oxygen and nutrients delivery, leading to ischaemia and degenerative changes.

2.1.9.2 Diabetic Neuropathy

(DN) Diabetic neuropathy is poorly understood and inadequately investigated. It is increasingly evident that neuropathy plays a significant role in many other aspects of diabetes mellitus including abnormal visceral functions, hormonal secretory regulation and non-neurologic complications of diabetes mellitus (Amthor et al., 1994). According to Viniket al.,(2000) DN refers to symptoms and signs of neuropathy in a patient with diabetes after the exclusion other causes of neuropathy has been made. It accounts for mosthospitalization in diabetic patients and is the reason often for most amputations (Bansal et al., 2006). What causes diabetic neuropathy is mostly unclear; however metabolic components and ischemia are thought to play a role (Malik, 1999). "Hyperglycaemia results in raised endothelial vascular resistance with reduced nerve blood flow. Hyperglycaemia equally brings about depletion of nerve myoinositol via a competitive uptake mechanism.Though, stimulating polyol pathway in the nerve by the action of enzyme aldose reductase results in buildup of sorbitol and fructose within the nerve and induces non-enzymatic glycosylation of structural

nerve proteins" (Zochodne, 2008). Hyperglycaemia also induces oxidative stress. Protein kinase C activation has been linked to vascular damage in DN. These changes result in abnormal neuronal, axonal, and Schwann cell metabolism, which result in impaired axonal transport, affecting almost all organs of the body (Solomon et al., 2010).

2.1.9.3 Skin Changes:

30% of patients with diabetes mellitus develop one type of skin disorder or the other (Piérard et al., 2013). The skin disorders that are frequently encountered include dermatitis herpetiformis, psoriasis, and urticaria. The skin disorders of a diabetic are often more threatening than that of non-diabetics, as such skin lesions in diabetics require prompt aggressive management to prevent more serious complications (Sreedev et al., 2002).

2.1.9.4 Diabetic Retinopathy:

This is the leading basis of legal blindness for individuals aged 20-74 (Klein, 1995). One way in which diabetes affects the eye is the formation of a cataract. Cataract formation is a cloudiness of the lens, which can decrease visual acuity, and occurs more frequently and at a younger age in individuals affected with diabetes (Pollreisz and Schmidt, 2010). Diabetics often experience a cycle of retinopathy as blood sugar level alternates between excessively high and low. When blood sugar level increases, much glucose is transported to the eye together with excessive fluid migration leading to an accumulation of the solution in the eyes causing luring of vision. When the blood sugar levels decrease typically vision will return to normal. This may be a continuous cycle if the blood sugar is not well controlled.

2.1.9.5 Diabetic nephropathy

The diabetic patient is at risk of serious infectious and large vessel disease in the kidneys, glomerular microvasculopathy and it poses serious threat to the victim's longevity (Bangstad et al., 1994). Diabetic nephropathy occurs in approximately one third of all type II diabetes (Rehman et al., 2005).

2.1.10 Treatment and Management of Diabetes

Mellitus Diabetes mellitus is a chronic metabolic disease which presently has no

definitive cure but can be managed, management of diabetes focus on controlling the change blood sugar level and keeping it as close to normal (norrmoglycemia) as possible, without causing hypoglycemia. This can be achieved using a combination of lifestyle modifications and the use of appropriate medication. Lifestyle modifications involve diet control of consumption of food highly rich in carbohydrate and fat while increasing the consumption of protein food, fruits and vegetables. Exercise is also a very crucial in the management of diabetes. Medications used in the management of diabetes and its complications are generally regarded as hypoglycaemics and are aimed at reducing excessively high blood glucose level as normal as possible. Oral antidiabetic medications are prescribed especially when diet and exercise fails to control the blood glucose at the desired level (Derek, 2001). However, insulin administration by injection has been the age-long treatment for diabetes especially type I and metformin the first line choice drug for type II (Rojas and Gomes, 2013).

2.2 MECHANISM OF ACTION OF SOME HYPOGLYCAEMICS

1. Stimulation of beta cells to increase insulin production and increase responsiveness of the beta cells to both glucose and glucose analogues. Example includes the sulphonylureas and meglitinides (Proks et al., 2002).

2. Increasing the sensitivity of muscles and other tissues to insulin. They act by binding to peroxisome proliferator-activator receptors, which are a group of receptor molecules inside the cell nucleus. The ligand for these receptors are free fatty acids and eicosanoids, when activated, they are transported to the DNA, where they initiates transcription of a number of genes that ensure insulin sensitivity. Example is the thiazolidinediones.

3. Decreasing gluconeogenesis by the liver. It is the first line drug of choice for the treatment of type II diabetes; in particular, in over-weight and obese people and those with normal kidney function (it is only effective in the presence of insulin). They are the biguanides an example of which is metformin.

4. Delaying the absorption of carbohydrates from the gastrointestinal tract; these act by inhibiting upper gastrointestinal enzymes that convert dietary starch and other complex carbohydrates into simple sugars that can be readily absorbed they are Alkaline Phosphataseha-glucosidase inhibitors (Kavishankar et al., 2011).

These treatments are quite beneficial in managing diabetes but they also have their setbacks such as resistance with time and adverse effects such as toxicity in some patients. Examples of such disadvantages as recognized by researchers are; Michael et al., (2005), Dey et al., (2002) and Defronzo, (1999) "observed that thiazolidinediones may result in liver toxicity; sulphonylureas might worsen heart disease, cause hypoglycemia and increase in weight; bloating, gas, diarrhea, and abdominal discomfort and pain are major complaints with glucosidase inhibitors". Michael et al., (2005), noted that due to drug resistance, the effectiveness of drugs like Sulphonylureas drop by 44% amongst patients within six years and that about two-thirds of the drugs recommended for use in children have not been proven effective and safe for this patient population.It is also observed that the rise of diabetes in rural areas and poor nations is due to the expensive nature of anti-diabetic therapy and medications.

These limitations of currently available oral hypoglycaemicagents have resulted in the noncompliance of diabetic patients to medications. These has favored deep research to discover new drugs that can manage type 2 diabetes more efficiently with little or no side effects (Ranjan and Ramanujam, 2002). The rise in the need for such drugs especially in the rural areas have led to the development of indigenous, inexpensive herbal preparations as anti-diabetics either as crude or purified drugs (Venkatesh et al., 2008).

One of such herbal preparation is the use of Vernonia amygdalina as a hypoglycaemic herbal preparation. Studies conducted using Research done using Vernonia amygdalina to treat streptozotocin-induced diabetic animals showed a blood glucose reduction of 50% when compared to untreated diabetic animals (Nwanjo, 2004). Allium cepa also has been shown to reduce glucose levels in dose dependent manners with the highest percentage of reduction at 300mg/kg (Ozougwu, 2011). It's being reported also that coconut water possesses hypoglycemic properties and can find usefulness in the management of diabetes mellitus (Muanya, 2009).

2.2.1 Some observed Mechanism of Hypoglycaemic Action of Herbal Preparations:

- Stimulation glycolysis glycogenesis in the liver (Miura, 2001).
- Protective effect on the destruction of the beta cell (Chan et al., 2008).
- Prevention of pathological conversion of starch to glucose (Osman et al., 2012).
- Cortisol lowering activities (Gholap and Kar, 2004)

- Inhibition of Alkaline Phosphatase and amylase (Heidari et al., 2005)
- Inhibiting oxidative stress leading to pancreatic beta cells dysfunction found in diabetes (Hideaki et al., 2005)
- Inhibition in renal glucose reaborsption (Eddouks et al., 2002)
- Enhancing insulin secretion from beta cells of islets or/and preventing insulin degradative processes and reduction in insulin resistance (Pulok et al., 2006)
- Providing essential elements such as calcium, zinc, magnesium, manganese and copper, necessary for beta cells regeneration (Langerhans et al.,2006).

2.3 Vernonia amygdalina

2.3.1 Name and Classification

The highly potent medicinal plant is botanically referred to as Vernonia amygdalina; however, in Nigeria various tribes and languages where it is found identify it in their locale as follows;

Oriwo (Edo, Nigeria)

Chusardoki (Hausa, Nigeria)

Ewuro (Yoruba, Nigeria)

Onugbu (Igbo, Nigeria)

Etidot (Cross River State Nigeria)

Onugbo (Urhobo, Nigeria)

Ityuna (Tiv, Nigeria)

In some other parts of Africa, it iis known as follows:

Awonoo, Awonwene, Jankpantire (Ghana)

Mululuza (Luganda)

Ndolé (Cameroon)

English name: Bitter leaf.

Fig 2: Picture of a fresh *Vernonia amygdalina* plant (In its natural habitat)

The plant is botanically classified under;

Domain:	Eukaryote
Kingdom:	Plantae
Subkingdom:	Viridae plantae
Phylum:	Tracheophyta
Subphylum:	Euphyllophytina
Class:	Spermatopsida
Subclass:	Asteridae
Orders:	Asteraces
Family:	Compositae
Subfamily:	Cichoriodeae
Tribe:	Vernonieae
Subtribe:	Vernoniinieae
Genus:	*Vernonia*
Specific epithal:	*Amygdalina*
Scientific name:	*Vernonia amygdalina* (Bonsi *et al.,* 1995)

2.3.2 Plant Description

Vernonia amygdalinais a shrub of about 2 to 5m high with green leaves, characteristic odour, a bitter taste, Petiolate leaf of about 6mm diameter and oblong-lanceolate to narrowly elliptic-lanceolate in shape, 7-15cm long, 3-7cm broad. The leaves is cuneate at the base and acuminate at the apex, it has inflorescence corymbiform with numerous capitula, its floret is usually white or bluish, it has pappus of white or russet colour (Mshana et al., 2000). Theplant grows better in a humid environment but is also drought tolerant. It grows on a number of ecological zones and is used as a hedge plant in some communities (Bonsi et al., 1995).

2.3.3 Origin and Distribution

Vernonia amygdalina is a tropical plant seen in the savanna and the forest regions, often forming dense growth bushes in Guinea savanna and Western Cameroun. It is widely distributed in the African tropics and found in countries like Nigeria, Ghana, Cameroon,

Kenya and Uganda. It can thrive with conditions of low water and can grow in a variety of ecological conditions producing a large mass of forage, (Bonsi et al., 1995).

2.3.4 Uses of Vernonia amygdalina

In the tropics where it is found, Vernonia amygdalina is used for a variety of purposes. Generally, its use in Nigeria is for two major reasons; first is for nutritional purposes and secondly for its health benefits. The leaves are eaten after being washed and crushed, it is used as vegetable in local dishes e.g. ofe onugbu especially in Eastern Nigerian states. As an herbal preparation, it is employed in the treatment of various kinds of diseases and illnesses. All portions of the plant are of pharmaceutical benefits; the roots are chewed against gastro-intestinal diseases, enteritis, and anthelmintic. The leaves are chewed to stimulate the digestive system, as well as they reduce fever, the dried flowers are employed in the treatment of stomach disorders (Burkill, 1985).

In Zimbabwe, the chopped roots of Vernonia amygdalina are used for the treatment of sexually transmitted diseases (njovhera) (Kambizi and Afolayan, 2001). Its antifertility/abortificientpotency was shown by Desta (1994). In Eastern Uganda, a survey report showed that it was by far the most widely quoted plant for the treatment of malaria (Tabuti, 2003).

The leaves of Vernonia amygdalina may be consumed either as a vegetable (in soups) or aqueous extracts as tonics for the treatment of various illnesses. In Ethiopia, the leaves of the plant are used to treat skin wounds by Zay people (Giday et al., 2003). The roots of Vernonia amygdalina have been used to treat gingivitis, toothache and consequently its antimicrobial activity was established (Elujoba et al., 2005).

2.3.5 Phytochemistry of Vernonia amygdalina.

The herbal plant has been studied over the years and found to contain several secondary metabolites which points to its use and potency. Leaf extract of Vernonia amygdalinawas found to contain reducing sugar, polyphenolics, terpenoids, saponins, alkaloids, cardiac glycosides steroids or triterpenes, anthraquinone and coumarins without cyanogenic glycosides (Ayoola et al., 2008).

Tannins, glycosides and saponins without flavanoids could be obtained from its root

and bark extracts (Nduagu et al., 2008). Its bitter taste was reported to be due to the presence of antinutritional factors such as alkaloids, saponins, tannins and glycosides (Arhoghro et al., 2009).

Phenolic compounds identified in Vernonia amygdalinacanbe grouped into flavonoides, tannins and caffeoylqunic acid (Salawu and Akindahunsi, 2007). Flavonoids protect the cell as antioxidant against reactive oxygen species (ROS) and free radicals. Antioxidant activity of Vernonia amygdalina was contributed by the flavonoids which can be extracted from the leaves by using methanol extraction.

2.3.6 Biological Activities of Vernonia amygdalina

2.3.6.1 Antibacterial activity of Vernonia amygdalina.

The plant demonstrates antimicrobial activity against lumen bacteria and protozoa Newbold et al., (1997). Acetone extract of Vernonia amygdalinapossesses antibacterial activity towards Bacillus cereus, Bacillus pumilus, Bacillus subtilis, Micrococcus kristinae, Staphylococcus aureus, Enterobacter cloacae and Escherichia coli growth according to Kambizi and Afolayan (2001).

2.3.6.2 Anti-parasitic activity of Vernonia amygdalina

The methanolic extract of V. amygdalina possessed antitrichomonas activity with 100% of inhibition against Trichomonasvaginalis (Hakizamungu et al., 1992).

2.3.6.3 Anti-malaria/anti-plasmodial activity of Vernonia amygdalina

The ethanol, petroleum ether, dichloromethane, ethylacetate, acetone-water and isoamyl alcohol extracts of Vernonia amygdalina, possesses antimalarial activity, and its effective against Plasmodium falciparum specie in vitro (Tona et al., 2004).

This antimalarial effect of Vernonia amygdalina is contributed by its active compounds, or more specifically" sesquiterpene lactones such as vernolepin, vernolin, vernolide, vernodalin and hydroxyvernodalin" which exhibited antiplasmodial activity (Tona et al., 2004).

2.3.6.4 Anti-coagulant and anti-thrombic activities of Vernonia amygdalina

Awe et al., (1998) reported that Methanolic extract of Vernonia amygdalinaat 100 and 200 mg/kg induced 40 and 50% inhibition against thrombosis in mice. Vernonia amygdalina caused reduction of blood pressure and Vernolepin isolated from the plant has been shown to have anti-platelet activity and inhibition of platelet aggregation but the mode of action such as the effect on thromboxane A2 formation and on the level of cyclic AMP in platelets is yet to be unraveled.

2.3.6.5 Anti-viral activity of Vernonia amygdalina

Vlietinck et al., (1995) showed that Ethanol extract of the fruit (which is rarely found on most of the Vernonia amygdalina shrub) possessed antiviral effect on polio virus. 2.3.6.6 Anti-oxidant activity of Vernonia amygdalina Vernonia amygdalina ethanol extract was shown to possess antioxidant activity from radical scavenging test (Ayoola et al., 2008). Total flavonoid and phenolic contents was found to be correlated positively with total antioxidant activity of the plant.

Boiling of the plant also reduced its reducing capacity and free radical scavenging property but enhances its taste and reduces its toxicity (Oboh, 2005).

2.3.6.7 Hypoglycaemic activity of Vernonia amygdalina

Aqueous and n-hexane/isopropanol extract of Vernonia amygdalina had been reported to enhance the glucose utilization by muscle and hepatic cell, however, adipose cells utilizes it much less (Erasto et al., 2007). Aqueous (hot water) extract of Vernonia amygdalina leaf (500mg/kg) reduced blood glucose concentration of both normoglycemic and hyperglycemic rats induced by alloxan (Osinubi, 2007). Consumption of crude Vernonia amygdalina and raw chewing by normal human subjects were found to control post prandial blood glucose without inducing severe hypoglycemia (Okolie et al., 2008).

2.3.6.8 Toxicity and Safety Use of Vernonia amygdalina

Toxicity studies showed that Vernonia amygdalina only had mild toxic effect when administered at very high concentration (Njan et al., 2008). More importantly, safe consumption of dosage needs to be identified for women at different stages or vitality of pregnancy to avoid abortion since it may induce uterine contraction (Ijeh et al., 2008).

Generally, Vernonia amygdalina is safe to consume and is good for health unless it is consumed in very large quantities and the potential danger of taking this plant is much lower than the other common vegetables (Ojiako and Nwanjo, 2006).

2.4 EXPERIMENTALLY INDUCED DIABETES MELLITUS

Experimental studies of Diabetes Mellitus have been carried out through the years by the use of induced diabetes on laboratory animals. Such researches have been carried out to monitor the trend, compliance, response and effect of proposed medications for the said disease. Experimentally, diabetes mellitus can be induced in an animal employing certain chemical substances of various mechanism of action that will result in sustained hyperglycaemia and eventually diabetes mellitus.

Two prominent chemical toxins used in research laboratories are - Alloxan Monohydrate – Streptozotocin. These substances have shown marked induction of hyperglycaemia in laboratory animals such as rat, mice and rabbit.

2.5 STREPTOZOTOCIN

2.5.1 Chemistry

Streptozotocin is a naturally occurring, broad spectrum antibiotic and cyto-toxic chemical that is selectively toxic to the pancreatic insulin secreting beta cells in experimental animals (Hayashi and Kojima, 2006). It is an N-nitrosoure derivative of D-glucosamine that was first isolated from streptomycesachromogenes (Lewis and Xu, 2008). It is an alkylating and genotoxic agent that has antibacterial, diabetogenic, carcinogenic and tumoricidal, properties. Streptozotocin is a stereoisomer appearing as a pale yellowish or off- white crystalline powder. It is soluble in water, ketones, and lower alcohols and moderately soluble in polar solvent. Streptozotocin injection results in the degeneration of the islets beta cells and causes clinical symptoms of diabetes mellitus (such as frequent urination, increase thirst e.t.c.) to be seen in rats with 24-96hours, following single intraveneous or intraperitoneal injection of 40mg/kg of Streptozotocin.

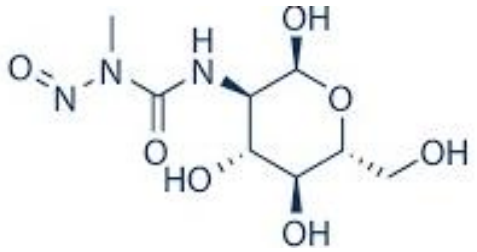


Fig. 3: Streptozotocin chemical structure. Source: (Lenzen, 2007)

2.5.2 Streptozotocin Mechanism of Action

The action of streptozotocin on B cells of the pancreas has been study broadlywith a clear insight to its mode of action. "The cytotoxic action of the diabetogenic agent is mediated through reactive oxygen species as cell undergoes oxidative stress.

According to Szkudelski, (2001) Streptozotocin is transported into B cell through a glucose transporter known as GLUT2, within the cell; it causes damage to the cell via alkylation of DNA. This damage results in activation of poly ADP-ribosylation, Poly ADP-ribosylation results in depletion of cellular NAD+ and ATP. Increased ATP dephosphorylations following the action of streptozotocin administration make available substrate for xanthine oxidase leading to the formation of superoxide radicals. Further, hydrogen peroxide and hydroxyl radicals are also generated. Streptozotocin releases toxic quantities of nitric oxide resulting in inhibition ofaconitase activity and causes DNA damage. As a result of this action, B cells undergo necrotic destruction. Streptozotocin by this action, affects the pancreas insulin secretion and causes a state of insulin dependent diabetes mellitus through its ability to induce a selective necrosis of the pancreatic related alkylating compound require their uptake into the cells." Typically nitrosourease are lipophilic, and tissue uptake is quick. However, due to the hexose substitution, streptozotocin is less lipophilic and this significantly affects the cellular distribution. Through the attachment of the methylnitrosourea moiety to the 2 carbon of glucose as a carrier molecule, stretozotocin is selectively accumulated in pancreatic beta cells and become a beta cell toxic and diabetogenic compound. The selective beta cell destruction by streptozotocin and the resulting diabetic metabolic state are clearly linked to glucose moiety in the chemical structure, which enables streptozotocin to enter the beta cell via the low affinity GLUT 2 glucose transporter in the plasma membrane (Elsner, et al., 2000).

2.5.3 Effect of Streptozotocin on Glucose Homeostasis.

The effects of streptozotocin on glucose homeostasis reflect toxin - induced abnormalities in pancreatic beta cell function. Initially, an inhibition of insulin biosynthesis and glucose-induced secretion as well as an impairment of glucose metabolism; both glucose oxidation and consumption are very prominent (Bedoye et al., 1996). On the other hand, streptozotocin has no direct inhibitory activity on glucose transport (Elsner et al., 2000) or upon glucose phosphorylation through glucokinase (Lenzen et al., 1987b). However, at later stages of functional beta cell impairment, deficiencies at the level of gene and protein expression and function of these structures become apparent (Wang and Gleichmann, 1998).

2.5.4 Stages of Streptozotocin Diabetes Induction

Diabetic induction occurs in a sequel of five stages according to Lenzen, (2007).

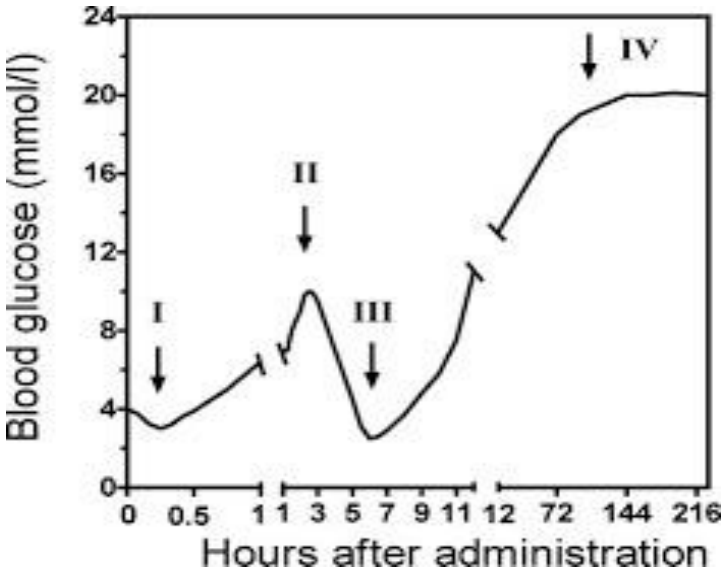


Fig. 4 Stages of streptozotocin action; Source: (Lenzen, 2007)

Stage 1(Compensation): As Streptozotocin induces destruction of Beta cells, resulting in a state of hypoinsulinaemia, the body responds to counter the hyperglycemic state by inducing an elevation in Insulin secretion, a phenomenon known as compensation. The commonest example of this phenomenon can be found in insulin resistance caused by obesity, which go with increased total insulin secretion (Polonsky et al., 1998) and increased acute glucose-

stimulated insulin secretion (GSIS) following an intravenous glucose challenge. Much of the elevation in insulin release undoubtedly comes from an increase in Beta-cell mass, which is often observed during autopsy studies in humans and animal models (Pick et al., 1998).

Stage 2 (Stable Adaptation): This stage is demonstrated by a loss of sensitivity to insulin by beta cells in a form of adaptation to the hyperglycaemic state. This is a stage of cell adaptation fasting levels between 5.0 and 7.3mMol/l (89-130 mg/dl). In stage 2, cells can no longer compensate as normal glucose levels are no longer be maintained. The stage is characterized by the loss of GSIS induced by glucotoxicity and Beta cell differentiation.

Stage 3 (Transient Early Decompensation): This is an unstable phase of early decompensation leading to an increase in glucose level relative to established diabetes of stage 4. This stage is short lived and eventually develops into the full blown diabetes.

Stage 4 (Stable Decompensation): This stage is demonstrated by a stable decompensation and severe Beta-cell dedifferentiation. It is characterized by a consistent rise in the blood glucose level that cannot be compensated by the body except by external source of insulin.

Stage 5 (Severe Decompensation): This stage shows severe decompensation and a significant reduction in Beta-cell mass, progressing to ketosis. One study shows that Streptozotocin does not induce diabetes or hyperglycaemia in humans however has been proven to cause diabetic induction in laboratory animals.

2.6 EXPERIMENTAL ALBINO RAT (Wistar Rat).

The Wistar rat is an outbred albino rat was first employed in 1906 at the *Wistar* Institute for extrapolatory medical research. Approximately half of strains are descendant from the original colony instituted by Henry Donaldson, a physiologist and scientific administrator, Milton J. Greenman, a genetic researcher and Helen Dean King, an embryologist. The Wistar rat is stands out in modern times amongst the animals used for laboratory research.

Figure 5; Wistar rat (*Rattus Norvegicus*) Albino breed.

The characteristics of the rat that has made it a choice animal for laboratory and experimental research is its unique anatomy and physiology that is as close as possible to that of humans. Also, it is readily available and can serve as a substitute in researches concerning human anatomy and physiology.

2.7 METABOLIC DISTURBANCES OF DIABETES

There are several metabolic complication associated with glucose imbalance observed in diabetes mellitus. These imbalances are revealed in the physiology of the body where its effect is manifested. The liver and the reticular endothelia cells are mostly affected among other body systems. In the liver, metabolic activities of certain enzymes are altered. While some inactive liver enzymes are activated, others are either inactivated or their activities are reduced. Liver function, kidney function, glycaemic pathways, antioxidant activity and lipid profile are also affected in the metabolic complications of the diseases.

2.7.1 Disturbances in Carbohydrate (Glucose) Metabolism

Carbohydrate metabolism goes through various metabolic pathways, some of which include; glycolysis, gluconeogenesis, glycogenesis and pentose phosphate pathway. During glycolysis, a pathway found in most organisms, a small amount of energy is generated as a glucose molecule is metabolized into two molecules of pyruvate. When circulating plasma glucose levels are high, some of this is stored as glycogen by a process called glycogenesis and when circulating plasma glucose is low, the stored glycogen is degraded to release glucose by a process known as glycogenolysis. Synthesized of glucose can also occur from non-

carbohydrate precursors by a mechanism known as gluconeogenesis. In pentose phosphate pathway glucose-6-phosphate an intermediate byproduct of glucose metabolism, is converted to ribose- 5-phosphate, the sugar from which nucleotides is synthesized, nucleic acids as well as other types of monosaccharides.NADPH, an important cellular reducing agent, is also produced by this pathway (McKee and McKee, 2011).

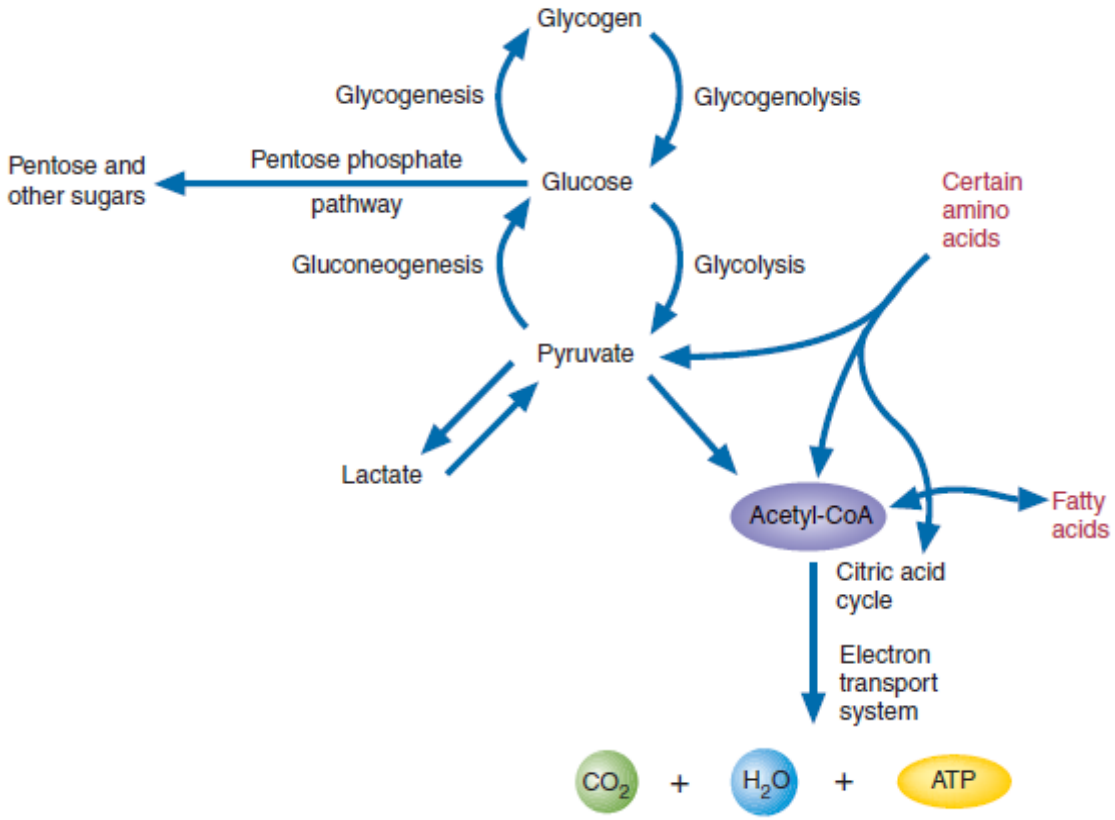


Figure 6: Major Pathways in Carbohydrate Metabolism.

Source: McKee and McKee, (2011)

2.7.1.1 Glycolysis and Gluconeogenesis

Glycolysis is the glucose metabolic pathway in which glucose is broken down into pyruvate/lactate within cells by a process of phosphorylation. Glycolytic pathway also leads to energy production in the form of ATP. Depending on the cell type,there are several rate-limiting steps in the glycolytic pathway such as the uptake of glucose, glucose phosphorylation, and/or conversion of fructose-6-phosphate (F6P) into fructose-1, 6-bisphosphate (F1, 6P2)."As such, glucose transporter-4 (GLUT4), glucokinase (GK), and 6-

phosphofructo-1-kinase (6PFK1) are of great importance in regulating the rate of glycolysis.

The reaction sequence in gluconeogenesis pathway is largely the opposite of glycolysis. Three glycolytic reactions (the stage catalyzed by pyruvate kinase and hexokinase, PFK-1) are irreversible; in gluconeogenesis alternate reactions catalyzed by different enzymes are used to bypass these obstacles. As it's the case with most metabolic pathways, gluconeogenesis outcome is affected largely by substrate availability, allosteric effectors, and hormones. Gluconeogenesis outcome is enhanced by high concentrations of lactate, glycerol, and amino acids. These substrates are usually readily available in high quantity in high-fat diet, during starvation. The four major enzymes in gluconeogenesis pathway, (pyruvate carboxylase, PEP carboxykinase, fructose-1,6-bisphosphatase, and glucose-6-phosphatase) are modulated to varying degrees by allosteric modulators. For instance, fructose-1,6-bisphosphatase is stimulated by citrate and inhibited by AMP and fructose-2,6-bisphosphate. Acetyl-CoA activates pyruvate carboxylase. (The concentration of acetyl-CoA, a substrate of fatty acid break down, is usually high during starvation).The figure below provides an overview of the allosteric control of glycolysis and gluconeogenesis.

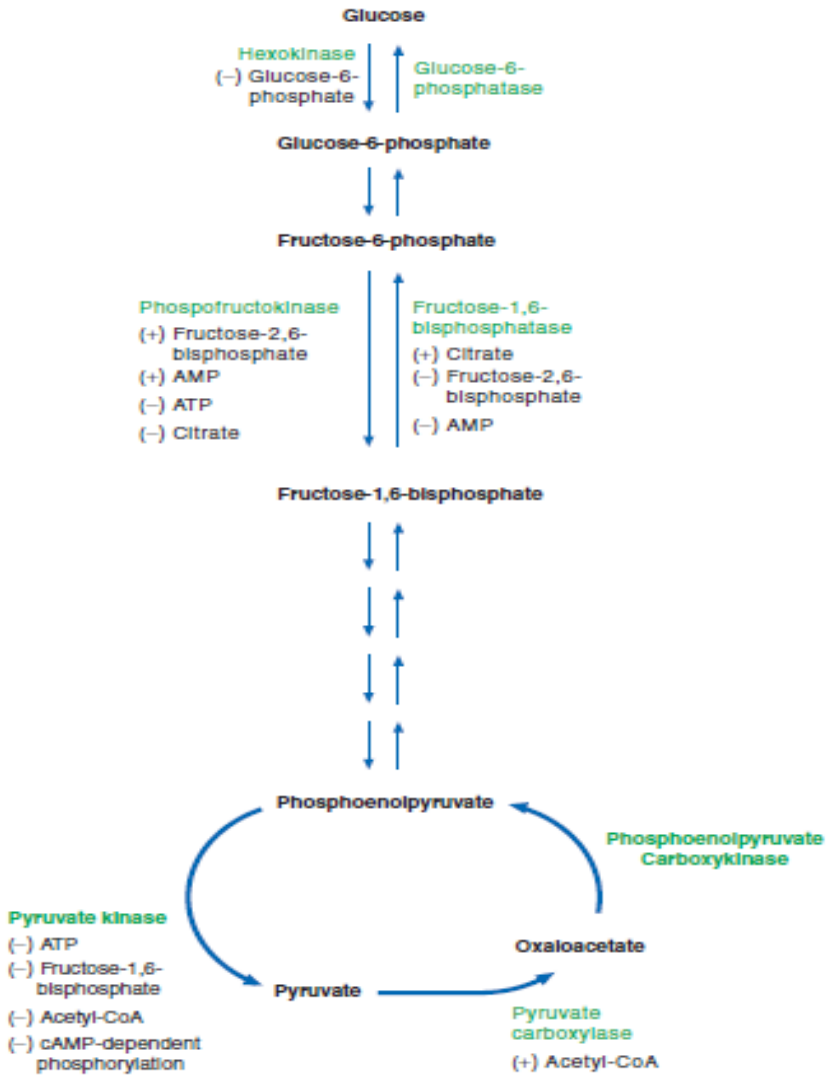


Figure 7: Allosteric Regulation of Glycolysis and Gluconeogenesis

Source: McKee and McKee, (2011)

2.7.1.2 Pentose Phosphate (Phosphopentose) Pathway

The first step of the pentose phosphate pathway, an irreversible committed step, is catalyzed by glucose-6-phosphate dehydrogenase. This step is controlled allosterically and the product of this reaction NADPH is a strong inhibitor. So in a state of high cytosol concentration of NADPH, the enzyme's activity is low. It is also allosterically controlled by fatty acid acyl esters of coenzyme A. The transcription of the gene for this enzyme is under hormonal regulation.

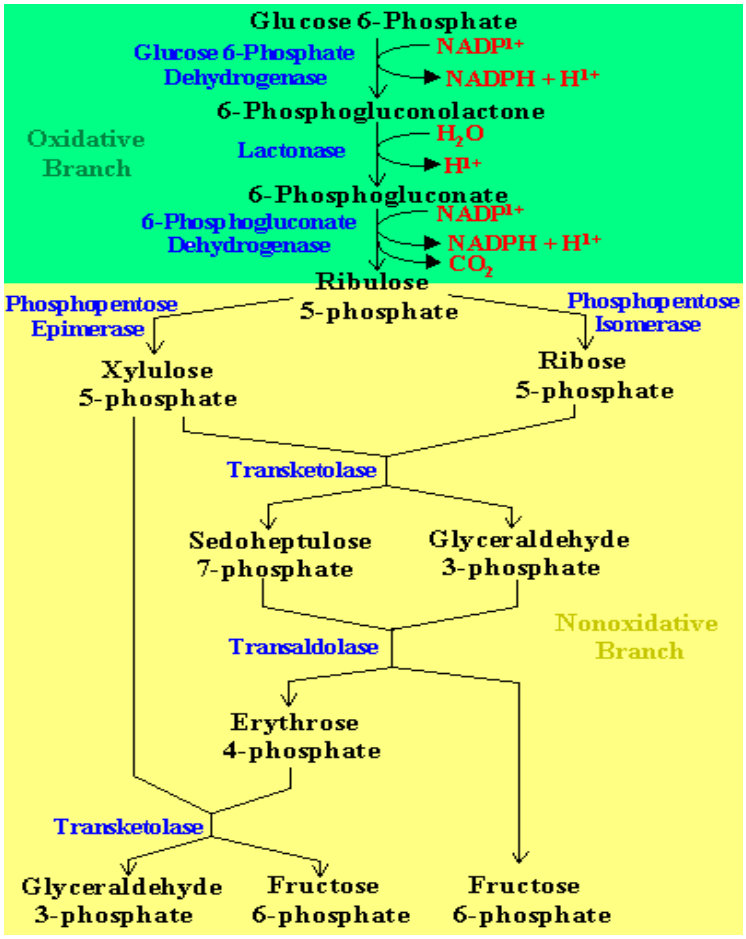


Fig. 8: Pentose Phosphate Pathway (Adapted from McKee and McKee, 2011).

2.7.2 Disturbances in Liver Functions

2.7.2.1 Alkaline Phosphatase Activities

Alkaline phosphatase (phosphate-monoester phosphor-hydrolase (alkaline optimum); EC 3.1.3.1) is a metallo-enzyme that exists as several tissue-specific 180 enzymes encoded by separate genes. This enzyme is expressed in many species (plant, bacteria and animals), catalyzes the hydrolysis of phosphomonoester, R-O-PO3, with little regard to the identity of the R group.

Alkaline phosphatase is the catalyst that hydrolysis of phosphate esters in alkaline buffer and yields an organic radical and inorganic phosphate. It is a hepatic enzyme associated with the catalytic breakdown of protein especially in the liver as such elevated level may be indicative of gluconeogenesis by protein breakdown. It is also indicative of hepatic disorders,

cholescystitis, (Corathers, 2006). A disturbance in alkaline phosphatase homeostasis is associated diseases of the liver and bones. Alkaline phosphatase in vertebrates is an ectoenzyme, and it's normally found bonded on the outer surface of the plasma membrane via a phosphatidyl inositol-glycophospholipid (GPI) anchor covalently attached to the c-terminus of the enzyme.

2.7.2.2 Aspartate Aminotransferases

Aspartate aminotransferases also known as serum glutamic oxaloacetate transaminase (SGOT) is a liver enzyme that participates in the synthesis of proteins. It catalyzes the reductive relocation of an amino group from aspartate to α-ketoglutarate to yield oxaloacetate and glutamate. Besides liver, it is also found in other organs like heart, muscle, brain and kidney. Any damage to these tissues often results in an elevated blood level of the enzyme. (Nathwani et al. 2005).The physiological range is usually between 7-40 U/L. It serves as an importance biomarker for detecting hepatocellular necrosis, however, it is not specific for it (Ozer et al., 2008) as it can also signify abnormalities in heart, muscle, brain or kidney (Dufour et al., 2000) and diabetes (Kunutsor et al., 2014). The ratio of serum AST to ALT is usually a useful to differentiate liver damage from other organ damage (Nathwani et al., 2005).

2.7.2.3 Alanine aminotransferases

Alanine aminotransferase also called serum glutamic pyruvic transaminase (SGPT) level is often relied upon as biomarker of hepatotoxicity. It plays an essential role gluconeogenesis and protein metabolism. It catalyzes the reductive relocation of an amino group to -ketoglutarate from alanine to pyruvate and glutamate. Physiological reference level is given as 5-50 U/L. Elevated level of this enzyme is released during liver damage as seen in diabetes (Vozarova et al., 2002). This enzyme being primarily from the liver makes its estimation a good biomarker for detecting abnormalities of the liver (Amacher, 2002). However, lower enzymatic activities are also found in skeletal muscles and heart tissue. This enzyme detects hepatocellular necrosis.

2.7.2.4 Lactate Dehydrogenase

This enzyme catalyzes interconversion of pyruvate and lactate to yield glucose in the presence of NAD/NADH. It is distributed widely within body cells and fluids, and shows high RBC/plasma ratio therefore it is elevated in plasma/serum following hemolysis. Lactate dehydrogenase level is increased in chronic diseases, hepatotoxicity or haemolysis and decreased in Clofibrate, fluoride (low dose) and in liver congestion.

It was discovered that Lactate dehydrogenase also increases due to cardiovascular complication in diabetes mellitus as a result of platelet specific interaction with blood vessels. This platelet-specific Lactate dehydrogenase isoenzyme pattern may have resulted from invivo platelet-vessel wall interactions in the diabetic patients whose platelets are known to be hyperaggregable similar to those of non-diabetic patients with vascular complications who also displayed a similarly elevated Lactate dehydrogenase-3/Lactate dehydrogenase-4 ratio. The measurement of the lactate dehydrogenase isoenzyme pattern will be helpful in assessing diabetic vascular complications (Nair et al.,1985).

2.7.2.5 Bilirubin Levels

Diabetes alters several metabolisms in body organs some of which include the liver and kidney function. Bilirubin comes from hemoglobin breakdown usually within the spleen, liver and bone marrow. Within the liver, breakdown of haemoglobin can be altered releasing free and conjugated bilirubin which poses a threat and in the kidney since evacuation of serum bilirubin is found in the urine, a phenomenon referred to as hyperbilirubinaemia.

In the liver, conjugation of bilirubin with glucoronic acid takes place to form a soluble compound. Conjugated bilirubin is excreted into the gastrointestinal tract, having mixed with bile, and subsequently can be passed out of the body in feces. The unconjugated form present in the circulation, pass through the kidneys to be excreted via urine.

Increased levels of circulating bilirubin in the serum or tissues results in a clinical state called jaundice. Jaundice occurs in toxic or infectious diseases of the liver, for example, hepatitis B, in bile duct obstruction and in rhesus incompatible babies.

High levels of conjugated is indicative of bile duct obstruction. Unconjugated or indirect bilirubin level is usually estimated by deducting the serum value of direct bilirubin from that of the total bilirubin value. High circulating plasma levels of unconjugated bilirubin is indicative of high levels of hemoglobin destruction or inadequate hemoglobin metabolism

by the liver.

2.7.3 Disturbances of Protein Metabolism in Diabetes

2.7.3.1 Serum protein

Proteins are compounds composed of carbon, hydrogen, oxygen, and nitrogen, which are aligned as strands of amino acids. They play an essential role in the maintenance of cell integrity, growth and functioning of the body, serving as the basic structural molecule of all tissues in the body, protein makes up nearly 17 percent of the total body weight.

2.7.3.2 Total protein.

This represent the sum of albumin and globulin, the normal range is about 7.5-8.0/100ml in humans. Total protein may be increased due to chronic infection, adrenal cortical hypo function, liver dysfunction, hypersensitivity states, sarcoidosis, dehydration, respiratory distress, haemolysis, alcoholism, leukemia. Total protein concentration may fall due to malnutrition, liver disease, diarrhea, severe burns, kidney disease and pregnancy.

Effects of diabetes on total protein level

Bodyweight reduction in diabetic rats clearly shows a loss or degradation of structural protein, this structural protein is known to contribute to body weight (Rajkumar and Govindarajulu, 1991). Ravi et al., (2004) reported that the characteristics loss of body weight associated with diabetes is due to excessive break down of tissue protein and an increased muscle wasting in diabetes. Dhanraj et al., (2007) reported that in the absence of insulin, protein production is not favored. Additionally, studies showed drop in serum total proteins in diabetic animals.

2.7.3.3 Albumin

Albumin is the most plentiful serum protein representing 55-65% of the total protein. It is produced in the liver and has a half-life of 2- 3 weeks. The normal concentration of albumin in the blood is about 3.4-5.4g/dl. The main biological functions of albumin are to maintain the

water balance in serum and plasma and to transport and store a wide variety of lingands e.g. Fatty acids, calcium, biliburin and hormones such as thyroxine. Albumin also provides an endogeneous source of amino acids. Hypoalbuminaemia is associated with the following conditions; analbuminaemia; impaired albumin synthesis in the liver; liver disease; malnutrition and malabsorbtion; generalized shock; burns or dermatitis; kidney diseases and intestinal disease. Hyperalbuminaemia has little diagnostic relevance except, perhaps in dehydration.

2.7.3.4 Globulin

The globulins are a family of globular proteins that have higher molecular weight and water solubility values than the albumins. Some globulins are formed in the liver while others are manufactured by immune systems. Albumin, globulins and fibrinogen are the main blood proteins. The normal concentration of globulins in the blood is about 2.6-4.6g/dl. The term "globulin" is sometimes used synonymously with globular protein. However, albumins are equally globular proteins nonetheless are not globulins. All other serum globular proteins are globulins.

2.7.3.5 Effects of diabetes on albumin-globulin level

Albumin and globulin combine together to make up the total protein. The hyperglycaemic effects of diabetes brings about a declination on the level of these parameters, this decrease brings about a disturbance in the maintenance of water balance in the serum and plasma, thereby causing an excessive depletion of water from the body and an increased intake of water.

2.8 DIABETES AND KIDNEY FUNCTION

2.8.1 Blood Urea Nitrogen and Creatinie

Bauer et al., (1982) reported that urea and creatinine are nitrogenous end products of metabolism, stating that urea is the principal metabolite resulting from dietary protein and tissue protein turnover while creatinine is the creation of muscle creatine catabolism.

Both are relatively lesser molecules (60 and 113 daltons, respectively) that dispense throughout total body water. Europe, clinically assays the entire urea molecule, but in the

United States only the nitrogen constituent of urea (the blood or serum urea nitrogen, that is, BUN or SUN) is estimated. The BUN, then, is approximately one-half (28/60 or 0.446) of the blood urea (Walker, 1990).

The usual range of urea in blood or serum is 5 to 20 mg/dl, or 1.8 to 7.1 mmol urea per liter (Bauer et al., 1982). The range is wide due to normal dissimilarities in protein intake, endogenous protein catabolism, state of hydration, liver urea production, and renal urea excretion (Bauer et al., 1982). A BUN of 15 mg/dl would signify meaningfully impaired function for a woman in the thirtieth week of gestation. Her greater glomerular filtration rate (GFR), extended extracellular fluid volume, and anabolism in the evolving fetus add to her relatively low BUN of 5 to 7 mg/dl. In distinction, the rough farmer who eats in excess of 125 g protein per day may have a normal BUN of 20 mg/dl (Bauer et al., 1982).

The normal serum creatinine (sCr) contrasts with the subject's body muscle mass and with the method used to measure it. In adult male, the normal range is about 0.6 to 1.2 mg/dl, or 53 to 106μmol/L by the kinetic or enzymatic method, and 0.8 to 1.5 mg/dl, or 70 to 133μmol/L by the older manual Jafféreaction. For the adult female, with her generally lower muscle mass, the normal range is 0.5 to 1.1 mg/dl, or 44 to 97?μmol/L by the enzymatic method (Mitch et al., 1980).

Technique

Multiple approaches for study of BUN and creatinine have evolved over the years. Most of the one currently in use are automated and give clinically dependable and reproducible outcome. There are two general techniques for estimating urea nitrogen. "The diacetyl, or Fearon, reaction yields a yellow chromogenusing urea, and this is measured by photometry. It has been employed for use in autoanalyzers and generally gives fairly accurate results. It still has restricted specificity, though, as showed by bogus elevations with sulfonylurea compounds, and by colorimetric intrusion from hemoglobin when whole blood is used (Bauer et al., 1982).

In the more precise enzymatic techniques, the enzyme urease converts urea to ammonia and carbonic acid. These products, which are proportionate to urea concentration in the sample, are analyzed in a range of systems, some of which are automated.

A system assesses the reduction in absorbance at 340 mm when the ammonia reacts with

alpha-ketoglutaric acid (Walker, 1990). Though that the test is presently performed mostly on serum, the term BUN is still retained by convention. Being that fluoride inhibits urease, the specimen should not be collected in tubes contaminated with sodium fluoride. Chloralhydrate and guanethidine have also been detected to increase BUN values (Walker, 1990).

1886 Jaffé reaction, in which creatinine is treated with an alkaline picrate solution to produce a red complex is still the foundation of most commonly used techniques for measuring creatinine (Narayanan and Appleton, 1980). This reaction is generic and subject to intrusion from many non creatinine chromogens, comprising acetone, acetoacetate, pyruvate, ascorbic acid, glucose, cephalosporins, barbiturates, and protein. It is sensitive equally to pH and temperature variations. Some alterations in the designed to nullify these sources of error is used in most clinical laboratories today. For example, the recent kinetic-rate modification, which isolates the brief time interval during which only true creatinine contributes to total colour formation, is the basis of the Astra modular system (Narayanan and Appleton, 1980).More specific, non-Jaffé assays have also been developed. The automated dry-slide enzymatic technique, measures ammonia produced following hydrolyses of creatinine by creatinine iminohydrolase. Its ease, precision, and speed, makes it well recommend for routine use in the clinical laboratory. Only 5-fluorocytosine impedes meaningfully with the test (Narayanan and Appleton, 1980).

Creatinine should be estimated in plasma or serum and not whole blood due to the fact that erythrocytes contain significant amounts of non creatinine chromogens. In order to reduce the conversion of creatine to creatinine, specimens should be fresh and the pH maintained at pH 7 throughout storage period. Urea synthesis mostly occurs in the liver, over 99%. Its chief source is via dietary protein. Within the gastrointestinal tract, the protein is broken down into peptides and amino acids, and most of them, greater than 90% transported to the liver. Within the liver cells, the amino acids are deaminated and transaminated. The resultant excess nitrogen feeds into the urea cycle to be incorporated into urea. Protein moieties not absorbed in the small bowel, together with the recycled urea, are transformed into ammonia by gastrointestinal flora predominantly in the colon. The ammonia diffuses through the portal circulation into the liver to get into the urea cycle.

2.8.2 Absorption, Metabolism, and Excretion of Urea

The quantity of urea generated varies with substrate delivery to the liver and the fitness of liver function. It is amplified by a high-protein diet, by gut bleeding (based on plasma protein level of 7.5 g/dl and a hemoglobin of 15 g/dl, 500 ml of whole blood is equivalent to 100 g protein), and by clinical state of the body that supports catabolic processes such as fever or infection, also by medications such as anti-anabolic drugs like tetracyclines (excluding doxycycline) and glucocorticoids. Conditions that lead to a decrease include; low-protein diet, malnutrition or starvation, and by compromised metabolic activity in the liver due to parenchymal liver disease or, infrequently, to congenital deficiency of urea cycle enzymes. The normal subject on a 70 g protein diet produces about 12g of urea each day.

This newly produced urea dispenses throughout total body water. Some of these get recycled via the entero-hepatic circulation. Usually, a small amount (less than 0.5 g/day) is lost via the gut, lungs, and skin; during exercise, a considerable fraction may be excreted in sweat. Majority of the urea, about 10gm lost per day is via the kidney in a process that starts with glomerular filtration. During high flow rates of urine (that is greater than 2 ml/min), 40% of the filtered consignment is reabsorbed, and when the flow rates less than 2 ml/min, reabsorption may rise to 60%. Low flow rate as observed during obstruction in urinary tract, allows more time for reabsorption and is frequently accompanied with increases in antidiuretic hormone (ADH), which increases the perviousness of the terminal collecting tubule to urea. During ADH-induced anti-diuresis, urea secretion adds to the intra-tubular concentration of urea. The subsequent build-up of urea in the inner medulla is critical to the process of urinary concentration. Reabsorption is also increased by volume contraction, reduced renal plasma flow as in congestive heart failure, and decreased glomerular filtration.

2.8.3 Metabolism and Excretion of Creatinine

Creatinine production starts with the transamidination from arginine to glycine to form glycocyamine or guanidoacetic acid (GAA). This reaction takes place principally in the kidneys, but also in the mucosa of the small intestine and the pancreas. GAA is then conveyed to the liver for methylation by S-adenosyl methionine, to produce creatine. Creatine goes into the circulation, and 90% of it is taken up and put in storage within muscle tissues.

Creatine phosphokinase (CPK), catalyzes most of the process in which muscle creatine is phosphorylated to creatine phosphate. Daily, roughly 2% of these stores are converted non-

enzymatically and irreversibly to creatinine. Thus, creatinine production principally reflects lean body mass. Because this mass changes little from day to day, the production rate is fairly constant. Total creatinine production drops with age in line with declining muscle mass. Unlike urea, creatinine is mostly unaffected by bleeding in the gut or by clinically catabolic process including fever and steroids drugs. However, the ingestion of cooked meat can raise the sCr because cooking converts the creatine in meat to creatinine.

Some drugs, especially the psychoactive phenacemide, can result in an increase the rate of production. Similar to urea, creatinine distributes all through total body water. Serum creatinine concentration in serum is a factor of the rate production and excretion. It may be slightly higher in the evening than in the morning, due most likely to dietary meat intake by day. In physiologic individuals, creatinine is excreted mainly by the kidneys. There is negligible extra-renal disposal or obvious metabolism. Being small in size (molecular weight of 113 daltons), it is easily filtered by the glomerulus. Dissimilar to urea, it is not reabsorbed or affected by rate of urine flow. It is typically secreted by the tubules in a small but noteworthy amount (up to 10% of total excretion). During exercise, excretion of urea and creatinine is increased without a corresponding change in serum concentration. Total creatinine excretion in a normal male is about 14 to 26 mg/kg/day, while in a normal female is about 11 to 20 mg/kg/day. There is decline in creatinine excretion with age and it is around 10 mg/kg/day in a 90-year-old man. It however should not fluctuate more than 10 to 15% in a given individual. The quantity excreted has been used as a rough index of the totality of daily urine collection. Measurement of urine creatinine excretion is used in calculating the creatinine clearance (cCr).

Short of the more precise but technically impractical inulin clearance, the cCr is the standard clinical tool for estimating GFR, particularly in the early stages of renal disease. In that setting, Cr and BUN are not very useful indices of GFR due to their parabolic relationship and to the wide range of normal.

2.8.4 Clinical Significance of Blood Urea Nitrogen and Serum Creatinine Level

Blood urea nitrogen and serum creatinine are screening tests of renal function. Because they are handled principally by glomerular filtration with little or no renal regulation or alteration in the course of declining renal function, they essentially reflect glomerular filtration

rate (GFR). Their relation to GRF however is not a straight line but rather a parabolic curve; their values remain within the normal physiological range until more than 50% of renal function is lost (Bauer et al., 1982). Within that range, however, a doubling of the values (for example, BUN rising from 8 to 16 mg/dl or sCr from 0.6 to 1.2 mg/dl) may mean a 50% fall in the GFR. Hence, in the early stages of renal disease, these assessments could create an incorrect sense of security. Random values above the midrange of normal should be corroborated by a normal cCr before one can confidently tell a patient that his or her kidney function is normal. At the other end of the curve, slight changes in kidney function can produce large increments in BUN and sCr. Here, these tests are generally adequate to follow a patient's course. Indeed, the reciprocal of the sCr plotted against time shows a straight-line progression of renal disease in each patient, and can be used to predict the beginning of end-stage renal disease. At all stages of renal insufficiency, the sCr is a much more reliable indicator of renal function than the BUN because the BUN is far more likely to be affected by dietary and physiologic circumstances not related to renal function. Patients with congestive cardiac failure for example, and intact kidneys commonly present with a BUN of 50 to 70 mg/dl and a sCr below 1.2 mg/dl. Of course, sCr may rise under some extra-renal factors, but seldom will it exceed 3 to 4 mg/dl (Narayanan and Appleton, 1980).

2.8.5 Extra renal factors affecting blood urea nitrogen and serum Creatinine

With so many limitations on the usefulness of the BUN, one wonders why the test survives. When taken with these Cr, it is a very useful clue to the presence of a pre-renal or post-renal component to azotemia.

Other factors being normal, a patient with asCr of 5.0 mg/dl would be expected to have a BUN close to 50 mg/dl. If the BUN is 100 mg/dl as an alternative, then the clinician should begin a search for extra-renal factors. It should be noted that a ratio of 10 to 1, can be best applicable in moderate to advanced renal failure.

Attending to these changeable complications of uremia can give a patient a reprieve from a premature sentence of end-stage renal disease. A low BUN/Cr ratio is indicative of insufficient protein intake, reduced urea production as seen in advanced liver disease, excessive excretion of urea as in sickle cell anemia, increased creatinine manufacture as in rhabdomyolysis, or more active elimination of urea than creatinine during dialysis. The BUN

subsists and finds wide use in the nutritional management of critically ill patients.

Urea nitrogen presence (UNA) accurately lets the intensivist know if the patient's nitrogenous requirements are being met. The UNA assessment needs the measurement of BUN at the onset and end of the period of surveillance as well as the entire urea excretion.

The BUN and creatinine, taken collectively, are essential screening tests in assessing renal disease. Though they may not on their own be conclusive indicators of renal function at any-given in time, they serve as very useful tool in monitoring the progression of the disease.

2.9 ANTIOXIDANT ACTIVITY IN DIABETES

2.9.1 Catalase

Catalase is an enzyme found in nearly all living organisms exposed to oxygen. It catalyzes degradation of hydrogen peroxide to water. It is a very significant enzyme in reduction reactions.

Catalase is found in high concentration in a compartment in cells called the peroxisome both in plants and animals (Chelikani et al., 2004). Catalase has the largest turnover number of enzymes, millions of molecules of hydrogen peroxide can be converted to water and oxygen by a molecule of catalase every second (Goodsell, 2004). Animals employ catalase in every organ function, this is especially so with the liver, (Boon et al., 2007).

Hydrogen peroxide is an injurious by-product of many metabolic processes in the body, to prevent damage; it must be converted speedily into less dangerous substance. Catalase is regularly used by cells to swiftly catalyze the degradation of hydrogen peroxide into less reactive gaseous oxygen and water molecules (Gaetani et al., 1996).

2.9.2 Superoxide Dismutase (SOD)

Superoxide Dismutase are enzymes that catalyses the dismutation of superoxide ion into oxygen as well as hydrogen peroxide. Hence, they are an imperative antioxidant defense in almost all cells exposed to oxygen (McCord and Fridovich, 1988). There are three main families of superoxide dismutase, contingent on the metal cofactor:

i. Cu/Zn (that binds both copper and zinc).
ii. ii. Fe and Mn (which binds iron or manganese).
iii. iii. Ni (which binds nickel). Three forms are present in humans, mammals and other

chordates.

SOD 1 is found in the cytoplasm, SOD 2 in the mitochondria and SOD 3 is extracellular. SOD 1 and SOD 3 comprise copper and zinc whereas SOD 2, the mitochondria enzyme contains manganese in its reactive Centre (Corpas et al., 2006).

Superoxide Dismutase serves a basic antioxidant role in cells. Mice lacking SOD 2 die few days after birth amid massive oxidative stress (Li et al., 1995). Mice lacking SOD 1 develop a wide range of pathologies plus hepatocellular carcinoma (Elchuri et al., 2005) and increased diabetic complications (Muller et al., 2006).

2.9.3 Lipid peroxidation

Reaction of oxygen with unsaturated lipids referred to as lipid peroxidation produces a varied multiplicity of oxidation products. The principal products of lipid peroxidation are lipid hydroperoxides (LOOH). Among the numerous diverse aldehydes which can be formed as secondary substrates following lipid peroxidation, "malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal (4-HNE) have been expansively studied by Esterbauer and his colleagues in the 80s. MDA seems to be the most mutagenic substance of lipid peroxidation.

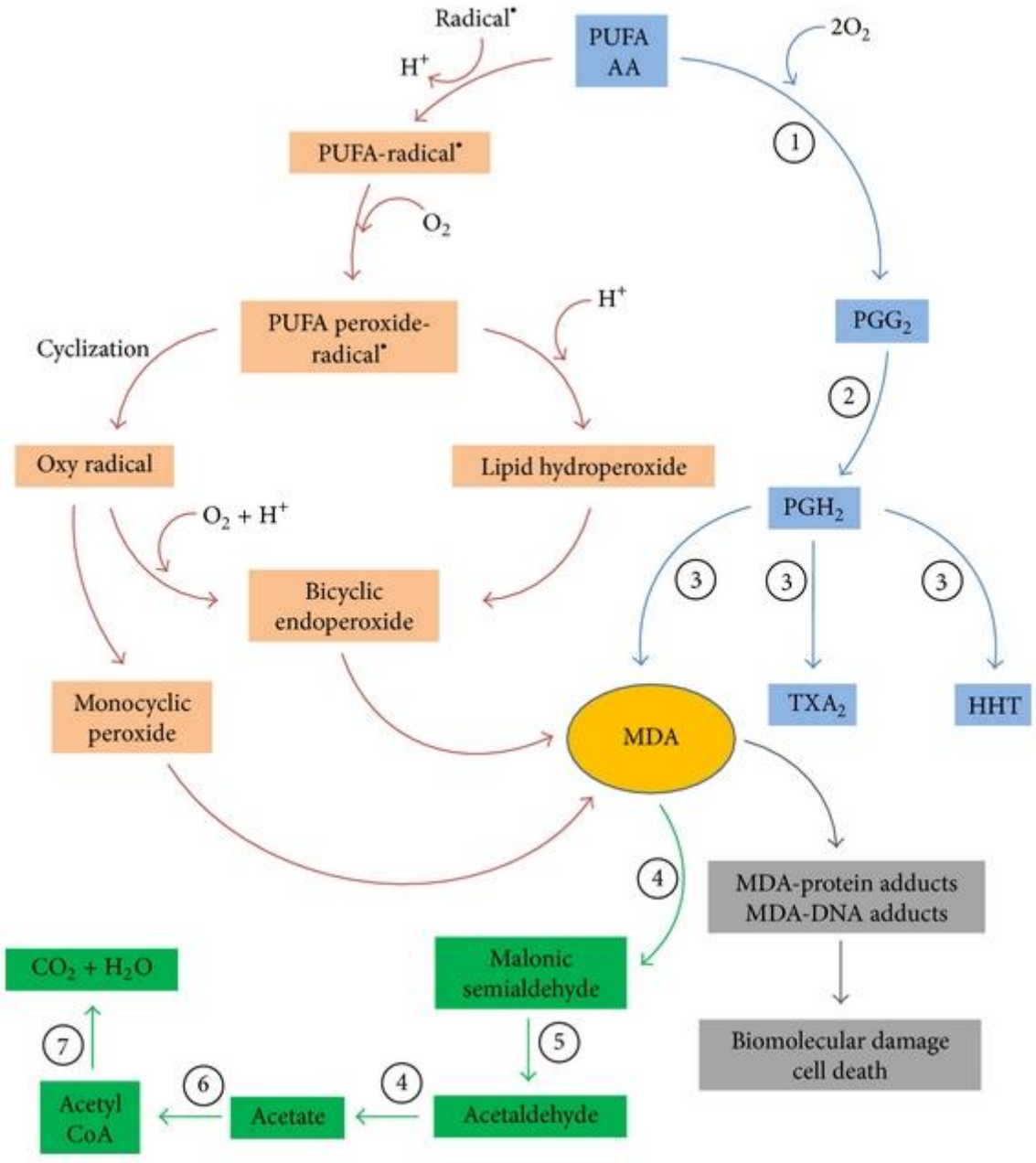
2.9.3.1 Malondialdehyde (MDA)

MDA has been extensively employed for years as an expedient biomarker for lipid peroxidation of omega-3 and omega-6 fatty acids for the reason of its facile reaction with thiobarbituric acid (TBA) (Esterbauer and Cheeseman, 1990). TBA test is based upon the reactivity of TBA toward MDA to produce an deeply colored chromogen fluorescent red adduct; this test was first employed by diet chemists to appraise autoxidative break down of fats and oils (Sinnhuber et al., 1958). Though, the thiobarbituric acid reacting substances test (TBARS) is particularly nonspecific which has led to significant disagreement over its use for quantification of MDA from in vivo samples. Malondialdehyde is one of the most common and dependable markers that define oxidative stress in clinical situations (Giera et al., 2012.), and because of MDA's great reactivity and toxicity underlying the point that this substance is very pertinent to biomedical research community.

MDA is a terminal-product produced by the breakdown of arachidonic acid and larger

PUFAs (Esterbauer et al., 1991), through enzymatic or non-enzymatic processes. MDA generation by enzymatic procedures is well known, however its biological functions and its likely dose-dependent double role have not been studied. Though malondialdehyde permeates membranes more readily compared to ROS, is more chemically stable and less toxic when compared to 4-HNE and methylglyoxal (MG) (Esterbauer et al., 1991).

Few papers have thus far reported that MDA would likely functions as signaling messenger and gene expression regulator: (i) a recent research reported that MDA acted as a signaling messenger as well as islet glucose-stimulated insulin secretion regulator (GSIS) mainly via Wnt pathway. The moderately high MDA levels (5 and 10μM) promoted islet GSIS, elevated ATP/ADP ratio and cytosolic Ca2+ level, and affected the gene expression and protein activity manufacture of the key regulators of GSIS (Wang et al., 2014); (ii) in hepatic stellate cells, "MDA prompt collagen-gene expression by up regulating specificity protein-1 (Sp1) gene expression and Sp1 and Sp3 protein levels" (Garcia-Ruiz et al., 2002). Sp1 as well as Sp3 can intermingle with and recruit a huge amount of proteins as well as the transcription initiation complex, histone modifying enzymes, and chromatin transformation complexes, which intensely propose that Sp1 and Sp3 are essential transcription elements in the transformation chromatin as well as the regulation of gene expression (Li and Davie, 2010). Malondialdehyde production on the other hand by non-enzymatic methods remains poorly understood notwithstanding their prospective therapeutic value, due to the fact that this MDA is thought to be produced under stress conditions and has high ability of reacting with several biomolecules like proteins or DNA that leads to the formation of adducts (Zarkovic et al., 2013; Blair, 2008), excessive MDA synthesis have been associated with diverse pathological states (Paggiaro et al., 2011). Recognizing in vivo malondialdehyde generation and its function in biology is vital as shown by the widespread literature on the compound.



Pathway for malondialdehyde formation and metabolism: (Ayala*et al.*, 2014)

2.9.3.2 MDA Formation and Metabolism

Malondialdehyde can be produced in vivo by break down of arachidonic acid (AA) and bigger PUFAs as a side product by enzymatic methodsthroughout the biosynthesis of thromboxane A2 (TXA2) and 12-l-hydroxy-5,8,10-heptadecatrienoic acid (HHT) (blue

pathway), or through non-enzymatic processes by bicyclic endo-peroxides produced for the period of lipid peroxidation (red pathway). Once produced, malondialdehyde can be enzymatically metabolized (green pathway). The main enzymes involved in the production and metabolism of MDA: (1) cyclooxygenases, (2) prostacyclin hydro-peroxidase, (3) thromboxane synthase, (4) aldehyde dehydrogenase, (5) aldehyde decarboxylase, (6) acetyl CoA synthase, and (7) tri-carboxylic acid cycle.

2.9.3.3 Malondialdehyde Production by Enzymatic Processes

Malondialdehyde can be generated in vivo as a byproduct by enzymatic procedures for the duration of the biosynthesis of thromboxane A2 (Li et al., 2012). TXA2 is an active biological metabolite of arachidonic acid synthesized by the action of the thromboxane A2 synthase, on prostaglandin endo-peroxide or prostaglandin H2 (PGH2) (Ekambaram et al., 2011). PGH2 formerly is produced by the actions of cyclooxygenases on AA (Yang and Chen, 2008).

2.9.3.4 MDA Production by Non-enzymatic Processes

Lipid peroxidation process results in the production of a combination of lipid hydroperoxides. The peroxyl radical in the hydroperoxides with a cis-double bond homoallylic to the peroxyl group allows their facile cyclization via intramolecular radical addition to the double bond and the synthesis of a new radical. The intermediate free radicals produced following cyclization can undergo the same process again to generate a bicycle endo-peroxides, related structurally to prostaglandins, and undergo cleavage to produce MDA via a non-enzymatic oxygen radical-dependent reaction. AA is the chief precursor of bicyclic endo-peroxide, which then go through further reactions with or without the involvement of other compounds to form MDA (Milne et al., 2008). It is however possible that other eicosanoids that can also be generated by non-enzymatic oxygen radical-dependent reaction (Robertset al., 2005) may be substrates of bicyclic endo-peroxide and malondialdehyde. Latest review has addressed the pathways for the non-enzymatic production of malondialdehyde under specific conditions (Onyango and Baba 2010).

2.9.3.5 Malondialdehyde Metabolism

As soon as malondialdehyde is produced, it can be enzymatically break down or can react with cellular and tissue proteins or DNA to generate adducts leading to biomolecular

injuries. Initial research showed that a probable biochemical path for MDA metabolism comprises its oxidation by mitochondrial aldehyde dehydrogenase followed by decarboxylation to produce acetaldehyde, which is oxidized by aldehyde dehydrogenase to acetate and further to CO2 and H2O (Figure 1) (Marnett et al., 1982). Phospho-glucose isomerase on the other hand is perhapsaccountablefor metabolizing cytoplasmic malondialdehyde to methylglyoxal (MG) and later to D-lactate by enzymes of glyoxalase system, using GSH as a cofactor (Agadjanyan et al., 2005.) Portions of malondialdehyde are pasted out in the urine as various enaminals (RNH-CH-CH-CHO) for example, N-epsilon-(2-propenal) lysine, or N-2-(propenal) serine (Esterbauer et al., 1991).

CHAPTER THREE

3.0 RESEARCH METHOD

3.1 MATERIALS

3.1.1 EQUIPMENTS AND CONSUMABLES

- **Syringes And Needles** (Lot: Ww-Ag-13024), Production Date-Oct 2013, Expiring Date-Oct 2018, (NAFDAC No-03-0777), Manufactured For Agary Pharmaceutical Limited By Wuxi Yushou Medical Applainces Co.Ltd
- **Hand Gloves** (Lot No 2116), NAFDAC No-03-3206, Production Date-04 2015, Expiring Date-04 2018, Manufactured For Longer Life Health Care Ltd, No 5 Udi Street Onitsha Anambra State Nigeria.
- **Incubactor** (Model TT 9052), Company Name- Techmel and Techmel, USA.
- **Glucometer** Accu Check www.acu-check.com
- **Aucku Check active** Strip (Lot No-24640133), Expiring Date-10 2016
- **Micropipette** (Microlux) Vol. Range 0-1000ul
- **Stop Watch** (Taksun: Ts-1809), Stop Timerq. C 2014-0907
- **Oven** (Dhg-9023a)
- **Centrifuge** Model 800 Made In China Zhengji
- England Labscience
- **Cotton Wool** (NAFDAC No-03-1113), Expiring Date 2019, Batch No-666 Manufacturing Date-2015
- **Distiller:** Model No. Tt2, Techmel Techmel USA.

3.1.2 CHEMICALS

- **Streptozotocin (STZ)** (Batch 1378), Address: Aldrich Sigma Co.3050, Spruce Str, St. Louis Mo 63103 U.S.A 314-771-5765
- **Acetic Acid** (Batch - 70419322), Sulphoric Acid (H2SO4) SL 704l419322, E .Merk, Darmstadt 2.5
- **H_2O_2** Guanghang Guanghua Chemical Factory Co. Ltd Shantou Guanghung China
- **Phenol** (Batch 1368),

- **Ethyl Acetate** (1502 batch 1356o517) Gato Perez, 33-P.1. Masden Gsa 08181sentmenat Spam. Shelf Life 5/2017
- **Hydrochloric acid** - Guanghua Chemical Factory, Shantou Guondghuo China
- **Magnisium Chloride** (Production: Moo6112), Batch No: 5h160911
- **Glucose** - 500g,
- **Adenosine Triphosphoric Acid Disodium Salt** (Atp), (Batch 1448), Address: Aldrich Sigma Co.3050 Spruce Street St. Louis Mo 63103 U.S.A314-771-5765
- **Dichlorophenol Indophenol Sodium Salt,** D35940808, Lot No 100743 (Batch 1341), Address: Sigma. Aldrich Co.3050 Spruce Street St. Louis Mo 63103 U.S.A 314-771-5765
- **Chloroform** - Plot D-22, Tarapur Midc, Boisar.Dist Thane 401 506
- **N-Butanol**, Guangdong Guanghua Sci-Tech Co., Ltd. Shatou, Guangdong, China, 515000
- **Benzene** (Batch No. 704 L419322). Gato Perez, 33-P.1.Masden Gsa 08181sentmenat-spam, Shelf Life 5/2017

3.1.3. REAGENTS

Liver Enzymes [ALT, AST, ALP] ALT= Batch-295830, **ALP**= Batch-213577. Randox Laboratory Ltd, 55 Diamond Road Crumlin Country Antrim United Kingdom. Expiring Date 2016 -02

LDH (Batch -43930), **UREA** (Batch -43724), **CREATININE** (Batch -43632). Teco Diagnostics, 1268 N. Lakeview Ave Anaheim, Ca 92807 USA. Expiring Date -2016, 03.

3.2 METHODS

3.2.1 Plant Collection

Fresh leaves of Vernonia amygdalina were acquired from Delta State University Abraka, third site; same was prepared and taken for authentication in Forestry Research Institute of Nigeria, Ibadan. After authentication, a herbarium number; FHI 110336 was given. The plants were transported to Emma-Maria Research Laboratory Abraka for extraction process.

3.2.2 Crude Extraction (Preparation)

The Vernonia leaves were spread out on flat surface and allowed to air-dry for a number of days. The dried leaves were then grinded and soaked in ethanol for 48 hours following which the extract was filtered and left to dry by evaporation. This represented the crude extract. The ensuing ethanol-free juice was further subjected to liquid-liquid fractionation with the aid of solvents of variable polarity from non-polar to highly polar according to Ekam et al., (2013). Solvents used include;

3.2.3 Fractionation of Crude Ethanolic Extract

The plant was fractionated following Ekam et al., (2013); Crude ethanolic extracts of Vernonia amygdalina was fractionated using a serial liquid-liquid separation method. The fractions gotten were: Benzene Fraction, Chloroform fraction, Ethyl acetate fraction and Butanol fraction. Fractionation procedure is as follow: 200ml of the crude extract of bitter leave was measured using a measuring cylinder into a separating funnel (500ml), held in place by the aid of a retort stand and clamp. 200ml of benzene was added to it, shake properly and allowed to stand 30 minutes. The mixture separated into two layers; one layer containing benzene soluble constituents of Vernonia amygdalina which was collected into a beaker, and the other layer comprising of non-benzene soluble filtrate. The resultant residue was left to stand in an open beaker in order for the benzene to evaporate. After drying, 200ml of the residue was measured into a separating funnel and 200ml of chloroform was added to it, shake properly and allowed to stand for separation. The mixture parted in two layers, a chloroform soluble phase and non-chloroform soluble residue. The non-chloroform soluble phase was collected, left to stand for evaporation of the chloroform to take place, it was then poured into another separating funnel for the next extraction phase using Ethyl acetate; the mixture separated into two phases and collected as above. 200ml of the residue from ethyl acetate fractionation was allowed to air-dry and further fractioned using 200ml of butanol and allowed to stand, separate and the fraction collected in a beaker. The fractions acquired were left to stand for evaporation to take place and dry to powder form. The various fractions were named by the solvent with which they were extracted. Each fraction was further subjected to Gas

Chromatography - Mass Spectrum (GC-MS) so as to identify the various chemical compounds in them. 3.2.4 Phytochemical Identification Fractionates were screened and identified using the qualitative chemical screening method. Phytochemicals identified were: Tannins, Glycosides, Alkaloids and Saponins. Phytochemical screening procedures were as follows;

3.2.4.1 Test for Tannins

One mililiter (1ml) of fractionate of the plant extract was mixed with 2ml of 2% solution of FeCl3. A blue-green or black coloration indicated the presence of tannins.

3.2.4.2 Test for Saponins

One mililiter (1ml) of fractionate from the plant extract was mixed with 5ml of distilled water in a test tube and shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

3.2.4.3 Test for Glycosides

Liebermann's test: One mililiter (1ml) of fractionate of the plant extract was mixed with each of 2ml of chloroform and 2ml of acetic acid. The mixture was allowed to cool using ice, and then cautiously concentrated H2SO4 was added. A change in colour from violet to blue, and then to green is indicative of the presence of steroidal nucleus, that is, glycone portion of glycoside.

3.2.4.4 Test for Alkaloids

One mililiter (1ml) of fractionate of the plant extract was mixed with two mililiter (2ml) of 1% HCl and heated gently. A Mayer's and Wagner's reagent was then added to the mixture, a resulting turbid precipitate is indicative of the presence of alkaloids.

3.2.5 Handling of Animals

Having obtained permission from the faculty's bioethics committee for use of animals for research, sixty five (65) adult male Wistar rats, weighing between 100-250g were used in this study. The rats were procured from the Emma-Maria Laboratory Animal unit, Abraka, were they were bred. They were then transported to the Animal house of the Faculty of Basic

Medical Sciences, Delta State University, Abraka, were they housed in an environment of normal ambient temperature and the lighting period was about 12hours daily. The humidity was between 40 and 60%, they were housed in stainless steel cages, fed with standard commercial pelleted feed (Vital feed, UAC, Lagos) and fresh drinking water ad libitum.

3.2.6 Induction and Treatment

3.2.6.1 Induction

Diabetes was induced with the aid of Streptozotocin dissolved in sodium citrate buffer. Before induction, the rats were fasted for 12 - 18hours, and their fasting blood glucose level noted. Streptozotocin was prepared by dissolving 2g of Sodium Citrate in 100ml of water to yield 0.1mole of citrate buffer; 0.6g of Streptozotocin was then dissolved in 10ml of citrate buffer to produce 60mg of Streptozotocin. 1ml of the resultant solution was injected into the animals through the lateral tail vein and their fasting blood glucose was assessed using ACCUCHEK active blood glucometer, 72hours after induction. A 50% increase in pre-induction blood glucose level was taken to be diabetic. Diabetes was not induced in animals for the normoglycaemic study.

3.2.6.2 Treatment group

The research was divided into two phases; the diabetic experimental study and the normoglycaemic study which comprised of 30 animals while diabetic study comprised of 35 animals.

Phase 1 animals were grouped into six (6) groups of five (5) animals;

- Group 1a **Control**
- Group 1b was treated with 300mg/kg of **Crude Vernonia amygdalina**
- Group 1c was treated with 300mg/kg of **Benzene fraction**
- Group 1d was treated with 300mg/kg of **Chloroform fraction**
- Group 1e was treated with 300mg/kg of **Ethyl acetate fraction**
- Group 1f was treated with 300mg/kg of **Butanol fraction**

The diabetic animals were grouped into seven (7) groups of five (5) animals each and each group were administered different treatments as follows;

- Group 2a (negative control) induced diabetes with 60mg of streptozotocin but **untreated**

- Group 2b was induced diabetes with 60mg of streptozotocin and treated with 50mg/kg of **Metformin**

- Group 2c was induced diabetes with 60mg of streptozotocin and treated with 300mg/kg of **Crude Vernonia amygdalina**

- Group 2d was induced diabetes with 60mg of streptozotocin and treated with 300mg/kg of **Benzene fraction**

- Group 2e was induced diabetes with 60mg of streptozotocin and treated with 300mg/kg of **Chloroform fraction**

- Group 2f was induced diabetes with 60mg of streptozotocin and treated with300mg/kg of **Ethyl acetate fraction**

- Group 2g was induced diabetes with 60mg of streptozotocin and treated with 300mg/kg of **Butanol fraction**

Observation

Weekly measurement of body fasting blood sugar level and body weight was done with the aid of a glucometer and an electronic weighing balance. Blood was collected from the tip of the animal's tail. Values obtained were rerecorded and expressed in mg/dl and gm respectively.

3.2.7 Sacrificing of the Rats and Sample Collection

The rats were sacrificed after an overnight fast so as to determine their final fasting blood sugar level prior to sacrificing. Anesthetic was administered by inhalation. Anesthesia was prepared by soaking Cotton wool in chloroform and placed in desiccators for 5 minutes to saturate the desiccator. The animal was then placed in the desiccator, covered and left for 10 minutes to weaken the animal. The animal was then removed and pinned on the board and was dissected using dissecting materials. The rats were put individually into the container and the lid replaced until they become anaesthetized. Each rat was placed on the dorsal surface. Laparotomy was done to expose the internal organs; blood was collected from the heart using 5ml syringes and same was put in a blood sample container. The pancreas organ were harvested, weighed and sectioned. Part of the organs was preserved in formo-saline in preparation for histopathology.

3.2.8 Determination of Body Weight and Organ

Weight Body weight of experimental animals was checked/determined at week 0 (before and after induction / before administration) and subsequent weeks and last day of experiment before sacrifice. Percentage weight change was later calculated as follows.

$$\text{Percentage weight change (\%)} = \frac{final - intialbodyweight\ (g)}{intialbodyweight\ (g)} X \frac{100}{1}$$

3.2.9 Biochemical Analysis

Biochemical analysis was carried out on the samples collected to determine the enzyme activity level of glucose metabolic pathways, Liver function, antioxidant activities, lipid peroxidation, renal function, and lipid profile as shown below;

3.3 ENZYMES OF GLUCOSE METABOLISM

3.3.1 Estimation of Glucose-6-Phosphate Dehydrogenase Activity

Glucose-6-phosphate dehydrogenase (G6PD, D-glucose-6-phosphate: oxido-reductase, EC 1.1.1.49) catalyzes the initial step in the pentose phosphate shunt, oxidizing glucose-6-phosphate (G-6-P) to yield 6-phosphogluconate (6-PG) and reducing agent, NADP to NADPH. This procedure is a modification of the spectrophotometric methods of Kornberg and Horecker (1955) and of Lohr and Waller (1974), involving the following reaction: The change in absorbance was measured at 340nm according to the following reaction;

$$\textbf{Glucose-6-phosphate + NADP}^{+} \xrightarrow{\textbf{G6PDH}} \textbf{Gluconolactone 6- phosphate + NADPH + H}^{+}$$

Nictotinamide adenine dinucleotide phosphate (NADP) is reduced by G6PD in the presence of G-6-P". The rate of formation of NADPH is proportionate to the activity of G6PD and this is estimated spectrophotometrically as increase in absorbance at 340nm. The unit of activity is defined as the amount of Glucose-6-phosphate dehydrogenase that forms 1µmol of NADPH per minutes at 30OC

Reagent used include; 100mM Tris- HCl, Buffer solution at pH 9.0, 1M MgCl2 solution(20.33g MgCl2.6H20/100ml distilled water).22.5mM NADP+solution (0.188g

MgCl.6H2O/100ml distilled water).33Mm Glucose 6-phosphate (G6P) solution (0.112g G6P disodium salt.2H2O/10ml distilled water).The lyophilized enzyme was dissolved with distilled water and diluted to 5 to 10u/ml with 50mM Tris-HCl buffer, pH 8.5. The working reaction mixture was prepared in a beaker by adding 25.20ml of Tris-HCl buffer, 1.20ml of MgCl2, 1.20ml of NADP+ and 2.4ml of G6P. 3.00ml of reaction mixture was pippetted into a curvette and incubated at 30OC for about 3 minutes. After incubation, 0.01ml of enzyme solution was added into the curvette and mix. The absorbance change at 340nm per minutes (ΔAbs340) was read in a linear curve.

Calculation

$$\text{Volume activity (U/ml)} = \frac{(\Delta \text{Abs340})\text{X}(3.00 + 0.01)\text{X D.f}}{6.22 \text{ X } 0.01}$$

$$\text{Specific activity (U/ml)} = \frac{\text{Volume activity (U/L)}}{\text{ProteinConcentration(mg/ml)}}$$

d.f: dilution factor, 6.22: millimolar extinction coefficient of NADPH (cm2/mol), Protein concentration; determined by Bradford's method (1976).

3.3.2 Estimation of Diaphorase (NADPH) Activity

The diaphorases are a ubiquitous class of flavin-bound enzymes that catalyze the reduction of various dyes which act as hydrogen acceptors of the form of reduced di- and tri-of, NADH and NADPH. Its activity is assayed based on the decolorization of 2,6 dichlorophenolindophenol which becomes chromogenic on reduction (Brower and Woodbridge, 1970; Nachlas et al., 1960). Spectrophotometrically, the change in absorbance is measured at 600nm according to the following reaction:

$$\text{NADPH + DCIP (OX) + H}^+ \xrightarrow{\text{Di-1}} \text{NAD (P)}^+ \text{ + DCIP}$$

One unit of activity is defined as the quantity of Di-1 that reduces 1µmol of DCIP per minute at 300C.Reaction solutions used were as follows; 500mM Tris-HCL Buffer solutions at pH 8.5, 13.1mM NADH solution (0-100g disodium salt. 3H20/10ml distilled water) and 1.2mM 2,6 dichlorophenolindophenol (DCIP) solution (2.0mg DCIP solution salt. 2H20/5ml dostilled water)

The reaction mixture was prepared by adding 3.00ml Tris-buffer, 2.28ml NADH

solution, 23.22ml H2O. 1.85ml of the reaction mixture was pipette into the cuvette and incubated at 300C for about 3min. After incubation, 1.5ml of DCIP solution and 0.01ml of enzyme solution were pippeted into the cuvette and mix. Absorbance change at 600nm per minute was read in linear portion of cuvette. Distilled water was used in place of enzyme solution for blank.

Calculation

$$\text{Volume activity (U/ml)} = \frac{(\Delta Abs(test) - \Delta Abs(blank)) \times (3{,}00 + 0.01)}{19 \times 0.01}$$

$$\text{Specific activity (U/mg protein)} = \frac{volumeactivity}{protein\ concentration\ (\frac{mg}{ml})}$$

3.3.3 Determination of Pyruvate Kinase

Pyruvate kinase (PK) is an enzyme that catalyzes the final step in the glycolytic pathway, it involves the transfer of a phosphate group from phosphor (enol) pyruvate (PEP) to ADP, producing one molecule of pyruvate and one molecule of ATP. Pyruvate kinase deficiency, resulting from a failing in pyruvate kinase expression or activity, is the second commonest cause of hemolytic anemia. The change in absorbance is measured at 340nm according to the following reaction;

$$\text{ADP + PEP} \xrightarrow{\text{PK}} \text{ATP + Pyruvate}$$

$$\text{Pyruvate + NADH + H}^{+} \xrightarrow{\text{LDH}} \text{lactate + NAD}^{+}$$

Calculation

$$\text{Volume activity (U/ml)} = \frac{(\Delta Abs(test) - \Delta Abs(blank)) \times (3{,}00 + 0.01)}{19 \times 0.01}$$

$$\text{Specific activity (U/mg protein)} = \frac{volumeactivity}{protein\ concentration\ (^{mg}/_{ml})}$$

- d.f, dilute factor
- 6.22, millimolar extinction coefficient of NAD ($cm^2/\mu/mol$)
- Protein concentration, determined Bradford's method.

Expected value of pyruvate kinase

Results vary depending on the testing method used. In general, a normal value is 179 ± 16 units per 100mL of red blood cells (Elghetany and Banki, 2011). Normal value ranges may vary slightly among different laboratories. Some laboratory employs different measurements or assessment different samples. While an abnormal result means a low level of pyruvate kinase confirms pyruvate kinase deficiency (Gallagher, 2011).

3.3.4 Estimation of Hexokinase Activity

The assay is centered on the reduction of NAD+ via a coupled reaction with glucose-6-phosphate dehydrogenase. Glucose phosphorylated initially by hexokinase in a reaction with ATP. The product, glucose-6-phosphate (G6P), then undergoes oxidation to yield 6-phosphogluconate in the presence of NAD+ in a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH). For the duration of this oxidation, an equimolar amount of NAD+ is reduced to NADH. Therefore the reaction can be monitored by estimating the rise in absorbance at 340nm.

$$\text{D-glucose+ ATP} \xrightarrow{\text{HK}} \text{glucose-6-p+ADP}$$

$$\text{Glucose-6-P + NAD} \xrightarrow{\text{ZF}} \text{gluconate-6-P + NADH + H}$$

One unit of activity reduces one micromole of NAD+ per minute at 30OC and PH 8.0 under the specified conditions. Leuconostoc mesenteroides glucose-6-phosphate dehydrogenase (Worthington code: ZF or ZFL). Dissolve at a concentration of 300IU/ml in above Tris.MgCl2 buffer. Store at 0-4^{O}C during use S sample enzyme solution was dissolved in Tris.MgCl2 buffer, PH 8.0 to obtain a rate of 0.02-0.04 ΔA/min

Procedure: The reaction mixture was prepared by adding 2.28ml TrisMgCl2 buffer, 0.50ml of 0.67m Glucose, 0.10ml of 16.5mM ATP, 0.10ml of 6.8mM NAD and 0.01ml of G-6-PDH in a cuvette and incubated in the spectrophotometer at 30OC for 6-8minutes to achieve temperature equilibration and establish blank rate, if any. 0.1ml of diluted enzyme solution was included to the reaction mixture and mixed thoroughly. Change in absorbance at 340nmper minute was recorded for 3-4minutes; Determine Absorbance/min from initial linear portion of the curve.

Calculation:

Units/mg protein = $\frac{\Delta A340/min}{6.22 \text{ X mg enzyme/ml reaction mixture}}$

3.3.5 Enzymatic Activity of Glucose6Phosphatase

Definitions: Purified Water - Water from a deionizing system, resistivity > or = 18M?.cm at 25°C, G 6-P: Glucose 6-Phosphate, G6Pase: Glucose 6-Phosphatase, Pi -Inorganic Phosphate, PPi -Inorganic Pyrophosphate.

Glucose 6-phosphatase catalyses the hydrolytic dephosphorylation of glucose 6-phosphate to produce glucose and inorganic phosphate/orthophosphate, thus allowing glucose in the liver to enter the blood, the principal route for hepatic gluconeogenesis; deficiency causes glycogen storage disease. The enzyme activity was analytically determined by quantifying the quantity of liberated inorganic phosphate following the method of Nordlie and Arion (1966) and Taussky and Shorr (1953).

(PPi or Nucleoside di or triposphate) + Glucose $\xrightarrow{\text{G6Pase}}$ G6 P + (Pi or Nucleoside mono or diphosphate)

$$G6\,P + H_2O \xrightarrow{\text{G6Pase}} Glucose + Pi$$

$$PPi + H_2O \xrightarrow{\text{G6Pase}} 2Pi$$

Procedure

Conditions: T = 37°C, pH = 6.5, A660nm, Light path = 1 cm

Method: Spectrophotometric Stop Rate Determination

Reagents Preparation:

i. 100 mM BIS-TRIS Buffer, pH 6.5 at 37°C (Buffer): 200 ml was prepared in purified water using BIS-TRIS, Sigma Aldrich Product Number B9754. Adjust the pH to 6.5 at 37°C.
ii. 200 mM Glucose 6Phosphate (Sub): 10ml was prepared in purified water using D-Glucose 6-Phosphate Sodium Salt, Sigma Aldrich Product Number G7879.

iii. 20% Trichloroacetic Acid (TCA): 10ml was prepared in purified water using Trichloroacetic Acid Solution, 6.1 N, Sigma Aldrich Product Number T0699.

iv. Phosphorus Standard Solution, 20 (Std) Use neat, Sigma Aldrich Product Number P3869.

v. 5M Sulfuric Acid Solution: 50ml was prepared in purified water using Sulfuric Acid, Aldrich Product Number 258105.

vi. 10% Ammonium Molybdate Solution. 10ml was prepared in Sulphuric acid above using Ammonium MolybdateTetrahydrate, Sigma Aldrich Product Number A7302.

vii. TausskyShorr Color Reagent (TSCR) was prepared by adding 10mls of Reagent 'vi' to 70 ml of purified water and stirred. Then 5g of Ferrous Sulfate Heptahydrate(Sigma Aldrich Product Number Sigma Aldrich Product Number F7002) was added and stirred until it was completely dissolved. The resultant solution was made up 100ml adding purified water.

viii. Glucose-6-Phosphatase Enzyme Solution (Enz): the sample enxyme solution was prepared freshly before use in a solution containing 1.0 - 2.0 units/ml in cold purified water.

Enzymatic Assay:

The assay was carried out by pipetting the following reagents in appropriate containers (in milliliters):

To the test tubes labeled Test and Blank, 3ml of TRIS buffer and 1ml of Glucose 6-phosphate substrate was pipette, mixed by swirling and allowed to equilibrate at 37oC for a minimum of 5 minutes then 0.1ml of sample enzyme was added to the Test- test tube, mixed immediately by swirling and both test tubes incubated at 37°C for exactly 5 minutes. After incubation, 0.90ml of TCA was added to both test tubes and 0.1ml of purified water was added to Blank test tube. Both test tube were emptied into a sample bottle, tightly capped and mixed by inversion. Thee mixture was incubated for 5 minutes at 25°C and centrifuged at 4,000 rpm for 10 minutes and the supernatant collected in the color development step.

A Standard curve was prepared by pipetting (in milliliters) into suitable containers as follows: Purified water was pipetted in test tubes labeled Standard blank (2.0ml), Standard 1 (1.8ml), Standard 2 (1.6ml), Standard 3 (1.4ml), Standard 4 (1.2ml) and Standard 5 (1.0ml).

After which increasing volume of standard phosphorus was added to all test tubes 0.0ml, 0.2ml, 0.4ml, 0.6ml, 0.8ml and 1.0ml respectively. The mixture was mixed by swirling and 2ml of TSCR was added to all test tubes and allowed to incubate at 25oC for 56 minutes. All samples were transferred into appropriate cuvettes and absorbance change recorded at 660nm for each.

Calculations

Calculate the ΔA_{660nm} of the standards as follows:

ΔA_{660nm} = (A_{660nm} Std A_{660nm} Std Blank)

Plot ΔA_{660nm} of the standards versus μmoles of phosphorus and obtain the slope (m) and y-intercept (b) of the linear regression. Use these in the calculations for the test reaction mixtures.

Calculate the ΔA_{660nm} of each test solution as follows:

ΔA_{660nm} = (A_{660nm} Test – A_{660nm} Test Blank)

Calculate the μmoles of Pi liberated as follows:

μmoles Pi = (ΔA_{660nm} Test b)/m

Calculate the units per mg of enzyme as follows:

Units/mg S= (μmoles Pi * 5.0 * df)/(T * 0.1 * 2.0) Where:

5.0 = the final volume (in milliliters) of the enzymatic reaction

0.10 = volume (in milliliters) of the enzyme solution used

2.0 = volume (in milliliters) of enzyme assay used in color development

df = dilution factor of the enzyme solution

3.4 Estimation of Liver Enzymes

3.4.1 Lactate Dehydrogenase (LDH)

Lactate dehydrogenase level in the serum is measured to determine the presence of

damaged tissues. The level of the enzyme activity may indicate the presence of oxidative stress, liver disease, pancreatitis or other disease that damage body tissues. Lactate dehydrogenase catalyses the reaction

$$\text{Pyruvate+NADH} \rightleftharpoons \text{Lactate} + \text{NAD}$$

The reaction velocity is determined by a decrease in the absorbance at 340nm resulting from oxidation of NADH. One unit causes the oxidation of 1micromole of NADH per minute at 25^{O}C and pH 7.3 under specified conditions.

Procedure

From the reagent kit provided,2.8ml of 0.2 M Tris·HCl at pH 7.3, 0.1 ml of 6.6mM NADH,0.1ml of 30mM Sodium Pyruvate, was pipetted into appropriate cuvettes labeled sample and blank and incubated for 4-5 minutes at 25^{O}C. 0.1ml of the sample was added to thetest tube labeled sample. The spectrophotometer was zeroed with the blank and absorbance was read at 340nm at interval of 60secs for 4 minutes.

Calculation

$$UI/L = \frac{\Delta A/Min \text{ X } totalvolume}{absorptivity \text{ X } sample\ volume}$$

3.4.2 Aspartate Amino Transferase (AST) Determination:

The first kinetic assay of AST for diagnostic purpose was described by (Karmen, et al; 1955). Using a couple reaction of malate dehydrogenase (MDH) and NADH; this assay system was critically evaluated and optimized in 1960 by (Henry, 1960). In 1977, the International Federation of Clinical Chemistry (IFCC) suggested a reference method for estimating AST activity centered upon Karmen's procedure. The AST makes use of the formulation suggested by the IFCC.

Principle

The enzymatic reaction sequence principle that was used in the assay of aspartate aminotransferase is as follows;

L-Asparate + 2-oxoghitarate AST Oxalacetate + L-GlutamateOxaloacetate + NADH = H^+ MDH malate + NAD^+ + H_2O

AST catalyzes the transfer of an amino group between L - aspartate and 2 - oxoghitarate. The oxaloacetate formed in the initial reaction is then reacted with NADH to yield NAD in the presence of malate dehydrogenase (MDH), AST activity was determined by measuring the degree of oxidation of NADH at 340nm, lactate dehydrogenase was included in the reagent to convert endogenous pyruvate in the sample to lactate for the period of the lag stage prior to measurement.

Manual Procedures for AST Determination:

1. AST working reagent was prepared according to instructions.
2. Spectrophotometer was zeroed at 340mm with distilled water.
3. For each sample and control, 1.0ml working reagent was added to cuvette and warmed to 37oC for 3 minutes.
4. 0.10ml of the serum was added to each tube respective and mixed gently. Absorbance was read and recorded at 1 minute, incubating continued at 37oc and absorbance was again recorded at 2 and 3 minutes.
5. Average absorbance per minute (DA/mim) was determined and multiplied by factor 1768 for result in U/L.

3.4.3 Alanine Amino Transferase (ALT)

Estimation the enzyme alanine aminotransferase is widely reported in a variety of tissue sources. Hepaticorigin is the main source of ALT and hence the employment of ALT determinations in studying hepatic disease. Elevated serum levels are seen in hepatitis, cirrhosis, and obstructive jaundice. In myocardial infarction, the level of ALT is only mildly elevated. UV method for ALT determination was first developed by Wroblewski and LaDue in 1956. The method was based on oxidation of NADH by lactate dehydrogenase (LDH). In 1980, IFCC suggested a reference method for the measurement of ALT based on the Wroblewski and LaDue technique. The ALT reagent conforms to the design suggested by the IFCC.

Principle

The enzymatic reaction sequence principle used in the assay of ALT is as follows: L-Alanine + 2-oxoglutarate. Alanine aminotransferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2,4dimitrophanylhydrazine (Reitman and Frankel; 1957).

Procedure for ALT Estimation

Spectrophotometer was zeroed at 546nm with distilled water. Two cuvettes were labeled reagent blank and sample. To the cuvette labeled sample, 0.1ml sample serum was pipette after which 0.5ml of the ALT reagent (R1) solution was pipette into both cuvettes and 0.1ml distilled water pipette into the blank cuvettes. All cuvettes were mixed and incubated for exactly 30min at 37oC. After incubation, 0.5ml of ALT reagent two (R2) was added into both cuvette, allowed to mix and stand for exactly 20mins, at 20 to 25oC. Afterward sodium hydroxide, 5.0ml was added into both cuvette as an activator and absorbance of sample (A sample) against the reagent blank was read after 5 minutes.

Results

Values are derived based on the absorptivity micromolar extinction coefficient of NADH at 340nm (0.00622). Units per litre (U/L) of AST/GOT activity are that amount of enzyme which oxidizes one umol/L of NADH per minutes.

3.4.4 Determination of Bilirubin Level

Direct and indirect bilirubin levels were measured to determine the in vitro quantity of serum bilirubin.

Principle of Test

Elevated levels of conjugated or direct bilirubin shows that bile is not being appropriately excreted; suggestive of the presence of an obstruction in the bile duct or gall bladder. Unconjugated or indirect bilirubin level can also be estimated by deducting the direct bilirubin level from the total bilirubin result. Elevated levels of unconjugated bilirubin suggest that much hemoglobin is being destroyed or that the liver is not actively metabolizing the

hemoglobin coming to it.

Levels of bilirubin can be measured using colorimetric method described by Jendrassik and Grof, (1938). Direct bilirubin reacts with diazotisedsuphanilic acid in alkaline medium to form a blue coloured complex. Total bilirubin is measured when caffeine present, there is release of albumin bound bilirubin, by the reaction with diazotisedsulphanilic acid. The product of the reaction and the intensity of the complex formed can be colourimetrically determined using a spectrophotometer to measure the level of absorbance and calculated to obtain the bilirubin level.

Procedure

For total bilirubin test: The reagent provided in the bilirubin kit; R1, R2, R3 and sample were mixed and allow to stand for 10min at 20-25^{o}C. Billirubin reagent (R4) to the sample and allow to stand for 5-30mins at 20-25^{o}C. Sample blank was prepared with the procedure above but without the sample. The absorbance of the sample against the sample blank was then read at 578nm wavelength.

For direct bilirubin: The reagent provided in the bilirubin kit; R1, R2, 0.9% NaCl and sample were mixed and allowed to stand 10min at 20-25^{o}C. Sample blank was prepared with the procedure above but without the sample. The absorbance of the sample against the sample blank was then read at 578nm wavelength.

Calculation

Total Bilirubin (µmol/l)	=	185 X A_{TB} (578nm)
Total bilirubin (mg/dl)	=	10.8 X A_{TB} (578nm)
Direct Bilirubin (µmol/l)	=	246 X A_{DB} (546nm)
Direct bilirubin (mg/dl)	=	14.4 X A_{DB} (546nm)

Where A = Absorbance

3.4.5 Assessment of Kidney Function

3.4.5.1 Urea (Colorimetric Technique)

Serum urea content in samples is estimated by the aid of an automated analyzer, "Blood Urea Analyzer, Beckman Coulter Inc., USA". The procedure requires a combination of reagents,

Hichem kit of reagents for blood urea nitrogen analyzer. The kit is supplied by Elan Diagnostics, USA (Adekomi, 2010).

3.4.5.2 Creatinine (Colorimetric Method)

Creatinine Analyzer-2 (Beckman Coulter Inc., USA) in combination with a particular kit of reagents (HichemCreatine Pak, Elan Diagnostics, USA) used to calculate creatinine content of the serum samples (Adekomi, 2010).

3.4.6 Total Protein

Principle: In an alkaline medium, cupric ion reacts with protein peptide bonds resulting in the production of a coloured complex.

Sample: Serum, heparinized plasma or EDTA plasma.

Materials: Biuret Reagent, Blank Reagent, Standard.

Reagent:

i. Biuret reagent (stock solution): 45g of sodium potassium tartarate was dissolve in 400ml of 0.2N (0.8%) NaOH, afterwhich 15g copper sulphate and 5.0g potassium iodide was then added, and the volume up made to 1litre with 0.2N NaOH.
ii. Biuret working solution: 200ml of the stock was diluted to 1litre with 0.2N NaOH containing 5g/l potassium iodide.
iii. Tartarate iodide solution: 9g of sodium potassium tartarate was dissolved in 1litre of 0.2N NaOH containing 5g/l potassium iodide.
iv. Standard protein: haemolysis free pooled sera were collected from experimental rats. The protein content was determined by Kjedahl's method (By boiling in the presence of sulphuric acid and a catalyst, proteins are digested). Store at 2-8^{o}C.

Procedure

Four test tubes were labeled 'Test' in which 5.0ml of working biuret reagent and 0.05ml was added,'Testblank' which contained 5.0ml Tartarate iodide solution and 0.05ml serum, 'Standaard' which contained 5.0ml working biuret reagent and 0.05 standard and 'Blank' containing 5.0ml biuret reagent and 0.05ml distilled water.All test tubeswere mixed and incubated for 10mins at 20-25^{o}C.Absorbanceof the sample (A sample) and of the standard (A standard) at 540nm (green filter), was measured, zeroing the spectrophotometer with the

reagent blank.

Calculation

When measurements were taken at 540nm, total protein concentration was calculated as follows:

Total Protein conc. (g/l) =190 multiplied by A sample

Total Protein conc. (g/l) =19 multiplied by A sample

When using standard

Total protein conc. = $\frac{\text{A sample}}{\text{A standard}}$ multiplied by standard concentration

3.4.7 Assay of Enzymatic Antioxidants

3.4.7.1 Assay of catalase (CAT) activity

Catalase activity was estimated in accordance with the method by Aebi (1983). 0.1 ml of the homogenates (supernatant) was pipetted into cuvette containing 1.9 ml of 50mM phosphate buffer, pH 7.0. Reaction was started by the addition of 1.0 ml of freshly prepared 30% (v/v) hydrogen peroxide. The rate of breakdown of H2O2 was estimated spectrophotometrically for differences at absorbance of 240nm. Activity of enzyme was expressed as units /mg protein.

3.4.7.2 Assay of Superoxide Dismutase (SOD) Activity

SOD activity was estimated in accordance with the method described by Misra and Fridovich (1972) and adapted by Isamah et al, in 1994. The assay principle was based on the ability of SOD to impede the autoxidation of epinephrine by superoxide radical (O2-). Briefly, the reaction mixture contained 0.2ml of the Stomach suspension; 2.5ml of 0.05ml carbonate buffer pH 10.2 was added. The reaction will start by adding 0.3ml freshly prepared 0.3Mm adrenaline. This was mixed by inversion. A reference curette containing 2.5ml of the buffer and 0.2ml of water and 0.3ml of the substrate (epinephrine). The increase in absorbance was monitored at 480nm every 30seconds for 150seconds. Percentage reduction in SOD activity is then calculated: One unit of SOD is expressed as the amount of enzyme needed to impede the autoxidation of epinephrine by 50% under the specific conditions. It was stated as u/mg

protein.

3.5 HISTOPATHOLOGICAL EXAMINATION

3.5.1 Preparation of Tissue for Microscopic Examination

The process of preparation of tissue for histological examinations is divided into a number of phases: it starts with the tissue to be processed being impregnated and the tissue embedded in a medium to offer support and appropriates ability for the microtomy cutting with the aid of differential concentration of alcohol from 70% to 100% to dehydrate, the tissue. Next, the tissue was treated with paraffin wax by means of an automatic tissue processor by the following schedule. The sample was embedded in paraffin wax at 70^{o}C and cut with a rotary microtone 4µ. The staining technique employed in this study was the haematoxylin and eosin staining techniques.

3.5.2 Photomicrography

Stained tissue images were captured using digital microscopic eyepiece 'Scoptek' Dcm 500, 5.0mega pixels connected to USB 2.0 computer.

3.6 STATISTICAL ANALYSIS

All the data are expressed as mean ± standard error of mean SEM. Statistical comparisons were performed using ANOVA and followed by Fisher's least significant difference (LSD). The SPSS software (version 20) was used in the statistical analysis using multiple comparison tests. A p-value of less than 0.05 ($p < 0.05$) was considered significant.

CHAPTER FOUR

4.0 RESULTS

The result obtained from the statistical analysis were divide in two parts; results from analysis on the plant in two sections; section 4.1.1 Phytochemical analysis, x-raying the plants phytochemicals and section 4.1.2 Gas Chromatography and Mass Spectrum screening of the plant fractions. Part two includes the result of the plants activity on some biochemical parameters studied in seven sections; Section 4.2.1 shows treatment with the crude fraction and extract of VA influences the animal body weight as well as the blood glucose level, Section 4.2.2 shows influence on glucose metabolism, Section 4.2.3 shows effect on lipid profile, Section 4.2.4 shows effect on liver function, Section 4.2.5; renal function test, section 4.2.6; peroxidation of lipids and section 4.2.7 shows effect on enzyme antioxidant activity. All results are presented in figures stated as Mean ± standard error of mean, presenting the influence of VA as stated below.

4.1 Analysis on the Plant Phytochemistry and Active Constituents

4.1.1 Phytochemical Screening of *Vernonia amygdalina* crude extract

Table 4.1: Qualitative analysis of the aqueous extract of *Vernonia amygdalina*

Chemicals	*Vernonia amygdalina* Del
Alkaloids	+ + -
Saponins	+ + +
Tannins	+ - -
Flavonoids	+ + +
Phenols	+ + +
Anthraquinones	+ + -
Cardiac glycosides	+ + +
Steroids	+ + +

Keys: + + + (Abundantly present), **+ + - (Moderately present)**, **+ - - (trace amount)**

Table 4.2 Quantitative analysis of the phytochemical extract of *Vernonia amygdalina* Del

Chemicals	fractionates extract	Crude extract
Alkaloids	5.9%	3.9%
Saponins	8.2%	8.3%
Tannins	2.3%	3.2%
Flavonoids	6.8%	7.6%
Phenol	6.0%	6.9%
Glycosides	5.2%	5.8%

Qualitative and quantitative assessment of the plant extract showed the presence of phytochemical constituent similar to the finding of Imaga and Bamigbetan, (2013).

Table 4.3Nutritional analysis of *Vernonia amygdalina* Del extract

Chemicals	Ethanol extract
Vitamin C (mg)	**2.9±0.02**
Protein (mg)	**2.5±0.02**
Crude fibre (%)	**1.2±0.01**
Lipid (%)	**27.7±0.02**
Ash content (%)	**6.0±0.01**
Carbohydrates (%)	**58.00±0.03**
Moisture content (%)	**20.00±0.01**

Nutritional assessment of the plant extract showed the presence of constituents similar to the finding of Imaga and Bamigbetan (2013).

Identification of Bioactive Constituents in *Vernonia amygdalina*

The VA fractions were analyzed with GC-MS for bioactive constituents. Results from the assay are as follows;

Analysis on Benzene Fraction

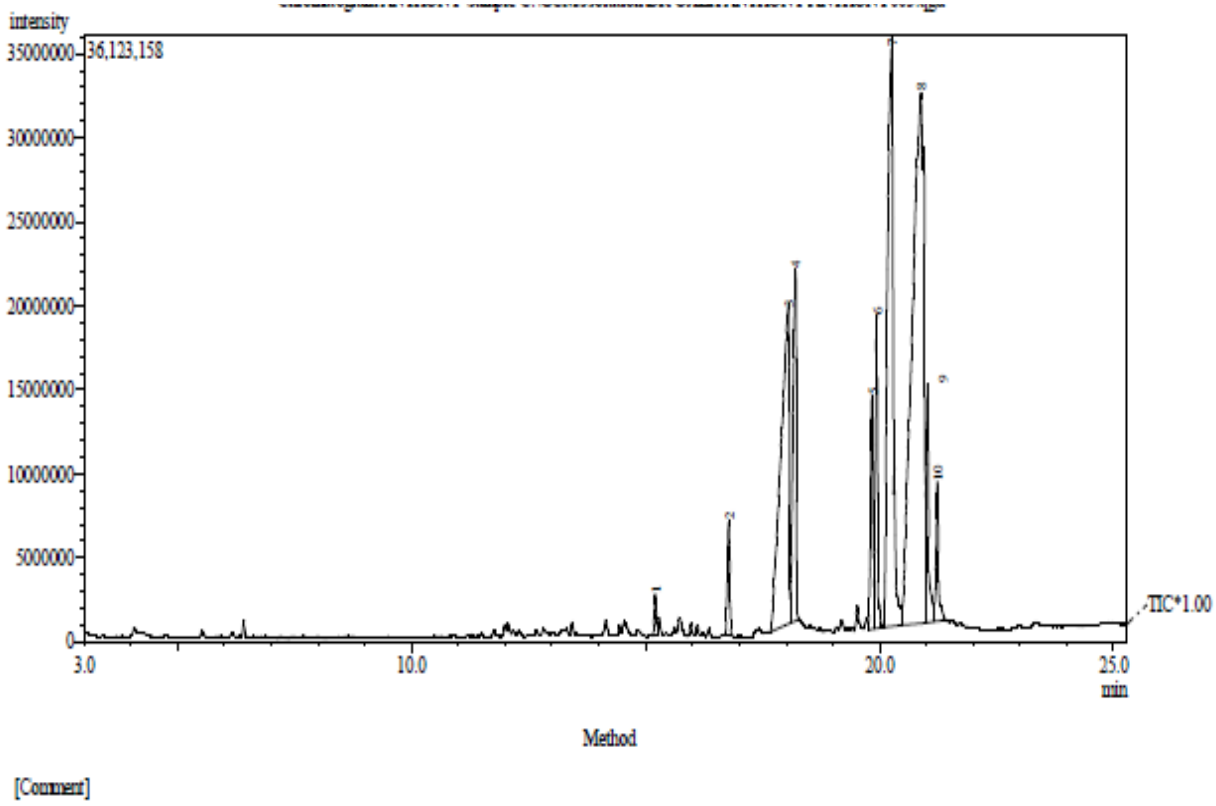


Fig. 4.1.4: GC-MS chromatogram of Benzene fraction of *VA*. Ion fragmentation pattern for spectral peak at t_R20.264mins and 20.879mins was specific for 15-MethylHexadeconoic acid and Octadecatrienoic acid respectively.

Analysis of Chloroform Fraction

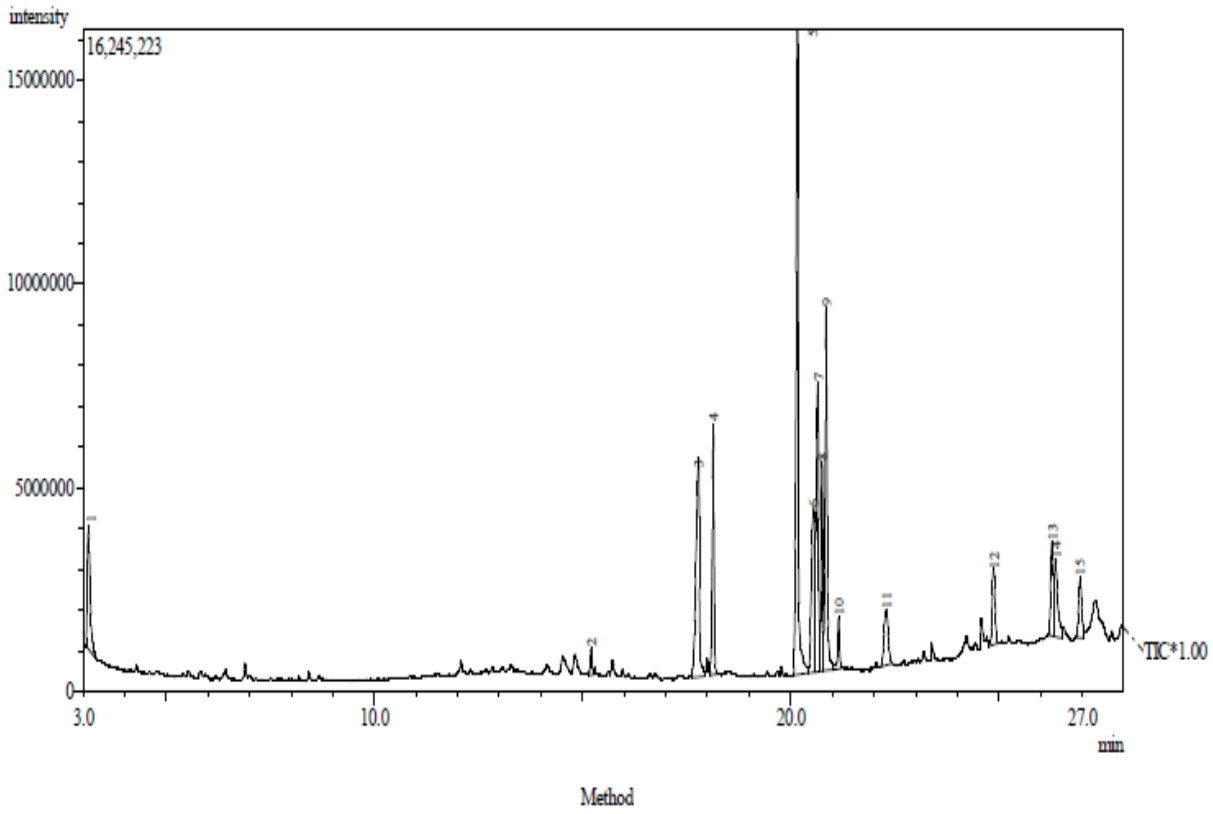


Fig. 4.1.5: GC-MS chromatogram of Chloroform fraction of *VA.*Ion fragmentation pattern for spectral peak at t_R 20.161mins was specific for 2-Hexadecen-1-ol.

Analysis of Ethyl Acetate Fraction

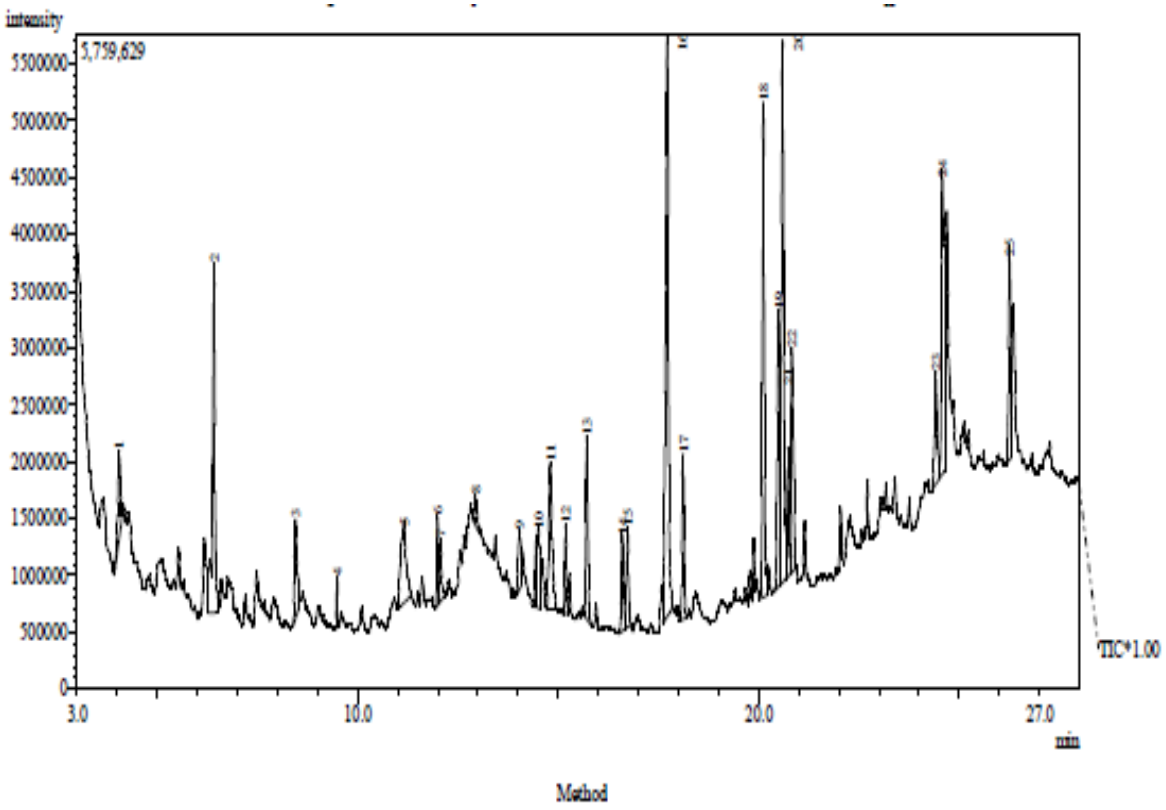


Fig. 4.1.6: GC-MS chromatogram of Ethyl acetate fraction of *Vernonia amygdalina*. Ion fragmentation pattern for spectral peak at t_R 17.722mins, 20.116mins and 20.595mins was specific for Hexadecanoic acid, Phytol (Hexadecen-1-ol) and 9,12,16-Octadecatrienal respectively.

Analysis of Butanol Fraction

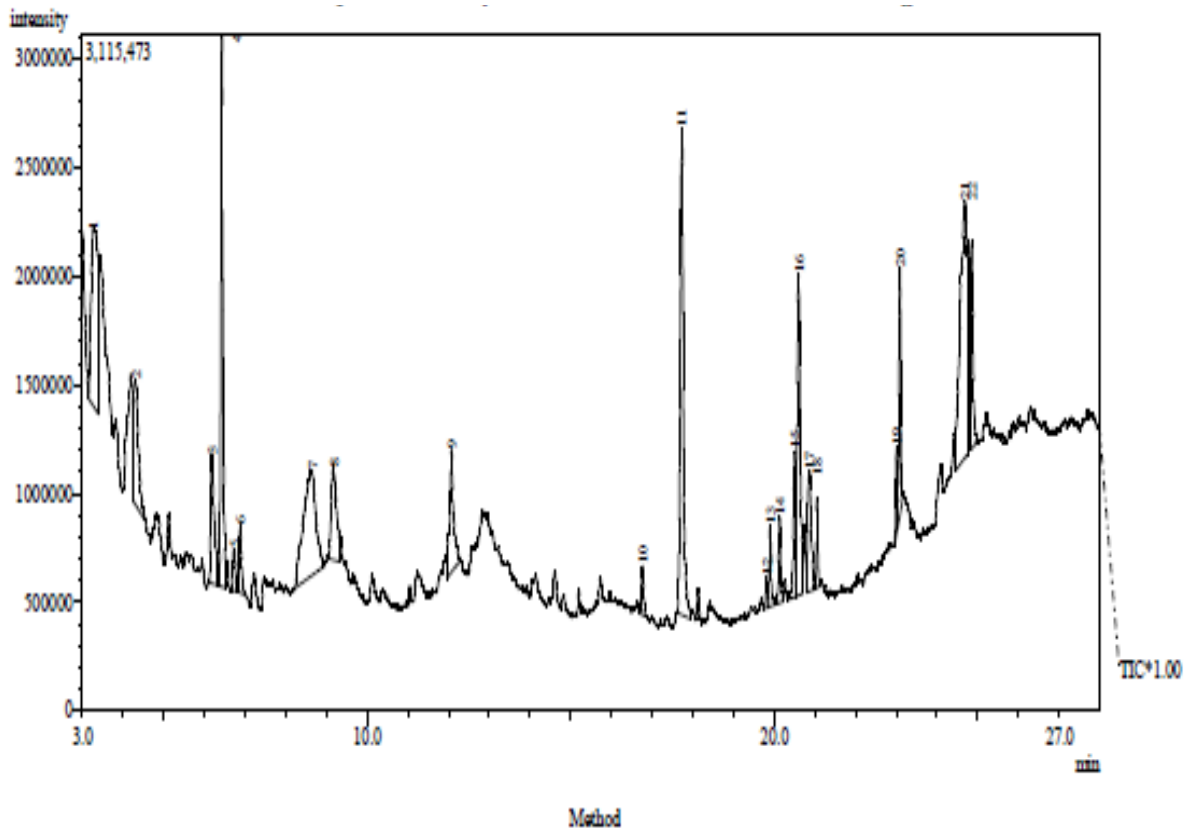


Fig. 4.1.7: GC-MS chromatogram of Butanol fraction of *Vernonia amygdalina.* Ion fragmentation pattern for spectral peak at t_R6.43mins and 17.729mins was specific for 2,3-Pentanedioneand Hexadecanoic acid respectively.

4.2 Effect of VA on biochemical parameters in experimental rats

Results from statistical analysis are presented in the figure below with the following designations;

All values designated ([a]) showed significant increase when compared to control
All values designated ([b]) showed significant decrease when compared with control

4.2.1 Effect of VA crude extract and fractions on the body weight of experimental animals

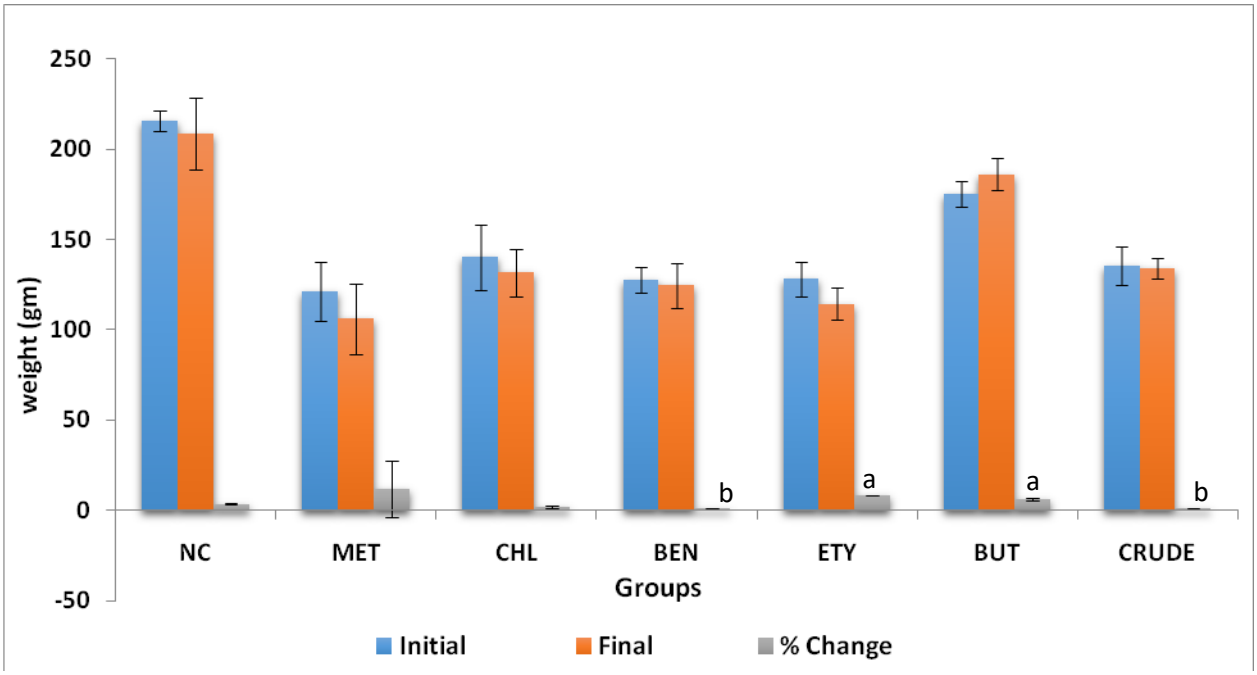


Fig. 4.5: Presenting the influence of VA fractionates on the body mass of treated STZ-induced diabetic animals stated as Mean±S.E.M.

Key: **NC**: Diabetic Untreated, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction.

(a) Significant increase when compared to diabetic control, (b) Significant decrease when compared with diabetic control

The influence of VA crude extract and fractions on the body mass of non-diabetic experimental rats

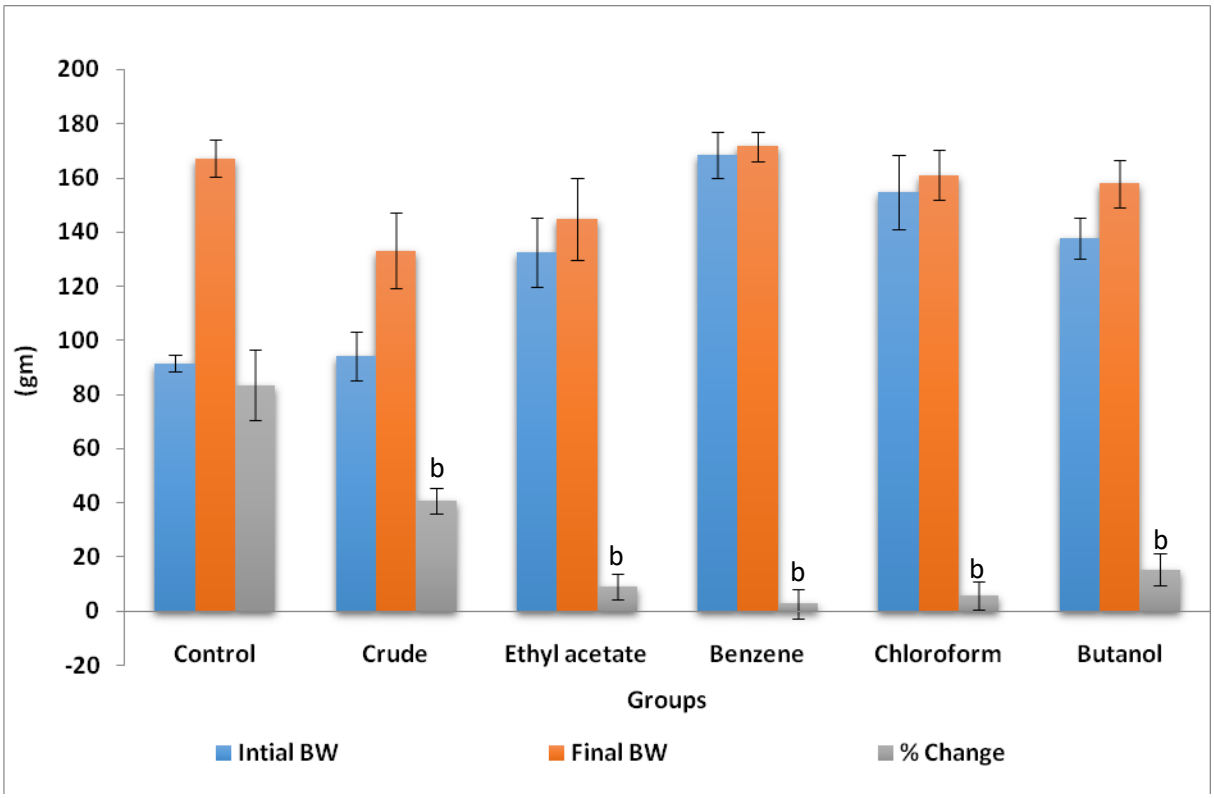


Fig. 4.6: Shows the influence of VA fractionates on the body mass of treated non-diabetic experimental animals stated as Mean±S.E.M.

Key: **NC**: Diabetic Untreated, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction.

(a) Indicates a significant increase when compared with diabetic control, (b) Shows significant decrease when compared with diabetic control.

DM affects metabolism of macromolecules and subsequently resulting in significant weight loss in diabetics.

4.2.2 Effect of VA crude extract and fractions on the fasting sugar level of investigational rats.

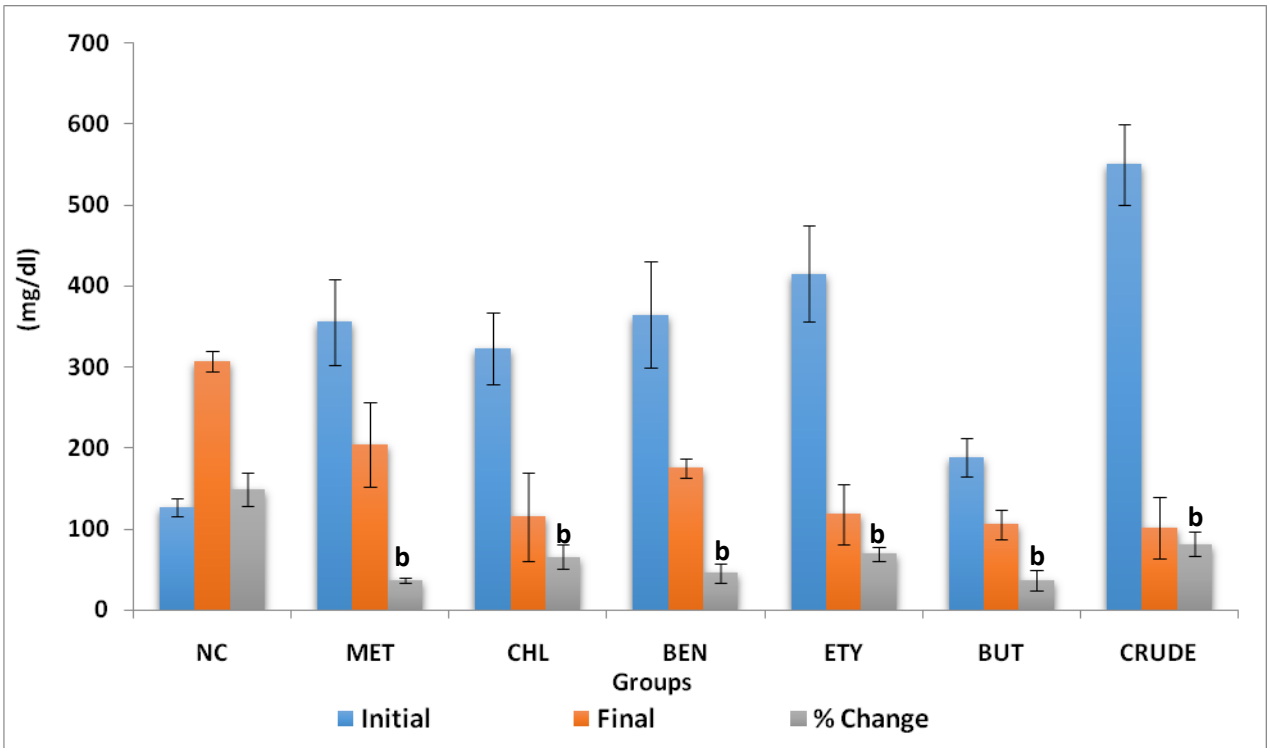


Fig. 4.7: Shows the effects of VA fractionates on the fasting blood sugar level of treated STZ induced diabetic investigational rats stated as Mean±S.E.M

Key: **NC**: Diabetic Untreated, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction.

(a) Indicates significan t increase when compared with diabetic control, (b) Shows significant decrease when compared with diabetic control.

Effect of VA crude extract and fractions on the fasting sugar level of non-diabetic experimental rats

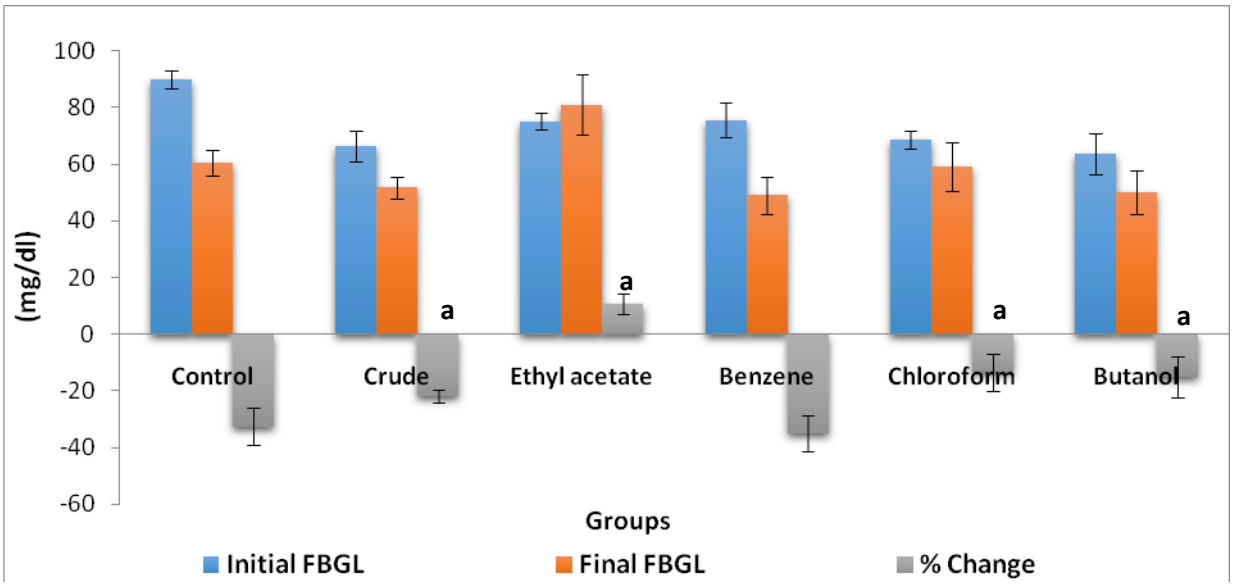


Fig. 4.8: Effect of VA crude extract and fractions on the fasting sugar level of non-diabetic experimental rats.

Key: **NC**: Diabetic Untreated, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction

(a) Indicates a significant increase when compared with diabetic control, (b) Shows a significant decrease when compared with diabetic control.

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4.2.3 Effect of VA crude extract and fractions on the enzymes of glucose metabolism of experimental animals

The important pathways involved in the metabolism of glucose; glycolytic, pentose phosphate and gluconeogenesis pathway were studied to ascertain the influence of treatment with VA crude extract and fractions in the management of diabetic rats.

4.2.3.1 Effect of VA crude extract and fractions on the enzymes of glycolytic pathway

Effect of VA on Hexokinase activity are presented in the figure below;

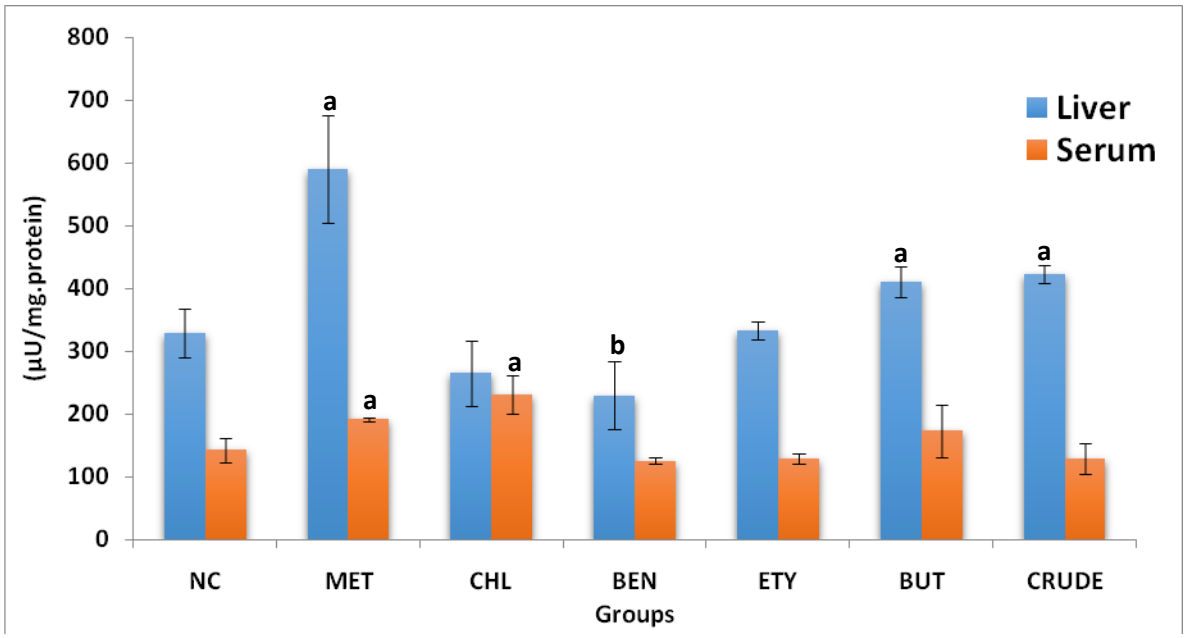

Fig. 4.9: Shows the effect of VA on hexokinase activity in liver and serum of treated STZ-induced diabetic investigational rats stated as Mean±S.E.M

Key: **NC**: Diabetic Untreated, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction.

(a) Symbolizes a significant increase when compared with diabetic control, (b) Indicates a significant decrease when compared with diabetic control.

Effects of VA on pyruvate kinase activity in diabetic experimental rats

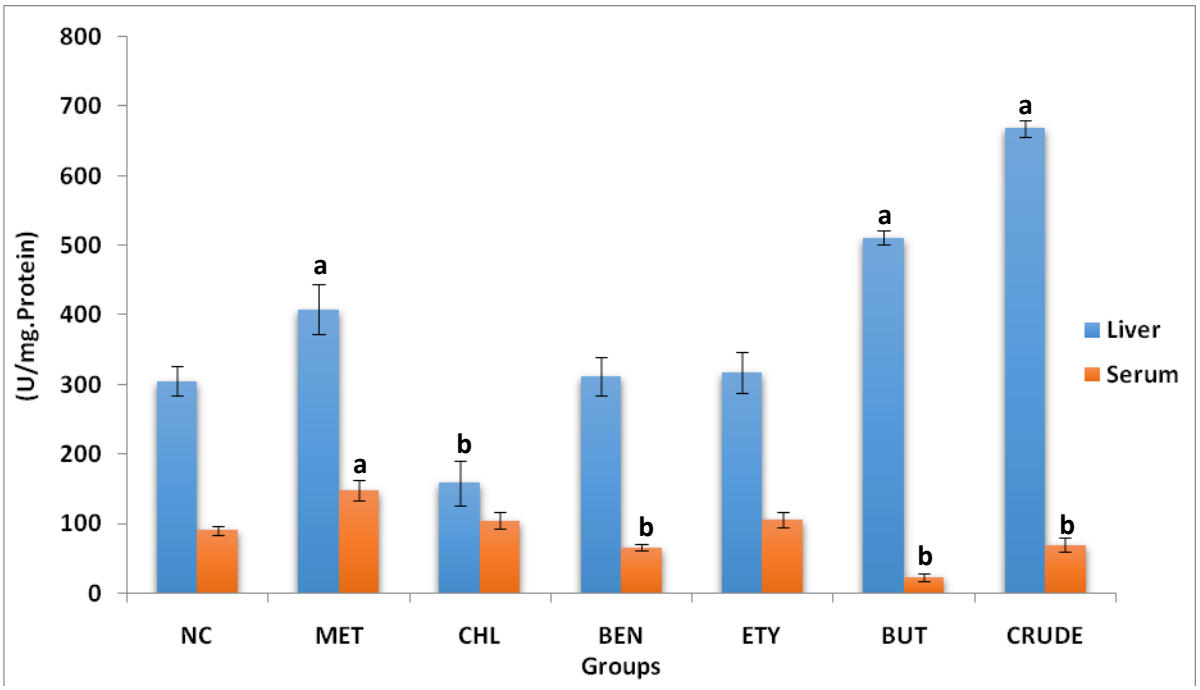

Fig. 4.10: Shows the effects of VAon the level of Pyruvate kinase activity in the liver and serum of treated STZ-induced diabetic investigational rats stated as Mean±S.E.M

Key: **NC**: Diabetic Untreated, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction

(a) Symbolizes a significant increase when compared with diabetic control, (b) Indicates a significant decrease when compared with diabetic control.

Effects of VA on Hexokinase and pyruvate kinase activity in non-diabetic experimental rats

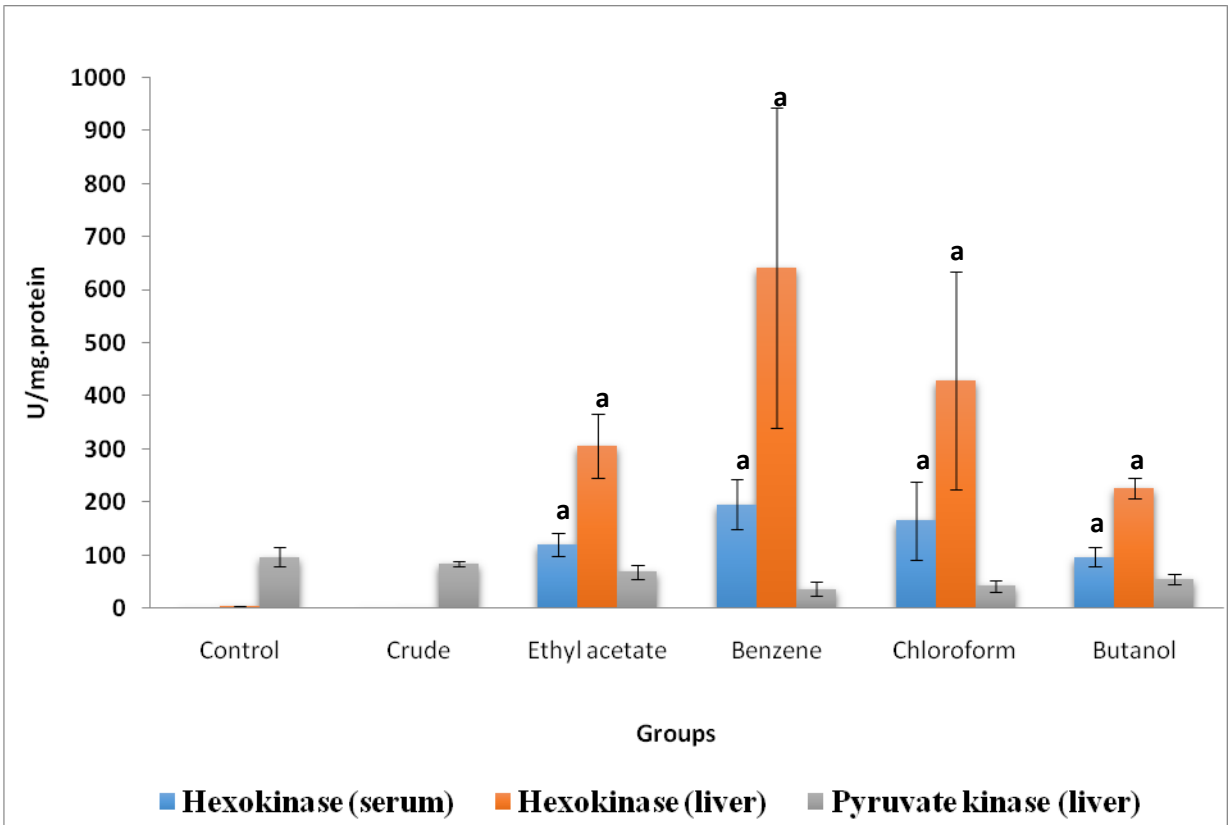

Fig. 4.11: Shows the effect of VA on Hexokinase and Pyruvate kinase activity in liver and serum of treated non-diabetic investigational rats stated as Mean±S.E.M

Key: **NC**: Diabetic Untreated, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction.

(a) Indicates a significant increase when compared with diabetic control, (b) Shows a significant decrease when compared with diabetic control.

4.2.3.2 Effect of VA crude extract and fractions on the enzymes of gluconeogenesis pathway.

The influence of VA on target marker enzymes of gluconeogenesis was assessed as presented in the figures below;

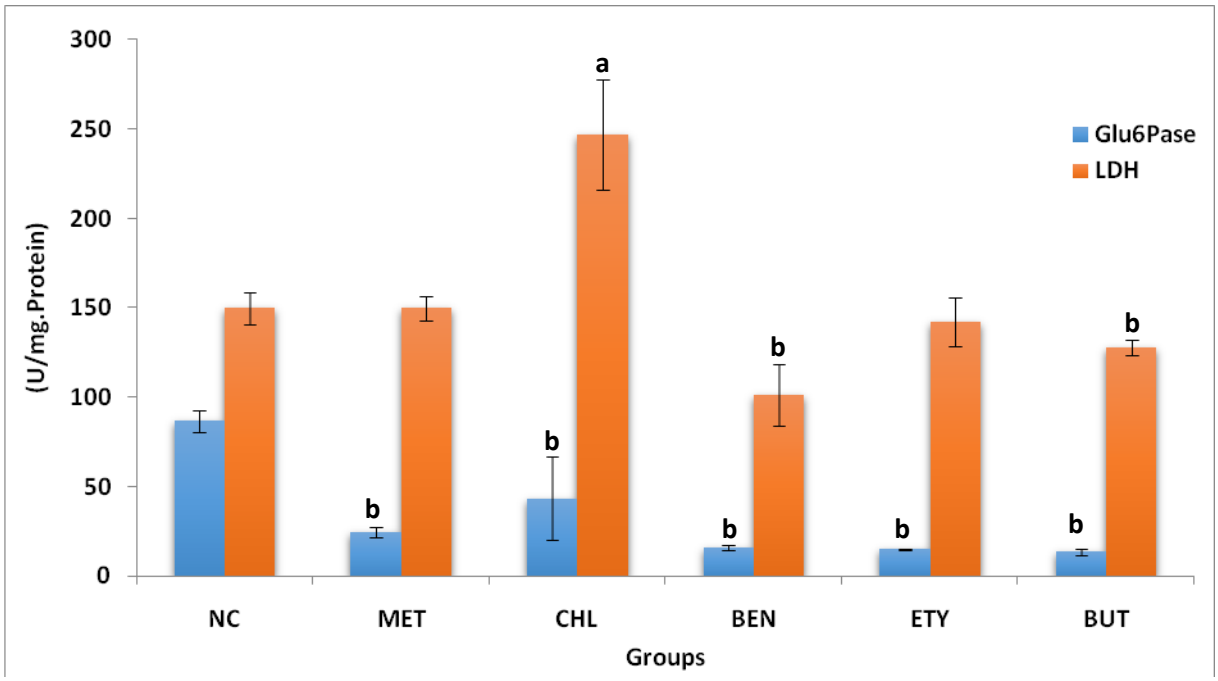

Fig. 4.12: Shows the effect of VA on the activities of lactate dehydrogenase as well asGlucose-6-phosphatasein serum and liver of treated STZ-induced diabetic experimental animals stated as Mean±S.E.M

Key: **NC**: Diabetic Untreated, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction.

(a) Indicates a significant increase when compared with diabetic control, (b) Symbolizes a significant decrease when compared with diabetic control.

DM causes a state of energy starvation and in a bid to maintain body functions, the body looks to non-carbohydrate sources for the generation of glucose for cellular metabolism, a process known as gluconeogenesis.

4.2.3.3 Effect of VA crude extract and fractions on the enzymes of the pathway of pentose phosphate.

The influence of V. amygdalina on target marker enzymes of the pathway was assessed as presented in the figures below;

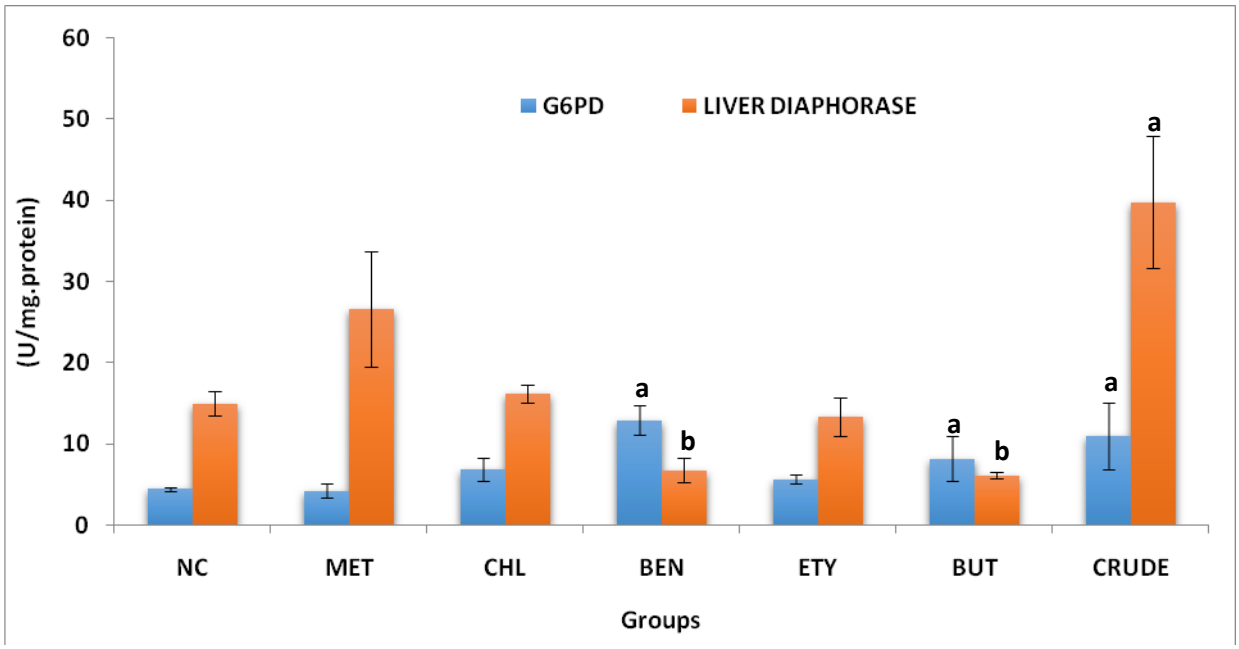

Fig. 4.13: Shows the effects of VA on Glucose-6-phosphate dehydrogenase (G6PDH) level, and Diaphorase (NADPH) activity in the serum and liver of treated STZ-induced diabetic investigational rats stated as Mean±S.E.M

Key: **NC**: Diabetic Untreated, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction.

(a) Symbolizes a significant increase when compared with diabetic control, (b) Shows a significant decrease when compared with diabetic control.

Effect VA on Glucose-6-phosphate dehydrogenase (G6PDH) level, and Diaphorase (NADPH) activity in non-diabetic experimental rats.

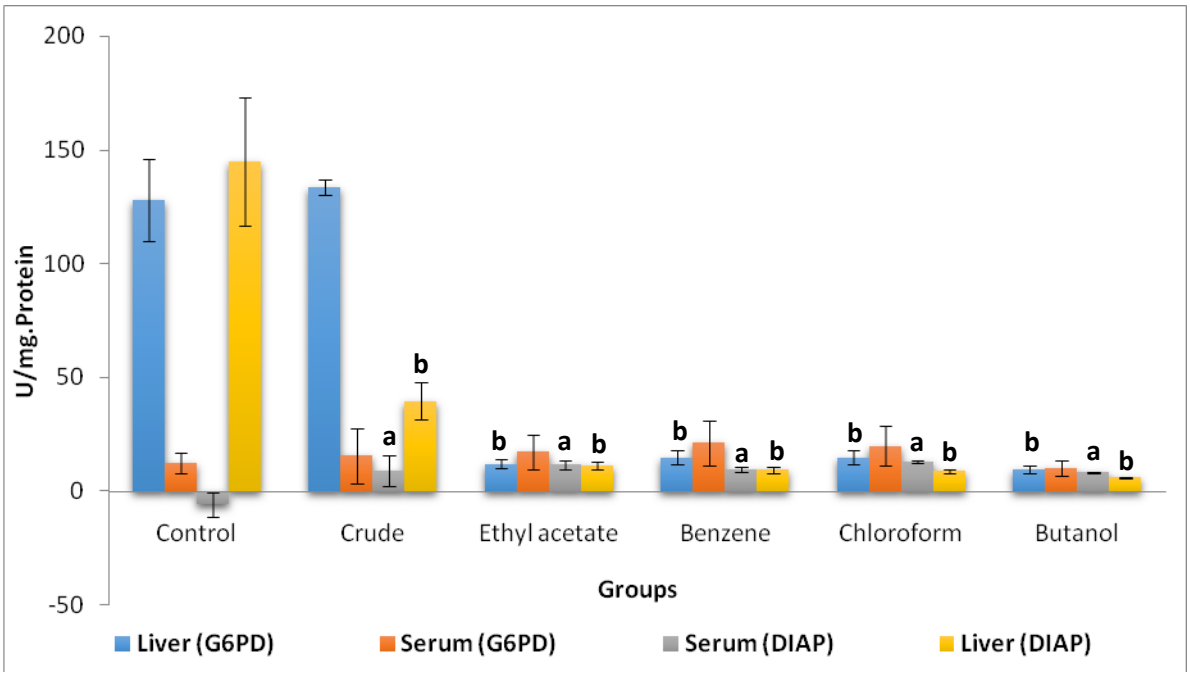

Fig. 4.14: Shows the effect of VA on Glucose-6-phosphate dehydrogenase (G6PDH) level and Diaphorase (NADPH) activity in the serum and liver of treated non-diabetic investigational rats stated as Mean±S.E.M

Key: **NC**: Diabetic Untreated, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction, **DIAP**: Diaphorase.

(a) Symbolizes a significant increase when compared with diabetic control, (b) Indicates a significant decrease when compared with diabetic control.

In DM, pathways such as the pentose phosphate shunt are initiated in an effort to rid the body of excess circulating glucose. The pathway consumes glucose via oxidative and non-oxidative reactions to generate ribose sugar, synthesis of glutathione and NADPH as energy. Important enzymes such as glucose 6-phosphate dehydrogenase (G6PDH) and diaphorase which are important to the reaction are impaired in diabetes.

4.2.4 Effect of VA crude extract and fractions on lipid profile of investigational rats.

The effect of VA on lipid metabolism was assessed using serum lipid as presented in the figures below;

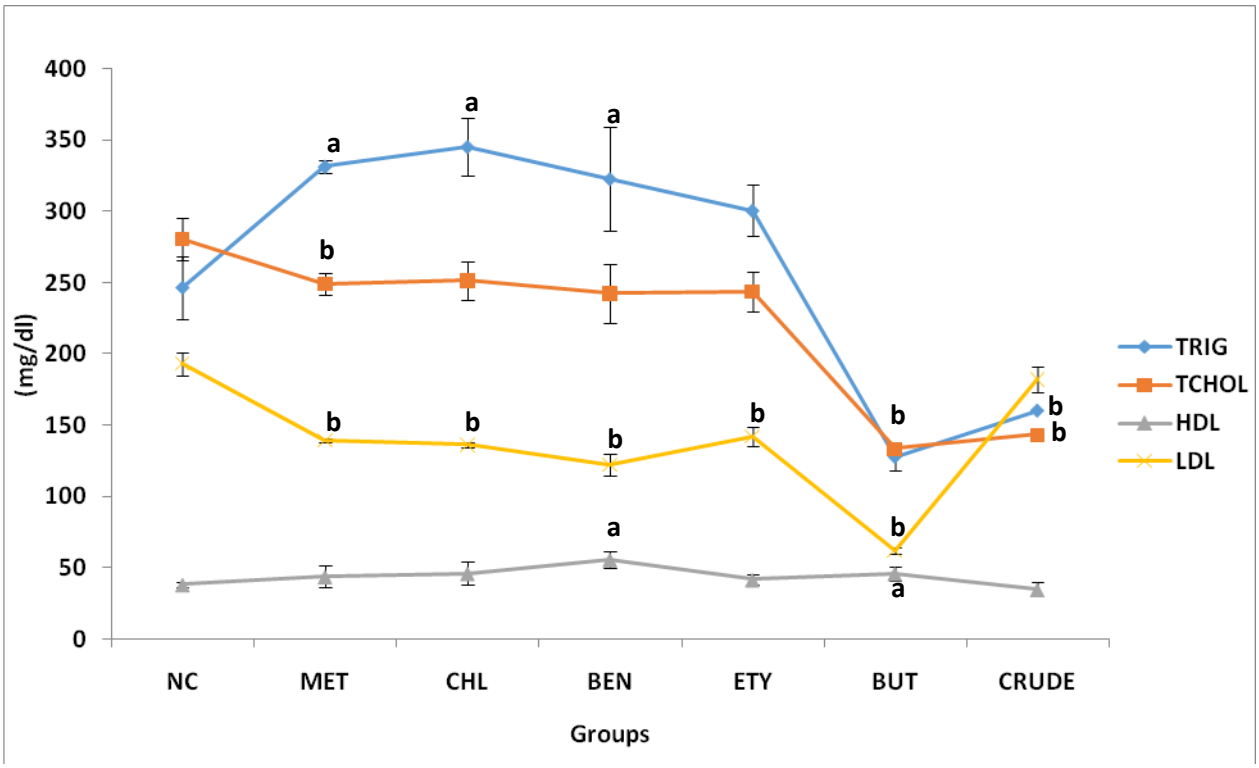

Fig. 4.15: Shows the influence of VA treatment on serum level of lipids in STZ-induced diabetic investigational rats stated as Mean±S.E.M.

Key: **NC**: Untreated Diabetic, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction, **TRIG**: Triglycerides, **TCHOL**: Total Cholesterol, **HDL:** High Density Lipoprotein, **LDL**: Low Density Lipoprotein.

(a) Shows a significant increase when compared with diabetic control, (b) Indicates a significant decrease when compared with diabetic control.

Effect of VA treatment on serum lipids in non-diabetic experimental rats.

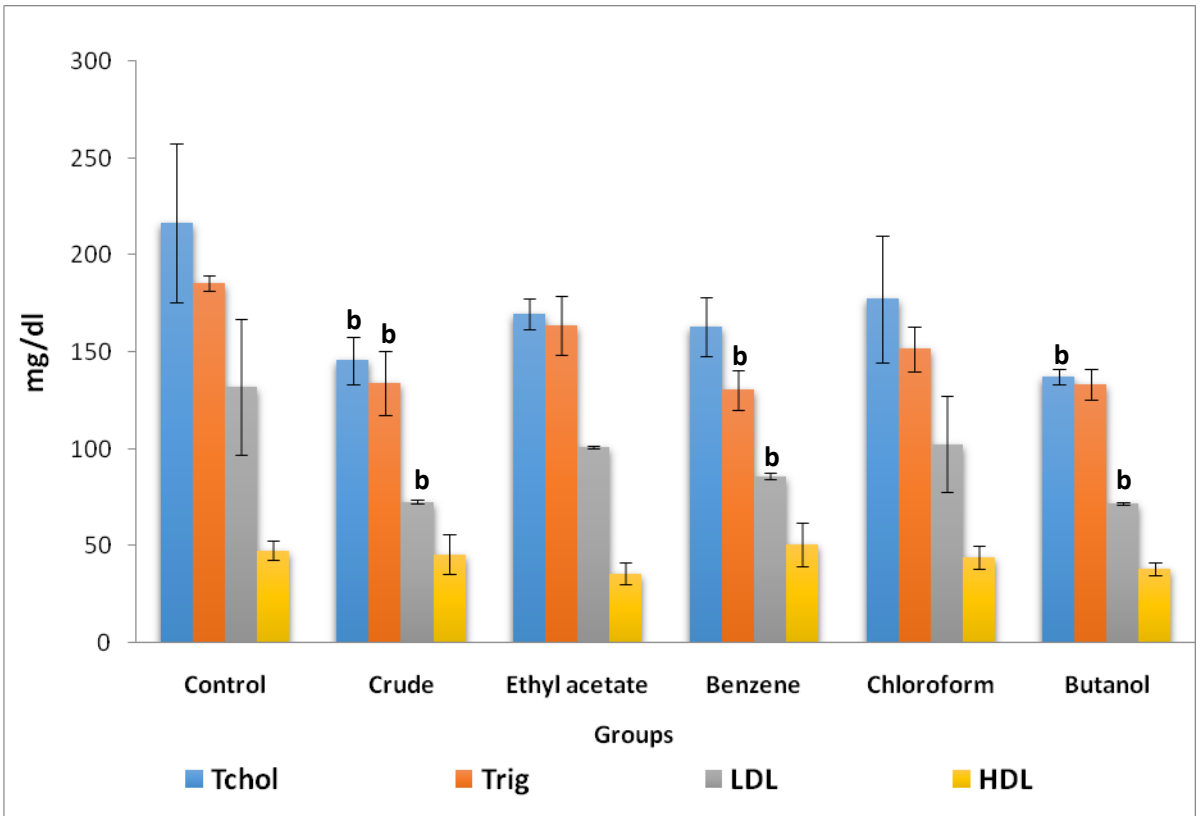

Fig. 4.16: Shows in the effect of VA treatment on serum lipids in non-diabetic investigational rats stated as Mean±S.E.M.

Key: **NC**: Untreated Diabetic, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction, **TRIG**: Triglycerides, **TCHOL**: Total Cholesterol, **HDL:** High Density Lipoprotein, **LDL**: Low Density Lipoprotein.

(a) Symbolizes a significant increase when compared with diabetic control, (b) Indicates a significant decrease when compared with diabetic control.

A common complication seen in DM is lipid dystrophy, resulting in hyperlipidemia leading to cardiovascular complications. Results from this investigation shows that VA improves lipid profile by its ability to reduce circulating bad cholesterol (LDL) while increasing good cholesterol level (HDL).

4.2.5 Effect of VA crude extract and fractions on liver function of investigational rats.

The cytoprotective and ameliorative effect of V. amygdalina on the hepatic assault characteristic in diabetes is as presented in the figures below;

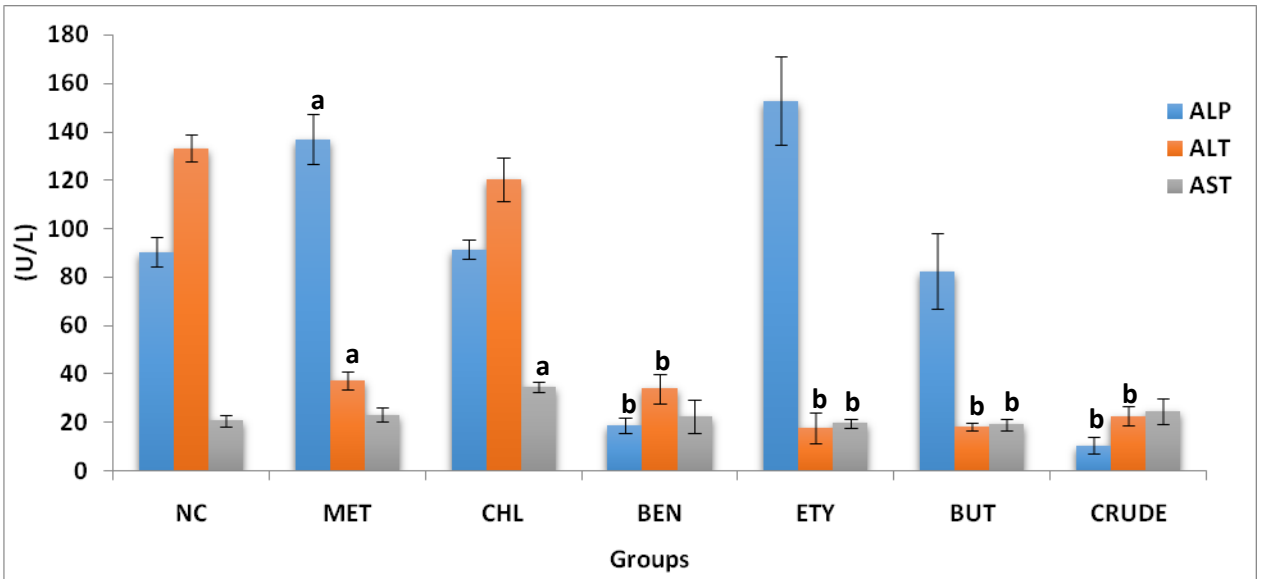

Fig. 4.17: Shows the effect of VA treatment on hepatic biomarker enzymes in STZ-induced diabetic investigational rats stated as Mean±S.E.M

Key: **NC**: Untreated Diabetic, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction, **ALP**: Alkaline Phosphatase, **AST**: Aspartate Transaminase, **ALT:** Alanine Transaminase.

(a) Significant increase when compared to diabetic control, (b) significant decrease when compared with diabetic control.

Effect of VA treatment on hepatic biomarker enzymes level of non-diabetic experimental rats

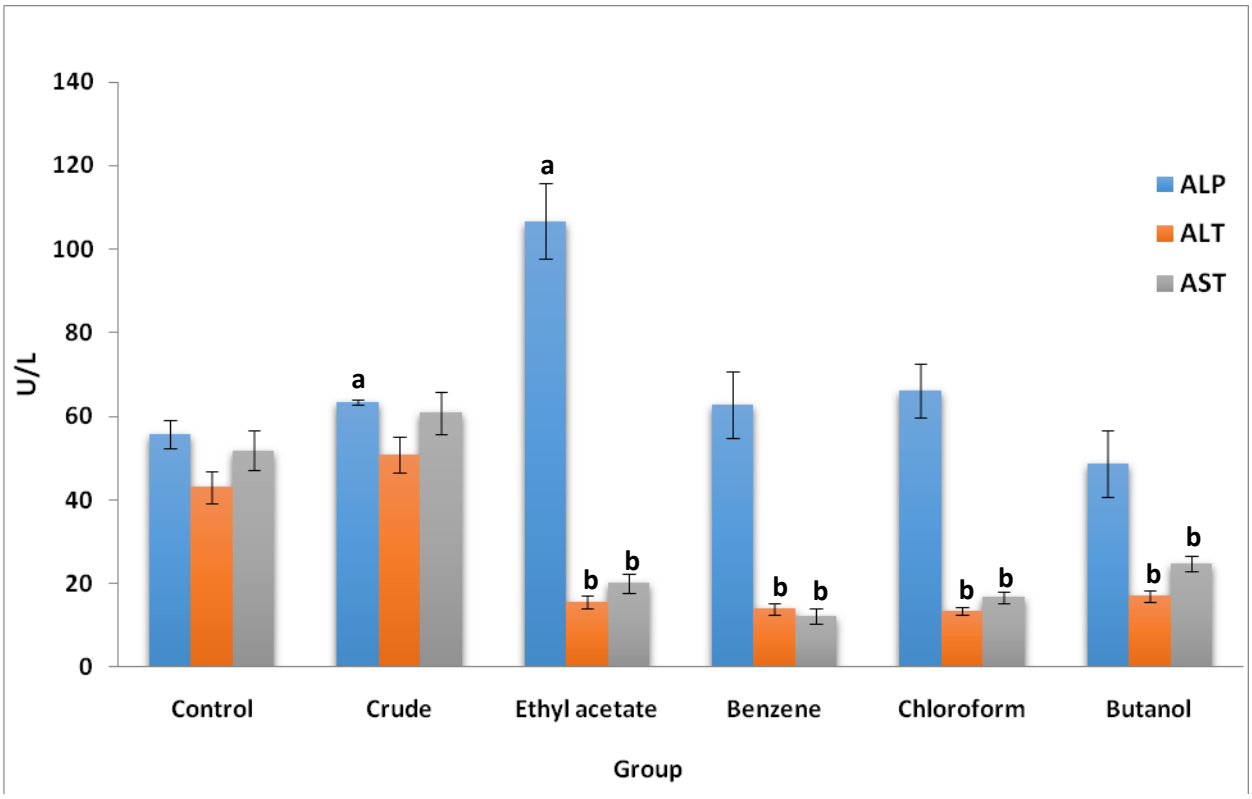

Fig. 4.18: Shows the effect of VA treatment on hepatic biomarker enzymes level in non-diabetic experimental animals stated as Mean±S.E.M

Key: **NC**: Untreated Diabetic, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction, **ALP**: Alkaline Phosphatase, **AST**: Aspartate Transaminase, **ALT:** Alanine Transaminase.

(a) Symbolizes a significant increase when compared with diabetic control, (b) Shows a significant decrease when compared with diabetic control.

The liver is the main organ assaulted by free radicals which destroys the cell membranes of hepatic cells, causing in leakage of liver enzyme into the blood; levels of circulating liver enzymes constitute the basic principle for liver function test. The hepato-protective and ameliorative effect of VA on hepatic complication was determined. The results showed a reduction in the level of circulating liver enzymes, suggesting that *Vernonia amygdalina* restores liver function by restoring the cellular integrity of liver cells, thus preventing further

enzyme leakage.

Effect of VA treatment on bilirubin level in STZ-induced diabetic experimental rats.

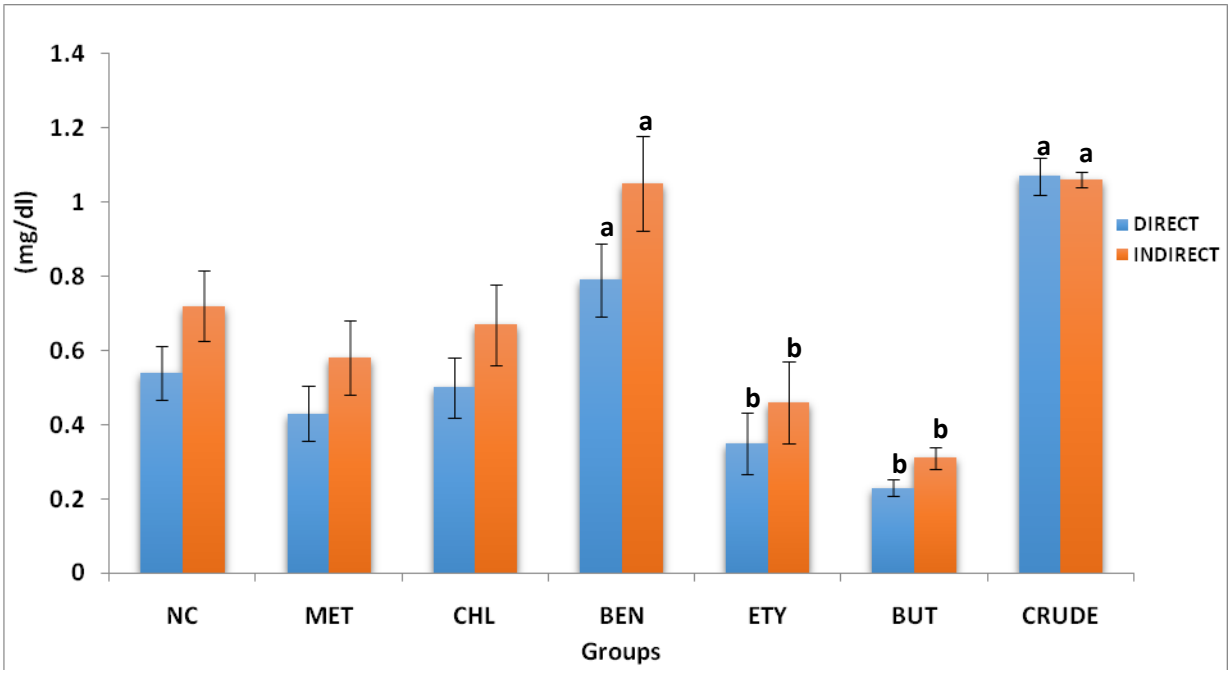

Fig. 4.19: Shows the influence of VA treatment on bilirubin level in STZ-induced diabetic investigational animals expressed as Mean±S.E.M.

Key: **NC**: Untreated Diabetic, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction

(a) Significant increase when compared to diabetic control, (b) Significant decrease when compared with diabetic control.

Effect of VA treatment on bilirubin level in non-diabetic experimental rats

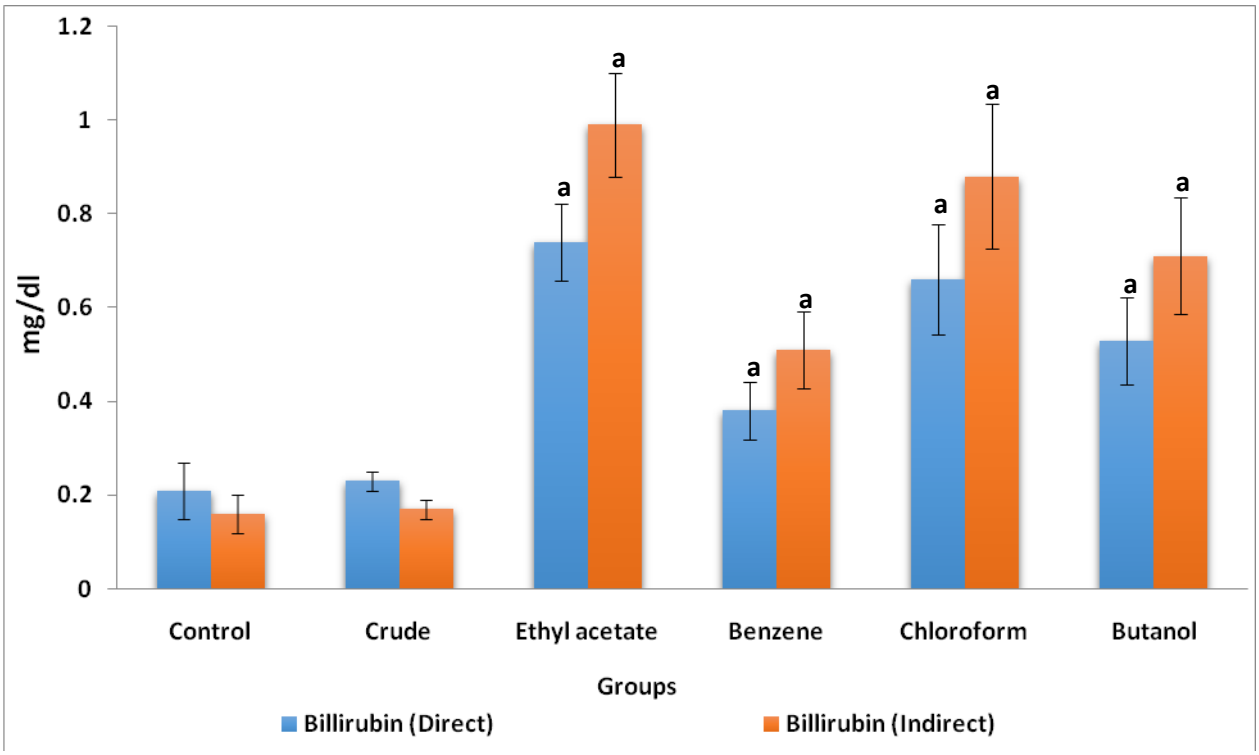

Fig. 4.20: Shows the influence of VA treatment on bilirubin level in non-diabetic experimental animals stated as Mean±S.E.M

Key: **NC**: Untreated Diabetic, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction.

(a) Significant increase when compared to diabetic control, (b) Significant decrease when compared with diabetic control.

Hepatocellular distortion can result in poor metabolism and conjugation of billirubin which usually reflects in blood. The finding suggests that VA fractions of ethyl acetate and butanol reduced circulating billirubin level.

Effects of VA treatment on total protein level in serum of STZ-induced diabetic experimental rats.

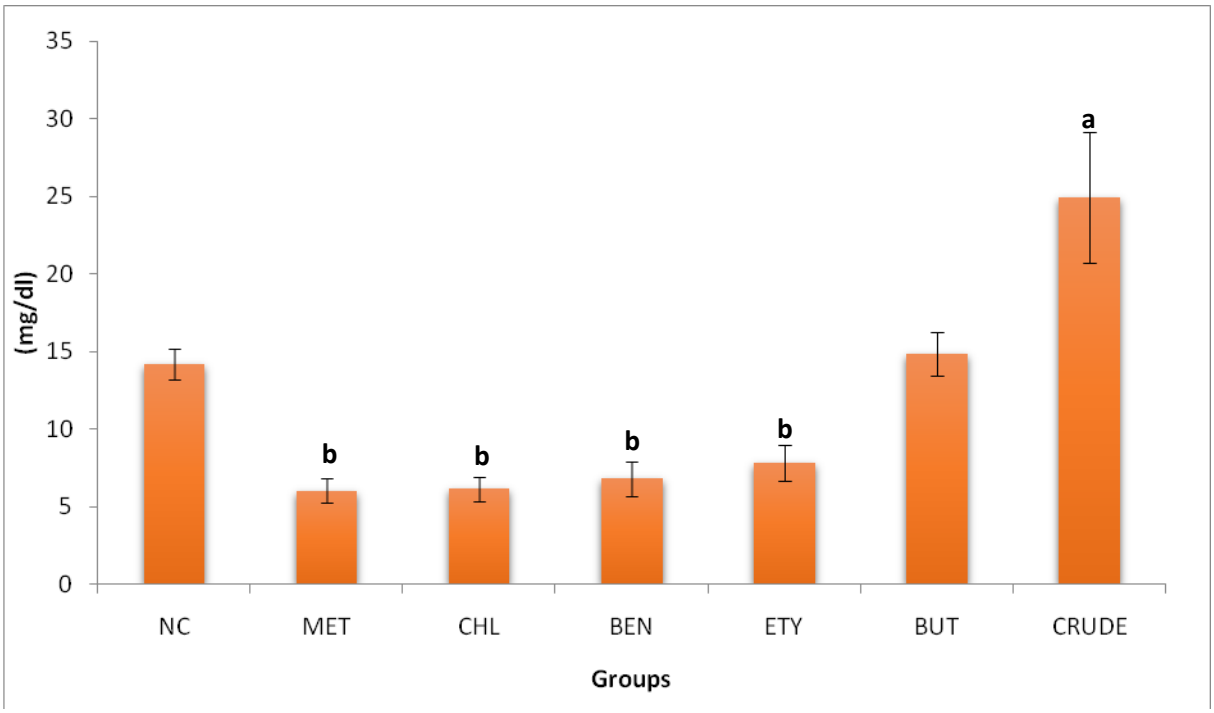

Fig. 4.21: Shows the influence of VA treatment on total protein level in the serum of STZ-induced diabetic investigational animals expressed as Mean±S.E.M

Key: **NC**: Untreated Diabetic, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction.

(a) Significant increase when compared with diabetic control, (b) Significant decrease when compared with diabetic control.

Effects of VA treatment total protein level in the serum of non-diabetic experimental rats

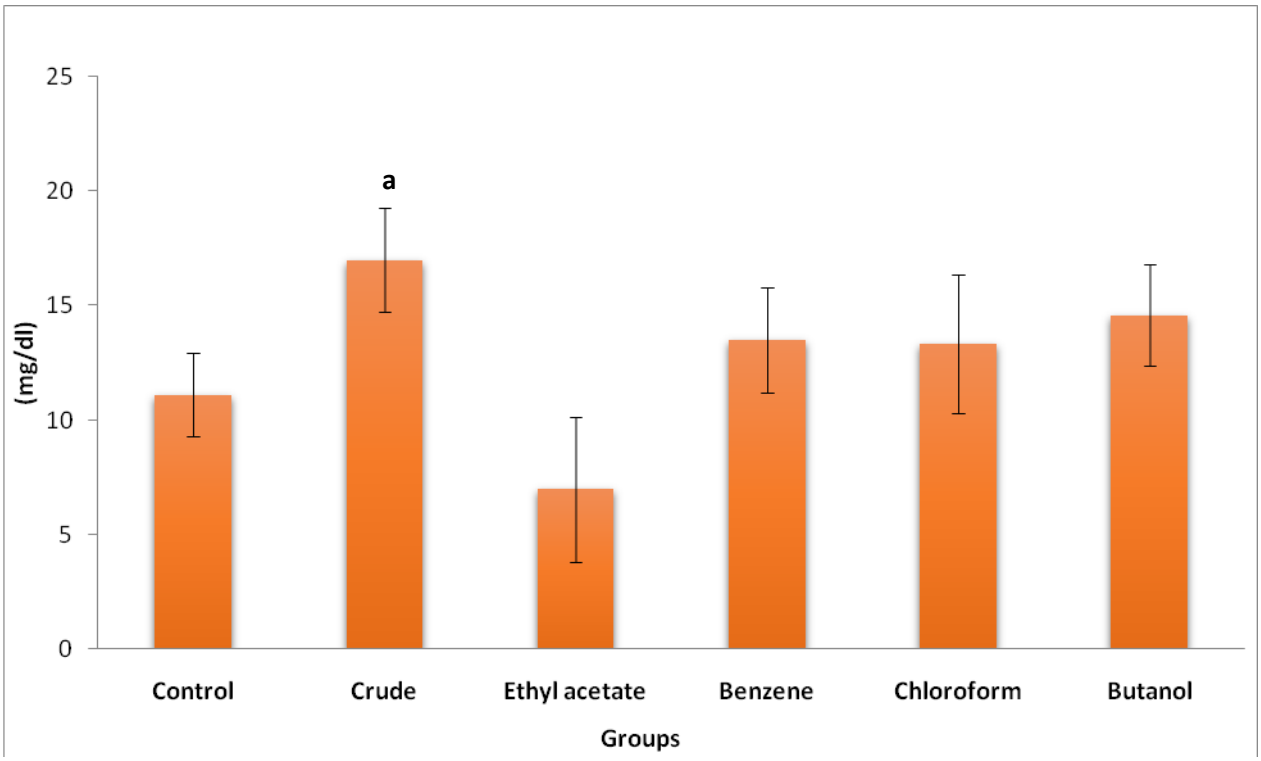

Fig. 4.22: Shows the influence of VA treatment on total protein level in the serum of non-diabetic experimental animals stated as Mean±S.E.M

Key: **NC**: Untreated Diabetic, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction.

(a) Significant increase when compared with diabetic control, (b) Significant decrease when compared with diabetic control.

The result from this investigation showed that VA fractions promoted protein sparing and decreased circulating total protein level.

4.2.6 Effect of VA crude extract and fraction on renal function of experimental rats

The cytoprotective and ameliorative effect of VA on renal assault characteristic in diabetes is as presented in the figures below;

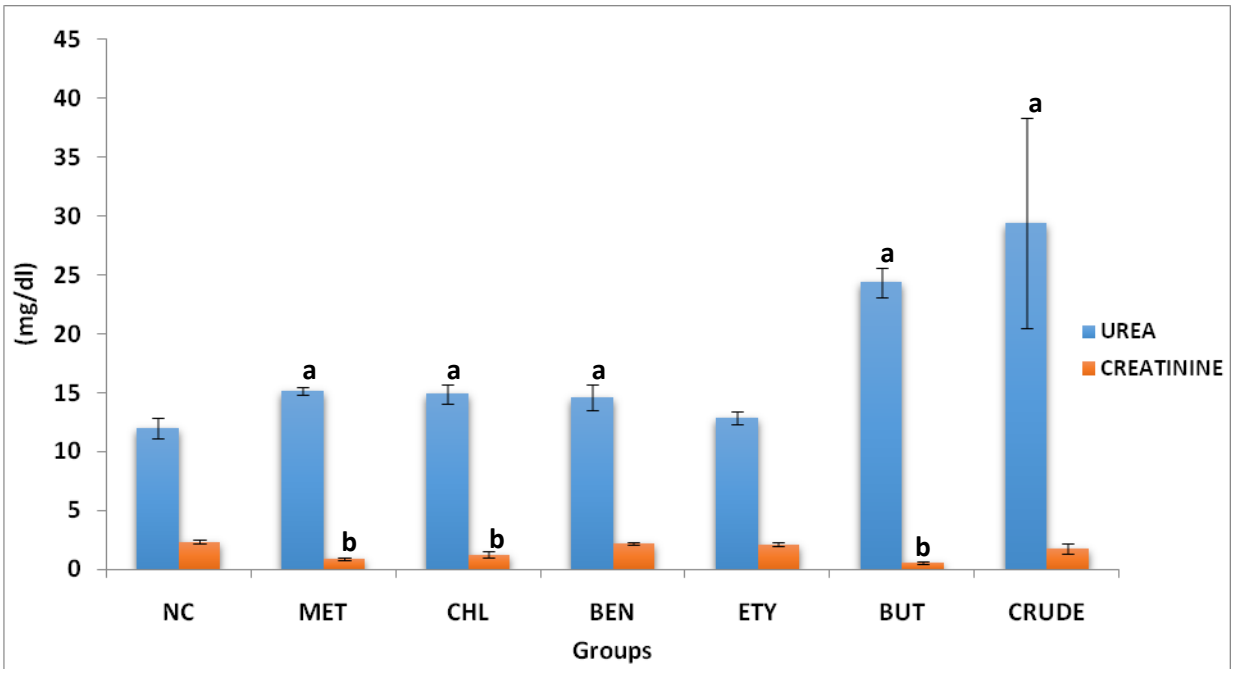

Fig. 4.23: Shows the effect of VA treatment on blood urea nitrogen and creatinine levels in STZ-induced diabetic investigational rats stated as Mean±S.E.M

Key: **NC**: Untreated Diabetic, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction.

(a) Indicates a significant increase when compared with diabetic control, (b) Symbolizes a significant decrease when compared with diabetic control.

Effects of VA treatment on blood urea nitrogen and creatinine levels in non-diabetic experimental rats

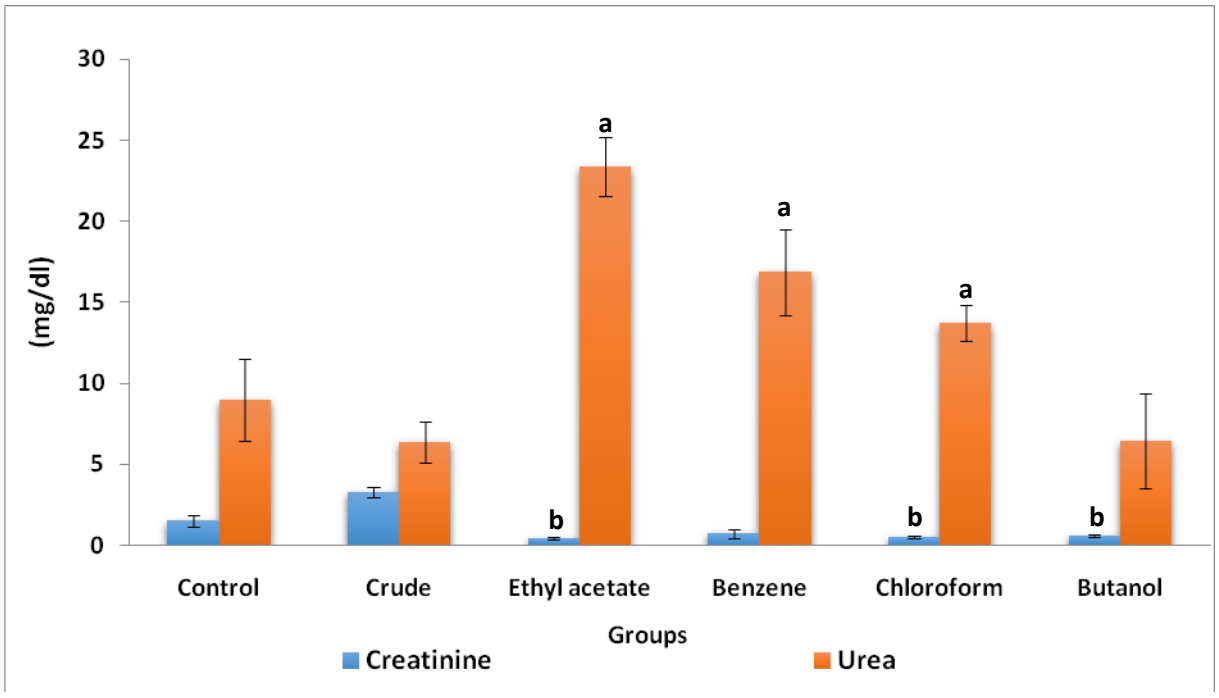

Fig. 4.24: Shows the effect of VA treatment on blood urea nitrogen and creatinine levels in non-diabetic investigational animals expressed as Mean±S.E.M

Key: **NC**: Untreated Diabetic, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction.

(a) Significant increase when compared to diabetic control, (b) Significant decrease when compared with diabetic control.

DM is associated with renal dysfunction, frequently manifesting circulation as increase in blood urea nitrogen and creatinine which are used as markers. Results from this study showed that VA fractions significantly reduced circulating levels of blood creatinine. Indicative of ameliorative diabetic nephropathy potency of the plant.

4.2.7 Effect of VA crude extract and fractions on lipid peroxidation of investigational animals

The influence of VA on lipid peroxidation characteristic in diabetes is as presented in the figures below;

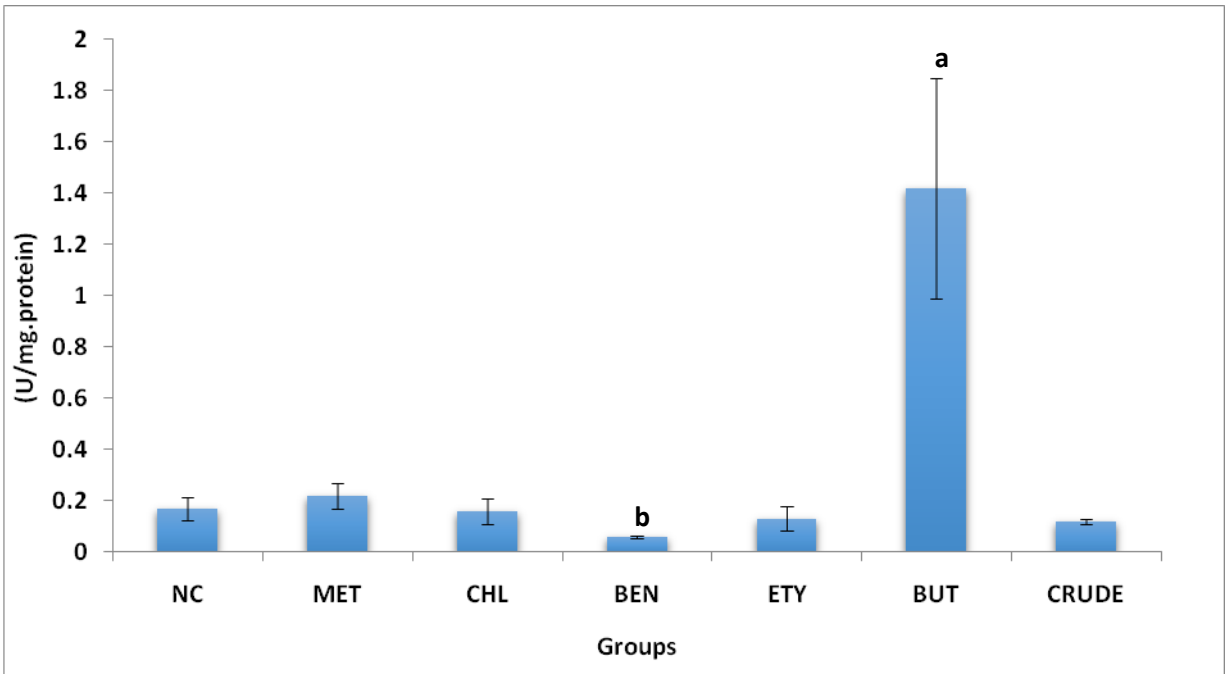

Fig. 4.25: Shows the influence of VA treatment on serum malondialdehyde in STZ-induced diabetic investigational rats stated as Mean±S.E.M

Key: **NC**: Untreated Diabetic, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction.

(a) Shows a significant increase when compared with diabetic control, (b) Symbolizes a significant decrease when compared with diabetic control.

Effects of VA treatment on serum malondialdehyde level in non-diabetic experimental rats

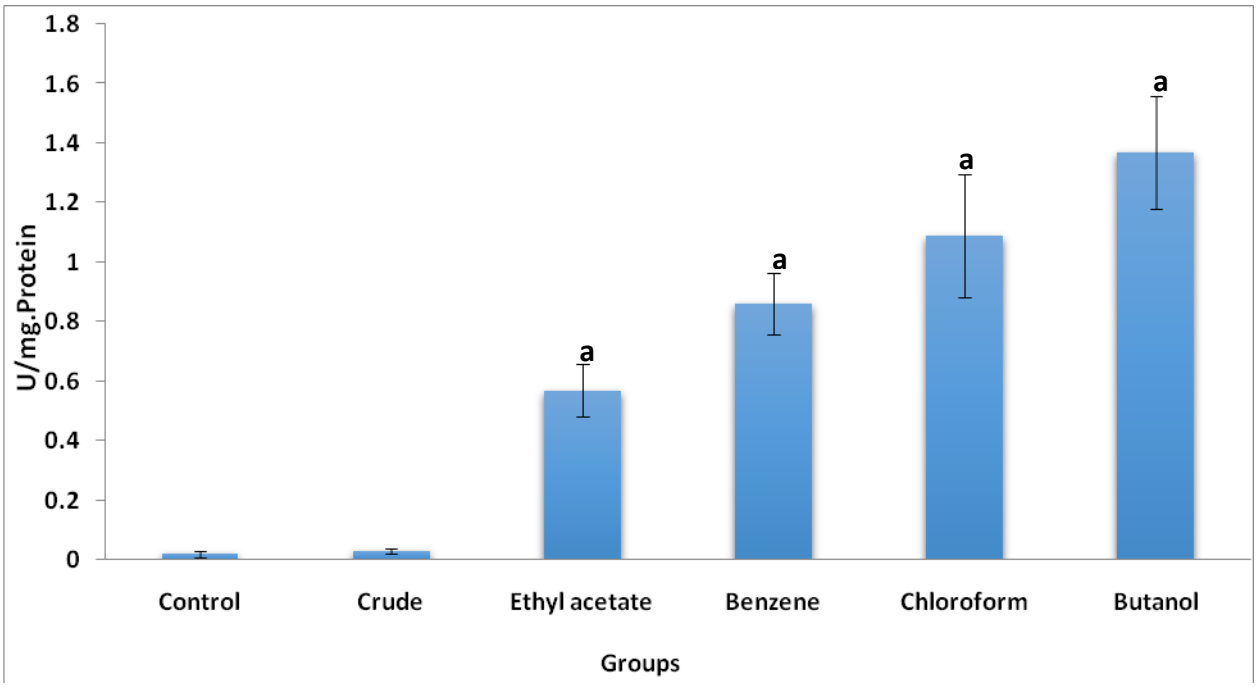

Fig. 4.26: Shows the effect of VA treatment on serum malondialdehyde level in non-diabetic experimental animals stated as Mean±S.E.M

Key: **NC**: Untreated Diabetic, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction.

(a) Significant increase when compared to diabetic control, (b) Significant decrease when compared with diabetic control.

Free radicals are often released into circulation in situations of metabolic dysfunction such as during DM. A byproduct of such metabolism is malondialdehyde. Its often employed in the laboratory in assessing the level of lipid peroxidation in diseases such as diabetes. Results from this study showed benzene fraction of VA bring about significant decrease in malondialdehyde level and consequentially, lipid peroxidation.

4.2.8 Effect of VA crude extract and fractions on antioxidant enzymes activity in investigational animals

The antioxidant property of V. amygdalina was determined by assessing its influence on the activity of antioxidant enzymes in diabetes is as presented in the figures below;

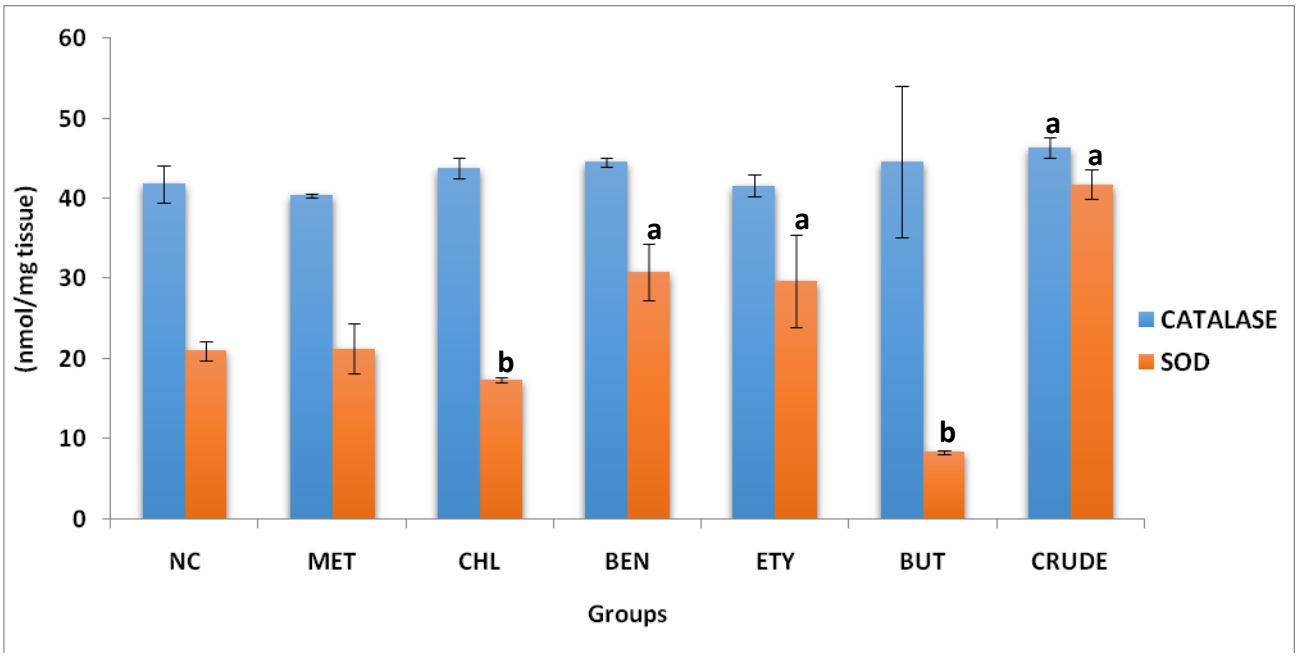

Fig. 4.27: Shows the effect of VA treatment on superoxide dismutase and catalase activities in STZ-induced diabetic experimental animals stated as Mean±S.E.M

Key: **NC**: Untreated Diabetic, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction. **SOD**: Superoxide dismutase.

(a) Significant increase when compared with diabetic control, (b) Significant decrease when compared to diabetic control.

Effects of VA treatment superoxide dismutase and catalase activities in non-diabetic experimental rats.

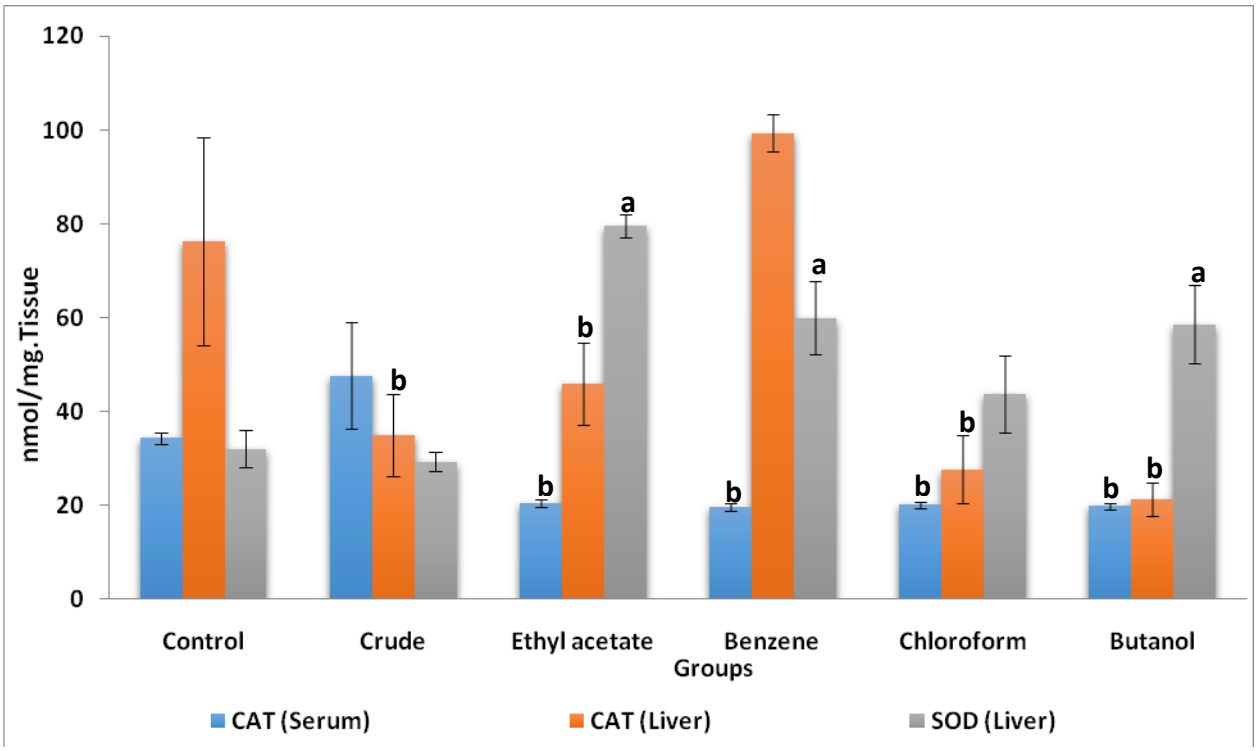

Fig. 4.28: Shows the effect of VA treatment on superoxide dismutase and catalase activities in non-diabetic experimental animals stated as Mean±S.E.M

Key: **NC**: Untreated Diabetic, **MET**: Metformin, **CHL**: Chloroform fraction, **BEN**: Benzene fraction, **ETY**: Ethyl acetate fraction, **BUT**: Butanol fraction. **SOD**: Superoxide dismutase. (a) Significant increase when compared with diabetic control, (b) Significant decrease when compared to diabetic control.

Free radicals are commonly generated as byproducts of defective cellular metabolism and the activity antioxidants like catalase and superoxide dismutase are often of great importance in ameliorating the toxic effects of these free radicals. Results from this study showed VA as clearly possessing antioxidant properties.

4.2.9 Effect of VA crude extract and fractions on the histological morphology of the pancreas in experimental rats.

The cyto-restorative property of V. amygdalina was determined by assessing its action on the microscopic anatomy of pancreas in diabetic animals as presented in the figures below;

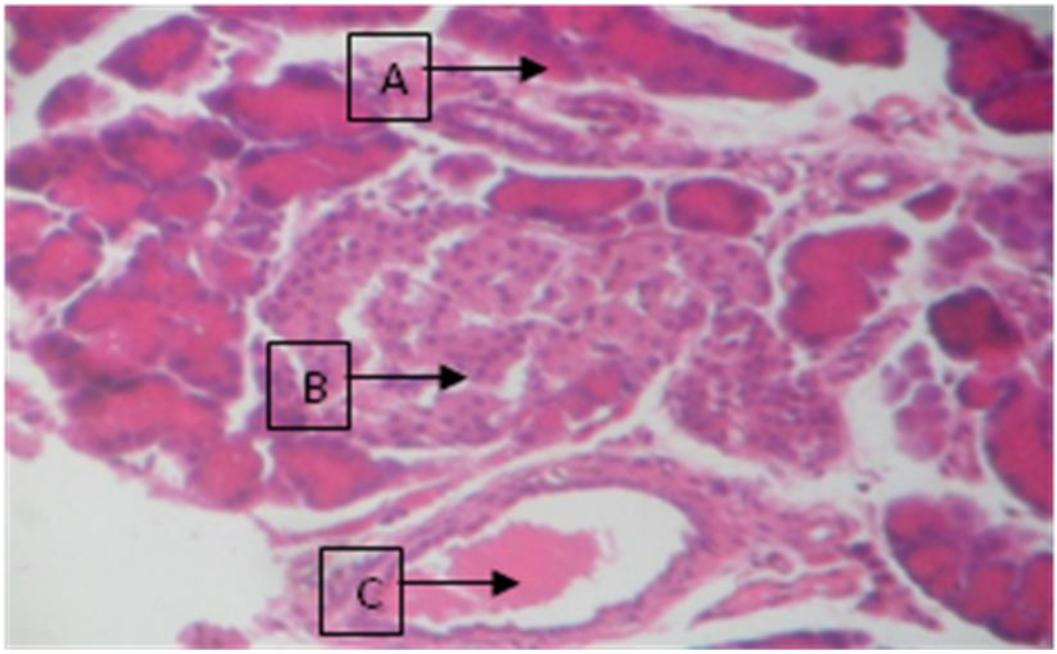

Figure 4.29: Control Rat pancreas made up of exocrine glands A,and B, islets cell and interlobular duct C (H&E x 100)

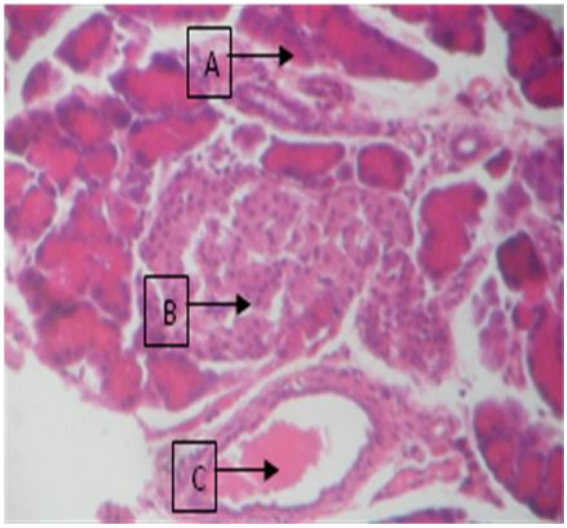

Figure 4.29: Control Rat pancreas composed of exocrine glands A, islets cell B and interlobular duct C (H&E x 100).

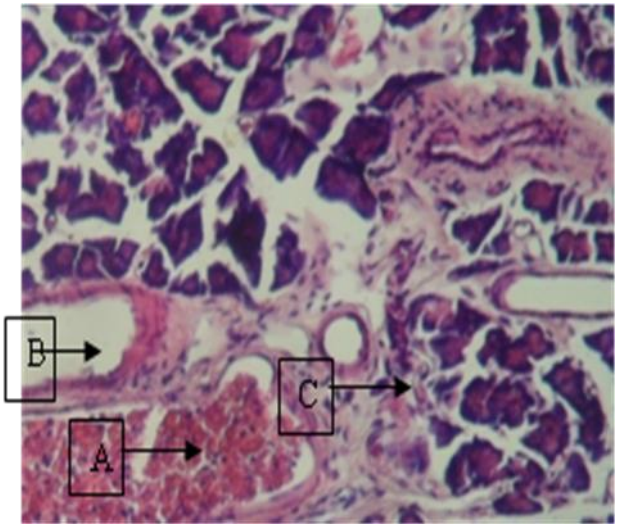

Figure 4.30: Negative control: Rat pancreas induced with STZ showing moderate interlobular vascular congestion-100A, B-hypertrophy and dilatation and C-interlobular penetrates of chronic inflammatory cells (H&E x 100)

The histologic examination was carried out to investigate the effect of diabetes on the pancreatic structure, and how treatment with VA brings about any alterations. Secretion of insulin requires a normal physiologic anatomy of pancreatic architecture. Figure 4.29 shows normal architecture of the pancreas. Figure 4.30 is the pancreatic histology after induction with STZ showing evidence of degeneration of pancreatic cells and consequent infiltration of inflammatory cells.

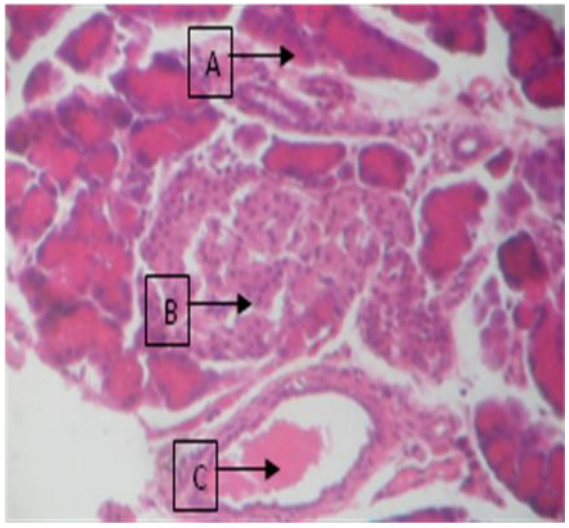

Figure 4.29: Control Rat pancreas composed of exocrine glands A, islets cell B and interlobular duct C (H&E x 100).

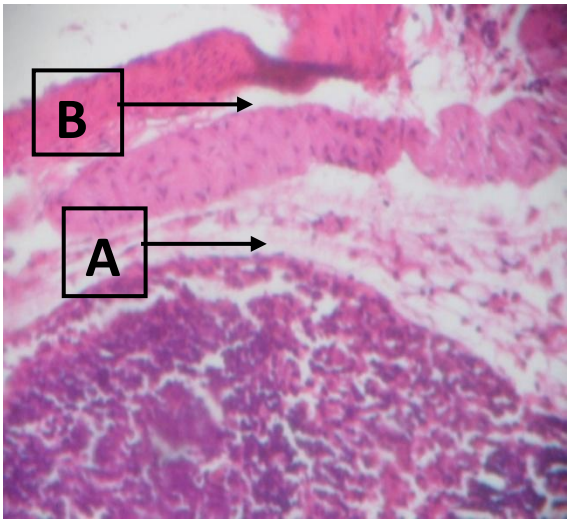

Figure 4.31: Rat Pancreas treated with crude extract of VA showing faintly resurgent islets A and showing mildly activated lymphoid aggregates B (H&E x 100)

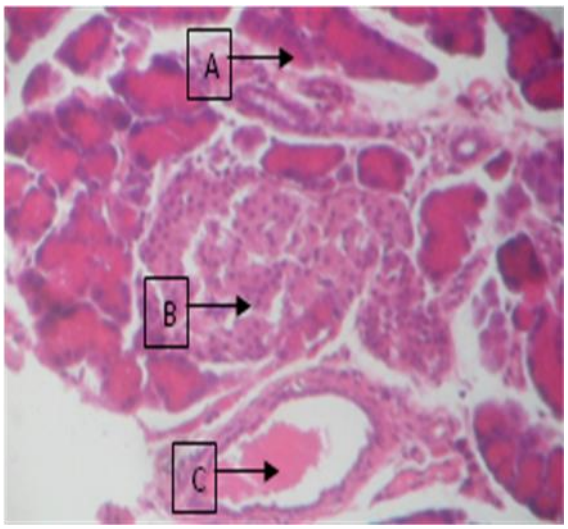

Figure 4.29: Control Rat pancreas composed of exocrine glands A, islets cell B and interlobular duct C (H&E x 100).

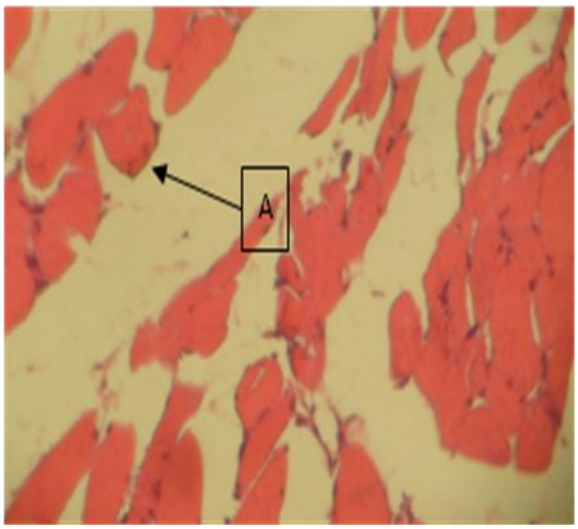

Figure 4.32: Rat pancreas induced diabetic with STZ and treated with Metformin showing ghost of stromal fibers A (H& E x 100)

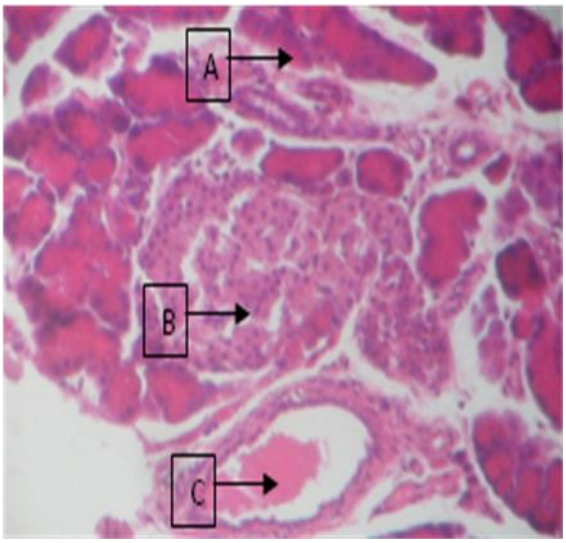

Figure 4.29: Control Rat pancreas composed of exocrine glands A, islets cell B and interlobular duct C (H&E x 100).

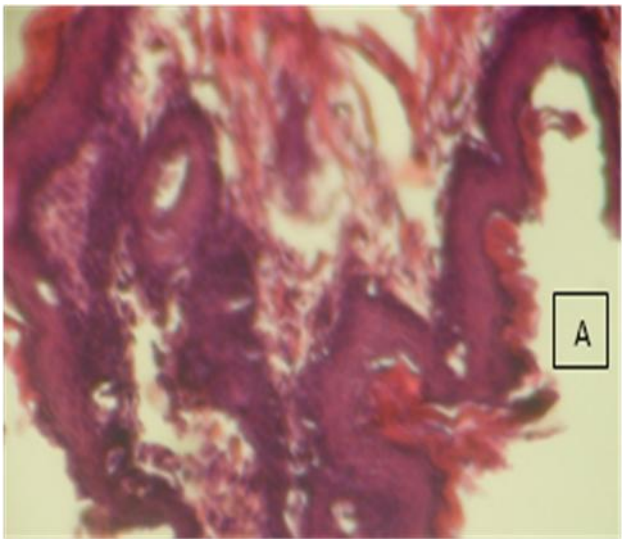

Figure 4.33: Rat pancreas induced diabetic with STZ andtreated with Benzene fraction of Vernonia amygdalina showing stratified squamous epithelium, keratinized A (H&E x 100)

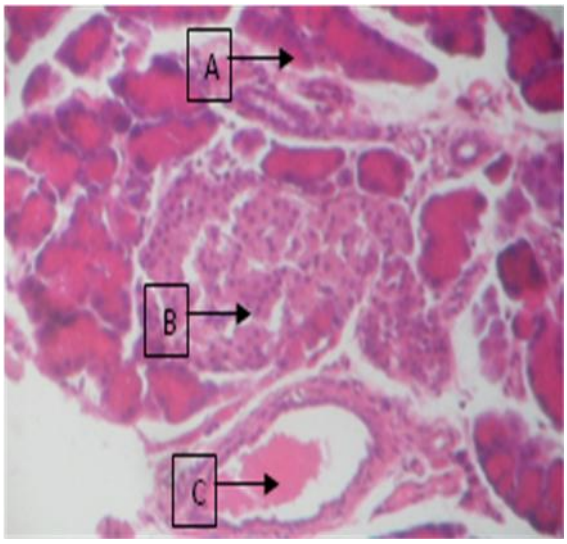

Figure 4.29: Control Rat pancreas composed of exocrine glands A, islets cell B and interlobular duct C (H&E x 100).

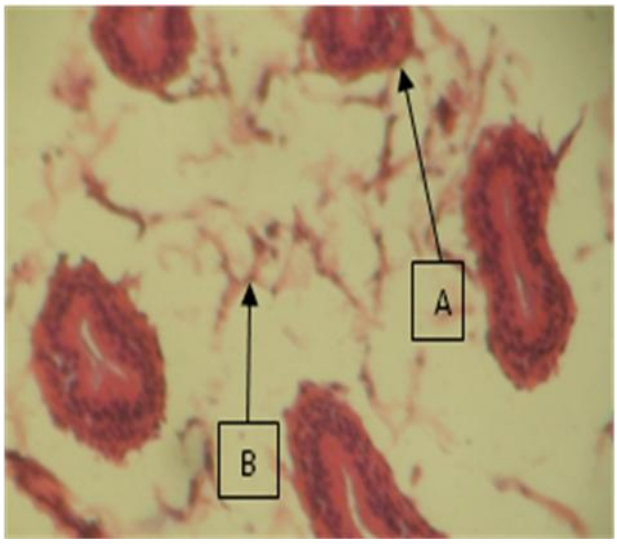

Figure 4.34: Rat pancreas induced diabetic with STZ and treated with Chloroform fraction of Vernonia amygdalina showing discrete, A-exocrine gland embedded in fat B (H&E x 100)

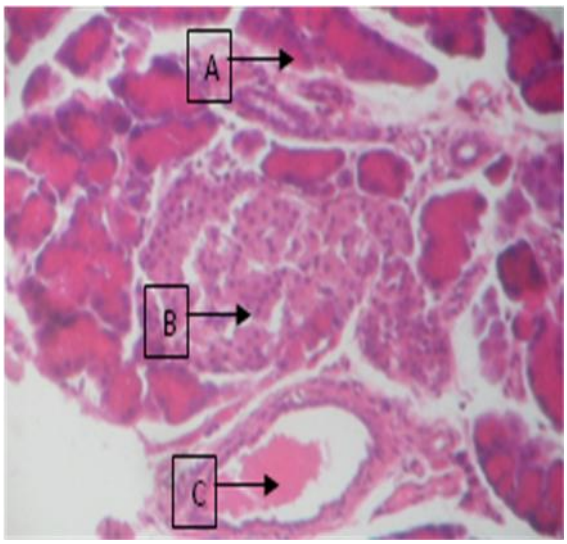

Figure 4.29: Control Rat pancreas composed of exocrine glands A, islets cell B and interlobular duct C (H&E x 100).

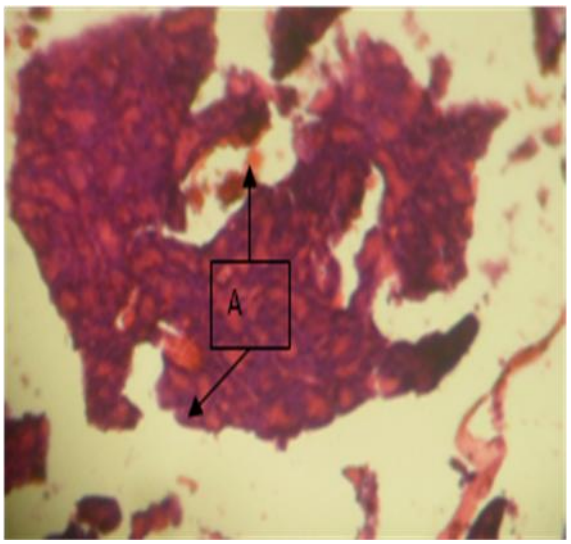

Figure 4.35: Rat pancreas induced diabetic with STZ andtreated with ethyl acetatefraction of Vernonia amygdalina showing moderate congestion A (H&E x 100)

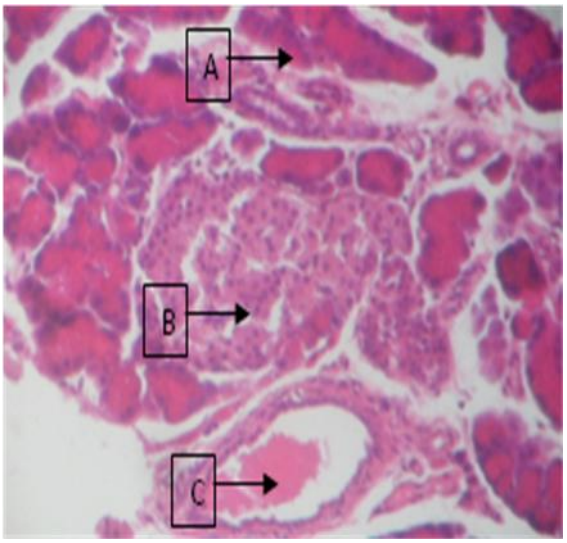

Figure 4.29: Control Rat pancreas composed of exocrine glands A, islets cell B and interlobular duct C (H&E x 100).

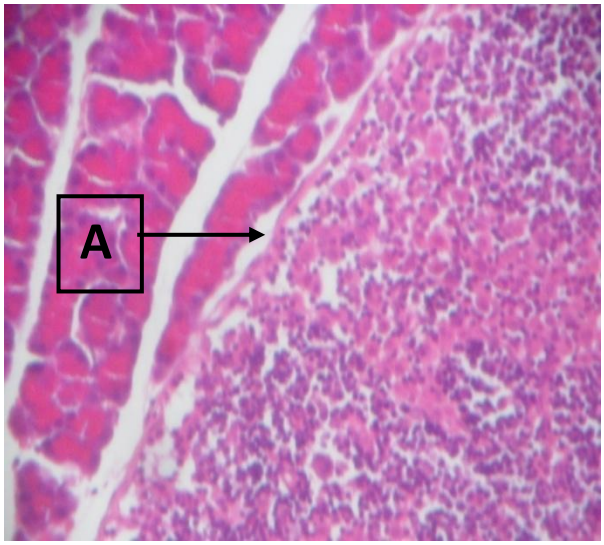

Figure 4.36: Rat Pancreas treated with 1000mg/kg of Butanol fraction from Vernonia amygdalina of extract showing markedly activated lymphoid aggregates A (H&E x 100)

CHAPTER FIVE

5.0 DISCUSSION

Streptozotocin has been shown to be cyto-toxic to the pancreas, selectively destroying pancreatic beta cells in mammals (Hayashi and Kojima, 2006) within a period of 72hrs (Elsner *et al.,* 2000) this was quite evident in the course of this study.

Herbal remedies with purported anti-diabetic potency are a subject of many researches for the enhanced management of diabetes.

Tissue (pancreas) histopathology examination showed that the pancreas cells of diabetic group reduced in size which could alter the release of insulin and thus, hinder glucose uptake. Treatment of diabetic animals with VA showed insignificant reviving of islet cells. They also showed increase in activation of adjacent lymphoid aggregates, due to infiltration of lymphocytes around the damaged pancreatic tissues which is seen as a harbinger of diseases, in the Islets cells it is referred to as insulitis (Peggy *et al.,* 2007). Inflammation or necrosis of cells results to increase in lymphoid follicles and neutrophils in response to tissue damage. The incidence of diabetes surged over the years with no defined measure of treatments, the fractions of Vernonia amygdalina as depicted from the result does not possess the ability to restore the integrity of pancreatic cells savaged by the diabetogenic compound. This is in contrast to the finding of Sunday *et al.,* (2012) who observed restorative influence of aqueous extract of VA on the pancreas tissue of diabetic rats induced with alloxan monohydrate a

known diabetogenic substance.

The insufficient production and/or absence of insulin coupled with the reduced sensitivity of its receptors to insulin lead to sustained hyperglycemia which is characteristic of diabetic condition. Blood glucose level regulation to restore euglycaemia is the target and mechanism of anti-diabetic therapy. In this study, the blood sugar lowering influence of VA and fractions showed significant reduction in blood glucose level with crude displaying 81.5% decrease in fasting blood glucose level of diabetic rats; fractions showed 69.7%, and 65.9% reduction, greater than that of metformin which showed 36.5% reduction. An earlier study by Nwanjo, (2005) purported the hypoglycaemic property of Vernonia amygdalina. The result of this study shows that administration of Vernonia amygdalina and fractions caused a significant decrease in fasting blood glucose level similar to Osinubi (2007) who observed a pronounced blood glucose lowering potential of Vernonia amygdalina and Ekam *et al.,* (2013) who observed that fractions from Vernonia amygdalina significantly reduced blood glucose levels of diabetic rats. This confirms the plants hypoglycaemic activity as well as that its fractions.

Diabetes causes alteration in the metabolism of macromolecules resulting to disturbances in body composition and body weight. Significant weight loss is usually evident in the progression of the disease. In this study, untreated diabetic rats showed a significant decline in their body weights while those that received VA extract and fractions displayed a significant restoration in their body weight. This finding contrasted with that of Ibrahim *et al* (2000) who reported a significant decrease in Wistar rats' body weight following prolong feeding on VA leaves, it however agreed with the report of Iwalokun *et al.,* (2006) who observed a body weight increase in mice treated with VA extracts, following exposure to acetaminophen induced toxicity.

Diabetes disturbs principally glucose metabolism which in turn causes alteration in the metabolism of other molecules in the body. The effect of these alterations is seen in the actions of metabolic enzymes in the glucose metabolic pathways; glycolytic, gluconeogenesis and pentose phosphate pathway.

Results from this work also demonstrated a significant increase in hexokinase activity in the serum as well as liver of diabetic rats that were placed on VA beyond that observed in untreated diabetic rats. Since hexokinase activity is dependent on the presence of glucose and necessary for glycolytic conversions and pentose phosphate pathway, this indicates glucose

utilization in the diabetic animals hence suggestive of glucose mobilization into the cell. This is similar to the findings of Shetti *et al.,* (2012), which purported that reduced sugar in diabetic rats treated with Phyllantus amarus was due to increased hexokinase and gluckokinase activity. However, Atangwho *et al.,* (2014) reported a decreased expression of hexokinase activity in rats treated with Vernonia amygdalina. Pyruvate kinase is a universally released enzyme catalyzing conversion of phosphoenol pyruvate to pyruvate and generating ATP in the process.

This is altered in hyperglycemia and leads to poor glucose utilization and metabolism (Steiner, 1966). The level pyruvate kinase activity in this study showed that rats treated with respective fractionates of the herb expressed higher level of pyruvate kinase activity and this was also similar with that seen in rats that received metformin. This suggests that the herb promotes glucose utilization in cells. This is similar and agrees. This suggests that Vernonia amygdalina promotes the activity of glycolytic enzymes hence promoting glycolysis and energy production.

Hepatic glucose production (HGP) is central to metabolic adaptation during self-starvation and its anomalous increase is a principal basis for assessing fasting hyperglycaemia in diabetic state, (Rizza, 2010; Lin and Accili, 2011). The expression of glucose 6-phosphatase (G6Pase) a key gluconeogenic target enzyme, at the cellular level was assayed in the liver tissue in order to determine the possible effect of the managements of glucose variation via gluconeogenesis. In this study, the exclusively hepatocyte-domiciled G6Pase enzyme was significantly reduced by the administration of the plant fractions when compared with untreated diabetic rats which showed higher G6Pase expression. This reduction suggests a suppression of gluconeogenesis and hepatic glucose production in the treated rats. This is in tandem with the findings of Atangwho *et al.,* (2014) who reported a striking reduction in the expression of important gluconeogenic target enzymes after 14 days administration of chloroform fraction of Vernonia amygdalina. The suppression of gluconeogenesis in treated rats complement the enhanced glycolysis as both pathways are reciprocally regulated (Berg *et al.,* 2002).

As a confirmation of the observed glucose utilization in this study, the action of glucose 6-phosphate dehydrogenase (G6PDH) an important enzyme in the pentose phosphate pathway and in NADPH-diaphorase level; a produce of the pathway were determined. Result showed that the expression of G6PDH was profoundly decreased in untreated diabetic rats, in

conformism with earlier report (Gupta *et al.*,1999) but turn out to be greatly expressed at the termination of the administration plant extract, this comparable to study by Atangwho *et al.,* (2014). According to Ugochukwu and Babady, (2003) the G6PDH enzyme is a documented sugar modulatory target of a number of anti-diabetic mediators and therapeutic plants. Glucose oxidation via the G6PDH pathway primarily produces or generates reducing power -NADPH- needed in synthetic (anabolic) reaction and deactivation of reactive oxygen species (ROS) in the cell (antioxidant action).

Similarly, there was an increase in the expression of NADPH-diaphorase by this pathway in rats treated with the plant, suggesting a potentiating of the pentose phosphate pathway. It is possible that the NADPH produced from this triggered glucose pathway is used to improve tissue storing of glucose in the mode of triglycerides, a process that needs NADPH for reductive removal of ROS in glutathione-dependent pathway. A previous study by Atangwho *et al.,* (2012) showed that the plant is able to heighten antioxidant enzymes activity in diabetic rat models. The elevated levels of NADPH through G6PDH expression could function as an enhancement of the tissue storing process, with the probable goal to clear free fatty acids out of the circulation and may have increased the disposal of glucose which could partly account for the normoglycemia seen in all Vernonia amygdalina fractions-treated diabetic rats in this study.

The herb and its fractionates from the foregoing, have an enhancing effect on glucose utilization as seen in its effects as increasing key enzyme activities of the glycolytic pathway especially enzymes like hexokinase and pyruvate kinase activity whose activity are principally concerned with glucose breakdown via glycolysis and are irreversible in their action. This is suggestive of its hypoglycemic mechanism of action; enhancing glycolysis. Glycolytic breakdown suggests the availability and mobilization of glucose into the cells of tissues since the reduction of blood glucose is matched with the progression of glycolysis. A progressive glycolysis matched with an increase in blood glucose would suggest gluconeogenesis but the findings of this study presents otherwise.

The hypoglycemic potency could be credited to simple phytochemical components of the herb. Major components of VA leave extract,,consist of sesquiterpene lactones (vernodalin, vernolide, hydroxyvernolide), and steroid glucosides (vernonioside A1-A4: for bitter tasting constituents and vernonioside B1-B3; for non-bitter related constituents) (Babalola *et al.*,

2001; Koshimizu *et al.,* 1994). Sesquiterpene lactone has been documented as being gotten from Ambrosia maritime extract, and is a potent hypoglycemic agent. It has been advocated that aloes hypoglycemic property is due to the bitter principle which stimulates production and/or discharge of insulin from beta-cells (Ajabnoor, 1990). Vernonia amygdalina, which contains the bitter principle as well as sesquiterpene lactones, bring about its hypoglycemic action via similar mechanisms, Atangwho *et al.,* (2014) reported. Other mechanism of actions including; inhibition of sugar absorption, higher sensitivity of receptors to insulin, insulinase inhibiting effect, and enhanced peripheral glucose uptake cannot be dismissed. Changes in lipid concentration, consequent disorders of lipid metabolism and lipid dystrophy have been observed in diabetes mellitus (Ononogbu, 1988).

Hyperlipidaemia and hypercholesterolaemia observed in diabetes could result to elevated level of lipid peroxidation. The increase in serum triglycerides, LDL-cholesterol and total cholesterol in diabetic controls study are in keeping with previous reports documenting elevated serum triglyceride and serum cholesterol levels in diabetic subjects (Oberley, 1988) and diabetic rats (Newairy *et al.,* 2002).

In this study administration of extract of V. amygdalina leaf and it fraction significantly reduced triglyceride, total cholesterol and LDL cholesterol when compared to the untreated diabetic rats. Levels of HDL cholesterol were increased in treated diabetic rats. Similar effect was observed in Vernonia amygdalina treated non-diabetic rats, suggesting that the plant crude leaf extract and fraction have the potentials to protect against artherosclerosis and its associated or incidental cardiovascular complications commonly prompted by chronic hyperglycaemia of diabetes. The hypolipidaemia observed in treated diabetic rats can be attributed to a restored lipid metabolism by the action of the phytochemical constituent of the plant which has been purported (Nwanjo *et al.,* 2005).

Hepatic complications are common in diabetes due to hyperglycaemia induced hepatotoxicity which presents with similar characteristics of typical liver toxicity in alterations in the activities of hepatic enzyme like Alanine Transaminase, Aspartate Transaminase, Lactate Dehydrogenase (LDH), Alkaline Phosphatase (ALP) and key liver biomarkers such as Bilirubin levels and Serum Proteins levels.

ALT, AST, LDH, and ALP activities were over expressed in liver of streptozotocin induced rats compared to the normoglycaemic control group while administration of Vernonia

amygdalina significantly decreased these enzyme levels. Similar hepato-protective effect by Vernonia amygdalina was reported by Leelaprakash *et al.,* (2011) in CCl4 hepatotoxcity. The reversal of elevated serum intracellular enzyme levels by Vernonia amygdalina extract and fractions in diabetic rats might be ascribed to the ability of the plant to cause stabilizing of the hepatic cell membrane precluding enzymes seepages. In addition, it was reported that the reversal of increased levels of transaminases almost usually predicts the repair of hepatocytes and regeneration of hepatic parenchyma (Jain *et al.,* 1989). This observation is consistent with earlier report on hepato-protective potentials of leaf extracts of Vernonia amygdalina in mice (Iwalokun *et al.,* 2006).

Ethyl acetate as well as butanol plant fractions significantly decreased the level of Serum bilirubin in treated diabetic rats while this effect was not found in the plant crude extract and other fractions. Serum protein play an essential part in cellular maintenance, growth and functioning of the body, acting as the intricate structural molecule of body tissues, its levels are essential of health markers in the body. Ravi *et al.,* (2004) reported that the characteristics loss of body weight associated with diabetes is due to excessive break down of tissue protein and an increased muscle wasting in diabetes. Similarly, hepatic problems in diabetes are connected with a change in the levels of serum albumin and total protein and they act as markers for hepatic damage (Rehman *et al.,* 2012; Al Ghamdi, 2001). Serum protein levels in untreated diabetic rats where seen to be significantly lower than that of healthy rats of the control group which can be credited to the diabetogenic action of streptozotocin and its effect on the liver. Administration of crude Vernonia amygdalina crude increased total serum protein while it fractions decreased serum protein levels in treated diabetic rats. Akah *et al.,* (2009), reported an increase in hepatic proteins following consumption of VA. This may result from effective control of protein metabolism due to the plant administration, as a pointer to early functional and secretory improvement of hepatic cells. Due to the high protein degradation in diabetes, there is increased availability of amino acids to liver, which nourish gluconeogenesis and accelerated ureagenesis, resulting in hypoproteinemia and hypoalbuminemia (Bhavpriya and Govindasamy, 2000) which may have been the case in the present study.

The level of antioxidant enzymes activities and lipid peroxidation can be employed in predicting the severity liver complications induced by diabetes. Antioxidants enzymes such as SOD and CAT necessarily act in metabolic pathways involving free radicals. Therefore, SOD

and CAT levels decrease in liver as seen in this study suggest the toxic effects of streptozotocin and diabetes on liver functions but the administration of Vernonia amygdalina counter the progress of diabetic complications on liver cells by blocking the decrease in antioxidants levels. Consequently, an elevation in antioxidant enzyme activities (SOD and CAT) after Vernonia amygdalina administration mayadd to the ameliorating effects of oxidative stress. Malondialdehyde (MDA) is a biomarker of oxidative stress and lipid peroxidation, an elevation in MDA level is indicative of the toxic consequence of diabetes on liver (Trush *et al.*,1982) but the counteractions of Vernonia amygdalina in bringing about reduction in MDA level shows a potential characteristics of VA in the repair of damaged liver tissues due to diabetes. Thus, the antioxidant activity of VA improves the liver functions by enhancing antioxidant enzyme activities. This potency was best expressed in the crude extract.

The kidneys keep up optimum chemical make-up of body fluid via acidification of urine and elimination of metabolic wastes such as urea, uric acid, and creatinine. The inadequate cellular glucose utilization in diabetes leads to increased gluconeogeneis.

Proteolysis and liver amino acid store feed the liver for gluconeogenesis thatentails proteins deamination and uric wastes generation such as urea and creatinine to be cleared off by the kidneys. In diabetic nephropathy however, there is compromise of the kidney excretory functions resulting in accumulation of metabolites in blood (Jaspreet *et al.,* 2000). In this study, there was a rise in blood urea nitrogen of all diabetic rats compared to the non-diabetic rats. Administration of Vernonia amygdalina extract and fractionates displayed no significant reversal of this elevated values in the diabetic rats. On the other hand, there were reductions in creatinine levels in the blood following administration of extract and fractions of VA to diabetic rats. Several factors such as food type, physical activities and starvation may influence urea level while making it a non-specific diagnostic assay. On the other hand, creatinine concentration is more specific and functions as superior diagnostic instrument for assessing renal competence (Loeb, 1991). This shows an improvement of renal capacity in diabetic rats. Comparable renal restoration capability was described by Atangwho *et al.,* (2007).

The anti-diabetic potency of Vernonia amygdalina exhibited in this study as well as in the reports of several researchers (Atangwho *et al.,* 2014; Akinola et al., 2009; Akah *et al.*, 2009; Nwanjo and Nwokoro, 2004) have been attribute to phytochemical constituents which are

purported to possess anti-diabetic properties. In this study, the specific bioactive elements of VA were isolated by fractionation using solvents varying in polarity. After chromatographic analysis (GC-MS), the fractions were found to contain the following compound with proven anti-diabetic properties;

Phytol (C20H4OO), an acyclic disterpene alcohol a constituent of chlorophyll commonly used as precursor for manufacturing. Vitamin E and Vitamin K1 synthetic ligands of retinoid X receptor (RXR) have shown anti-diabetic activity in mice, apparently owing to the fact that they stimulate the transcriptional activity off peroxisome proliferator-activated receptor (PPAR) gamma /RXR heterodimers, much like thiazolidinedione drugs. It is thus, reasonable to suspect that phytanic acid may have utility for treatment and prevention of human type 2 diabetes (Elmaza *et al.*, 2013; McCarthy, 2001).

Hexadecanoic Acid (Palmitic Acid; C16H32O2) was found to rapidly induced GLUT4 translocation and stimulate glucose uptake in rat skeletal muscle cell (acute effect). Phosphorylation of AMP - activated protein kinase (AMPK), and extracellular signal-related kinase A/2 (ERK1/2) was enhanced by Palmitic acid in a time - dependent manner (Jing *et al.*, 2011).

Stearic acid (C18H36O2): Valeric Lynn in her theses for a Ph.D in Physiology, stated that feeding diabetic mice with high fat diet enriched with stearic acid, had significant reduction in blood glucose level thereby, emphasizing the argument that the risks and benefits of fat in diet depend on the chemical structure, rather than the chemical class of fat ingested. The valuable influence of stearic acid seems to be connected with a reduced absorption of dietary fat (Reeves, 2012).

Benzoate salts is identified as 2-(6(3R)-3-aminopipcridin-L-yl)-3-methyl = 2,4-dioxo-3,4-dihydropyrimidin-L(2H)-yl) methyl) benzonitricmonobenzoate. (C18H2N5O2.C7H6O2); inhibits the enzymatic activity of dipeptidyl peptidase-4 (DPP-4).DPP-4 contributes to glucose level regulation by degrading incretins such as Glucagon-like peptides (GLP-1) (Barnett, 2006). Incretins are a group of metabolic hormones that stimulatesthe decrease in blood glucose level by causing an increase in the amount of insulin released from the pancreatic beta cells to control postprandial hyperglycaemia (Drucker and Nauck, 2006).

Butylphenol; administration of 2,6-di-t-butyl-phenol (DH-581) to subjects with hypercholesterolemia and hypoxia associated with diabetes mellitus, showed a decreased in

cholesterol and phospholipids concentration in the serum of all subjects. Carbohydrate-accentuated lipemia diminish as a result of DH-581 administration.

Serum TG concentration also fell during DH581 administration in subjects with lipemia and diabetes mellitus (John *et al.,* 1969).

Oxirane; Phenylalkyloxirane carboxylic acids and esters are a new class of potent hypoglyceamic substance. Sodium 2-(5-(4-chlorophenyl)-pentyl)-oxirane-2-carboxylate (B807-27) produces a dose dependent hypoglyceamic effect when administered orally or intravenously to fasted diabetic laboratory animals (rats); induced with streptozotocin. The minimal dose for lowering blood glucose significantly in rats is 15nmol/kg. Hence, the substance B807-27 is approximately five times more potent than tolbutamide and 30 times more potent than the biguanide buformin with respect to lowering blood glucose levels. B807-27 differs from the sulphonylureas in that it fails to stimulate insulin secretion. In contrast to the biguamide, the substance decreases rather than increases blood lactate concentration and blocks both fatty acid oxidation and gluconeogenesis (Wolf *et al.,* 1982).

Octadecatrienoic acid; Peroxisome proliferator - activated receptor gamma (PPAR?) found in adipose tissue play an important role in the control of adipogenesis. PPAR?Activators are known to have potent anti-hyperglyceamic activity and used to treat insulin resistance associated with diabetes. Thus, numerous natural occuring and synthetic agonists of PPAR? Are employed in the management of glucose disorders. In a study by Takahashi et al.,(2015), they found that 13-oxo-9(Z), 11(E), 15(Z) - octadecatrienoic acid (13-oxo-OTA), a linolenic acid, activated PPAR? Andprompt the mRNA expression of PPAR? Target genes in adipocytes, thusencouragingseparation. Additionally, 13-oxo-OTA induced secretion of adiponectin and stimulated glucose uptake in adipocytes.

5.1 SUMMARY OF FINDINGS

In this study, treatment with fractions of Vernonia amygdalina has shown;

i. Reduction of blood sugar level in diabetic animal as shown by its effect on the glucose metabolic pathways (enzymes).
ii. Improvement in body weight in diabetic rats.
iii. Hepatoprotective potential as indicated by its effect on liver enzymes (AST, ALP, ALT), bilirubin and protein sparing ability.

iv. Cyto-protection against lipid peroxidation by improvement in antioxidant activity.

v. Improvement in renal function indicated by the effect on renal biomarkers (Blood Urea Nitrogen and creatinine).

vi. Anti-lipemic activity indicated by its effect on serum levels of Cholesterol, HDL, LDL and triglycerides.

vii. Potent hypoglycaemic phytochemicals such as; sesquiterpene lactones (vernodalin, vernolide, hydroxyvernolide), steroid glucosides (vernonioside).

viii. Bioactive chemicals with known anti-diabetic property such as; Phytol, Hexadecanoic Acid, Stearic acid, Benzoate salts, Butylphenol, Oxirane and Octadecatrienoic acid

5.2 CONTRIBUTION TO KNOWLEDGE

i) In this study, VA revealed protein sparing ability, thus can check body protein breakdown (loss of weight) often observed in DM and at the same time, providing substrate to cells for energy generation. This can considerably reduce diabetic complications while enhancing patients health

ii) VA this study also showed good ability to decrease dyslipidemia often seen in DM. This action is essential in the management of DM as it helps to decrease elevated levels of serum keto-acids leads to diabetic keto-acidosis which often complicates DM.

iii) The effect of VA in decreasing blood lipid levels in non-diabetic rats as observed in this study, shows that the plant possess the potentials to protect against artherosclerosis and its associated or incidental cardiovascular complications usually induced by hyperlipidaemia which is not of diabetic origin.

iv) In this study, VA treatment resulted in improved renal glomerulus filtration, evident by its effect on serum creatinine. This is very critical in diabetes management which often present with renal complications resulting from poor filtration of metabolic waste products.

v) In this study, bioactive compounds such as phytanic acids, hexadecanioc acid, benzoate salts and oxirane gotten from VA extract and fractions that were

subjected to gas chromatography mass-spectrum analysis have great potential for synthesizing the next generation of anti-diabetic drugs.

5.3 CONCLUSION

In this study, the hypoglycaemic property of Vernonia amygdalina has been confirmed and some mechanism of action of the plant demonstrated. The ability of plant to ameliorate hepatic and renal complications arising from diabetes mellitus as well as dyslipidemia was also elucidated. Thus, alternative and complimentary approach to diabetes mellitus management in human may include the use of the leaf extract and fractions of Vernonia amygdalina.

5.4 RECOMMENDATION

Clinical trials involving the use of Vernonia amygdalina crude extracts and fractions as well as the use of the identified bioactive chemicals (which may be commercially available) would advance the search and synthesis of new drugs to enhance diabetes mellitus management.

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APPENDIX I

ETHICAL APPROVAL

RESEARCH AND BIOETHICS COMMITTEE FACULTY OF BASIC MEDICAL SCIENCES, DELTA STATE UNIVERSITY, ABRAKA.

OUR REF: RBC/FBMS/DELSU/14/04 **DATE** 12/2/2014

Ojieh Anthony Emeka
Department of Physiology,
Delta State University,
Abraka.

Dear Sir,

RE: APPLICATION FOR PERMISSION FOR THE USE OF ANIMAL IN CARRYING OUT A RESEARCH

This is to convey approval for the use of Wistar rats in the research study titled: *"Antidiabetic Effect of Vernonia amygdalina fractionates in Streptozotocin Induced Diabetes"*. The committee granted the approval during their February meeting that held on the 10th of February, 2014.

Congrats.

Dr. I. Onyesom
(Chairman)

APPENDIX II

SOLVENT AND SOLVENT PROPERTIES

Name	Solvent Polarity Index (0-9)	Solubility In Water At 25^0c	Formula/Structure	Biological Uses
Chloroform	4.1	0.815%	$CHCL_3$	It is used in pesticides formulations (Payner, 1998). It is used as a solvent for fats, oils, rubber, alkaloids, waxes, gutta-perchia and resins (Payner, 1998). It was previously used as anesthetics agents in hospitals and clinics, especially during surgery in Europe and America (Gordon and laing, 2002)
Ethyl Acetate	4.4	8.7%	$C_4H_80_2$	It is used in confectionery, perfumes and fruits because it evaporates at a fast rate leaving but the scent of the perfume on the skin. In the field of entomology, ethyl acetate is an effective asphyxiant (poison) for use in insect collector as its vapors are a respiratory tract irritant whose vapors can kill insect quickly without destroying, leaving it intact for study. In the laboratory, it is often

				used for column chromatography and extraction. It is commonly used to clean circuit boards and in some nails vanish removers (acetone and acetonitrile are also used. It is used to decaffeinate coffee beans and tea leaves. Ethyl acetate is the most common ester in wine, being the product of the most common volatile organic acid – acetic acid, and the ethyl alcohol generated during the fermentation (Robinson, 2006).
Benzene	2.7	0.18%	C_6H_6	It was initially used to decaffeinate coffee. It was also used for degreasing metals. It is used in the industries in manufacturing plastics, lubricants, rubber, synthetic fibers and dyes.
N Butanol	4	0.43%	$C_4H_{10}O$	It is used as an artificial flavorant in the united states in butter, fruits, cream, whiskey, ice cream (Hall and Oser, 1965). It used in the manufacturing of pharmaceuticals, polymers, pyroxylin, plastics, herbicides

	and butyl xanthate (Monich, 1968). It is used as an ingredient in perfumes and as a solvent for the extraction of essential oils (Mellan, 1950; Doolittle, 1954)

APPENDIX III

GC-MS RESULT TABLES

Sample – Methanol Fraction				
S/N	NAMES	R.Time	Area%	Height %
1	3,3 – Dimethyl-2-pentane	3.276	9.86	4.95
2	1-methylbutyl	4.306	4.24	3.45
3	Propanoic acid	6.183	3.77	3.60
4	3,4-Dimethyl-2-pentanone	6.430	9.37	15.25
5	3,4-Anhydro-d-galactosan	6.726	0.78	1.16
6	2,5-Dimethyl-4-hydroxyl-3-hexane	6.884	1.09	1.85
7	1,2,3,4-Butanetetrol	8.649	10.70	2.89
8	1,3-cyclohexane	9.175	4.03	2.58
9	Phthalic acid	12.074	3.91	3.38
10	Tridercanoic acid	16.765	0.80	1.34
11	Octadecanoic acid	17.729	12.12	13.38
12	11,14-Eicosadienoic acid	19.808	0.35	0.84
13	9,12,15-Octadecatrienoic acid	19.903	1.15	2.24
14	Phytol	20.131	1.38	2.40
15	9,12-Octadecadienoic acid	20.504	2.80	4.02
16	9,12,15-Octadecatrienoic acid	20.597	8.22	8.84
17	Hexadecanoic acid	20.864	3.33	3.33
18	Hexadecanoic acid	21.052	1.08	2.50
19	9,12-Octadecadienoic acid	23.006	0.96	2.37
20	11,14,17-Eicosatrienoic acid	23.085	2.99	6.87
21	1-Hexene	24.683	13.84	7.09
22	di-n-octyl phathalate	24.855	3.23	5.69
23	Total		100.00	100.00

Benzene				
S/N	Names	R.time	Area%	Height%
1	3,3-Dimethyl-2-pentane	15.207	0.56	1.43
2	Propanoic acid -2-methyl	16.777	1.66	3.95
3	Hexadecanoic acid	18.055	16.48	11.17
4	Eicosanoic acid	18.202	7.03	12.19
5	9,12-Octadecadienoic acid	19.838	3.48	8.11
6	15-Tetracosenoic acid	19.938	4.55	10.92
7	Phytol	2.264	21.05	20.58
8	9,12,15-Octadecatrienoic acid	20.879	39.16	18.45
9	Octadecanoic acid	21.025	3.76	8.34
10	Ethyl tridecanoic	21.235	2.27	4.86

Chloroform				
S/N	NAMES	R. Time	Area%	Height%
1	1,2-Xylene	3.130	5.61	4.57
2	1-Octadecyne	15.206	0.74	1.00
3	n-Hexadecanoic acid	17.784	10.89	8.11
4	Hexadecanoic acid	18.140	7.11	9.26
5	Phytol	20.161	21.73	23.83
6	9,12-Octadecatrienoic acid	20.548	8.51	6.08
7	9,12,15-Octadecatrienoic acid	20.655	10.54	10.67
8	9,12-Octadecadienoic acid	20.755	5.67	7.74
9	9,12,15-Octadecatrienoic acid	20.851	10.80	13.38
10	Octadecanoic acid	20.152	1.37	1.91
11	Tetratetracontane	22.298	3.51	2.09
12	Eicosane	24.876	3.11	2.80
13	9,12-Octadecadienoyl acid	26.279	4.13	3.49
14	7,10,13-Hexadecatrienal	26.364	3.54	2.86
15	Tetracosane	26.954	2.80	2.22

ETHYL ACETATE				
S/N	NAMES	R.Time	Area%	Height%
1	1-Undecene	4.053	2.11	1.92
2	4-propoxy-2-butane	6.417	7.44	7.51
3	5H-L-pyrindine	8.447	2.42	2.13
4	3-Tridecene	9.488	0.50	1.11
5	Phenol	11.157	3.95	1.73
6	3-Tetradecen	11.988	1.07	1.93
7	Phthalic acid	12.066	1.15	1.36
8	Benzenemethanol	12.930	0.66	0.63
9	Tratrazole	14.034	1.57	1.27
10	Oxirane	14.509	2.01	1.74
11	3,5-Octadienoic acid	14.802	4.42	3.18
12	l-octadecyne	15.185	1.28	1.90
13	Propanedionic acid	15.176	3.13	3.94
14	Acetic acid	16.602	1.86	2.09
15	Methyl tetradecanoate	16.732	1.90	2.17
16	Hexadecanoic acid	17.722	14.35	12.49
17	Octadecanoic acid	18.112	2.99	3.53
18	Phytol	20.116	7.87	10.62
19	9,12-octadecadienoic acid	20.495	6.31	5.96
20	9,12,15-octadecatrienoic acid	20.595	12.12	11.64

21	Pentadecadien-L-o1	20.728	1.96	2.78
22	9,12,15-Octadecatrienoic acid	20.823	4.74	4.83
23	Z-10-pentadecen-L-01	24.416	2.58	2.44
24	Hexadecanoic acid	24.577	7.31	6.53
25	9,12-Octadecadienoyl chloride	26.250	4.28	4.59

APPENDIX IV

RESULT TABLES

Table1: Effect of *Vernonia amygdalina* on the body weight and fasting blood glucose level of Diabetic experimental animals.

Group	BODY WEIGHT			FASTING BLOOD GLUCOSE		
	Initial	Final	% Change	Initial	Final	% Change
Diabetic	215.6±5.963	208.4±20.034	3.71±0.325	126.6±10.764	307.4±12.18	148.54±20.436
Metformin	121.33±16.333	106.03±19.526	11.9±15.765	355.67±52.639	204.67±52.106	36.53±2.769
Crude	135.4±10.86	133.85±5.65	1.125±0.01	550.00±50.05	102±38	81.45±15.23
Chloroform	140.2±17.976	131.63±13.298	1.88±0.45	323.4±44.399	115.6±54.772	65.85±14.498
Benzene	127.75±7.087	124.42±12.547	0.93±0.064	364.25±65.383	175.25±12.086	45.59±11.861
Ethyl Acetate	128.03±9.628	114.26±8.968	8.31±0.07	415±59.319	118.33±37.423	69.65±8.875
Butanol	175.22±7.191	186±8.953	6.27±0.409	188.5±23.591	105.67±17.682	37.31±12.361

Table 2: Effect of *Vernonia amygdalina* on the body weight and fasting blood glucose level of Normoglycaemic experimental animals.

Group	BODY WEIGHT			FASTING BLOOD GLUCOSE		
	Initial	Final	% Change	Initial	Final	% Change
Control	91.5±2.98	167.2±6.7	83.5412.79	90±3.21	60.33±4.41	-32.61±6.62
Crude	94.3±8.89	133.07±13.92	40.93±4.81	66.33±5.49	51.67±3.71	-21.89±2.38
Ethyl acetate	132.66±12.74	144.80±15.12	9.10±4.95	75.00±2.912	81.00±35.46	10.71±51.47
Benzene	168.52±8.43	171.65±5.47	2.80±5.56	75.40±6.12	49.00±6.47	-35.17±6.33
Chloroform	154.66±13.64	161.02±9.10	5.87±5.33	68.50±3.23	59.17±13.66	-13.39±19.62
Butanol	137.74±7.64	157.87±8.69	15.40±5.92	63.67±7.14	50.00±12.85	-15.27±23.33

Table 3: Effect of *Vernonia amygdalina* on the activity of glycolytic enzymes in diabetic experimental animals.

Group	HEXOKINASE		PYRUVATE KINASE	
	Liver	Serum	Liver	Serum
Diabetic	328.56±38.818	142.36±18.759	304.98±20.755	90.72±6.288
Metformin	589.87±86.236	191.49±3.533	407.92±36.296	147.72±15.37
Chloroform	264.65±52.117	230.17±30.633	158.55±31.823	104.18±12.064
Benzene	229.66±54.215	125.13±4.949	311.36±27.477	65.78±4.656
Ethyl Acetate	333.73±14.307	128.44±8.083	317.43±29.646	105.74±11.855
Butanol	410.45±24.692	173.56±41.735	511.19±10.616	23.65±5.508
Crude	422.92±13.42	129.23±24.55	668.3±11.95	69.52±10.75

Table 4: Effect of *Vernonia amygdalina* on the activity of glycolytic enzymes in normoglycaemic experimental animals.

Group	HEXOKINASE		PYRUVATE KINASE
	Liver	Serum	Liver

Control	3.78±1.66	0.98±0.2	97.14±17.91
Crude	0.890±0.26	1.63±0.21	84.3±3.94
Ethyl Acetate	444.21±47.85	220.81±41.20	69.23±13.14
Benzene	461.82±58.70	222.12±23.84	36.63±13.54
Chloroform	450.11±49.14	186.23±20.99	42.99±10.94
Butanol	345.70±29.40	156.97±26.68	55.35±10.57

Table 5: Effect of *Vernonia amygdalina* on the activity of gluconeogenesis enzymes in diabetic experimental animals.

Group	Glucose 6-Phosphatase	Lactate Dehydrogenase
Diabetic	86.65±6.33	149.8±8.74
Metformin	24.6±2.91	149.7±6.80
Chloroform	43.36±23.23	247.01±31.05
Benzene	16.09±1.37	101.32±17.15
Ethyl Acetate	14.89±0.33	142.13±13.42
Butanol	13.5951±1.76	127.91±4.51

Table 6: Effect of *Vernonia amygdalina* on the activity of Pentose Phosphate pathway enzymes in diabetic experimental animals.

	Glucose 6-Phosphate Dehydrogenase	NADPH-Diaphorase
Diabetic	4.43±0.272	14.96±1.52
Metformin	4.21±0.905	26.55±7.09
Chloroform	6.84±1.448	16.2±1.071
Benzene	12.92±1.78	6.76±1.45
Ethyl Acetate	5.65±0.498	13.33±2.296
Butanol	8.15±2.772	6.07±0.393
Crude	10.95±4.1	39.76±8.1

Table 7: Effect of *Vernonia amygdalina* on the activity of Pentose Phosphate pathway enzymes in normoglycaemic experimental animals.

	Glucose 6-Phosphate Dehydrogenase		NADPH-Diaphorase	
Group	**Liver**	**Serum**	**Liver**	**Serum**
Control	127.85±18.17	12.67±4.54	5.7±0.39	144.97±38.08
Crude	133.61±3.31	15.55±12.3	39.76±8.1	9.19±6.79
Ethyl acetate	12.07±1.93	17.34±7.69	11.35±1.63	11.75±2.00
Benzene	14.95±3.27	21.16±10.07	9.43±1.32	9.74±1.09
Chloroform	14.81±3.23	19.89±8.62	8.81±0.85	12.78±0.50
Butanol	9.65±1.67	10.23±3.39	5.98±0.36	8.26±0.39

Table 8: Effect of *Vernonia amygdalina* on the level of serum billirubin in diabetic experimental animals.

	Direct Billirubin	Indirect Billirubin
Diabetic	0.54±0.072	0.72±0.095
Metformin	0.43±0.075	0.58±0.1
Chloroform	0.5±0.082	0.67±0.109
Benzene	0.79±0.097	1.05±0.129
Ethyl Acetate	0.35±0.082	0.46±0.109
Butanol	0.23±0.023	0.31±0.031
Crude	1.07±0.05	1.06±0.02

Table 9: Effect of *Vernonia amygdalina* on the level of serum billirubin in normoglycaemic experimental animals.

	Direct Billirubin	Indirect Billirubin
Control	0.21±0.06	0.16±0.04
Crude	0.23±0.02	0.17±0.02
Ethyl acetate	0.74±0.164	0.99±0.219
Benzene	0.38±0.061	0.51±0.082
Chloroform	0.66±0.233	0.88±0.311
Butanol	0.53±0.093	0.71±0.124

Table 10: Effect of *Vernonia amygdalina* on the activity of antioxidant enzymesin diabetic experimental animals.

	Catalase	Superoxide Dismutase
Diabetic	41.82±2.27	21.03±1.18
Metformin	40.39±0.28	21.29±3.10
Chloroform	43.77±1.27	17.4±0.33
Benzene	44.57±0.53	30.8±3.513
Ethyl Acetate	41.65±1.39	29.71±5.76
Butanol	44.61±9.46	8.38±0.23
Crude	46.35±1.25	41.81±1.8

Table 11: Effect of *Vernonia amygdalina* on the activity of antioxidant enzymes in normoglycaemic experimental animals.

	Serum Catalase	Liver Catalase	Liver Superoxide Dismutase
Control	34.36±1.22	76.4±22.11	32.06±4.01
Crude	47.72±11.43	35.08±8.79	29.34±2.01
Ethyl acetate	20.49±0.723	46.05±17.640	79.61±2.420
Benzene	19.62±0.882	99.41±4.052	60.03±15.361
Chloroform	20.18±0.711	27.69±7.223	43.72±8.125
Butanol	19.86±0.646	21.23±3.554	58.67±16.417

Table 12: Effect of *Vernonia amygdalina* on the level of malondialdehydein Diabetic experimental animals.

	Liver	Serum
Diabetic	0.17±0.045	0.38±0.043
Metformin	0.22±0.05	0.29±0.071
Chloroform	0.16±0.05	0.24±0.044
Benzene	0.06±0.005	0.37±0.021
Ethyl Acetate	0.13±0.047	0.26±0.065
Butanol	1.42±0.429	1.48±0.354
Crude	0.12±0.01	0.68±0.09

Table 13: Effect of *Vernonia amygdalina* on the level of malondialdehyde in normoglycaemic experimental animals.

	Brain MDA	Liver MDA
Control	0.03±0.005	0.02±0.01
Crude	0.04±0.01	0.03±0.01
Ethyl acetate	0.46±0.235	0.57±0.177
Benzene	2.47±0.211	0.86±0.207
Chloroform	2.80±0.101	1.09±0.413
Butanol	0.82±0.437	1.37±0.380

Table 14: Effect of *Vernonia amygdalina* on the level of Total Protein in diabetic experimental animals.

	SERUM	LIVER
Diabetic	14.2±0.99	14.59±1.65
Metformin	6.02±0.79	6.7±0.67
Chloroform	6.13±0.76	8.98±1.04
Benzene	6.79±1.10	12.32±0.65
Ethyl Acetate	7.8±1.17	10.21±1.12
Butanol	14.83±1.37	12.863±0.99
Crude	24.93±4.24	14.21±1.33

Table 15: Effect of *Vernonia amygdalina* on the level of Total Protein in diabetic experimental animals.

Control	11.11±0.91	12.92±1.36
Crude	17±1.14	10.926±0.332
Ethyl acetate	6.98±3.17	9.4924±0.92424
Benzene	13.50±2.30	11.6926±0.39548
Chloroform	13.33±3.04	12.6952±1.49619
Butanol	14.58±2.22	13.9238±1.46936

Table 16: Effect of *Vernonia amygdalina* on the liver function ofdiabetic experimental animals.

	Alkaline Phosphatase	Alanine Transaminase	Aspartate Transaminase
Diabetic	90.24±6.09	133.12±5.426	20.84±2.323
Metformin	136.82±10.431	37.32±3.606	23.33±3.01
Chloroform	91.49±4.139	120.14±8.933	34.68±1.981
Benzene	19.01±3.212	33.95±6.231	22.34±6.807
Ethyl Acetate	152.77±18.33	17.99±6.35	19.76±1.935
Butanol	82.37±15.56	18.18±1.668	19.21±2.491
Crude	10.59±3.37	22.72±3.96	24.56±5.41

Table 17: Effect of *Vernonia amygdalina* on the liver function ofnormoglycaemic experimental animals.

	Alkaline Phosphatase	Alanine Transaminase	Aspartate Transaminase
Control	55.66±3.43	43.13±3.82	51.8±4.75
Crude	63.35±0.56	50.9±4.36	60.89±5.09
Ethyl acetate	106.74±9.036	15.67±1.567	20.05±2.330
Benzene	62.74±7.920	13.94±1.367	12.14±1.830
Chloroform	66.14±6.474	13.50±0.862	16.74±1.444
Butanol	48.72±7.857	17.01±1.474	24.74±1.921

Table 18: Effect of *Vernonia amygdalina* on the Lipid profile of diabetic experimental animals.

	Triglyceride	Total Cholesterol	HDL	LDL
Diabetic	246.58±21.982	280.19±14.753	38.06±2.075	192.814±8.2816
Metformin	331.51±4.554	249.01±7.393	43.65±7.637	139.058±1.1548
Chloroform	345.36±19.977	251.15±13.666	45.98±8.07	136.098±1.6006
Benzene	322.81±36.029	242.21±20.988	55.58±6.006	122.068±7.7762
Ethyl Acetate	300.55±17.972	243.44±13.765	41.56±3.581	141.77±6.5896
Butanol	127.67±9.079	133.5±4.281	45.83±4.654	62.136±2.1888
Crude	160.19±17.19	143.31±10.45	34.66±5.08	181.88±9.12

Table 19: Effect of *Vernonia amygdalina* on the Lipid profile of normoglycaemic experimental animals.

	Triglyceride	Total Cholesterol	LDL	HDL
Control	216.82±40.87	185.76±4.02	131.888±34.986	47.78±5.08
Crude	145.67±12.47	134.3±16.64	72.95±-0.818	45.86±9.96
Ethyl acetate	164.00±15.056	169.73±8.098	131.888±34.986	35.94±5.753
Benzene	130.53±10.250	163.13±15.328	72.95±-0.818	50.90±11.484
Chloroform	151.67±11.722	177.44±32.803	100.99±-0.6662	44.41±5.884
Butanol	133.56±8.016	137.39±4.066	86.124±1.794	38.50±3.245

Table 20: Effect of *Vernonia amygdalina* on the Renal function of normoglycaemic experimental animals.

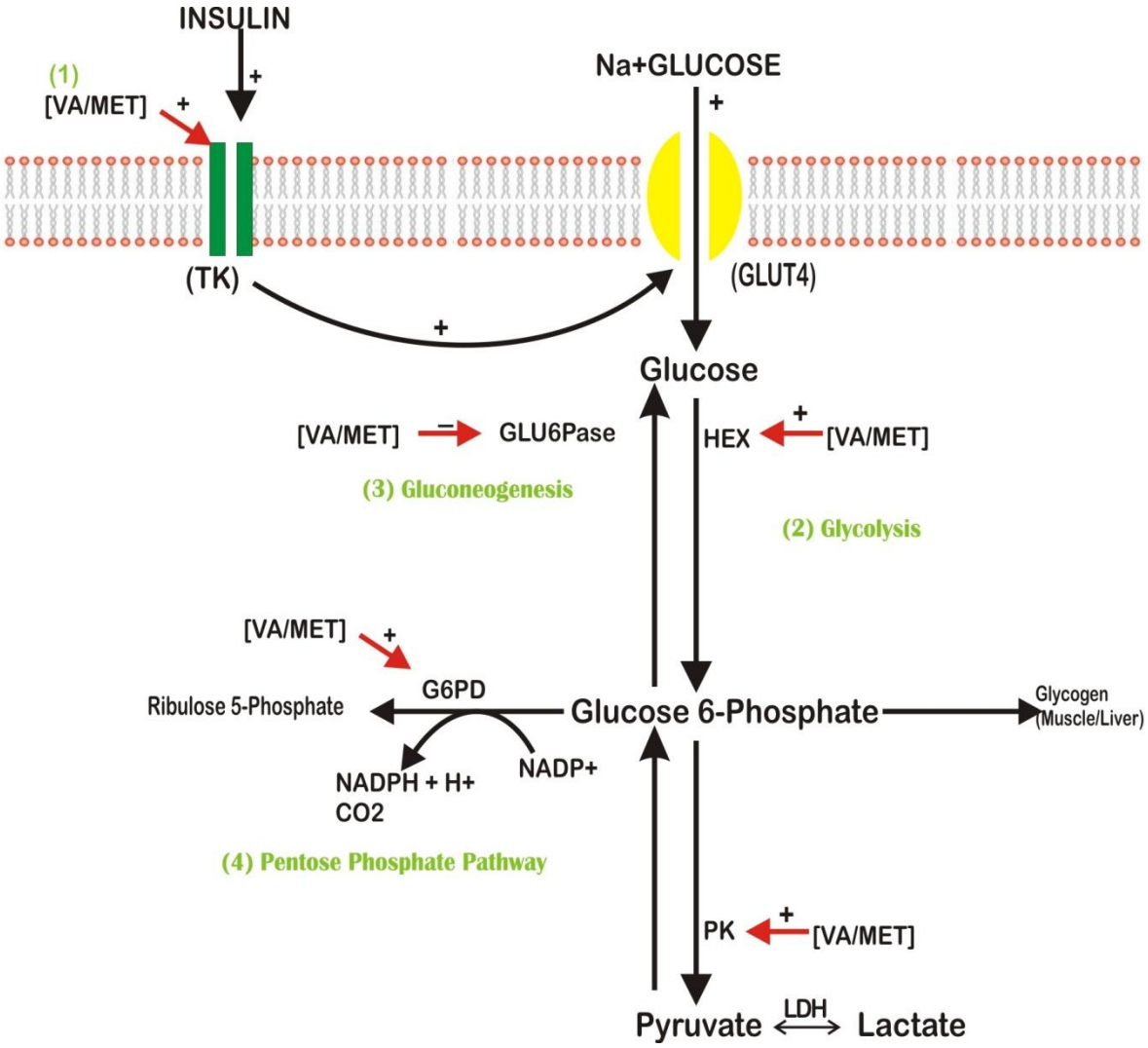
	UREA	CREATININE
Diabetic	11.95±0.876	2.33±0.178
Metformin	15.13±0.321	0.91±0.128
Chloroform	14.88±0.78	1.27±0.248
Benzene	14.62±1.068	2.19±0.076
Ethyl Acetate	12.87±0.592	2.12±0.148
Butanol	24.36±1.234	0.57±0.073
Crude	29.41±8.9	1.73±0.44

Table 20: Effect of *Vernonia amygdalina* on the Renal function ofnormoglycaemic experimental animals.

	CREATININE	**UREA**
Control	1.52±0.35	8.95±2.54
Crude	3.28±0.3	6.35±1.26
Ethyl acetate	0.46±0.071	43.37±1.798
Benzene	0.74±0.267	36.87±2.661
Chloroform	0.50±0.088	33.70±1.101
Butanol	0.59±0.064	26.47±2.912

APPENDIX V

PROPOSED MECHANISM OF ACTION OF VERNONIA AMYGDALINA EXTRACTS AND FRACTIONS IN DIAABETIS MELLITUS



KEYS:

VA: *Vernonia amygdalina*, **MET**: Metformin, **TK**: Tyrosin Kinase (Insulin Receptor), **GLUT4**: Glucose Transporter 4, **Glu6pase**: Glucose 6-Phosphatase, **G6PD**: Glucose 6-Phopsphate Dehydrogenase, **HEX**: Hexokinase, **PK**: Pyruvate Kinase, **CO_2**: Carbon Dioxide, **NADPH**: Nicotinamide Adenine Dinucleotide Phosphate, **Na^+**: Sodium, –: Imparment/Inhibition, +: Stimulation/Improvement.

(1) Improved insulin receptor sensitivity

(2) Improved glycolysis

(3) Impaired gluconeogenesis

(4) Enhanced pentose phosphate pathway