AMELIORATIVE EFFECTS OF FRACTIONATED EXTRACTS OF *VITEX SIMPLICIFOLIA* LEAF ON BLOOD GLUCOSE AND SOME PHYSIOLOGICAL PARAMETERS IN ALLOXAN- INDUCED DIABETIC WISTAR RATS

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JULY, 2016

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A
DOCTOR OF PHILOSOPHY DEGREE IN HUMAN PHYSIOLOGY

DEPARTMENT OF HUMAN PHYSIOLOGY,

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ZARIA, NIGERIA

JULY, 2016

DECLARATION

I declare that the work in this thesis entitled "Ameliorative effects of fractionated extracts of *Vitex Simplicifolia* leaf on blood glucose and some physiological parameters in alloxan induced diabetic Wistar rats" has been carried out by me in the Department of Human Physiology,ABU,Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

MOHAMMED ALI SALIM	_____	_____
Name	Signature	Date

CERTIFICATION

This thesis entitled AMELIORATIVE EFFECTS OF FRACTIONATED EXTRACT OF *VITEX SIMPLICIFOLIA* LEAF ON BLOOD GLUCOSE AND SOME PHYSIOLOGICAL PARAMETERS IN ALLOXAN INDUCED DIABETIC WISTAR RATS by MOHAMMED ALI SALIM meets the regulations governing the award of the degree of Doctor of Philosophy of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ACKNOWLEDGEMENTS

First and foremost my gratitude goes to my supervisors Dr Aliyu Mohammed, Dr Yusuf Tanko and Professor A.A.U. Dikko for their intellectual guidance and supervision of this enormous task. I am so happy to be privileged to share in there deep wealth of knowledge.

My sincere thanks and appreciation goes to the entire staff of Human Physiology Department, Bayero University, Kano especially Sale Musa Kachako of the Animal house, Sani Gwarzo the Departmental Secretary and Drs I.U.Yarube,Sharazad Ahmad and Mahdi Dissi.

My gratitude goes to Professor M.Y. Gwarzo, Dean, Faculty of Allied Health Sciences for suggesting the topic. I would also like to thank Ayuba Suleiman of Chemistry Department, Musa Belli of Central laboratory Complex and Dr A.A. Imam of Biochemistry Department, Bayero University Kano.

I am great full to the entire staff of Human Physiology Department, Ahamadu Bello University, Zaria. I also appreciate the contributions of my internal examiners Dr I. G. Bako, Professor A.G. Bakari and Professor A.U. Zezi in making this work a reality.

I heartily thanked and appreciate my beloved brothers Farouk, Sa'eed, Munir and Saddik for their support and encouragement. May the gentle souls of Alhaji, Jaddi and Mama rest in perfect peace.

To my father, I thank you for the sense of pride, hard work and independence, I learn from you.

Lastly, I appreciate the patience and support of my wife Mariya and daughters Aisha and Fatima.

DEDICATION

This work is dedicated to my brother Farouk Ali Salim for the positive role he played in my intellectual development

ABSTRACT

Herbal medicine is the oldest form of health care known to mankind. A concoction of *Vitex simplicifolia* leaf is widely used in sub Saharan Africa for treatment of diabetes mellitus and a lot other diseases. The study investigated the hypoglycemic, hypolipidemic, antioxidant and hepato-renal toxicity of fractionated extract of *Vitex simplicifolia* leaf on alloxan-induced diabetic rats. The plant extract was fractionated in solvents of varying polarities (aqueous, methanolic, hexane and ethyl acetate). 250, 500 and 1000mg/kg of body weight of the fractionated extracts were administered to the diabetic rats, aqueous (groups 4, 5 and 6), methanolic (groups 7, 8 and 9), ethyl acetate (groups 10, 11 and 12), hexane (groups 13, 14 and 15). Groups 1 and 2 were normal and diabetic control, while group 3 was administered glibenclimade (reference drug). Fasting blood glucose, lipid profile, liver glycogen content, superoxide dismutase (SOD), vitamins A, C and E, AST, ALP, ALT, unconjugated bilirubin and serum electrolytes; sodium, chloride, carbonate, urea and creatinine were determined. The result of the study showed that there was significant decrease ($P< 0.05$) in fasting blood glucose level, with the aqueous fraction showing percentage reduction of 72,75 and 81%, n-hexane 58,71 and 77%, ethyl acetate 54,70 and 71%, glibenclimade (reference drug) 65% and methanol 8, 16 and 17% at 250,500 and 1000 mg/kg of body weight respectively. Oral administrations of fractionated extracts aqueous, ethyl acetate and N-hexane of *Vitex Simplicifolia* for 21 days significantly increase liver glycogen content, however, the methanolic fraction showed no significant change. All the fractionated extracts showed no significant changes in lipid profile , SOD, vitamins A, E and C. Aqueous, methanolic and ethyl acetate fractions significantly increase ALP ($P<0.05$) and significantly decrease ($P<0.05$) AST. However, the n- hexane fraction showed significant increase in ALP,

AST and unconjugated bilirubin. Aqueous fraction of *Vitex simplicifolia* showed no significant change in Na, K and chloride but significantly increase urea and significantly decrease ($P<0.05$) creatinine and HCO_3. But the methanol and ethyl acetate fraction significantly increased urea, sodium and chloride but showed no significant change in HCO_3, potassium, chloride and creatinine .The histopathological studies of the liver showed mild to moderate sinusoidal lymphocytosis with prominent kupfer cells and focal portal inflammation in the test groups. The kidneys of the test groups showed mild to moderate distal tubular epithelial cell oedema and mild interstitial inflammation. In conclusion, the four fractionated extracts of *Vitex simplicifolia* leaf extract were observed to posses' potent hypoglycaemic activity but have no hypolipidemic and antioxidant potential. However, it was also observed to possess a moderate hepato-renal toxicity.

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LIST OF ABBREVIATIONS

ACTH:	Adrenocorticotrophic hormone
ADA:	American diabetes association
AGEs:	Advanced glycation end products
ALP:	Alkaline phosphatase
ALT:	Alanine aminotransferase
AOPPs:	Advance oxidation protein products
AST:	Aspartate transaminase
BMI:	Body mass index
CTGF:	Connective tissue growth factor
CVD:	Cardiovascular disease
DM:	Diabetes mellitus
FCPD:	Fibrocalculous pancreatic diabetes
GDM:	Gestational diabetes mellitus
GPX4:	Glutathione peroxidase 4
GWAS:	Genome wide association studies
HCG:	Human chorionic gonadotrophin
HDL:	High density lipoprotein
HNE:	4-hydroxynonenal
HPL:	Human placental lactogen
IDDM:	Insulin dependent diabetes mellitus
IDF:	International diabetes federation
IR:	Insulin resistance
Iso LGs:	Isolevuglandin
LDL:	Low density lipoprotein

MDA:	Malondialdehyde
MRDM:	Malnutrition related diabetes mellitus
NDDG:	National diabetes data group
NIDDM:	Non insulin dependent diabetes mellitus
NOx:	Nitric oxide metabolic pathway
NRDS:	Neonatal respiratory distress syndrome
OGCT:	Oral glucose challenge test
OGTT:	Oral glucose tolerance test
ONE:	4-oxononenal
PDPD:	Protein-deficient pancreatic diabetes
PKC:	Protein kinase C
PUFA:	Polyunsaturated fatty acid
ROS:	Reactive oxygen species
SOD:	Superoxide dismutase
SP 1:	Schwangerschafts-protein 1
T1 DM:	Type 1 diabetes mellitus
T2 DM:	Type 2 diabetes mellitus
TBARS:	Thiobarbituric acid-reactive substances
TNF:	Tumor necrosis factor
WHO:	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Preamble

Diabetes Mellitus (DM) refers to a group of common metabolic disorders that share the phenotype of hyperglycaemia and characterized by elevated blood glucose concentration caused by insulin deficiency, often combined with insulin resistance. Type 2 Diabetes mellitus is more prevalent and accounts for about 90- 95% of all diagnosed cases of diabetes (W.H.O, 2011). Type 2 diabetes mellitus is characterized by fasting hyperglycaemia and an excessive, prolonged rise in the plasma glucose concentration after glucose or meal ingestion (DeFronzo *et al.,* 1989). The interest in natural products for use as medicine has acted as the catalyst for exploring methodologies involved in obtaining the required plant materials for pharmacological screening and drug development. Herbal medicine or phytomedicine refers to the use of a plants seeds, roots, leaves, bark or flower for medicinal purposes (Lewis, 1981). A medicinal plant is any plant which one or more of its organ contain substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs (Lewis, 1981).

Medicinal plants are of great importance to the health of individuals and communities. It is estimated that there are between 200,000 – 700,000 species of tropical flowering plants that have medicinal properties (Atata *et al.,* 2003) and these medicinal properties lie in some chemical substance they contain (Oladunmoye, 2007). The high cost of prescription medication coupled with the desire of returning to natural or organic remedies has led to an increase in the use of herbal medicine (Yach, 2004). In Germany, roughly 600 – 700

plant based medicines are available and are prescribed by approximately 70% of German Physicians (Hawkins and Ehrlich, 2009).

Vitex simplicifolia is a shrub with soft hairy branches. Leaves are stiff, dull green with grey brown mid-rib above .It is a small tree of the savanna and across Africa to Egypt. Its fruits are shining green with white spots. *Vitex simplicifolia* (family- LABIATAE) commonly known as *Dinyar Biri* (Hausa) and *Ngumeehi* (Fulfulde) the plant is widely spread from Mali to west Cameroon (Kenneth, 1974).

Young sticks are used as tooth-stick in Nigeria; bark in decoction is used as a lotion in Ivory Coast on edema, skin and for toothache (Burkill, 2000).

The WHO definition of traditional medicine was revised in 2003 as the health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illness or maintain well being (WHO, 2003). Countries in Africa, Asia and Latin America use traditional medicine to help meet some of their primary health care needs. For example in Africa, up to 80% of the population use traditional medicine for primary health care. The WHO, however, also notes that its use is spreading in popularity in industrialized countries. For example, in the United States, 158 million adults use alternative medicine (a field which incorporates traditional medicine but is broader in scope) (WHO, 2003). The WHO also notes, that inappropriate use of traditional medicines or practices can have negative or dangerous effects and that further research is needed to ascertain the efficacy and safety of several of the practices and medicinal plants used by traditional medicine systems (WHO, 2003).

1.2 Statement of Research Problem

The existence of experimental animal models of a disease, aids not only the understanding of the pathophysiology of such disease, but also the development of drugs for its treatment. According to World Health Organization (WHO), there are approximately 171 million diabetics worldwide, this number has double in the last few years and is expected to double once again in the year 2025 (Beretta, 2001). Diabetes is a major degenerative disease in the world today, affecting over 150 million people and having complications which include hypertension, atherosclerosis and microcirculatory disorders (Ogbonnia *et al.*, 2008). The prevalence of diabetes is on the increase globally and in African communities due to the ageing of the population and drastic lifestyle changes accompanying urbanization and westernization (Sobngwi *et al.,* 2001). Also, studies from five West African communities in Nigeria and Ghana have identified genes within populations that create susceptibility to diabetes (Rotimi *et al.*, 2010). The prevalence of diabetes mellitus in Nigeria is 1.9% with more than 1.5 million cases (IDF, 2015). Hence, it represents a growing burden of health care systems of African countries, most of which already face difficult economic conditions. The disease remains incurable and can only be controlled with drugs; hence, a scrupulous control is needed to help reduce hyperglycemia and the risk of long-term complications, which are known to be the major causes of morbidity and mortality (Rotimi *et al.,* 2010). In Nigeria, information available from the indigenous traditional healers indicates that, a decoction of the chopped stem barks and leaf of *Vitex simplicifolia* is prepared and taken orally for treatment of diabetes and other disease conditions. The plant extracts have been used as medication for infertility, liver disease, stiffness, hypertension, cancer, febrifuge, as tonic

galactagogue to aid milk production in lactating mothers, sedative, digestive regulator and treatment of eye, kidney and as supplement for lack of vitamin A and B (Sofowora, 1993; Burkill, 2000).

1.3 Justification

Type 2 Diabetes mellitus is more prevalent and accounts for about 90- 95% of all diagnosed cases of diabetes (WHO, 2011). Type 2 diabetes mellitus is characterized by fasting hyperglycaemia and an excessive, prolonged rise in the plasma glucose concentration after glucose or meal ingestion (DeFronzo *et al.,* 1989). The high cost of prescription medication coupled with the prevalent economic hardships faced in the developing world requires the need to look in to natural or organic remedies that are cheaper.

Although parts of this plant are used by traditional healers of various ailments information on the toxicity of the extract in man and animal are lacking. There is paucity of scientific study to establish the scientific basis of its use and like many other herbal remedy, there is little or no information about its possible side effects or toxicities.

This study, therefore, was aim at elucidating the hypoglycemic and anti- oxidant effects of fractionated leaf extract of *Vitex simplicifolia* on alloxan induced diabetic rats. This is important since science requires the validation of drugs by medicinal practitioners and drug regulatory authorities demand that all potential drugs should pass through a rigorous series of study and scrutiny (Abdulrahaman, 1992).

1.4 Aim and Objectives

1.4.1 Aim

To carry out antidiabetic and some physiological parameters of fractionated extracts of *Vitex simplicifolia* leaf in alloxan- induced diabetic wistar rats.

1.4.2 Specific objectives

1. To determine the effects of the fractionated leaf extracts of *Vitex simplicifolia* on blood glucose levels of Alloxan induced diabetic rats.
2. To determine effects of the fractionated leaf extracts of *Vitex simplicifolia* on lipid profile in alloxan- induced diabetic animals.
3. . To determine effects of the fractionated leaf extracts of *Vitex simplicifolia* on liver Glycogen content in alloxan- induced diabetic animals
4. To determine the levels of oxidative stress biomaker Superoxide dismutase in alloxan- induced diabetic rats
5. To determine effects of the fractionated leaf extracts of *Vitex simplicifolia* on serum anti oxidants vitamins A,C and E in alloxan- induced diabetic rats
6. To determine the toxic effect ,if any, of these fractionated leaf extracts on the liver enzymes i.e. AST, ALP, ALT and Bilirubin
7. To determine the effect of the fractionated leaf extracts on serum electrolytes level i.e. Na,Cl,K,HCO_3^- and Urea
8. To determine the toxic effect of the fractionated leaf extracts, if any, on the liver and kidneys of the experimental animals through histopathological studies.

1.5 Research Hypothesis

1. The fractionated leaf extracts of *Vitex simplicifolia* does not exert any hypoglycaemic and hypolipidemic effect in alloxan-induced diabetic wistar rats

2. The fractionated leaf extracts of *Vitex simplicifolia* does not increase liver glycogen content in alloxan-induced diabetic wistar rats

3. The fractionated leaf extracts of *Vitex simplicifolia* does not increase the level of serum liver enzymes and serum electrolytes in alloxan-induced diabetic wistar rats

4. The fractionated leaf extracts of *Vitex simplicifolia* does not have any effect on SOD, anti-oxidant vitamins and histopathology of liver and kidneys in alloxan-induced diabetic wistar rats

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Diabetes Mellitus

Diabetes mellitus is a group of anatomic and chemical problems resulting from a number of factors in which an absolute or relative deficiency of insulin and/or its function is present. It tends to run in families; is associated with accelerated atherosclerosis, and predisposes to certain specific microvascular abnormalities including retinopathy, nephropathy and neuropathy. It doubles the risk of stroke, increases the risk for heart attacks 2- to 3-fold, and for peripheral vascular problems, particularly in the feet, 50-fold. There are other problems, such as the lessening of resistance to infection, especially if the diabetes is poorly managed (Marble *et al.*, 1985).

Diabetes mellitus (DM) is the most widespread complex metabolic disorder among the world‘s population currently affecting around 250 million people globally (Srinivasan *et al.*, 2008). In early 1930‘s, Himsworth documented that two types of diabetes existed (Himsworth, 1935). This complicated metabolic syndrome is due to either insulin insufficiency or impaired action of the insulin hormone or both (American Diabetes Association, 2012). This disease is classified as insulin- dependent or type 1 diabetes mellitus (T1DM) and non-insulin dependent diabetes mellitus or type 2 diabetes mellitus (T2DM) of which both types are associated with high levels of morbidity and mortality (Alberti, 2004). The epidemic of diabetes mellitus is accompanied by an increase in the incidence of diabetic complications including cardiovascular disease (CVD), retinopathy,

nephropathy and microangiopathy, neuropathy and several other complications (Kumar and Clark, 2007).

The American Diabetes Association has classified diabetes mellitus into two main classes, type 1 and type 2. Type 1 diabetes results from beta cell destruction and this usually results in a complete insulin deficiency in the body. This type is usually diagnosed in children and adolescents. Type 2, the most common form of diabetes, is a more progressive form of the disease that is typically diagnosed in adults and is characterized by an insulin secretory defect. Type 2 diabetes account for 90-95% of all diagnosed cases of diabetes (Zimmet *et al.*, 2001). Complications of diabetes include the progressive damage, dysfunction and failure of various organs including the kidneys, nerves, heart, eyes and blood vessels (National Diabetes Fact sheet, 2007). Diabetes can also affect the limbs, particularly the feet. Cardiovascular disease, retinopathy, nephropathy, peripheral vascular disease and peripheral neuropathy are co-morbidities of diabetes. In addition to these serious complications, diabetes can often cause life-threatening events such as diabetic ketoacidosis and hypersomolar (nonketotic) coma resulting from biochemical imbalances. Infections, such as influenza or pneumonoia are also a serious concern for patients with diabetes, as they are more likely to die as a result of infection than individuals who do not have diabetes (National Diabetes Fact Sheet, 2007). Diabetes has also been associated with an increase in complications such as heart disease and stroke. In 2004, heart disease was noted in more than two thirds of all diabetes-related death certificates in the elderly. Stroke was included in 16% of the diabetes-related death certificates in the same population, suggesting that diabetes impacts cardiovascular health over time. Mortality from heart disease is on average, three

times greater in adults with diabetes, and the risk for stroke is 2 to 4 times higher in persons with diabetes (National Diabetes fact sheet, 2007). Hypertension is considered one of the largest co morbidities of diabetes, with nearly 75% of adults with diabetes reporting high blood pressure and/or use of prescription medications for hypertension in 2003-2004. Diabetes is the leading cause of other complications including blindness from diabetic retinopathy, kidney failure and resulting dialysis and non-traumatic limb amputation. Nerve damage occurs in 60-70% of the diabetes population (National Diabetes fact sheet, 2007).Many studies of mortality in diabetes have made no distinction between the major forms of the disease. Furthermore, under reporting makes accurate assessment of the impact of diabetes on mortality and life expectancy impossible if only death certificates are available. In spite of under reporting, diabetes ranks as the seventh leading cause of death in USA (Finch and Zimmet., 1988*).*

Several prospective studies have documented excessive risks of cardiovascular death among diabetics compared to non-diabetic.The incidence of cardiovascular death was increased among the diabetics, and in each the relative risk for women exceeded that of men, but the absolute risk of ischemic heart disease mortality among diabetic men was greater in several of them. Overall mortality and cause-specific mortality were determined from a 9-year follow-up of men and women aged 44-77 in U.S.A survey. All age related mortality rates were more than twice as high in diabetic men and women as in the corresponding non diabetic groups, with most of the increase attributable to cardiovascular disease. Ischemic heart disease was 2.8 times as frequent in men and *2.5* times as frequent in women. Ischemic heart disease counted for approximately one-half of the deaths among diabetic men and one third of those among diabetic women. Other

cardiovascular disease (including renal disease) deaths were 2.34 and 1.93 times as frequent in diabetic men and women, respectively. Non cardiovascular causes accounted for 26% of the excess of deaths among diabetic men and 22% among women. The risk of death increased with increasing duration of diabetes. Thus, each additional 10 years of diabetes was associated with a 24% increase in risk for cardiovascular disease death (Finch and Zimmet., 1988*).*

Excessive mortality among diabetics has also been found in the Pima Indians among whom diabetic nephropathy accounted for about *25%* of the deaths is diabetics. Ischemic heart disease, while much more common among the diabetics, accounted for a much lower proportion of the diabetic deaths. In Nauru the age-standardised mortality among diabetic men and women was increased approximately four-fold over that of the population with normal glucose tolerance *(*Finch and Zimmet., 1988*).* Large variations in mortality among diabetics from different countries have been found, with much higher mortality rates in Western Europe (predominantly cardiovascular disease) than in Hong Kong and Japan (primarily renal disease). This study is notable in so far as hypertension emerged as one of the most predictive factors for increased mortality among diabetics after adjustment for age, sex, and duration of diabetes.

Proteinuria is a strong predictor of mortality in NIDDM. It was shown that abnormal albumin excretion at levels below those normally detected by dipstick predict higher mortality rates including higher rates of cardiovascular and renal disease. Among the Pima Indians proteinuria is associated with virtually all of the excess risk of mortality that is attributable to NIDDM (Finch and Zimmet., 1988*).*

2.1.1 Classification of diabetes mellitus

In mid-sixties a set of diagnostic criteria for diabetes was published among others with wide variation both between and within countries (WHO, 1965). Since then and before 1978, a variety of descriptive terms were used to classify diabetes, some based on the age of onset and others, the stage or the degree of severity of the disease. Substantial differences in the diagnosis criteria used by diabetes experts were also found (West, 1975). This leads to confusion which hindered the assessment of data from studies of the natural history of the disease and its complications. The NDDG [USA] then created a new classification based on clinical or descriptive observations from epidemiologic studies of large populations to provide uniform designations and a frame work for collecting investigative and epidemiological data on diabetes (NDDG, 1979). A similar but more inclusive classification adopted by the WHO Expert Committee on Diabetes Mellitus in 1980 (WHO, 1980) and modified in 1985 (WHO, 1985*)* has received general acceptance. Diabetes is sub classified as: Type I, insulin dependent diabetes mellitus [IDDM]; Type II, non-insulin dependent diabetes mellitus [NIDDM]; malnutrition-related diabetes mellitus [MRDM]; and other types of diabetes. Each category is described briefly below (Keen *et al.,* 1982). IDDM or type I diabetes mellitus, which involves about 15 % of the diabetic population, occurs primarily in young patients, but may be seen at any age. It is usually characterized by an abrupt onset of symptoms, although present evidence suggests that its evolution may involve an antecedent period of slowly developing autoimmune damage to the pancreatic B cells *(*Leslie *et al.,* 1989*).* NIDDM or type II diabetes mellitus, which occurs in 80 to 85 % of the diabetic population, is usually encountered in adults, but may occur in young patients. NIDDM is

recognized as having a strong genetic basis, as evidenced by studies of identical twins and by familial transmission of diabetes in an autosomal dominant inheritance pattern (Taylor, 1989). Malnutrition-related diabetes mellitus is a clinical subgroup that is included in the WHO classification but not in that of the NDDG. It occurs predominantly among young adults in tropical, developing countries. Distinctive clinical features and cause, and the great number of cases in certain regions, led to the creation of this new major class of diabetes. Clinical studies have suggested the existence of at least two subclasses: FCPD and PDPD (Abu-Bakare *et al*, 1986).

There were other types of diabetes mellitus which was formerly termed secondary diabetes. This is a heterogeneous subclass that includes many specific disorders that induce diabetes including pancreatic disease, hormones, drugs or chemicals, certain genetic syndromes and insulin receptor abnormalities (Keen *et al.*, 1982*).* The designation of impaired glucose tolerance applies to individuals whose plasma glucose concentrations after an oral glucose load lie between normal and values diagnostic of diabetes (Alberti, 2004). The term avoids the social, economic, and psychologic disadvantages associated with such formerly used designations as chemical, latent or subclinical diabetes.

Gestational diabetes mellitus occurs in approximately 2 % of pregnancies. It is a consequence of the diabetogenicity of the gravid state in women with marginal insulinogenic capacity; it generally reverts to normal following parturition. If unrecognised or untreated, gestational diabetes may increase the risk of perinatal morbidity and mortality (Hadden, 1986*).*

To identify the statistical risk classes for glucose intolerance, two additional terms are used. These are not part of the diagnostic identifications for patient records but are useful in research studies. The term previous abnormality of glucose tolerance refers to individuals with previous diabetes or ICT who regained normal glucose tolerance (NDDG, 1979). Most frequently, individuals in this class include patients with former gestational diabetes or acute hyperglycemia whose metabolic status has returned to normal following appropriate treatment. These individuals are at increased risk for developing diabetes with stress or weight gain.

The potential abnormality of glucose tolerance was formerly termed prediabetes or potential diabetes and applies to individuals at substantially greater risk for diabetes than the general population (WHO, 1980*).* The category includes first-degree relatives of patients with diabetes, an identical co-twin of a patient with IDDM or NIDDM, HLA-identical siblings, or islet-cell antibody positive individuals. Also included are obese individuals and individuals who have delivered a large infant.

2.1.1.1 Type 1 diabetes mellitus (IDDM)

Type 1 diabetes mellitus (T1DM) is a T-cell mediated autoimmune disease, caused by interplay between inherited and environmental factors. A triggering factor, or combination of several factors, leads to initiation of autoimmunity against pancreatic beta cells. Subsequent destruction of the majority of the beta cells by the immune system causes the diabetic phenotype of hyperglycaemia. T1DM is commonly associated with microvascular complications including nephropathy and retinopathy and it also predisposes to CHD (Adler *et al.,* 2003)

Both environmental and inherited precipitating factors are implicated in T1DM pathogenesis. Dizygotic twin co-incidence is about 10%, while monozygotic twin co-incidence is 50%, although it may be higher in life-long comparisons (Ozcan *et al*., 2006) suggesting a major impact of the environment. The genetic component of T1DM, investigated through genome-wide associations studies (GWAS), has led to an ever-growing list of known risk loci. The one major genetic predictor of T1DM is variation in the human leukocyte antigen (HLA) region, representing a predicted odds-ratio value of 6.8 (Moretta *et al.*, 2002) with some alleles conferring protection and some conferring increased risk. Some of the other genes shown to be associated with T1DM are the insulin gene, PTPN22, the IL-2 receptor and CTLA-4. However, they only confer minor odds-ratio risks, and the total T1DM incidence explained by genetic risk loci is in the order of few percent.

In comparison, environmental factors play a crucial role in defining the risk of T1DM, illustrated by twin emigrant studies, showing that the twins acquire the T1DM incidence at the location they live in (Buschard *et al.*, 1987). The importance of the environment for the development of T1DM has given rise to the hygiene hypothesis stating that the reduced load of infections of the modern (Western) lifestyle can lead to a hyperactive immune defence that is more prone to develop autoreactivity. This hypothesis is supported by the observations that a range of infections and infection-mimics reduce the incidence of T1DM in spontaneous diabetic animal models (NOD mice) (Palmer *et al*, 1989). Indeed, many lines of evidence indicate that regular presentation of gut bacterial products is needed for normal autoimmune response.

Viral infection in early life has also been proposed to directly trigger T1DM, although the identity of the specific virus responsible is still debated. Especially entero virus infection is known to relate to the development of T1DM, perhaps through a mechanism of antigen mimicry of T1DM auto antigen (Kullin *et al.*, 2000). A numbers of dietary factors have also been proposed as risk generating factors. Cow´s milk protein, particularly milk albumin, has been proposed (Preissing and Rigby, 2009) and a gluten-rich diet is known to have several adverse effects on the immune balance. This link between gluten and T1DM is also seen in the increased incidence of celiac disease (autoimmune gluten intolerance) in T1DM patients. Indeed, a gluten-free diet can prevent diabetes in NOD mice, as can a gluten-rich diet. Finally, hyperactivity of the pancreatic beta cells has been linked to an increased T1DM risk. The detrimental effects of hyperactivity are well known in the development of T2DM, but may also extend to T1DM.

The burden of diabetes is increasing globally, particularly in developing countries (WHO, 2011). In Finland the incidence and prevalence of T1DM in children and adolescents rates among the highest in the world. Overall, the yearly incidence of childhood diabetes in Finland from the year 1987 to 1989 was an estimated 35.2 per pupil. In boys it was 38.4 making it higher than in girls at 32.6 (Simpson *et al*, 2003). According to the Diabetes Mondiale 2000, project launched by WHO in 1990 to expand the surveillance of T1DM worldwide, the incidence of diabetes is rising at an alarming rate and even faster, on average 4.1% annually, between 1992-2005. From 1997 to 2001, the incidence of T1DM in 0-14-year old Finns was 49.1 per 100,000/year, and there was a slight male predominance with a male/female ratio of 1.08. It is worth noting that several studies on childhood-onset T1DM including the age group of 15-19 show that the peak in the

incidence is during puberty between 10 and 14 years further growing into adolescence (Zimmet *et al,* 2001)

DM is an increasing problem in sub-Saharan Africa, especially Nigeria, with type 2 DM being the most common. It is becoming more prevalent owing to the increasing rates of obesity, physical inactivity, and urbanization. The Framingham study revealed that the burden of DM, which also increases the risk for CVD, has remained fairly constant over the last 50 years (Nathan *et al.,* 2005). Studies have shown that the risk of CVD mortality is higher in diabetic men compared with non-diabetic men having controlled for other CVD risk factors

Similar to other developing countries, Nigeria is experiencing a rise in the incidence of DM, as shown by an increase from 2.0% in 1992 to 2.2% in 1997. The current projected prevalence estimate of DM in Nigeria, based on the International Diabetes Federation figures, is 4.04 % (International Diabetic Federation, 2015, Musa *et al, 2014*)

DM comes along with the added cost of care disability from its complication and loss of productivity to the nation. Although data is scanty on the actual economic burden of DM in Nigeria, it can however be assumed to approximate that of countries with similar socio economic and geographical profiles.

Although there is no nationwide population-based study on the complications of DM, anecdotal hospital-based reports suggest that it is on the rise. A study in Jos, Nigeria, found 75% prevalence of peripheral neuropathy in diabetics; whereas a study in Kano, Nigeria, found 36% prevalence of diabetic retinopathy (Ugoya *et al.,* 2006, lawan and Mohammed.*2013)*

These complications include retinopathy, neuropathy, peripheral vascular disease, and diabetic foot.

The direct medical cost of treating a single diabetic patient in Cameroon was US$489in 2001(Mbanya *et al*., 2006).An IDF estimate reports the ratio of cost of care for diabetics in Nigeria (age 20-70 years) compared with non-diabetics as approximately double making it one of the countries with the most expensive costs of diabetes care in Sub-SaharanAfrica. (Mbanya *et al.,* 2006).

2.1.1.2 Type 2 diabetes mellitus (NIDDM)

Type 2, the most common form of diabetes, is a more progressive form of the disease that is typically diagnosed in adults and is characterized by an insulin secretory defect. Type 2 diabetes may account from 90-95% of all diagnosed cases of diabetes. The causes of Type 2 diabetes and the differential impact it has on certain subgroups of the population are important to examine. While the known causes of diabetes such as diet and exercise play key roles, other less examined reasons such as culture and ethnicity may play an even greater role in determining environment and thus diet and exercise of the individual. While a genetic predisposition to diabetes may increase an individuals' chances of diagnosis, individual factors, such as diet and exercise, decreases this chance. Just as diabetes differentially affects the population, there is also a variation in adherence to diabetes medication within the diabetic population (WHO, 2011).

The world prevalence of type 2 in 2010 among adults aged 20-79 years is estimated at 6.4%, affecting 285 million adults (Shaw *et al*., 2010). Between 2010 and 2030, there is an expected 70% increase in numbers of adults with diabetes in developing countries and

a 20% increase in developed countries (Shaw *et al.*, 2010). Each year more than 231,000 people in the United States and more than 3, 96 million people worldwide die from diabetes and its complications (IDF, 2009). This number is expected to increase by more than 50 percent over the next decade (WHO, 2011). In 2010, estimated global healthcare expenditures used for the treatment and prevention of diabetes and its complications was about 376 billion US Dollar (USD). By 2030, this number is projected to exceed some 490 billion USD (IDF, 2010). These costs are mainly due to treatment of concomitant CVD (Gilmer *et al*., 2005). It has been shown in several studies that a clustering of features, such as high plasma glucose obesity, dyslipidemia (high triglyceride and total cholesterol levels low, high density lipoprotein (HDL) cholesterol levels and hypertension, referred to as insulin resistance or the metabolic syndrome, is a marker of increased risk for the development of type 2 diabetes as well as for CVD (Taskinen, 2003). Environment and lifestyle factors are the main causes of the dramatic increase in type 2 diabetes (Diamond, 2003). Genetic factors probably identify those most vulnerable to these changes.

NIDDM is one of the most important non-communicable diseases in the world, with a prevalence ranging from less than 2% in rural India to as much as 30% in certain American Indian and Pacific Island populations (Zimmet, 1982). The disease generally presents in middle life and is diagnosed either as an incidental finding or following presentation with thirst, polyuria and recurrent infections. It is a cause of substantial morbidity and mortality, frequently due to atherosclerotic complications, and in certain developing countries is assuming equal importance to infectious diseases. It is widely assumed that NIDDM has a multifactorial aetiology in which the role of environmental

factors is to hasten the progression of NIDDM in a genetically predisposed individual (Zimmet, 1982).

The importance of the genetic contribution to NIDDM has been established by the study of certain inbred populations, almost 100% concordance of disease in monozygotic twins and by familial clustering. The study of inbred populations has suggested that the genetic component of NIDDM is due to a single gene inherited in an autosomal dominant fashion (Hitman *et al*, 1990). Progress in identifying specific genetic factors involved in NIDDM has been slow and no consistent evidence has emerged supporting a major aetiologcal role for any of the genes so far studied. This may be due in part to methodological problems encountered in the identification of such disease susceptibility genes. Less well known is the mode of inheritance of NIDDM genes, how many genes may contribute to the disease, the way in which they may interact, and none of the genes that contribute to NIDDM has been identified. This lack of knowledge is largely attributable to the nature of NIDDM. Family studies suggest a strong genetic component in the aetiology of NIDDM, with evidence for a major gene of co-dominant effect. A gene-dosage effect, whereby diabetes develops earlier in people with two susceptibility genes than in those with one susceptibility gene is likely (Serjeantson et *al*, 1992). The search for the diabetes gene has led to the cloning and characterization of many genes involved in controlling glucose homeostasis. These include the insulin, insulin receptor, glucose transporter, amylin and glucokinase genes. Molecular techniques have permitted rapid screening of these genes in NIDDM patients and controls. Finally, there is now a rather contradictory genetic literature for NIDDM, with weak disease associations reported and refuted for most candidate genes (Serjeantson *et al.*, 1992). Many longitudinal studies have reported

that increased BMI is a strong risk factor for type 2 diabetes (Almdal *et al.,* 2008). A strong positive association between obesity and type 2 diabetes is found both in men (Knowler *et al.,* 1991), and women (Haffner *et al.,* 1997). Obesity is associated within creased risk of developing insulin resistance and type 2 diabetes. In obese individuals adipose tissue releases increased the amount of non-esterified fatty acids, glycerol, hormones, pro-inflammatory cytokines and other factors involved in the development of insulin resistance. When insulin resistance is accompanied by dysfunction of the beta cells, the following fall in insulin secretion results in failure to control blood glucose level leading to type 2 diabetes. Many genes interact with the environment leading to obesity and in some also to diabetes. Many genes have been shown to be involved in determining the whole range of BMI in a population, with each gene only explaining a few hundred grams difference in body weight (Hebebrand and Hinney, 2009). Genes responsible for obesity and insulin resistance interact with environmental factors such as increased fat/ calorie intake and decreased physical activity resulting in the development of obesity and insulin resistance followed ultimately by the development of type 2 diabetes (Kahn *et al.,* 2006).

Unfavourable blood lipids have been reported as a risk factor for type 2 diabetes by several prospective studies (Meisinger *et al*., 2002). An inverse relationship between HDL cholesterol and risk of type 2 diabetes has been documented in several studies (Meisinger *et al*., 2002). Some prospective studies found low HDL cholesterol to be a stronger risk factor for type 2diabetes in women only (Njolstad *et al.,* 1998). Only one previous study measuring non-fasting triglycerides found an independent risk of type 2 diabetes connected to elevated triglyceride levels (Almdal *et al.,* 2008). High plasma

triglycerides and low plasma HDL cholesterol levels are both seen in the insulin resistance syndrome, which is a prediabetic state (Taskinen, 2003), suggesting that non fasting triglycerides and HDL cholesterol levels reflect the degree of insulin resistance. The mechanisms suggested are increased circulating levels of free fatty acids due to increased insulin levels and increased chylomicron-assembly and secretion in the gut, the latter process being a result of localized insulin resistance in the intestine. Cross sectional studies have shown that high BMI is associated with a higher level of total cholesterol and unfavourable lipids pattern, with low concentrations of HDL cholesterol and high triglycerides concentrations. Longitudinal studies have shown BMI change over time to be positively associated with changes in total cholesterol, triglycerides, and low density lipoprotein (LDL) cholesterol and negatively associated with HDL cholesterol change (Czernichow,2002). Apart from triglycerides, all these lipids have been shown to convey diabetes risk independently of BMI, but how they interact have been little studied.

2.1.1.3 Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance with first recognition during pregnancy (Kun, 2006). The definition applies irrespective of whether insulin or dietary only treatment is utilized and whether the condition persists after pregnancy. It does not exclude the possibility that unrecognized glucose intolerance may have antedated or begun concomitantly with the pregnancy. Most often this abnormal glucose tolerance returns to normal postpartum. Reclassification of maternal glycemic status should be performed at least 6 weeks after delivery. The prevalence of GDM ranges from 1 to 14 % in all pregnancies, however it depends on the diagnostic criteria used and the ethnic background of the population being tested. Previous reports

from Hungary reported that the frequency of GDM was between 1-3%. More recent reports from many countries however detected higher frequencies up to 7.7%. The glucose intolerance of GDM is usually mild but perinatal mortality associated with this complication is 4 to 5 times increased compared to the general pregnant population. Macrosomia, intrauterine growth retriction, neonatal cardiomymiopathies, hypoglycaemia, jaundice, polycythemia, neonatal respiratory distress syndrome (NRDS) and hypocalcaemia may also affect newborns of GDM mothers. Offsprings of women with GDM are at increased risk of obesity, glucose intolerance, and diabetes in late adolescence and young adulthood (Kun, 2006). GDM is associated with an increased frequency of maternal hypertensive disorders and need for caesarean delivery. Moreover, GDM also considerably increases the woman's risk of developing manifest diabetes later in life. Thus, it is important to recognize and treat this disease. Obesity and other factors that promote insulin resistance appear to enhance the risk of type 2 diabetes after GDM, while markers of islet cell-directed autoimmunity are associated with an increase in the risk of type 1 diabetes.

The mechanisms involved in the development of this temporary diabetic state are still partly unknown. Among the possible explanations are reduced insulin secretion, increase insulin degradation, increased secretion of hormones with anti-insulin effect (human placental lactogen (HPL), human chorionic gonadotrophin (HCG) prolactin(PRL) Schwangerschafts-protein 1, beta-1- glikoprotein (SP 1), estrogen, progesterone, cortisol and adrenocorticotropic hormone (ACTH)), reduced tissue sensitivity to insulin, or a combination of two or more of these mechanisms (Kun, 2011). As glucose tolerance in pregnancy decreases in parallel with increasing levels of pregnancy-related hormones and

of cortisol (until 37 week of gestation), it has been suggested that one or more of these hormones might be implicated in bringing about alterations in carbohydrate metabolism. Recently, the role of tumor necrosis factor (TNF) alfa and leptin has been suggested as they have numerous effects on carbohydrate metabolism. Pregnancy is a state of insulin resistance. In late pregnancy fasting serum insulin concentration is almost doubled compared to postpartum levels, both in normal pregnant and in gestational diabetic women. However, the insulin response to oral glucose or a mixed meal is significantly greater in normal pregnant women than in the gestational diabetic subjects. GDM women compared to normal pregnant controls have relative insulin-deficiency. An increased insulin demand of normal pregnancy is assured by the increase in the number and the size of islets of Langerhans (Kun, 2011).

If insulin production in unable to compensate for the insulin resistance (IR) of pregnancy, GDM will develop. Most GDM cases develop between the 24 and 28 weeks of gestation. This is the period when in the absence other risk factors screening of GDM should be performed in all pregnant patients by a 75-gram OGTT (oral glucose tolerance test). Undiagnosed and untreated patients with GDM as well as their fetuses are at a higher risk of pregestional diabetes. Obstetric and perinatal outcomes in newborns of gestational diabetic women are related to metabolic control and fetal surveillance during pregnancy. If gestational diabetic subjects receive optimal care during gestation, the risk of maternal complications and perinatal morbidity is similar to those observed in normal pregnancies . Since GDM has (Kun and Tornoczky, 2010) no signs or symptoms, it can only be recognized by screening. GDM is screened by a number of different methods. Screening for GDM is universally recommended despite the lack of consensus about the optimal

screening method. The American Diabetes Association (ADA) recommends a two-step approach: All pregnant women should be screened for GDM between 24 to 28 weeks of gestation using a 50-gram 1-hour oral glucose challenge test (GCT) with a threshold for further testing of 140 mg/dl (7.8mmol/l) or higher. The diagnosis of GDM is based on the 100-gram OGTT. Two or more of the venous plasma glucose concentrations must be met or exceeded the values below for the diagnosis: fasting: 5.3 mmol/l, 1-h: 10.0 mmol/l, 2-h: 8.6 mmol/l, 3-h: 7.8 mmol/l. There is a controversy regarding the screening method for GDM. There are rational arguments both for a universal screening and for a selective screening within high risk groups. Selective screening for GDM is recommended according to the ADA (Kun and Tornoczky, 2010)

The prevalence of elevated blood pressure is doubled among overweight patients compared to normal-weight subjects. Furthermore, the observations suggest that the association between hypertension and insulin resistance (IR) is independent of obesity: relative IR was found not only in obese but also in lean hypertensive subjects compared to normotensive ones. Physiological changes during pregnancy lead to a decrease in insulin-sensitivity. As gestation advances, a progressive increase in insulin production to glucose can be found in the maternal pancreas. It has been shown that obesity plays a role in the development of IR in pregnancy. Patients with GDM are more insulin-resistant compared to control pregnant subjects with normal glucose tolerance. GH has been associated with hyperinsulinemia. No similar associations were reported regarding the relationship between PE and IR either in normoglycemic pregnancies or in GDM. PE is associated with a failure of trophoblast invasion to the spiral arteries of the placenta and it may lead to impaired uteroplacental perfusion and to the release of several vasoactive

factors into the maternal circulation that finally causes endothelial dysfunction, vasoconstriction, and hypertension (Kerenyi *et al.,* 2009).

2.1.2 Diagnosis of diabetes mellitus

Classical symptoms, fasting plasma glucose, elevated postprandial or post glucose values and oral glucose tolerance test (OGTT) are the four diagnostic areas that will be considered here. Criteria for the diagnosis of diabetes mellitus have been developed by both the NDDG and the World Health Organisation based on data from epidemiologic surveys in different parts of the world. These diagnostic standards provide a basis for uniformity in research and data collection. Further, the plasma glucose concentrations now considered diagnostic of diabetes provide better prediction for subsequent development of the specific complications of diabetes than those in the previously recommended criteria (Hundal *et al*, 2000).

In clinical practice, suspicion of diabetes is gleaned from history and physical findings. Symptoms such as fatigue, thirst, polyuria, weight loss, and recurrent infection are frequent clues. A family history of diabetes, obesity, unfavourable obstetrical experiences, premature atherosclerosis, neuropathic disorders are indicators of probable diabetes mellitus. Urine glucose testing is frequently performed as a screening test, but is not acceptable for diagnostic purposes. A positive urine test can be a helpful indicator, but may give misleading results during pregnancy because of increased renal blood flow or in the presence of renal glycosuria. Patients with high renal glucose thresholds or elderly patients with decreased renal blood flow may not have glycosuria in spite of elevated blood glucose concentrations (Hundal *et al*, 2000).

In normal subjects, the upper normal limit of fasting plasma glucose is 6.4mmol/L (115 mg/dl). Diabetes can be diagnosed reliably when fasting plasma glucose concentrations are 7.8 mmol/L (140 mg/dl). Immediate confirmation of the diagnosis can be obtained by determining the glycated haemoglobin concentrations simultaneously. If the glycated haemoglobin concentration is greater than normal, the diagnosis is confirmed by an elevated ambient glucose concentration preceding the fasting plasma glucose determination. Otherwise, second diagnostic plasma/blood glucose is required on a second occasion in a symptomatic subjects. With few exceptions, fasting plasma glucose values correlate well with the rise in plasma glucose concentrations observed after a meal or glucose load, although it is less sensitive and less specific for the diagnosis of diabetes than the post-glucose load glucose (Hundal *et al*, 2000).

A two-hour postprandial plasma glucose concentration of 1 mmol/L (200 mg/dl) or 10 mmol/l (180 mg/dl) for whole blood may be indicative of diabetes if certain precautions have been observed. Because glucose utilization is impaired in persons consuming low-carbohydrate or weight-reduction diets, the postprandial glucose test should be performed in those consuming unrestricted diets providing greater than 150 g of carbohydrate daily for at least 3 days. Additional precautionary measures include eliminating drugs that reduce glucose tolerance, having individuals maintain normal physical activity, and avoiding the test in ill or stressed persons. The test is most reliably performed using a standard oral glucose load. In patients with liver or kidney disease, partial gastrectomy, orthyrotoxicosis, elevated postprandial plasma glucose may also be observed. Diabetes can also be found in subjects with normal fasting values, although this is unusual in clinical practice (Harris *et al.,* 1985)

The oral glucose tolerance test (OGTT) is the most sensitive test for the diagnosis of diabetes. Without scrupulous care in standardizing the test and preparing the patient, however, the OGTT can be misleading since some factors can interfere with normal glucose tolerance and result in a hyperglycemic curve. This test is not required for diagnostic purposes when the fasting plasma glucose is unequivocally elevated. The OGTT as adopted by the WHO requires two plasma glucose values; fasting and 2-h after a 75 g glucose load. The intervening samples at ½, 1, and 1½ hours are not used. The scheme provides a simple and accurate method for obtaining diagnosis criteria. There is insufficient evidence from the available studies to justify the use of the more complicated NDDG criteria. Both sets of diagnostic criteria specify that a fasting plasma glucose 7.8 mmol/l (140 mg/dl) and / or a 2-h of 11.1 mmol/l [200 mg/dl] are diagnostic of diabetes mellitus in the non pregnant adult (Harris *et al.*, 1985). If only the 2-h value is elevated, then a second test is required for confirmation. The latter value represents the concentration observed in the bimodal distribution of two-hour plasma glucose in epidemiological studies, and is the glucose concentration at which characteristic micro vascular lesions such as diabetic retinopathy appeared in such studies (Harris *et al.*, 1985)

2.2 Oxidative Stress

Oxidative stress is defined as a "state in which oxidation exceeds the antioxidant systems in the body secondary to a loss of the balance between them (Halliwell, 2007). It not only causes hazardous events such as lipid peroxidation and oxidative DNA damage, but also physiologic adaptation phenomena and regulation of intracellular signal transduction. From a standpoint, if biomarkers that reflect the extent of oxidative stress were available, such markers would be useful for physicians to gain an insight into the

pathological features of various diseases and assess the efficacy of drugs. Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage (Halliwell, 2007). Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA (Valko *et al.*, 2007). Further, some reactive oxidative species act as cellular messengers in redox signaling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signaling. In humans, oxidative stress is thought to be involved in the development of cancer, Parkinson's disease, Alzheimer's disease, atherosclerosis, heart failure, myocardial infarction, fragile X syndrome, Sickle Cell Disease, lichen planus, vitiligo, autism infection (Pohanka, 2013) and chronic fatigue syndrome (Gwen *et al*., 2005). However, reactive oxygen species can be beneficial, as they are used by the immune system as a way to attack and kill pathogens. Short-term oxidative stress may also be important in prevention of aging by induction of a process named mitohormesis (Gems and Partridge, 2008).

Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses, such as glutathione (Schafer and Buettner, 2001).The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis (Lennon *et al*., 1991). Production of reactive oxygen species is a particularly destructive aspect of

oxidative stress. Such species include free radicals and peroxides .Some of the less reactive of these species (such as superoxide) can be converted by oxidoreduction reactions with transition metals or other redox cycling compounds (including quinones) into more aggressive radical species that can cause extensive cellular damage (Valko *et al*., 2005). DNA damage induced by ionizing radiation is similar to oxidative stress, and these lesions have been implicated in aging and cancer. Biological effects of single-base damage by radiation or oxidation, such as 8-oxoguanine and thymine glycol, have been studied. Tandem DNA lesions are formed at substantial frequency by ionizing radiation and metal-catalyzed Hydrogen peroxide reactions. Under anoxic conditions, the predominant double-base lesion is a species in which C8 of guanine is linked to the 5-methyl group of an adjacent 3'-thymine (Collis *et al*, 2008). Most of these oxygen-derived species are produced at a low level by normal aerobic metabolism. Normal cellular defense mechanisms destroy most of these. Likewise, any damage to cells is constantly repaired. However, under the severe levels of oxidative stress that cause necrosis, the damage causes ATP depletion, preventing controlled apoptotic death and causing the cell to simply fall apart (Lelli *et al*, 1998).

Oxygen is essential for energy metabolism and respiration but it has been implicated in many diseases and degenerative conditions (Ceconi *et al.,* 2003). Activation of oxygen may occur by two different mechanisms: absorption of sufficient energy to reverse the spin on one of the unpaired electrons and monovalent reduction. Non activated oxygen is a bi-radical. It can be activated by either reversing the spin on one of the unpaired electrons to form the singlet state or by reduction. In the monovalent reduction of oxygen, superoxide (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and finally, water

(H_2O) is formed. Superoxide forms the hydroxyl radical (OOH^-) which is a powerful oxidant unit's protonated form (Gebicki and Bielski, 1981; Ceconi *et al.,* 2003). Numerous enzymes (peroxidases) use hydrogen peroxide as a substrate in oxidation reactions involving the synthesis of complex organic molecules. Haber and Weiss (1994) identified the hydroxyl radical as the oxidizing species in the reaction between H_2O_2 and ferrous salts: $Fe^{2+} + H_2O_2 = Fe^{3+} + OH + OH$. Most oxygen is consumed by the cytochrome oxidase enzyme in the mitochondrial electron transport system. Isolated mitochondria produce H_2O_2 and O_2^- in the presence of NADH (Loschen *et al.,* 1974). The various Fe-S-protein and NADH dehydrogenase have also been implicated as possible sites of super-oxide and hydrogen peroxide formation (Waterfall *et al.,* 1997). Various oxidative processes including oxidation hydroxylations, dealkylations, deaminations, dehalogenation and desaturation occur on the smooth endoplasmic reticulum. Mixed function oxygenases that contain a heme moiety add an oxygen atom into an organic substrate using NADPH as the electron donor. The generalized reaction catalyzed by cytochrome P450 is: $RH + NADPH + H^+ + O_2$ $ROH + NADP^+ + H_3O$. Superoxide is produced by microsomal NADPH dependent electron transport involving cytochrome P450 (Valko *et al.,* 2007).

Compartmentalized enzymes involved in the B-oxidation of fatty acids and glyoxylic acid cycle includes glycolate oxidase, catalase and various peroxidases. Glycolate oxidase produces H_2O_2 in a two-electron transfer from glycolate to oxygen. Xanthine oxidase, urate oxidase and NADH oxidase generate superoxide as a consequence of the oxidation of their substrates. The xanthine oxidase reaction is often used *in vitro* as a source of superoxide producing one mole of superoxide during the conversion of

xanthine to uric acid (Fridorich, 1970). A superoxide generating NADPH oxidase activity has been clearly identified in plasmallema-enriched fractions (Valko *et al.,* 2007). These flavopoteins may produce superoxide by the redox cycling of certain quinones or nitrogenous compounds, while NADPH oxidase reduces Fe^{3+} to Fe^{2+} converting it to a form that can be transported. Dysfunction of NADPH oxidase results in the formation of superoxide radical (Maxwell and Lip, 1997).

2.2.1 Oxidative stress and diabetes mellitus

Oxidative stress plays a pivotal role in cellular injury from hyperglycemia. High glucose level can stimulate free radical production. Weak defense system of the body becomes unable to counteract the enhanced ROS generation and as a result, a condition of imbalance between ROS and their protection occurs which leads to domination of the condition of oxidative stress (Halliwell and Guttaridge, 2007). A certain amount of oxidative stress/ROS is necessary for the normal metabolic processes since ROS play various regulatory roles in cells (Gomes *et al*., 2015). ROS are produced by neutrophils and macrophages during the process of respiratory burst in order to eliminate antigens (Freitas *et al*., 2010). They also serve as stimulating signals of several genes which encode transcription factors, differentiation, and development as well as stimulating cell-cell adhesion, cell signaling, involvement in vasoregulation, fibroblast proliferation, and increased expression of antioxidant enzymes (Thannickal and Fanbury, 2002). However over and/or uncontrolled production of ROS is deleterious. Due to oxidative stress the metabolic abnormalities of diabetes cause mitochondrial superoxide overproduction in endothelial cells of both large and small vessels, as well as in the myocardium (Giacco and Browlee, 2010). Oxidative stress acts as mediator of insulin resistance and its

progression to glucose intolerance and installation of diabetes mellitus, subsequently favouring the appearance of atherosclerotic complications, and contributes to rise in many micro- and macrovascular complications (Negre-Salvayre *et al*., 2009). Hyperglycaemia causes tissue damage through multiple mechanisms including increased flux of glucose and other sugars through the polyol pathway, increased intracellular formation of advanced glycation end products (AGEs), increased expression of the receptor for AGEs and its activating ligands, activation of protein kinase C isoforms, and over activity of the hexosamine pathway (Brownlee, 2005). Atherosclerosis and cardiomyopathy in type 2 diabetes are caused in part by pathway-selective insulin resistance, which increases mitochondrial ROS production from free fatty acids and by inactivation of anti atherosclerosis enzymes by ROS. Diabetics differ significantly in their sensitivity to ROS. Inflammatory damage that characterizes type 1 diabetes is mediated at least in part through islet ROS, and in type 2 diabetes, the high nutrient flux and consequent ROS production appear to mediate loss of β-cell function. In insulin-sensitive tissues including muscle, liver, and heart, high fatty-acid flux leads to oxidative damage, whereas noninsulin-sensitive tissues including the eye, kidney, and nervous system are exposed to both high circulating glucose and fatty acid levels and, consequently, ROS-induced diabetic complications (Sivitz and Yorek,2010).

2.2.2 Lipid peroxidation

Lipids are reported as one of the primary targets of ROS. Hydroperoxides have toxic effects on cells both directly and through degradation to highly toxic hydroxyl radicals. They may also react with transition metals like iron or copper to form stable aldehydes, such as malondialdehyde (MDA), that damage cell membranes (Halliwell and Chirico,

1993). Peroxidation of lipids produces highly reactive aldehydes, including MDA, acrolein, 4-hydroxynonenal (HNE), 4-oxononenal (ONE) and isolevuglandins (IsoLGs) (Guo *et al*., 2012). It has been reported that peroxyl radicals can remove hydrogen from lipids, producing hydroperoxides that further propagate the free-radical pathway. MDA has been documented as a primary biomarker of free radical mediated lipid damage and oxidative stress .Significant changes in lipid metabolism and structure have been reported in diabetes, particularly in patients with vascular complications (Fowler, 2008). Increased level of MDA in diabetics suggests that peroxidative injury may be involved in the development of diabetic complications.The increase in lipid peroxidation is also an indication of decline in defence mechanisms of enzymatic and nonenzymatic antioxidants (Saddala *et al*., 2013). Oxidized lipids are able to produce MDA as a decomposition product and the mechanism is thought to involve formation of prostaglandins, like endoperoxides, from polyunsaturated fatty acid (PUFA) with two or more double bonds (Pandey and Rizvi, 2011). Increased MDA level in plasma, serum and many others tissues has been reported in diabetic patients (Moussa, 2008). Baynes 1991, reported that lipid peroxidation in diabetes induced many secondary chronic complications including atherosclerosis and neural disorders. It was observed that there is greater serum lipid peroxidation evaluated in terms of MDA in hyperglycemic mice and proposed that the increase in lipid peroxidation exacerbated the occurrence of myocardial infarction through NADPH oxidase activation. Lipid peroxidation of cellular structures is thought to play a key role in atherosclerosis. Significantly higher values of thiobarbituric acid-reactive substances (TBARS) in the red blood cells as well as in serum and decreased erythrocyte antioxidant enzyme activities have been reported in diabetic condition (Singh

and Shin, 2009). Increased lipid peroxidation presents a close relationship with the high glycemic levels and oxidative stress in diabetes mellitus. Recently, a clinical study performed by Bandeira *et al*., (2013) aimed at characterizing blood oxidative stress in diabetic patients reported a significant higher lipid peroxidation which showed a close relationship with high glucose levels as observed by the fasting glucose and HbA1c levels.

2.2.3 Protein oxidation

Proteins are the important vital biomolecules of the cell. They are involved in many physiological functions including cell signaling and transport across the cells. Proteins are another potential target of ROS, whose structure and function can be affected by modification. There are many side chain targets for protein oxidation including cysteine, methionine, and tyrosine. Carbonyls are the oxidation product of proteins and are reported as the potent biomarker of oxidative stress (Suzuki and Miyata, 1999). They represent the stable end product generated upon formation of transient radical species, such as chloramines and nitrogen/carbon radical, which are induced by oxidant stimuli. Glycation has been reported to induce the formation of protein carbonyls, such as ketoamine derivatives, thus generating reactive radicals and perpetuating a vicious cycle (Levine *et al*., 1990). Increased protein carbonyl content has been reported in different cells and plasma of the diabetic patients. Damage of proteins followed by accumulation of their oxidation products affects cellular physiology adversely (Suzuki and Miyata, 1999). Increased glycol- and lipooxidation are reported as one of the major factors in the accumulation of nonfunctional damaged proteins (Sakul *et al*., 2013). Gradinaru *et al*., (2013) reported the significance of the oxidative and glycoxidative protein damage in

subjects with prediabetes and type 2 diabetes mellitus. AGEs, low density lipoprotein susceptibility to oxidation (oxLDL) and nitric oxide metabolic pathway products (NOx), are documented as important biomarkers for evaluating the association between diabetes and protein status in diabetic patients (Gradinaru *et al*., 2013). AGEs are formed through nonenzymatic amino-carbonyl interactions between reducing sugars or oxidised lipids and proteins, amino phospholipids, or nucleic acids (Monnier, 2003). The generation of AGEs may lead to intracellular modifications of proteins, including those involved in the regulation of gene expression (Barrbosa *et al*., 2008). Many studies on animals as well as on humans have frequently reported the relationship between hyperglycaemia, oxidative stress, and formation of AGEs. AGEs are capable of modifying the circulating proteins in the blood that have receptors for AGEs. Activating them is followed by inducing the production of inflammatory cytokines and growth factors in endothelial cells (Bandeira *et al*., 2013). Advanced oxidation protein products (AOPPs) are the recently investigated marker of protein oxidation during oxidative stress which represents the overall status of the protein in the cell/tissue (Pandey and Rizvi, 2010). In chronic oxidative stress, AOPPs are formed by reactions between plasma proteins and chlorinated oxidants. Their increased levels are reported during type 2 diabetes. Significant positive association between plasma levels of AOPPs and TBARS during diabetes indicates that proteins are equally targeted by ROS as the lipids (Savini *et al.*, 2013). Oxidation of proteins in diabetics affects many physiological functions (Piwowar, 2010). Increased protein carbonyls as well as AOPPs level in diabetic patients underlie the importance of the protein conformational changes in the pathogenesis of diabetic nephropathy. AOPPs known as proinflammatory and prooxidative compounds that accumulate in aging

patients with diabetes may play a major role in increasing prevalence of endothelial dysfunction and subsequent cardiovascular diseases. AOPPs contain abundant dityrosines which allow crosslinking, disulfide bridges, and carbonyl groups and are mainly formed by chlorinated oxidants, hypochloric acid and chloramines resulting from myeloperoxidase activity (Piwowar, 2010). Several studies have pointed out that AOPPs and oxidative stress markers increase in adult subjects with type 2 diabetes with and without micro-/macrovascular complications (Gradinaru *et al*., 2013).

2.3 Antioxidants

Antioxidant is a molecule that inhibits the oxidation of other molecules. It terminates chain reactions by removing free radical intermediates, and inhibits other oxidation reactions. Antioxidant defense mechanisms involve both enzymatic and non enzymatic strategies. Common antioxidants include the vitamins A, C, and E, glutathione, and the enzymes superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. Other antioxidants include lipoic acid, mixed carotenoids, coenzyme Q10, several bioflavonoid, antioxidant minerals (copper, zinc, manganese, and selenium), and the cofactors (Folic acid, Vitamins B1, B2, B6, B12). They work in synergy with each other and against different types of free radicals. Vitamin E suppresses the propagation of lipid peroxidation; vitamin C, with vitamin E, inhibits hydro peroxide formation; metal complexing agents, such as penicillamine, bind transition metals involved in some reactions in lipid peroxidation (Feher *et al*., 1987) and inhibit Fenton and Haber-Weiss-type reactions; vitamins A and E scavenge free radicals (Baynes,1991). Extensive studies of pharmacological interventions based on biological antioxidants have been carried out (Oberley, 1988). Discrepancies in observed biomarkers for oxidative stress continue to be

seen in the many review, especially in the activities of SOD, catalase, and glutathione peroxidase in experimentally diabetic animals. Decreased levels of glutathione and elevated concentrations of thiobarbituric acid reactants are consistently observed in diabetes. In addition, changes in nitric oxide and glycated proteins are also seen in diabetes.

2.3.1 Superoxide dismutase (SOD)

SOD is the antioxidant enzyme that catalyses the dismutation of superoxide anion (O2−) into hydrogen peroxide and molecular oxygen (Faraci and Didion, 2004). SOD plays important protective roles against cellular and histological damages that are produced by ROS. It facilitates the conversion of superoxide radicals into hydrogen peroxide, and in the presence of other enzymes is converted into oxygen and water. All mammalian tissues contain three forms of SOD: Cu-Zn-SOD, Mn-SOD, and extracellularEC-SOD, and each of them is a product of a distinct gene. Cu-Zn-SOD or SOD 1 (EC 1.15.1.1) is localized in cytosol, Mn-SOD or SOD 2 (EC 1.15.1.1) in mitochondria and EC-SOD or SOD 3 (EC 1.15.1.1) in extracellular space (Oury *et al*., 1994). Superoxide reacts rapidly with nitric oxide (NO), reducing NO bioactivity and producing the oxidative peroxynitrite radical (Guzik *et al*., 2002). SOD, a major defender against superoxide, in the kidneys during the development of murine diabetic nephropathy and down regulation of renal SOD (SOD 1 and SOD 3) may play a key role in the pathogenesis of diabetic nephropathy (Fujita *et al*., 2009). Over expression of SOD or the supplements of antioxidants including SOD mimetics, targeted to overcome oxidative stress, reduce ROS, and increase antioxidant enzymes, has been shown to prevent diabetes mellitus (Wang *et al*, 2011).

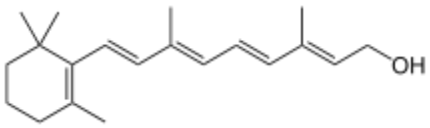
EC-SOD is found in the extracellular matrix of various tissues including pancreas, skeletal muscle, and blood vessels, and is the major extracellular scavenger of superoxide radicals. The higher level of EC-SOD resulted in a 6-fold increase in the total superoxide dismutase activity of the islets; therefore, superoxide radicals secreted to the extracellular space does not contribute to the β-cell destruction (Sandstrom *et al*., 2002). The elevated level of SOD is shown to reduce oxidative stress; decrease mitochondrial release of cytochrome C and apoptosis in neurons; and, in mice, prevent diabetes-induced glomerular injury, thus suggesting a major role of SOD in the regulation of apoptosis (Kowluru *et al*., 2006). Decline in the level of SOD in diabetic tissue and blood has been reported in many studies (Giugliano *et al*, 1995). Recently Kim (2013) reported that diabetic skin tissues express a relatively small amount of extracellular protein and concluded that extracellular SOD is related to the altered metabolic state in diabetic skin, which elevates ROS production. Study performed by Lucchesi *et al*., (2013) to observe the oxidative balance of diabetic rats reported diminished activity of SOD and other anti oxidative enzymes in the liver tissue.

2.3.2 Antioxidant vitamins

Vitamins A, C, and E are diet-derived and detoxify free radicals directly. They also interact in recycling processes to generate reduced forms of the vitamins. Tocopherol is reconstituted when ascorbic acid recycles the tocopherol radical; dihydroascorbic acid, which is generated, is recycled by glutathione. These vitamins also foster toxicity by producing prooxidants under some conditions. Vitamin E, a component of the total peroxyl radical-trapping antioxidant system (Weber *et al*., 1997), reacts directly with peroxyl and superoxide radicals and singlet oxygen and protects membranes from lipid

peroxidation. The deficiency of vitamin E is concurrent with increased peroxides and aldehydes in many tissues. There have been conflicting reports about vitamin E levels in diabetic animals and human subjects. Plasma and/or tissue levels of vitamin E are reported to be unaltered (Martinoli *et al*., 1993), increased (Asamaya *et al*., 1994), or decreased (Garg *et al*., 1996) by diabetes. Discrepancies among studies in terms of preventive or deleterious effects of vitamin E on diabetes induced vascular aberrations may arise from the variety of examined blood vessels or the administered dose of vitamin E.

2.3.3.1 Vitamin A



Chemical structure of retinol, one of the major forms of vitamin A

Description

Vitamin A is a general term that refers to fat-soluble compounds that are similar in structure and biologic activity to retinol. Vitamin A also refers to dietary precursors of vitamin A (Child *et al.,* 1999). The precursors of vitamin A (retinol) are the carotenoids (most commonly beta-carotene). The term retinoid refers to any compound that is structurally similar to retinal (aldehyde), retinol (alcohol), or any other substance that exhibits vitamin A activity. Retinoic acid, which is a metabolite of retinol (Child *et al.,* 1999), is such a substance that is often studied. Synthetic compounds within the vitamin A family have similar structures as the natural form, but may have few or no functions that the natural vitamin posses (Gleesson *et al*,1987). Most compounds within the vitamin A family are soluble in fat and essential to numerous processes within the body. There

have been several water-soluble retinoids, extracted from plasma, bile, and other tissue (Gleesson *et al,* 1987). For the purposes of this literature review, any discussion of vitamin A will focus on those retinoids with fat-soluble properties. The main discussion will involve retinol. Retinol is chemically a "pale yellow crystalline solid (Child *et al.*, 1999). The solid and its metabolites exist in nature as various isomers. The biologic metabolites of retinol are unique in that, they contain five conjugated double bonds within their six-carbon ring (B-ionone) and isoform specific side chains (Gleesson *et al*, 1987). The double bonds contribute special properties; for example, the double bonding in retinol plays a unique role in multiple vision processes, which will be discussed in a subsequent section of this review. As mentioned earlier, most retinoids are soluble in organic solvents and fat. However, oxidation and polymerization are all detrimental to retinoids; therefore, the compounds must be protected from light, oxygen and high temperature.

Dietary Function

Vitamin A is essential for numerous intrinsic processes. The most well-known and understood process is that of vision. The 11-cis retinal form of vitamin A is essential for the neural transmission of light into vision (Gleesson *et al*, 1987). Epithelial cells are highly dependent on retinoic acid and are commonly used to treat a variety of skin diseases. A developing fetus is also highly dependent on retinoic acid, as it is essential to the growth of the eyes, lungs, ears and heart (Child *et al.,* 1999). The retinoids are not only the most active form of vitamin A, but also a current area of interest to many scientists. The role of vitamin A as an antioxidant is debatable. Vitamin A has been shown to possibly have some antioxidant characteristics. However, the carotenoids such

as beta-carotene have in recent years received more attention from the scientific community because of the harmful role they may play as pro-oxidants (Haymes, 1991). A great deal more research is needed that addresses the role of vitamin A as an antioxidant to determine the exact role the vitamin and precursors play.

Sources

Retinol, the active form of vitamin A, is rarely found in foods. Instead, precursors to retinol, fatty acid retinyl esters, are found in the human diet. The esters are commonly found in foods of animal origin, such as egg yolks, liver, fish oil, whole milk and butter (Child *et al.,* 1999). Plants can synthesize the carotenoids, but cannot convert them to retinoids; this process occurs in the human body (Gleesson *et al*, 1987). The carotenoids are red, yellow, and orange in color and substantial in number (over 400 types). It is estimated that only 10% of the pigments have "vitamin A activity", with beta-carotene having the greatest activity, followed by the alpha and gamma forms (Child *et al.,* 1999). Fruits and vegetables that appear bright orange or yellow in color are usually high in carotenoids. All green vegetables also contain substantial amounts of carotenoids, but the orange or yellow color is masked by chlorophyll (Child *et al.,* 1999). The wide variety of vitamin A precursors allow for adequate amounts of the vitamin in all diet types.

Metabolism

Absorption and Bioavailability

Seventy to ninety percent of vitamin A from the diet is absorbed in the intestine. The efficiency of absorption for vitamin A continues to be high (60-80%) as intake continues to increase. Greater than 90% of the retinol store within the body enters as retinyl esters that are subsequently found within the lipid portion of the chylomicron (Gleesson *et al*,

1987). Absorption of vitamin A is very rapid, with maximum absorption occurring two to six hours after digestion (Gleesson *et al,* 1987). Within the intestinal lumen, the vitamin is incorporated into a micelle and absorbed across the brush border into the enterocytes. Within the enterocyte, precursors of vitamin A (carotenoids) are converted to active forms of the vitamin. The newly formed products and additional precursors are then packaged into chylomicrons and readied for transport throughout the body (Child *et al.,* 1999).

TRANSPORT

After leaving the enterocytes, chylomicrons which carry retinyl esters, carotenoids, and unesterfired retinol along with triglycerides, are circulated first through the lymphatic system and then through the general circulation. Upon arriving at extra-hepatic cells, chylomicrons release triglycerides; however, vitamin A remains within the chylomicron. The vitamin A is then incorporated into a chylomicron remnant (Child *et al.,* 1999). The chylomicron remnant then travels back to the liver, where it is taken up and further metabolized or stored. When needed, retinol is mobilized from the liver and requires the use of a carrier for transport through the blood. Retinol-binding protein (RBP) is the specific carrier used to transport all-trans retinol in the plasma. The all-trans isoform accounts for more than 90% of all plasma vitamins A. This specific carrier is manufactured and secreted by the parenchymal cells of the liver (Child *et al.,* 1999). Each mole of retinol released binds equivocally with RBP to form holo-RBP. This compound then binds with a molecule of transthyretin (TTR), formerly known as prealbumin. This newly formed retinol-RBP-TTR complex is not filtered by the glomerulus, but instead, freely circulates throughout the plasma. Tissues are then able to take the retinol up as needed via cellular retinoid-binding protein (11). Retinoic acid is

believed to be manufactured by the cells as needed. Therefore, transport of retinoic acid is likely not substantial. Instead, the cell possesses intra-cellular proteins that regulate the amount of retinoic acid produced. The proteins also help to determine the intracellular usage of retinoic acid (Child *et al.*, 1999).

Storage

Approximately, 50 to 85% of the total body retinol are stored in the liver when vitamin A status is adequate (Gleesson *et al*, 1987). Retinol returning to the liver is re-esterfied before storage. Because of this, over 90% of the retinol is stored in the form of retinyl esters. The retinol is stored in hepatic stellate (star-shaped) cells along with droplets of lipid (Gleesson *et al*, 1987). This constitutes the fat-soluble property of vitamin A. The size of stellate cells increases linearly with increasing retinol levels. Once hepatic stellate cells are saturated with all the retinol they can hold, hypervitaminosis can result. (Gleesson *et al*, 1987). The precursor to vitamin A, beta-carotene, can be stored in adipose cells of fat depots throughout the body. To date, the only side effect of excess beta-carotene supplementation appears to be yellowing of the skin. Serum levels of beta-carotene are an indicator of recent intake and not body stores (Child *et al.*, 1999).

Excretion

The kidneys are the main paths of RBP and retinol excretion from the body. This is achieved mainly via renal catabolism and glomerular filtration (Gleesson *et al*, 1987). Those persons suffering from renal disease often experience elevated serum levels of RBP and retinol and therefore, must be more aware of vitamin A toxicity.

Physiological Role

As previously mentioned, vitamin A is essential to vision. Within the photoreceptor cells of the retina are the rods, which detect small amounts of light and are specialized for motion detection and vision in dim light, and the cones that are specialized for color vision in bright light (Gleesson *et al*, 1987). Both rods and cones posses specialized outer segment disks that contain high amounts of rhodopsin and iodopsin respectively. These compounds are often referred to as the "visual pigment" (Gleesson *et al*, 1987). Photoreceptor cells detect light and undergo a series of reactions, which send signals to the brain, where they are deciphered as a particular visual image. A second very important function of vitamin A involves retinoic acid. Acting as a hormone, retinoic acid first binds to retinoic acid receptors. The receptors then interact with specific nucleotide sequences of DNA. The interaction directly affects gene expression and transcription, which in turn, control cellular development and body processes (Child *et al.*, 1999). For example, epithelial cells depend on retinoic acid for structural and functional maintenance. This role of vitamin A is important for growth mechanisms in a manner that is not completely understood (Child *et al.*, 1999). Retinoic acid is especially important in heart, eye, lung and ear development (Gleesson *et al*, 1987). The development of gap junctions between cells is also affected by retinoic acid (Child *et al.*, 1999). Besides the previously mentioned functions, vitamin A plays a role in numerous other processes. Vitamin A is thought to play a key role in glycoprotein synthesis. Once formed, glycoproteins are important in multiple cellular processes including: communication, recognition, adhesion, and aggregation. Reproductive processes, bone development, along with maintenance, and immune system function (Gleesson *et al*, 1987) are

dependent upon different isoforms of vitamin A. Retinoids are most commonly used in the treatment of skin diseases. The role the retinoids play in epithelial cell formation is very important in the treatment of skin cancer, acne, and acne-related diseases (Gleesson *et al*, 1987).Vitamin A also has antioxidant properties. However, beta-carotene has been noted as having pro-oxidant properties. Despite these discrepancies, vitamin A is known to help repair damaged tissue and therefore may be beneficial in counter-acting free radical damage (Gleesson *et al*, 1987).

Deficiency

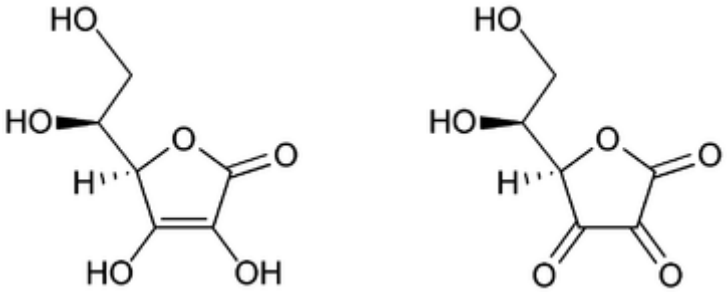
Deficiency of vitamin A is very rare in the United States, unless confounding malabsorption conditions such as steatorrhea or diseases of the liver, pancreas, or gallbladder are present. In contrast, vitamin A deficiency is prominent in young children (<5 years old) living in third world countries (Gleesson *et al*, 1987). At birth, many neonates experience low plasma vitamin A content, but the levels are corrected with a diet sufficient in vitamin A (Child *et al.*, 1999). Symptoms of vitamin A deficiency include metaplasia (changing of normal tissue into abnormal tissue), poor growth, xerophthalmia (dry corneas), and keratinization of epithelial cells resulting in a loss of differentiation (Gleesson *et al*, 1987). If vitamin A deficiency has not been chronic leading to permanent debilitation, the symptoms can often be reversed through supplementation.

Toxicity

The use of acne medicines (i.e. Acutane) has led to birth defects and even death (Gleesson *et al*, 1987) in children born to mothers using these compounds (Child *et al.*, 1999). This has helped make the public more aware of the toxic properties of vitamin A.

In adults, a condition known as hypervitaminosis exhibits itself after chronic ingestion of the vitamin in doses that are ten times the RDA (10 mg RE). Symptoms of vitamin A toxicity include: anorexia, headache, bone and muscle pain, vomiting, alopecia, liver damage, and coma. These symptoms slowly reside as vitamin A intake levels are reduced (Gleesson *et al*, 1987). To date, the only side effect of excess beta-carotene has been yellowing of the skin, most commonly in the fatty areas of the hands and palms. The yellowing disappears as beta-carotene intake decreases. This commonly ingested dietary precursor to vitamin A has yet to exhibit any signs of toxicity even at levels as high as 180 mg per day (Child *et al.,* 1999). Researchers believe that the presentation of unbound retinol to the cell is a major factor in toxicity. Excessive intakes of vitamin A saturate RBP and instead of retinol being transferred bound to RBP, it is transferred to the tissue via plasma lipoproteins. When retinol reaches the tissue by a carrier other than RBP, it is hypothesized that the retinol is released and causes toxic side effects (Child *et al.,* 1999).

2.3.3.1 Vitamin C



Ascorbicacid

(reduced form)

Description

Vitamin C, also referred to as ascorbic acid or ascorbate, belongs to the water-soluble class of vitamins. Humans are one of the few species who lack the enzyme to convert glucose to vitamin C (Groff *et al*, 1995). Ascorbic acid (AA) is an odorless, white solid having the chemical formula $C_6H_8O_6$. The vitamin is easily oxidized to form dehydroascorbic acid (DHAA), and thus oxidation is readily reversible. Vitamin C is a generic name for all compounds that exhibit the same biologic activity as AA. Consequently, the term includes both AA and DHAA. The importance of vitamin C was first was discovered in 1747. During the 16th century, numerous sea voyagers died due to the disease known as scurvy. James Lind found that men suffering from scurvy were cured when given oranges and lemons and he published his findings in the Treatise of the Scurvy in 1753. He developed a hypothesis based upon the results he observed; although his ideas were incorrect, he was the first person to understand the importance of what would later be called vitamin C. These findings were not widely accepted by the rest of the world and scurvy continued to lead to wide spread death throughout the 19th century (Jacob, 1999). Finally, in 1907 scurvy was induced in laboratory animals and this opened a new opportunity to understand the disease. Around 1930, two scientists working independently isolated and published their findings on vitamin C. The men found that vitamin C prevented and treated scurvy. The term ascorbic acid was adopted to describe its ability to prevent scurvy. The vitamin was then synthesized in the laboratory during 1933 (Bucci, 1998).

Sources

A wide variety of food that exists contains vitamin C. A well-balanced diet easily obtains the DRI for vitamin C. It is widely known by the general public today that the best sources of vitamin C are citrus fruits and their juices. Fruits with high vitamin C content include, but are not limited to oranges, lemons, peaches, strawberries, bananas and grapefruit. A wide variety of other foods also contain sufficient quantities of vitamin C. Cabbage, broccoli, cauliflower, leaf lettuce, tomatoes, potatoes, and beans also have relatively high (7 mg/100 g to 163 mg/100g) vitamin C content (Bucci,1998)

Absorption and Bioavailability

Transport of vitamin C is a saturable and dose dependent process that occurs by active transport. At the intestine and cells AA is oxidized to DHAA, which is more quickly transported across the cell membrane. Once inside the tissue or intestinal epithelium, the vitamin is reduced back to AA. The degree of intestinal absorption decreases as intake of AA increases. Intakes of 1 to 1.5 grams results in 50% absorption, but at intakes over 12 grams, only 16% of the vitamin is absorbed. In contrast, an intake of less than 20 mg, has a 98% absorption rate (Groff *et al,* 1995). Absorption of vitamin C is greater when several individual doses of vitamin C, in quantities less than one gram, are taken throughout the day rather than one megadose (Jacob, 1999). Eighty to ninety-five percent of the vitamin C found in foods is absorbed (Groff *et al*, 1995). Furthermore, the bioavailability of synthetic and "natural" forms of the vitamin differ very little despite the claims made by manufacturers (Groff *et al*, 1995). Vitamin C absorption can be impaired by a number of factors. A single large dose saturates the enzyme kinetics for vitamin C, leading to excess AA in the intestinal lumen, which causes numerous gastrointestinal

problems. Pectin and zinc also inhibit AA absorption, but this mechanism is not well understood. A high iron concentration in the gastrointestinal tract may cause oxidative destruction and in turn impair uptake (Groff *et al*, 1995).

Physiological Role

Vitamin C has been studied for many years. It participates in numerous biochemical reactions, suggesting that vitamin C is important for every body process from bone formation to scar tissue repair (Groff *et al*, 1995). The only established role of the vitamin appears to be in curing or preventing scurvy. Vitamin C is the major water-soluble antioxidant within the body. The vitamin readily donates electrons to break the chain reaction of lipid peroxidation. The water-soluble properties of vitamin C allow for the quenching of free radicals before they reach the cellular membrane. Tocopherol and glutathione also rely on AA for regeneration back to their active isoforms. The relationship between AA and glutathione is unique. Vitamin C reduces glutathione back to the active form. Once reduced glutathione will regenerate vitamin C from its DHAA or oxidized state. The prophylactic effects of vitamin C as an antioxidant during exercise, when free radical formation is high, will be discussed in future sections of this literature review. A well-known function of AA is the role it plays in hydroxylation reactions that are essential for the formation of collagen. Vitamin C is important in collagen formation as it allows for a tight cross-linking of the triple helix, thereby resulting in stabilization of the peptide. Evidence also suggests that AA may be involved in collagen gene expression. However, this mechanism is not well understood. Carnitine synthesis prefer to use vitamin C as the reducing agent (Groff *et al*, 1995). Carnitine facilitates the beta-oxidation of fat, through its role transporting long chain fatty acids from the cytoplasm

into the mitochondrial matrix of cardiac and skeletal muscle. High concentrations of AA are found in adrenal and brain tissue where they are fairly resistant to AA depletion. Vitamin C is directly involved in the enzyme activity of two copper dependent mono-oxygenases, which are important in the formation of norepinephrine and serotonin (Jacob, 1999). Furthermore, AA regulates the activity of some neurons within the brain. Some of these functions include neurotransmitter membrane receptor synthesis, and neurotransmitter dynamics. Indirectly, AA plays important regulatory roles throughout the entire body due to its involvement in the synthesis of hormones, hormone-releasing factors, and neurotransmitters (Groff *et al*, 1995). Animal models have also shown that AA is an important factor in development of the nervous system, specifically in the maturation of glial cells and myelin (Jacob, 1999).Vitamin C is important to a host of numerous other functions within the body. The vitamin is an important aid in the absorption and conversion of iron to its storage form. Bile acid formation, and hence cholesterol degradation are highly dependent on AA. Some hypothesize that vitamin C may even have a hypocholesterolemic effect. This has been suggested because the enzyme needed for the first step in bile acid synthesis, cholesterol 7-alpha hydroxylase, is dependent upon the presence of vitamin C. Ascorbic acid may also has vasodilatory and anticlotting effects within the body by stimulating nitric oxide release. Physiological effects such as an antihistamine modified bronchial tone, and insulin responses have been linked to AA. The protection of neural and endothelial tissue, along with effects on cellular tone can also be attribute to vitamin C. Multiple other mechanisms of function for vitamin C have been proposed, but experimental results addressing these topics are variable. Other possible functions for vitamin C include regulation of cellular nucleotide

concentrations, immune function, and the endocrine system. Vitamin C has been proposed by some to have pharmacological benefits in preventing cancer, infections, and the common cold. However, these benefits have yet to be reported in the scientific literature. The role of vitamin C in preventing cancer is controversial, but has been studied for cancers of the oral cavity, uterus, esophagus, bladder, and pancreas. The research is at best equivocal and more studies are needed to further address the role of vitamin C in preventing cancer.

Toxicity

The saturable kinetics of vitamin C make toxicity more likely when multiple large doses (~1gram) are consumed throughout a day versus one single dose. A common symptom of unabsorbed vitamin C left in the gastrointestinal tract is osmotic diarrhea (Groff *et al*, 1995). Vitamin C can be transformed in the body to oxalate, which is a common constituent of kidney stones. Doses up to 10 grams have shown to be associated with a higher prevalence of oxalate excretion, but the level does not fall outside of the normal range. As a precaution, people who are prone to kidney stones may want to avoid large doses (10 times the DRI or greater) of the vitamin (Groff *et al*, 1995). People who lack the control to regulate iron uptake should also avoid large doses of the vitamin. As stated earlier, vitamin C enhances iron absorption, which can lead to toxicity of iron in some people. Furthermore, excess ascorbate in the urine and feces can falsify laboratory tests such as glucose in the urine and fecal occult blood test.

Prevention of Oxidative Stress / Lipid Peroxidation

Vitamin C has the ability to sequester the singlet oxygen radical, stabilize the hydroxyl radical, and regenerate reduced vitamin E back to the active state. These functions work

to halt peroxidation of cellular lipid memebranes (Howald *et al*, 1975). Despite these functions, the studies involving AA and lipid peroxidation are disappointing at best. Vitamin C has been shown to induce a lower-frequency fatigue (indicates less muscle damage) when compared with those deficient in the vitamin (Jakeman and Maxewell, 1993). A second study found that vitamin C does control reactive oxidant species formed during exercise. If not controlled, these species have the ability to react with cell membranes and damage them, initiating lipid peroxidation. In 1992, Kaminski *et al*.(1992) examined the relationship between AA given to 19 subjects for three days before exercise (and seven after) and the muscle damage induced by two bouts of eccentric exercise that were 3 weeks apart. The authors concluded that AA reduced muscle damage, however they did not measure any indices of oxidative stress other than muscle soreness (Kaminski *et al*.1992) Supplementation with two 500-gram dosages of vitamin C for one day is associated with a decreased shift from pre-exercise to post exercise prooxidant activity when compared to a placebo. The same dosage given over a two-week period did not elicit as great a change in peroxidation activity as a one-day supplementation provided (Alessio and Blasi, 1997). This difference may be related to the fact that a one-day dose of vitamin C, such as that used in this study, helped to regenerate other antioxidants in the body (i.e. Vitamin E). The authors hypothesize that a two-week period of vitamin C supplementation may replenish other antioxidants and then lead to prooxidant properties within the body (Alessio and Blasi, 1997), likely via the Fenton reaction. As shown here there is an obvious lack of research addressing antioxidants and exercise, particularly vitamin C. The existing preliminary data

addressing vitamin C to-date seems promising, but also demonstrates the need for further research in this area.

2.3.3.3 Vitamin E

Vitamin E is the collective name for a set of eight related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidant properties (Herrera and Barbas, 2001, Packer *et al.,* 2001). Of these, α-tocopherol has been most studied as it has the highest bioavailability, with the body preferentially absorbing and metabolising this form (Brigelius-Flohé *et al.,* 2007). It has been claimed that the α-tocopherol form is the most important lipid-soluble antioxidant, and that it protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Herrera and Barbas, 2001). This removes the free radical intermediates and prevents the propagation reaction from continuing. This reaction produces oxidised α-tocopheroxyl radicals that can be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol (Wang and Quinn, 1999). This is in line with findings showing that α-tocopherol, but not water-soluble antioxidants, efficiently protects glutathione peroxidase 4 (GPX4)-deficient cells from cell death (Seiler *et al.,* 2008). GPx4 is the only known enzyme that efficiently reduces lipid-hydroperoxides within biological membranes (Seiler *et al.,* 2008).

However, the roles and importance of the various forms of vitamin E are presently unclear, (Brigelius-Flohé *et al.,* 2007, Atkinson *et al.,* 2008), and it has even been suggested that the most important function of α-tocopherol is as a signaling molecule, and that it has no significant role in antioxidant metabolism (Azzi and Angelo, 2007 , Zingg and Azzi., 2004). The functions of the other forms of vitamin E are even less well-

understood, although γ-tocopherol is a nucleophile that may react with electrophilic mutagens, (Brigelius-Flohé *et al.,* 1999) and tocotrienols may be important in protecting neurons from damage (Sen *et al.,* 2006). It is a naturally occurring antioxidant, essential for normal reproduction in many animals.

Chemistry of Vitamin E

Vitamin E refers to a group of ten lipid-soluble compounds that include both tocopherols and tocotrienols (Brigelius-Flohé *et al, 1999,* Traber and Atkinson, 2007). Alpha-tocopherol, the most biologically active form of vitamin E, is the second-most common form of vitamin E in the diet. This variant can be found most abundantly in wheat germ oil, sunflower, and safflower oils (Brigelius-Flohé *et al,* 1999). As a fat-soluble antioxidant, it stops the production of reactive oxygen species formed when fat undergoes oxidation (Herrera and Barbas, 2001; Packer *et al.,* 2001). Alpha-Tocopherol is an important lipid-soluble antioxidant. It performs its functions as antioxidant in the glutathione peroxidase pathway and it protects cell membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Herrera and Barbas, 2001; Traber and Atkinson, 2007). This would remove the free radical intermediates and prevent the oxidation reaction from continuing. The oxidized α-tocopheroxyl radicals produced in this process may be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol (Wang and Quinn, 1999). Other forms of vitamin E have their own unique properties; for example, γ-tocopherol is a nucleophile that can react with electrophilic mutagens (Brigelius-Flohé *et al,* 1999).

Biochemical function of Vitamin E

Most of the functions of vitamin E are related to its antioxidant property. It prevents the nonenzymatic oxidations of various cell components; unsaturated fatty acids by molecular oxygen and free radicals such as superoxide and hydrogen peroxide.

Vitamin E has many biological functions, the antioxidant function being the most important and best known. Other functions include enzymatic activities, gene expression, and neurological function(s). The most important function of vitamin E has been suggested to be in cell signaling (and it may not have a significant role in antioxidant metabolism) (Zingg and Azzi, 2004; Azzi and Angelo, 2007).

As an antioxidant, vitamin E acts as a peroxyl radical scavenger, preventing the propagation of free radicals in tissues, by reacting with them to form a tocopheryl radical, which will then be reduced by a hydrogen donor (such as vitamin C) and thus return to its reduced state (Traber and Stevens, 2011). Being fat-soluble, it is incorporated into cell membranes, which protects them from oxidative damage. Vitamin E has also found use as a commercial antioxidant in ultra high molecular weight polyethylene (UHMWPE) used in hip and knee replacements, to help resist oxidation (UHMWPE, 2009).

As an enzymatic activity regulator, for instance, protein kinase C (PKC), which plays a role in smooth muscle growth, can be inhibited by α-tocopherol. α-tocopherol has a stimulatory effect on the dephosphorylation enzyme, protein phosphatase 2A, which in turn, cleaves phosphate groups from PKC, leading to its deactivation, bringing the smooth muscle growth to a halt (Schneider, 2005).

Vitamin E plays a role in neurological functions and inhibition of platelet aggregation (Atkinson *et al.,* 2008). Vitamin E also protects lipids and prevents the oxidation of polyunsaturated fatty acids (Whitney and Sharon, 2011). So far, most human supplementation studies about vitamin E have used only α-tocopherol. This can affect levels of other forms of vitamin E, e.g. reducing serum γ- and δ-tocopherol concentrations. Moreover, a 2007 clinical study involving α-tocopherol concluded supplementation did not reduce the risk of major cardiovascular events in middle-aged and older men (Sesso *et al.,* 2008).

2.4 *Vitex Simplicifolia*



Plate 1: *Vitex simplicifolia* plant (M.A. Salim, personal photograph, February, 2014)

2.4.1 Morphology

Vitex simplicifolia commonly known as *dinyar biri* in Hausa belongs to the largest family of genus vitex (Labiatae). *Vitex simplicifolia* Oliv (Verbenaceae) is a perennial small tree which grows to olive sized, purple-black, and cupped in a calyx like an acorn. The thin pulp clings to the stone, which contains 3 to 4 seeds (Burkill, 2000). The leaves yield an essential oil of such sweet and penetrating fragrance that it is recommended for commercial development. The genus Vitex L. is comprised of about 300 species almost all occurring in the tropics. A tree about 30-35 ft tall.Bark brown, branchlets subterete or obtusely quadrangular, young parts subtomentose, glabrous when mature, nodes annulate, internodes, about 2-6 cm long. Leaves palmately compound with3-5 foliolate, rarely simple, leaflets lanceolate-elliptic or lanceolate-oblong, middle leaflets 8-12 × 2-5cm across, lateral leaflets 5-6 x2-2.5 cm across, petiolules 0.5-1cm long, base acute or cuneate, margin entire, apex acuminate, chartaceous, thick, dark greenglabrous both above and beneath,lateral veins 8-10 on either side of the midrib, ascending obscure, impressed above, prominent beneath, reticulate veinlets, petiole slender, canaliculated about 4-5 cm long. Inflorescence supra-axillary and terminal compound panicles, about 8-16 x 6-9 cm across, peduncles, slender, obtusely quadrangular, pubescent, about 3-7 cm long, bracts linear lanceolate, pubescent, about 1.5 x0.5 mm across (Burkill, 2000). Flowers are bisexual, numerous and sessile. Calyx campanulate, faintly or minutely 5 toothed, teeth acute, green with pink tinge, margins ciliate, pubescent outside, about 5 × 4mm across, Corolla infundibular, 5 lobed, 2-lipped, whitish blue or purple, upper lip 2-lobed, light blue, lobes ovate, apex acute, about 4 x 3 mm across, lower lip 3 lobed, midlobes obovate, apex obtuse, about 5 x 4 mm across, lateral lobes ovate, Corolla tube,

narrow, pubescent outside. Stamens 4, didynamous, exserted, filaments slender, filiform, about 2.5-4 mm long, exserted, anthers obovoid or subglobose, black, 2-celled, Ovary bicarpellary, 4 lobed, ovoid or globose, style slender, glabrous, stigma bilobed, acute and equal. Fruit is drupaceous, globose, about 8 mm in diameter, green, glabrous, slightly depressed, black when ripe (Hooker, 1885). Vitex simplicifolia species flowers are complete, bisexual, i.e. with functional male (androecium) and female (gynoecium), including stamens, carpels and ovary. Pollination is entomophilous i.e., by insect and is by dispersal by birds or animals (Rajendra and Daniel, 2002).

2.4.2 Climate and distribution

Vitex simplicifolia is widely distributed from Egypt to Guinea, is common in savanna forests of Ghana, Mali, Togo, and Cameroon, and is to be found as far to the east as Sudan and Egypt and of the nearly 70 *Vitex* species found scattered across tropical Africa, at least some are of local commercial and nutritional importance. These small and rugged trees are essential wild foods. In season, they become bespangled by an abundance of blackish fruits, which passersby eagerly gather up. Part of the harvest is eaten there and then, but most goes to market for sale. The reason may be due to new comers who loathe the pungent scent and brown stain on the lips while almost everyone loves the "chocolate" flavor. Unknown beyond Africa, these particular *Vitex* species, from the mint family (Labiatae), are essentially unknown to science too. But they are so useful that without them life would be even harder in many places between Senegal and South Africa. The genus Vitex L. is comprised of about 300 species almost all occurring in the tropics. A substantial proportion of the genus occurs in South-East Asia, and seems to be particularly rich in species and morphological variation on the South-East Asian

mainland. The genus is widespread in Eastern Malesia and the Pacific, but the number of species decreases sharply south and west from New Guinea. Members of the genus are common in swamps and primary rainforests and are often prominent in secondary, coastal or fire-influenced vegetations or the drier evergreen forests in parts of Java and the monsoon forest in the Philippines (Whitten *et al*., 1996).

2.4.3 Medicinal use

In Burkina Faso, the plant is used for internal or external use to treat various diseases like skin diseases, dermatitis, bilharzia, migraines, fever, aches, amoebiasis, sore teeth, colic, infant tetanus (Nacoulma,1996). Ethnobotanical investigations have revealed that this plant is also used in the treatment of skin infections and wounds healing. In Burkina Faso, infectious diseases are the leading cause of infant mortality (2.37%) and maternal (14.6%), therefore they constitute public health problems. The healing process is an immune response that begins after injury and takes place in three stages: vascular and inflammatory stage, phase of tissue repair and phase of maturation. A drug having simultaneously the potential antioxidant and antimicrobial activities may be a good therapeutic agent to accelerate cicatrization and wound healing (Heike *et al*., 1999). Aromatherapy is now considered to be another alternative way in healing people, and therapeutic values of aromatic plants lie in their volatile constituents such as monoterpenoids, sesquiterpenoids and phenolic compounds that produce a definite physiological action on the human body (Bruneton, 1993). The bark of *vitex simplicifolia* is used in medicines for treatment of dropsy, swellings, oedema, gout, oral treatments, pain-killers; skin, mucosae, fruit-pulp and young twig is used as food. In agri-horticulture it is used as shade-trees across Africa. Young twigs are used as tooth-sticks in Nigeria;

bark decoction is used as a lotion in Ivory Coast on edemas, skin infections, and for toothache (Burkill, 2000).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment

Clinical chemistry analyzer, Glucometer (Accu chek active), Glucometer strip, Spectrophotometer (Newlife England 752s), Cuvette, Freezer (Thermocool-super deluxe), filter paper, measuring cylinder, beaker, Funnel, Micropipette (Switzerland, Socorex), Test tubes, Test tubes racks, hand gloves, syringe, Sample bottles, Cotton wool, Razor blades (tiger), Centrifuge machine (Gulfex Medical And Scientific England 800D), Weighing balance (gallenhamp England EL00000096), Weighing balance (Ohaus Corporation, Pine Brook, NJ USA Scout Pro Spu402), Water bath (Grant Instruments (Cambridge) LTD OLS 200), (Microscope Vamed Engineering Nigeria Limited Axiostar plus 1097-707), Slide Draying Bench (Windaus Labortechnic GmbH & CO. KG Clausthal-Zellerfeld D38678), Tissue embedding system (Vamed Engineering Nigeria LTD LG 0016) and Tissue embedding system (Vamed Engineering Nigeria LTD. LG 0016).

3.1.2 Chemicals and reagents

The chemicals and reagent used in the study includes the following;

Aspartate Aminotransferase (AST) kit- Randox laboratories Ltd United Kingdom.

Alanine Aminotransferase (ALT) kit- Randox laboratories Ltd, United Kingdom.

Alkaline phosphatase (ALP) kit- Randox Laboratories Ltd, United Kingdom.

Bilirubin kit- Randox Laboratories Ltd United Kingdom.

Urea kit- Randox Laboratories Ltd, United Kingdom.

Sodium kit- Teco Diagnostics Lakeview ave. Anaheim USA.

Potassium kit- Teco Diagnostics Lakeview ave. Anaheim USA.

Chloride kit- Teco Diagnostics Lakeview ave. Anaheim USA.

Biocarbonate kit- Teco Diagnostics Lakeview ave. Anaheim USA.

Creatinine kit- Randox Laboratories Ltd, United Kingdom.

Lipid profile reagent kit - Randox Laboratories Ltd, United Kingdom.

Others are; Liver glycogen, Trichloroaceticacid, Anthrone, Potassium Lipid hydroxide (KOH), Superoxide dismutase, Carbonate buffer, Adrenaline, Vitamin A, Absolute buffer, Heptane, Vitamin C, DNP (dinitro phenyl hydrazine), Sulphuric acid, Thiourea, Vitamin E, Absolute ethanol, Xylene, Acetone, Bathophenanthroline, Ferric chloride Othorphosphoric acid and Tocopherol stock solution

3.1.3 Collection and preparation of plant material

Fresh leaves of *Vitex simplifolia* were collected on 20th of January, 2015 from the botanical garden of Bayero University, Kano with the assistance of Herbarium keeper. The leaves were authenticated by the Chief Technologist in the Department of Biological Sciences, Bayero University Kano (Herbarium number 242). The leaves were dried under room temperature and then grounded using pestle and mortar to a semi powdered form, 2.2Kg was obtained.

3.1.4 Experimental animals

One hundred and eleven (123) adult Wistar rats weighing between 160 –240g were obtained from the animal house of Department of Physiology, Bayero University, Kano and kept in cages at a room temperature for two (2) weeks to acclimatize and allowed access to food and water *ad libitum*. The principles of laboratory animal care (NIH publication number 85 – 23, revised, 1985) guidelines were followed.

3.2 Methodology

3.2.1 Induction of diabetes mellitus

Diabetes mellitus was induced in overnight fasted rats by a single intraperitoneal injection (i.p) of 150 mg/kg body weight of Alloxan monohydrate (Etuk *et al.*, 2010). Hyperglycaemia was confirmed by the elevated blood glucose levels, determined after 72hrs and then confirmed after 7^{th} day of injection. The rats found with elevated glucose level of 400 mg/dl and above were used for the study (Masiello *et al.*, 1998).

3.2.2 Acute toxicity studies and determination of LD_{50}

The lethal dose was determined by Lorke's method.

Phase 1: Nine wistar rats were used. They were divided into three groups of three animals each. Each groups of animals were administered different doses (10, 100, and 1000 mg/kg) of the extracts and then observed for 24 hours to monitor their behavior as well as mortality.

Phase 2: Three animals were used. They were divided into three groups of one animal each. The animals were administered higher doses (1600, 2900 and 5000 mg/kg of the extracts and observed for behavior as well as mortality. (Lorke, 1983)

Then LD_{50} is calculated by the formula: $LD_{50} = \sqrt{(D_0 x D_{100})}$

D_0 = Highest dose that gave no mortality,

D_{100} = Lowest dose that produce mortality.

3.2.3 Experimental design

A total of seventy five (75) wistar albino rats were used for the study. The rats were divided into fifteen (15) groups of five (5) each. Extracts were administered orally using 1ml syringe.

Group 1 - Normal untreated rats

Group 2 – Diabetic untreated rats (Diabetic control)

Group 3 – Diabetic rats administered 10 mg/kg of Glibenclamide (Prasanna *et al., 2012)*

Groups 4,5 and 6 – Diabetic rats administered 250, 500 and 1000 mg/kg body weight of aqueous leaf extract of *Vitex simplifolia* respectively.

Groups 7, 8 and 9 – Diabetic rats administered 250, 500 and 1000 mg/kg body weight of methanolic leaf extract of *Vitex simplifolia* respectively.

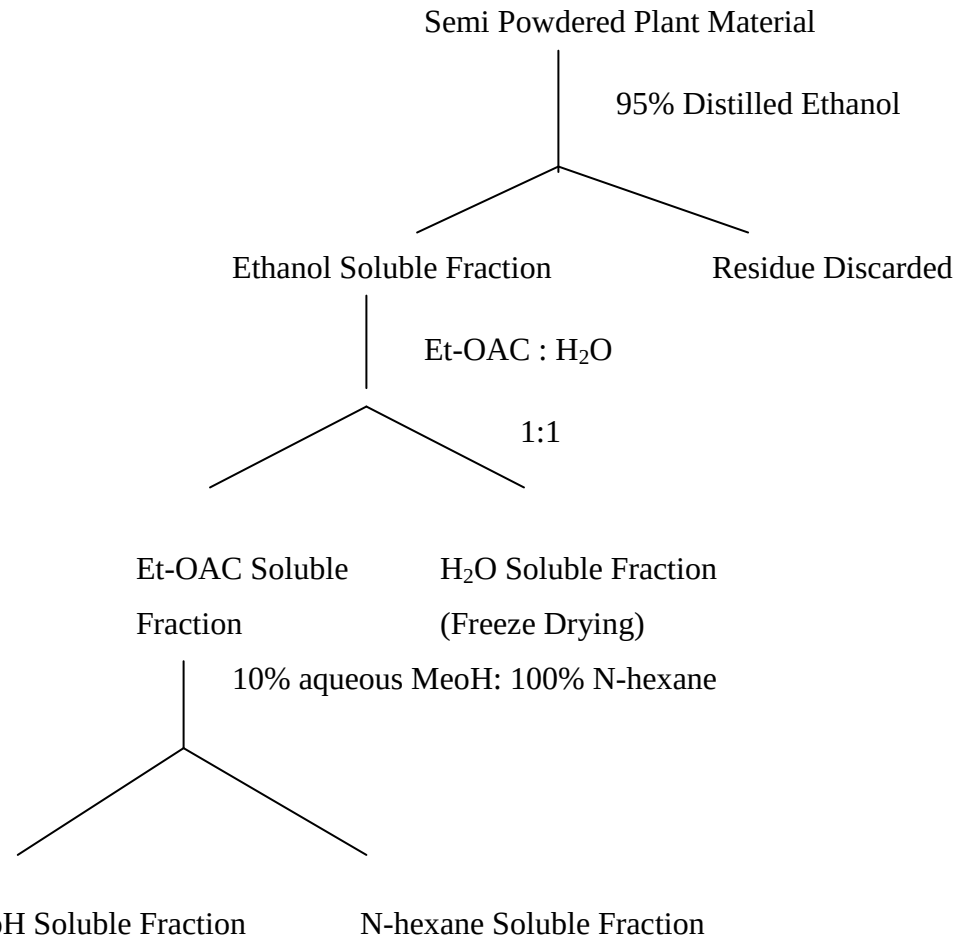
Groups 10, 11 and 12 – Diabetic rats administered 250, 500 and 1000 mg/kg body weight of ethyl acetate extract of *Vitex simplifolia* respectively.

Groups13, 14 and 15 –Diabetic rats administered 250, 500 and 1000 mg/kg body weight of N hexane leaf extract of *Vitex simplifolia* respectively.

Animals were treated for a period of three (3) weeks

3.2.4 Extraction and fractionation

Powdered materials of the plant were extracted with 95% ethanol at room temperature for seven days. The filtrates from this extract were concentrated under vacuum to yield crude ethanol extract. The latter was further fractionated using solvents of varying polarities (H_2O, CH_3OH, Ethyl acetate and hexane) to generate respective fractions (Prassanna *et al*, 2012)



3.2.5 Samples collection

At the end of the experimental period, the animals were deprived of food overnight and were anaesthetized using chloroform and bled by cardiac puncture 24 hrs after the last treatment. Blood was collected in tubes containing potassium oxalate and sodium fluoride mixture for the estimation of blood glucose. The serum was prepared by centrifugation at 3,000g for 15 minutes in a bench centrifuge. The separated serum was kept at 4^{o}C until when it was used in the estimation of lipid profile (HDL – cholesterol, Total cholesterol, Triglycerides and LDL – cholesterol), plasma Vitamin A, C, E, Superoxide dismutase, ALP, AST, ALT and Bilirubin

After the blood was collected, the animals were dissected; the liver and the kidney were removed and preserved in 9% formalin and taken to the Histopathology Department of Aminu Kano Teaching Hospital for histopathological analysis.

The liver was also removed and placed in 5% Trichloroaceticacid (TCA) and homogenized for liver glycogen content.

3.2.6 Determination of glucose level

Glucose dehydrogenase in the strip converts the glucose in the blood sample to glucunolactone. This reaction liberates two elements that react with a coenzymes (PQQ) electron acceptor. The complete reaction creates a harmless electrical current that the meter interprets as blood glucose. According to the manufacturer, the test is linear up to 33.3mmol/l (600mg/dl). This method will accurately measure glucose levels down to 0.6mmol/l (10mg/dl) (National diabetes association, 2012).

Procedure: The tail of the experimental rats was prick with a lancet and on the test strip there is a test area containing sensitive chemical. When blood is applied to the test area, the glucose dehydrogenase enzyme (Mut.Q-GDH_2) reacts with the blood glucose. The subsequent chemical reaction changes the colour of the test area. The meter registers this colour change and converts the signal obtained into a blood glucose result.

3.2.7 Determination of liver function test

The serum samples were used to test for four parameters, (indicators of liver damage) including ALP, AST, ALT and Bilirubin.

AST determination (Karmen, 1955): In 1955, Karmen described the first kinetic assay of AST for diagnostic purposes. This method was improved by many investigators primarily Henry (1960) and now forms the basis of many national and international recommended procedures. The AST reagent is based on the recommendation of IFCC (1986)

Principle: The principle of the assay is that AST present in the sample catalyses the transfer of amino group from L-aspartate to 2-oxoglutarate forming oxaloacetate and L-glutamate. Then oxaloacetate in the presence of NADH and malate dehydrogenase (MDH) is reduced to L-malate. In this reaction NADH is reduced to NAD. The reaction is monitored by measuring the rate of decrease in absorbance at 340nm due to the oxidation of NADH to NAD. And finally, addition of lactate dehydrogenase (LDH) to the reagent is necessary to achieve rapid and complete reduction of endogenous pyruvate so that it does not interfere with the assay.

Procedure: The sample (i.e. the serum) and the AST reagent were applied into the sample cups and fixed onto the clinical chemistry analyzer machine. The machine was set at 37^{o}C and wavelength of 340nm. The volumes of the sample and reagent were 30µL and 300µL respectively. The assay time was set at 60seconds. After the assay time had elapsed, the absorbance was taken from the analyzer digital readout and the activity of AST was calculated as follows:

AST (in U/L) = Absorbance/min.× Factor

Factor = TV×1000/6.3×SV×P where;

TV = Total reaction volume in cm^3

SV = Sample volume in cm^3

6.3 = Millimolar absorption coefficient of NADH at 340nm

P = Cuvvete path length in cm.

ALT determination (Wroblewski and Ladue, 1956; Henry, 1960 and Bergmeyer, 1978): Wroblewski and Ladue first described a method for determining ALT using LDH and NADH. This method was later modified by Henry and Bergmeyer to optimize substrate conditions and eliminate side reactions. The principle and the methodology are the same as in the determination of AST above. After the assay, ALT activity was also calculated as in AST.

ALP determination (Moss and Herdensen, 1994): The principle of this determination is that; under optimized conditions ALP presents in the sample catalyses the conversion of P-nitrophenol phosphate to P-nitrophenoxide and inorganic phosphate in the presence of magnesium under alkaline PH. At this PH, P-nitrophenoxide has an intense yellow color.

The reaction is monitored by measuring the rate of increase in absorbance at 405nm which is proportional to the activity of ALP in the serum.

Procedure: As in the method for AST, the ALP reagent and the samples were loaded onto the clinical chemical analyzer. The temperature was set at 37^{o}C and the wavelength at 405nm. In this case the sample and the reagent volumes were also 30µL and 300µL respectively while the assay time was 120 seconds. ALP activity was calculated using the following formula:

ALT (in U/L) = Absorbance/min. × TV × 1000/EF × P; where;

TV = Total reaction volume in cm^3

SV = Sample volume in cm^3

EF = Extinction Coefficient of P-nitrophenoxide at 405nm= 18

P = Cuvvete path length in cm.

Determination of direct bilirubin (Zilver and Pannall, 1979): Principle: The method for direct bilirubin determination as outlined by Zilver and Pannal is called acid diazo method. Conjugated bilirubin reacts with sulphanilic acid to produce an acid azobilirubin, the absorbance of which is proportional to the concentration of direct bilirubin in the sample and can be measured at 550nm.

Procedure: The blank, sample and reagent were applied to the clinical analyzer set at 37^{o}C and wave length of 550nm. The incubation time was set at 300 seconds. The absorbance of the blank was set at 500nm. After the assay, the direct bilirubin concentration was calculated using the formula;

Direct bilirubin (g/L) = Abs. of unknown /Abs. of calibrator × calibrator value.

Determination of total bilirubin (Zilver and Pannall, 1979): Principle: The total bilirubin reagent is a modification of Pearlman and Lee method in which a surfactant is used as a solubilizer. Conjugated and solubilized unconjugated bilirubin reacts with diazotized sulphanilic acid to produce an acid azobilirubin, the absorbance of which is proportional to the concentration of total bilirubin present in the sample.

Procedure: The procedure for the determination of total bilirubin is similar to the one described above for direct bilirubin. However, the value for unconjugated bilirubin was derived by subtracting direct bilirubin from total bilirubin.

3.2.8 Determination of serum lipid profile

Total cholesterol: Serum total cholesterol is determined using enzymatic (CHOD-PAP ASSAY) catorimetric method (Allian, 1974).

Principle: Cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quineimine is formed from hydrogen peroxide and 4-amino antipyrine in the presence of phenol and peroxidase.

$$\text{Cholesterol ester} \xrightarrow{\text{Cholesterol estarase}} \text{Cholesterol + fatty acid}$$

$$\text{Cholesterol + } O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Chlostene-3-one + } H_2O_2$$

$$2H_2O_2\text{+ phenol + 4-amino antipyrine} \xrightarrow{\text{Peroxidase}} \text{quinoneimine + } 4H_2O$$

Procedure: Three set of test tubes were set and labeled blank, standard and test. Distilled water (5UL) was added to blank; CAL standard (5UL) was added to standard and serum (5UL) to the test. Reagent (500UL) was then added to each of the test tubes. The content of each test tube was mixed and incubated for 10 minutes at +20 to +25^{o}C or 5 minutes at 37^{o}C after which the absorbance of the test sample were read against the reagent blank at 500nm wave length and timer was started simultaneously and the absorbance was read again after 60 minutes.

Calculation: total cholesterol concentration in the test samples was calculated using a factor given in the diagnostic kit.

$$\text{Concentration of cholesterol in sample (mmol/l)} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{conc of standard}$$

Serum triglyceride: Serum triglyceride was determined using enzymatic (CHOP-PAP) calometric method (Jacobs and Vandermark, 1960).

Principle: Triglyceride was determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen peroxide, 4-amino phenazone and 4-chlorophenol under the catalytic influences of peroxidase

$$\text{Triglyceride} + H_2O \xrightarrow{\text{lipoprotein Lipases}} \text{glycerol} + \text{fatty acid}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase}} \text{glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{Glycerol-3-phosphate oxidase}} \text{acetone dihydroxy acetone} + \text{phosphate} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + HCl + 4H_2O$$

Procedure: To each test tube labeled test, standard and reagent blank so distilled water (5UL) was added to the standard (5UL) was added to the standard and serum (5UL) was added to the test. Reagent (500nl) was added to each test tube. The content of each tubes was thoroughly mixed and incubated for 10 minutes at 20-25^{o}C or 5 minutes at 37^{o}C, after which the absorbance of the test, samples and standard were read against the reagent blank at 500nm wave length

Calculation: Triglyceride concentration was calculated using standard formula provided in the test kit.

$$\text{Triglyceride concentration} = \frac{\text{A sample}}{\text{A standard}} \times \text{conc of standard in mmol/l}$$

Serum high density lipoprotein (hdl) cholesterol: Serum high density lipoprotein (HDL) cholesterol was determined using (CHOP-PAP) method (Lopes *et al*., 1977).

Principle: Low density lipoprotein (LDL and VLDL) and chylomicron fractions are precipitate quantitatively by the addition of phosphotungstic acid in the presence of magnesium ion. After centrifugation, the cholesterol in the HDL fraction, which remains in the supernatant, is determined.

Procedure: Phosphotungstate (100UL) was added to 1cm^3 serum in a centrifuge tube and mixed. Magnesium chloride (50UL) was added and against mixed. The tube was centrifuged at 4,000rpm for 10 minutes or 12,000rpm for 2 minutes and the supernatant

was collected. To each test tube labeled blank, standard and test. 50UL of distilled water was added to blank, 50UL of standard supernatant to test. To each test tube 500UL of chol reagent was added. The content of each test tube were thoroughly mixed and incubated for 10 minutes at 20-25^{o}C or 5 minutes at 37^{o}C, after which the absorbance of the test sample was measured at 500nm wavelength against the within 60 minutes in a spectro photometer.

Serum low density lipoprotein (LDL) cholesterol: Serum low density lipoprotein (LDL) cholesterol was determined using Friedwald formular (Friedewald *et al.,* 1972). Low density lipoprotein cholesterol is difficult to determine directly but can be determined by an indirect method using the following formula

LDL cholesterol in mmol/L

LDL = Total cholesterol - HDL cholesterol – (Triglyceride / 2.2)

3.2.9 Determination of liver glycogen content

Liver glycogen was determined using the destruction by Alkali method (Good *et al*., 1933). Extraction of glycogen from the liver with trichloroacetic acid (TCA) was done as follows; 200mg sample of liver was weighed and finely grounded with 20ml of 5% TCA in a mortar. The precipitate of protein was filtered off and the clear filtrate was used for analysis.

Procedure: 2cm^{3} of liver extracts were pipette into a test tube calibrated at 20ml, 2ml of 10-KOH were added and the tubed placed in boiling water for 1 hour. After it was cooled, 1ml of glacial acetic acid was added to neutralize the excess alkali and the fluid

brought up to the 20ml mark with water 2ml of the solution were added slowly to 4ml of athrone reagent reagent in a test tube, which was placed in boiling for exactly 10 minutes for colour development and cooled with tap water. The absorbance was read using a spectrophotometer at 650nm against a blank which was prepared by subjecting 2ml of 5% TCA to the same procedure.

Calculation: Glycogen concentration was determined using glucose standard prepared as follows. 2ml of 5% TCA were heated to 100^{o}C with 2ml of 10N KOH and the solution was neutralized with 1ml of glacial acetic acid as described above. Then the 4ml of the solution containing varying concentration of glucose were added. After dilution to 20ml with water the procedure continued in the usual way. The result was obtained from a calibration curve.

3.2.10 Determinaton of oxidative stress biomarkers

Determination of superoxide dismutase (SOD) activity: The level of SOD activity was determined by the method of Mc cord and Fridorich (1969).

Principle: The ability of superoxide dismutase to inhibit the auto oxidation of epirephrine at PH 10.2 makes this reaction a basis for simple assay for dismutase. Superoxide (O_2) radical generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adenochrome and the yield of adenochrome produced per O_2 introduced increased with increasing PH and also increased with increasing concentration of epinephrine. This result lead to the proposal that auto oxidation of epinephrine proceeds by at least two distinct pathways only of which is a free radical reaction involving superoxide (O_2) radical and hence inhabitable by superoxide dismutase (Mc cord and Fridorich, 1969).

Procedure: 1ml of sample was diluted in 9ml of distilled water to make a 1 in 10 dilution. An aliquot of 0.2ml of the diluted sample was added to 2.5ml of 0.05m carbonate buffer (PH 10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3ml of freshly prepared 0.3mm adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5ml buffer, 0.3ml of substrate (adrenaline) and 0.2ml of fresh water. The increase in absorbance at 480nm was monitored every 30 seconds for 150 seconds.

Calculation: increase in absorbance per minute = $\frac{A_3 - A_0}{2.5}$

Where

A_0 = absorbance after seconds

A_3 = absorbance 150 seconds

% inhibition = $100 - 100 \times \frac{\text{Increase in absorbance for substrate}}{\text{Increase in absorbance of blank}}$

Determination of vitamin A: Principle: Vitamin A is extracted from serum with petroleum ether; the orange carotene is usually measured directly at this point using a spectrophotometer (Bessey *et al*., 1946) the nearly colourless vitamin A generally is determined next by formation of blue or violet colour after irradiation with long wave length ultra violet light at 326nm.

Procedure: To a test, 2ml of serum was added followed by addition of 2ml of absolute ethanol and 5ml of heptane was added and shook for 5 minutes and centrifuge for 10 minutes at 2000 rpm. Then 3ml of heptane layer was transferred to a 10mm covet and

then the absorbance (A_1) was read in spectrophotometer against heptane at 326nm. The covet was then exposed to long wave length ultra violet for 1 hour and after one hour, the absorbance (A_2) was read again at 326nm.

Calculation:

$$\frac{A_1 - A_2}{\text{Absorbance of standard}} \times \text{Conc of standard (125mg/dl)} = \text{Conc of vitamin (mg/dl)}$$

Determination of vitamin C: Principle: Vitamin C is heated in acid in the presence or absence of metal salts to form keto products which in turn form colored derivatives upon reaction with 2, 4-dinitrophenyl hydrazine (Roe and Kuether, 1943).

Procedure: To 12ml of 6% trichloroacetic acid, 3ml of serum was added and shake, it is then centrifuged for 5 minutes and the supernatant is decanted into a clean test tubes. One spatula tip of norit was added and mixed thoroughly. 4ml of the filtrate was used. In 2 tubes (black and test), 1 drop of thiourea was added to each test tube. To test 1ml of DNP reagent was added and mix for 5 seconds. Then incubate for 3 hours at 37^oC. At the end of the incubation, the tubes are ten placed in an ice bath and their 5ml of 85% sulphuric acid was slowly added and mix and then 1ml of DNP reagent was added to the blank. Allow the tube to stand for 30 minutes. The absorbance was read against the blank is 19mm covets at 540nm.

Calculation:

$$\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Conc of standard (2mg/dl)}$$

Determination of vitamin E: Principle: Tocopherol is oxidized by the ferric chloride and pink complex of ferrous ion with bathophenan throline is determined spectrophotometrically (Fabianek *et al*, 1968).

Procedure: 0.4ml of serum was added in test tube, equal volume of absolute ethanol was added for protein precipitation, and then mixed. 0.4ml xylene was added and mixed for at least 30 minutes and centrifuge for 5min at 3000rpm. The upper xylene layer is collected (containing extracted tocopherol) with dropper. The exact volume of 0.2ml of serum xylene extract is pipetted into a test tube containing 0.1ml of BA, 0.1ml of ferric chloride is added and followed by 0.1 of orthophosphoric acid, the content of the tubes are then mixed thoroughly. The absorbance was then read at 536nm after zeroing with blank (0.2ml xylene instead of serum xylene extract).

Calculation:

$$\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Conc of standard (20mg/dl)}$$

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3.2.11 Kidney function test

Urea (Weatherburn, 1967): Principle: Urea in serum is hydrolyzed to ammonia in the presence of urease. The ammonia is then measured photometrically by Berthelot's reaction.

$$\text{Urea} + H_20 \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

$$NH_3 + \text{hypochlorite} + \text{phenol} \longrightarrow \text{indophenols (blue compound)}$$

Procedure: Three test tubes were labelled as test (T), standard (S) and blank (B). To the three test tubes, 100µl of Reagent 1 (sodium nitroprusside and urease) were pipetted followed by 10 µl of serum, standard and distilled water to their respective test tube. The test tubes were mixed and incubated at 37^{o}C for 10 min. 2500 µl of reagent 2 (diluted phenol 1:6) and reagent 3 (diluted sodium hypochlorite: 22ml in 750ml of distilled water) were added to all the tubes. They were mixed immediately and incubated at 37^{o}C for 15 min. The absorbance of the test (A test) and standard (A standard) were measured against the reagent blank at 546nm.

Creatinine (Bartels and Bohmer, 1972): Principle: Creatinine in alkaline solution reacts with picric acid to form a coloured complex the amount of the complex formed is directly proportional to the creatinine concentration.

Procedure: Into 3 test tubes labelled test (T), standard (S) and blank (B), 1ml working reagent (equal volume of picric acid and sodium hydroxide) was pipetted followed by 0.1ml of serum, standard and distilled water to their respective tubes. The tubes were mixed and incubated for 30 seconds and the absorbance of the standard and test (A_1) was measured. After exactly 2 minutes absorbance A_2 of the standard and sample was measured again at 492nm.

Sodium (Kraut and Madias, 2007): Principle: This was calculated according to Kraut and Madias (2007) using the formula;

$$(Na^{+} + K^{+}) - (Cl^{-} + HCO_3^{-}) \leq 20$$

$$Na^{+} = 20 - K^{+} + Cl^{-} + HCO_3^{-}$$

Procedure: Three test tubes were labelled test (T), standard (S) and blank (B). 1.0 ml of filtrate reagent was added to all test tubes, 50 µl of samples to all test tubes and distilled water to the blank. The test tubes were vigorously and mixed continuously for 3 minutes. The tubes were centrifuged at high speed (1,500g) for 10 minutes and were measured against the reagent blank at 550nm.

Potassium (Henry, 1974): Principle: The amount of potassium is determined by using sodium tetraphenylboron in a specifically prepared mixture to produce a colloidal suspension. The turbidity of which is proportional to potassium concentration in the range of 2 - 7 mEq/L.

Procedure: Three test tubes were labelled test (T), standard (S) and blank (B). 1000 µl of Potassium reagent (sodium tetraphenylboron) was added to all the tubes followed by 10 µl of serum, standard and distilled water to their respective tubes. The tubes were mixed and incubated for 3 minutes at room temperature. The absorbance of the test (A test) and standard (A standard) were measured against the reagent blank at 500nm.

Chloride (White, 1970): Principle: Chloride ions form a soluble, non-ionized compound, with mercuric ions and will displace thiocyanate ions from non-ionized mercuric thiocyanate. The released thiocyanate ions react with ferric ions to form a colour complex that absorbs light at 480 nm. The intensity of the colour produced is directly proportional to the chloride concentration.

$Hg(SCN)_2 + 2CI^- \rightarrow HgCI2 + 2SCN^-$

$3SCN^- + Fe^{3+} \rightarrow 4\ Fe(SCN)_3$ red complex

Procedure: Three test tubes labelled test (T), standard (S) and blank (B) were pipetted with 1.5ml of chloride reagent (mercuric nitrate, mercuric thiocyanate, mercuric chloride and ferric nitrate). This was then followed by addition of 0.01ml serum, chloride calibrator (sodium chloride) and distilled water to the test tubes labelled T, S and B respectively. The tubes were mixed and incubated for 5 minutes at room temperature. The absorbance of the test (A test) and standard (A standard) were measured against the reagent blank at 480nm.

Bicarbonate (Forrester *et al.,* 1976): Principle: Phosphoenol pyruvate carboxylase (PEPC) catalyzes the reaction between phosphoenol pyruvate and carbon dioxide (bicarbonate) to form oxalacetate and phosphate ion. Oxalacetate is reduced to malate with simultaneous oxidation of an equimolar amount of reduced nicotinamide adenine dinucleotide (NADH) to NAD; the reaction is catalyzed by malate dehydrogenase (MDH). This results in a decrease in absorbance at 340 nm that is directly proportional to CO_2 concentration in the sample.

$$\text{Phosphoenol Pyruvate} + HCO_3^- \xrightarrow{\text{PEPC}} \text{Oxalate} + H_2PO_4$$

$$\text{Oxalate} + \text{NADH} \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}$$

Procedure: To the three test tubes labelled T (test), S (standard) and B (blank) containing 1000µl of Carbon dioxide reagent [PEP, magnesium sulfate, NADH, MDH (porcine), PEPC (microbial), sodium oxamate, buffer, Ph 7.0, non-reactive fillers and stabilizers with sodium azide], 5 µl of serum, standard (sodium bicarbonate in an aqueous solution) and distilled water were added respectively. The tubes were mixed and incubated for 5

minutes at room temperature. The absorbance of the test (A test) and standard (A standard) were measured against the reagent blank at 340nm.

$$\text{P-nitro phenyl phosphate} + H_2O \xrightarrow{\text{ALP}} \text{Phosphate} + \text{p-nitorphenol}$$

3.2.12 Histopathological studies (Avwioro, 2010)

The liver biopsies were fixed with 10% formal saline and then transferred to a cassette, a container designed to allow reagents to freely act on the tissue inside. This cassette is immersed in multiple baths of progressively more concentrated ethanol (to dehydrate the tissue with ascending grade of alcohol), cleared with toluene, infiltrated with molten paraffin wax. During this 12 to 16 hour process, paraffin will replace the water in the tissue, turning soft, moist tissues into a sample miscible with paraffin, a type of wax. This process is known as tissue processing. The processed tissue is then taken out of the cassette and set in a mold. Through this process of embedding, additional paraffin is added to create a paraffin block which is attached to the outside of the cassette. The process of embedding then allows the sectioning of tissues into very thin (2 - 7 micrometer) sections using a microtome. The microtome slices are the tissue ready for microscopic examination. The slices are thinner than the average cell, and are layered on a glass slide for staining. Tissue was dewax and hydrated, stained in Erich's haematoxylin for 15mins, rinsed in water, differentiated in 1% HCl in 70% alcohol for 1min, rinsed in water, counterstained with 1% eosin for 1min, rinsed in water again and finally dehydrated, cleared and mounted.

3.30 Phytochemical Screening

a. Test for anthraquinones (Felgis, 1975): 5g of each of the leaf and the leaf of *Vitex simplicifolia* was shaken with 10ml of benzene, filtered and 5cm^3 of ammonia solution was added to the filtrate. The mixture was shaken. The presence of a pink red or violet color in the ammonical lower phase indicates the presence of free anthraquinones.

b. Test for alkaloids (Sofowora, 1993): 0.5g of the leaf and bark powders were stirred with 5cm^3 of 1% hydrochloric acid in a steam bath. 1cm^3 of the filtrate was treated with a few drops of Meyer's reagent and picric acid. The turbidity or precipitation was taken as evidence for the presence of alkaloids in the plant.

c. Test for saponins (Earl, 1961): 0.5g of both the leaf and bark powders were dissolved in 5cm^3 of distilled water and shaken vigorously for about 30 seconds. Then it was allowed to stand for 30 minutes. Appearance of honey comb froth indicates the presence of saponins.

d. Test for saponins (Earl, 1961): The plant samples were dissolved in distilled water in a ratio of 1:4. A few drops of 10% ferric chloride was then added. A green solution indicates the presence of flavonoids.

e. Test for Terpenes (Sofowora, 1993): The plant materials were dissolved in chloroform, 1cm^3 of acetic anhydride and concentrated Sulphuric acid were

added by the side of the tubes. A reddish violet color indicates the presence of terpenes.

f. Test for tannins (Earl, 1961): 2.5g of each plant material was stirred with 10cm^3 of distilled water, filtered and few drops of ferric chloride reagent added to the filtrate. A blue – black, green or blue green precipitate indicates the presence of tannins.

3.3.1 Phytochemical Quantification

Some of the major phytochemicals like alkaloid, tannins and saponins were quantitatively determined as follows:

a. Quantitative estimation of alkaloids (El – Oleamy *et al.*, 1994). Five (5) grams of *Vitex simplicifolia* leaf were extracted in methanol. From the extract, 10cm^3 was placed in 250cm^3 separating funnel and 5cm^3 of dilute H_2SO_4 and water were added. The extract was shaken twice with 10cm^3 $CHCl_3$. The combined $CHCl_3$ extract was transferred to a second separating funnel containing 5cm^3 of dilute H_2SO_4 and 10cm^3 of distilled water. The $CHCl_3$ layer was discarded after shaking and the aqueous acidic layer was transferred to the content of the first separating tunnel. The extract was basified with ammonia solution and was shaken for 30 seconds. The alkaloids were completely extracted by successive portions of $CHCl_3$. The combined $CHCl_3$ extract was shaken with 5cm^3 of water and was run through a plug of cotton wool previously moistened with $CHCl_3$. The content was covered with a little anhydrous sodium sulphate, which was later washed in 5cm^3 of $CHCl_3$.

The filtrate was then place into 250cm^3 conical flask after which the chloroform was distilled completely, followed by addition of 5ml of neutral alcohol, which was evaporated on boiling water bath for 15min. The residue was dissolved in 2cm^3 of chloroform and 20cm^3 of N/50

H_2SO_4. The content was warmed to remove $CHCl_3$. The excess acid was titrated with N/50 NaOH using methyl red as indicator, a color change from pink to yellow was observed. The alkaloid content of the samples was then calculated using the following formula:

Alkaloid content:

Alkaloid content = cm^3 taken of N/50 NaOH X 0.00 578 X 100/10

b. Quantitative estimation of Tanins (El-Oleamy, *et al.*, 1994) from the water extract of *Vitex simplicifolia,* 5cm^3 was placed into a stopered conical flask, 25cm^3 of 0.1N iodine and 10cm^3 of 5% NaOH was added. The resulting mixture was kept in the dark for 15min. Ten (10) cm^3 of water was used to dilute the mixture and was acidified with 10cm^3 4% H_25O_4. The mixture was titrated with 0.1N sodium thiosulphate solution using starch solution as indicator. The titration value was corresponded to the sum of tannins and pseudotannins concentration A.

Another 25cm^3 of the extract was placed in a stopered conical flask followed by 15cm^3 of 1% gelatin. The volume was made up with water and filtered. Aliquot of 20cm^3 was placed in a volumetric flask, 25cm^3 of 0.1N iodine and 10cm^3 of 4% NaOH were added,

mixed and kept in the dark for 15 min. the mixture was diluted with 10ml of water and acidified with 10ml of 4% H_2SO_4. This was finally titrated with 0.1N sodium thiosulphate using starch solution as indicator. The titration value obtained was corresponded only to the pseudotannins concentration B. Blank experiment was carried out simultaneously using distilled water.

The tannins and pseudotannins content of the samples was then calculated using the following formula:

$$\% \text{ Pseudotannins} = \frac{\text{Blank Exp. B X } 0.029}{5 \text{ (Volume taken)}} \times 100$$

% of true tannins A – B = g % w/v.

c. Quantitative estimation of saponins (El-Oleamy, *et al.* 1994).

Three and a half grams (3.5g) of the plant samples were placed in $100cm^3$ conical flask and $21cm^3$ of 50% alcohol was added. The content was refluxed for 30min. The boiling alcohol extract was filtered while hot through a coarse filter paper. Two (2) grams of charcoal was added, boiled and filtered. Equal volume of acetone was added to the cooled extract. The separated saponin was collected by decantation and was dissolved in $5cm^3$ of boiling 95% alcohol. The content was filtered while hot through insoluble matter and was kept at room temperature.

The supernatant liquid was decanted and the precipitated saponin was suspended in 20cm^3 of alcohol and filtered. The filter paper was immediately transferred to a dessicator containing anhydrous calcium chloride where the saponin was dried and weighed. The supernatant was also filtered off and dried.

3.4 STATISTICAL ANALYSIS

Generated data was collated and cleaned using Microsoft excel while analysis was done using the statistical package for social sciences (SPSS) version 20. Quantitative variables were subjected to a one-way analysis of variance (1-Way ANOVA) Descriptive statistics were summarized using mean and standard error of mean (SEM), and presented in tables. Association between variables was considered significant at 95% confidence interval (i.e. at 5% alpha level of significance). A post hoc analysis was done using bonferroni's multiple comparison tests to compare for significance between the groups.

CHAPTER FOUR

4.0 RESULTS

4.1 Acute Toxicological Study

Table 4.1: Phase I LD_{50}, (Oral) of the aqueos fraction of *Vitex simplicifolia* leaf extract

Group	No. of Animals	Doses (g/Kg)	No. of Death
1	3	10	0
2	3	100	0
3	3	1000	0

Table 4.2: Phase 2 LD_{50}, (Oral) of the aqueos fraction of *Vitex simplicifolia* leaf extract

Group	No. of Animals	Doses (mg/Kg)	No. of Death
1	1	1600	0
2	1	2900	0
3	1	5000	0

Table 4.3: Phase I LD_{50}, (Oral) of the methanolic fraction of *Vitex simplicifolia* leaf extract

Group	No. of Animals	Doses (g/Kg)	No. of Death
1	3	10	0
2	3	100	0
3	3	1000	0

Table 4.4: Phase II LD_{50} (Oral) of the methanolic fraction of *Vitex simplicifolia* leaf extract

Group	No. of Animals	Doses (mg/Kg)	No. of Death
1	1	1600	0
2	1	2900	0
3	1	5000	0

Table 4.5: Phase I LD_{50}, (Oral) of the ethyl acetate fraction of *Vitex simplicifolia* leaf extract

Group	No. of Animals	Doses (g/Kg)	No. of Death
1	3	10	0
2	3	100	0
3	3	1000	0

Table 4.6: Phase II LD_{50} (Oral) of the ethyl acetate fraction of *Vitex simplicifolia* leaf extract

Group	No. of Animals	Doses (mg/Kg)	No. of Death
1	1	1600	0
2	1	2900	0
3	1	5000	0

Table 4.7: Phase I LD_{50}, (Oral) of the n-hexane fraction of *Vitex simplicifolia* leaf extract

Group	No. of Animals	Doses (g/Kg)	No. of Death
1	3	10	0
2	3	100	0
3	3	1000	0

Table 4.8: Phase II LD_{50} (Oral) of the n-hexane fraction of *Vitex simplicifolia* leaf extract

Group	No. of Animals	Doses (mg/Kg)	No. of Death
1	1	1600	0
2	1	2900	0
3	1	5000	0

The lethal dose (LD_{50}) determination was conducted using the method of Lorke (1983) through oral route in rats in two different phases. In both phases no signs of toxicity or mortality were observed after 24 hours of the administration.

Table 4.9: Results of phytochemical screening of the crude ethanol extract of the *Vitex simplicifolia leaf*

Phytochemicals	Qualitative	Quantitative
Alkaloids	Present	5.90%
Cardiac glycosides	Present	2.23%
Phenols	Absent	-
Flavonoids	Present	12.08%
Saponins	Present	9.59%
Tannins	Present	2.30%
Steroids	Absent	-
Terpenoids	Absent	-
Phytates	Present	0.44%
Oxalates	Present	0,74ug/g
Cyanates	Present	0.414mg/g

4.2 Effects of Fractionated Extracts of *Vitex simplicifolia* Leaf on Different Parameters

Table 4.10: Percentage changes in fasting blood glucose of alloxan induced diabetic rats treated with 250mg/kg of aqueos, methanolic, ethyl acetate and n hexane fractionated extracts of *Vitex simplicifolia* leaf.

Group	Mean Initial FBG(mg/dl)	Mean Final FBG(mg/dl)	Change (mg/dl)	% Change
Positive control	471.7±20.0	165.0±20.0	306.6.±0.00	65
Diabetic+250mg/kg AGVSF	600.0± 0.00	168.0±4.0	432.0±4.0	72
Diabetic+250mg/kg MNVSF	439.0±13.3	404.0±2.0	35.0±1.5	8
Diabetic+250mg/kg ETVSF	553.0±21.0	254.0±2.0	299.0±19.0	54
Diabetic+250mg/kg NHVSF	525.0±43.3	221.0±20.0	304.0±23.3	58

Key

AGVSF- Aqueos fraction of *Vitex simplicifolia* leaf

MNVSF-Methanolic fraction of *Vitex simplicifolia* leaf

ETVSF-Ethyl acetate fraction of *Vitex simplicifolia* leaf

NHVSF-N-hexane fraction of *Vitex simplicifolia* leaf

Table 4.11: Percentage changes in fasting blood glucose of alloxan induced diabetic rats treated with 500mg/kg of aqueos, methanolic, ethyl acetate and n hexane fractionated extracts of *Vitex simplicifolia* leaf.

Group	Mean Initial FBG(mg/dl)	Mean Final FBG(mg/dl)	Change (mg/dl)	% Change
Positive control	471.7 ± 20.0	165.0 ± 20.0	306.1 ± 0.00	65
Diabetic+500mg/kg AGVSF	557.0 ± 42.5	139.0 ± 4.0	418.0 ± 38.5	75
Diabetic+500mg/kg MNVSF	460 ± 0.5	386.0 ± 2.0	74.0 ± 1.5	16
Diabetic+500mg/kg ETVSF	435.0 ± 16.5	131.0 ± 2.0	304.0 ± 14.5	70
Diabetic+500mg/kg NHVSF	541.0 ± 59.0	156.0 ± 20.0	385.0 ± 39.0	71

Values are presented as mean ± standard error of mean.

AGVSF- Aqueos fraction of *Vitex simplicifolia* leaf

MNVSF-Methanolic fraction of *Vitex simplicifolia* leaf

ETVSF-Ethyl acetate fraction of *Vitex simplicifolia* leaf

NHVSF-N-hexane fraction of *Vitex simplicifolia* leaf

Table 4.12: Percentage changes in fasting blood glucose of alloxan induced diabetic rats treated with 1000mg/kg of aqueos, methanolic, ethyl acetate and n hexane fractionated extracts of *Vitex simplicifolia* leaf.

Group	Mean Initial FBG(mg/dl)	Mean Final FBG(mg/dl)	Change (mg/dl)	% Change
Positive control	471.7 ± 20.0	165.0 ± 20.0	306.6 ± 0.00	65
Diabetic+1000mg/kg AGVSF	557.5 ± 42.5	108.0 ± 4.0	449.5 ± 48.5	81
Diabetic+1000mg/kg MNVSF	440 ± 0.5	416.5 ± 2.0	23.5 ± 1.5	17
Diabetic+1000mg/kg ETVSF	435.0 ± 165.5	127.0 ± 2.0	308.0 ± 163.5	71
Diabetic+1000mg/kg NHVSF	541.0 ± 59.0	125.0 ± 20.0	416.0 ± 39.0	77

Values are presented as mean ± standard error of mean.

AGVSF- Aqueos fraction of *Vitex simplicifolia* leaf

MNVSF-Methanolic fraction of *Vitex simplicifolia* leaf

ETVSF-Ethyl acetate fraction of *Vitex simplicifolia* leaf

NHVSF-N-hexane fraction of *Vitex simplicifolia* leaf

Table 4.13: The effect of oral administration of aqueous fraction of leaf extract of *vitex simplicifolia* on liver glycogen content and lipid profile on alloxan induced diabetic rats.

Groups	Glycogen (mg/g)	Total Chol (mmol/l)	Triglyceride (mmol/l)	HDL (mmol/l)	LDL (mmol/l)
1	3.28 ± 0.09	0.24 ± 0.03	0.12 ± 0.05	0.20 ± 0.30	0.20 ± 0.04
2	1.96 ± 0.27	0.27 ± 0.01	0.11 ± 0.03	0.12 ± 0.02	0.10 ± 0.01
3	2.88 ± 0.02	0.14 ± 0.11	0.08 ± 0.01	0.13 ± 0.06	0.70 ± 0.04
4	2.27 ± 0.02	0.26 ± 0.06	0.19 ± 0.28	0.14 ± 0.28	0.15 ± 0.03
5	2.38 ± 0.46[b]	0.24 ± 0.47	0.14 ± 0.03	0.18 ± 0.06	0.08 ± 0.02
6	3.79 ± 0.29	0.23 ± 0.01	0.07 ± 0.02	0.68 ± 0.03	0.07 ± 0.01

Grp 1: Normal control, Grp 2: Negative control, Grp 3: Positive control, Grps 4, 5 and 6 received 250,500 and 1000mg/kg of extract, respectively. Total chol = Total cholesterol. Values are presented as mean ± standard error of mean. [b] = significantly different ($p<0.05$) from the Negative control.

Table 4.14: The effect of methanolic fraction of leaf extract of *Vitex simplicifolia* on liver glycogen content and lipid profile on alloxan induced diabetic rats.

Groups	Glycogen (mg/g)	Total Chol (mmol/l)	Triglyceride (mmol/l)	HDL (mmol/l)	LDL (mmol/l)
1	3.280 ± 0.09	0.24 ± 0.01	0.12 ± 0.05	0.20 ± 0.03	0.02 ± 0.03
2	1.960 ± 0.24	0.27 ± 0.01	0.11 ± 0.03	0.12 ± 0.02	0.10 ± 0.01
3	2.880 ± 0.02	0.14 ± 0.11	0.077 ± 0.03	0.13 ± 0.06	0.07 ± 0.04
4	3.323 ± 0.63	0.23 ± 0.21	0.127 ± 0.04	0.13 ± 0.02	0.06 ± 0.02
5	2.450 ± 0.05	0.24 ± 0E7	0.116 ± 0E-7	0.12 ± 0E-7	0.068 ± 0E7
6	2.240 ± 0E7	0.23 ± 0.0E7	0.042 ± 0.02	0.21 ± 0.01	0.151 ± 0.03^{ab}

Grp 1: Normal control, Grp 2: Negative control, Grp 3: Positive control, Grps 4, 5 and 6 received 250,500 and 1000mg/kg of extract, respectively. Total chol = Total cholesterol. Values are presented as mean ± standard error of mean. ^{a} = significantly different ($P < 0.05$) from the Normal control, ^{b} = significantly different ($P < 0.05$) from the Negative control.

Table 4.15: The effect of ethyl acetate fraction of leaf extract of *Vitex simplicifolia* on liver glycogen content and lipid profile on alloxan induced diabetic rats.

Groups	Glycogen (mg/g)	Total Chol (mmol/l)	Triglyceride (mmol/l)	HDL (mmol/l)	LDL (mmol/l)
1	3.28±0.09	0.24±0.01	0.12±0.05	0.20±0.03	0.02±0.03
2	1.96±0.24	0.27±0.01	0.11±0.03	0.12±0.02	0.10±0.01
3	2.88±0.02	0.14±0.11	0.08±0.03	0.13±0.06	0.07±0.04
4	1.91±0.34	0.12±0.01	0.06±0.012	0.06±0.012	0.21±0.02
5	2.10±0.10	0.12±0E-7	0.11±0.001	0.04±0.01	0.21±0.02
6	3.60±0.20[b]	0.07±0.05	0.13±0.04	0.09±0.01	0.23±0.02

Grp 1: Normal control, Grp 2: Negative control, Grp 3: Positive control, Grps 4, 5 and 6 received 250,500 and 1000mg/kg of extract, respectively. Total chol = Total cholesterol. Values are presented as mean ± standard error of mean. [b] = significantly different ($p<0.05$) from the Negative control.

Table 4.15: The effect of N hexane fraction of leaf extract of *Vitex simplicifolia* on glucose, liver glycogen content and lipid profile on alloxan induced diabetic rats.

Groups	Glycogen (mg/g)	Total Chol (mmol/l)	Triglyceride (mmol/l)	HDL (mmol/l)	LDL (mmol/l)
1	3.28±0.09	0.23±0.26	0.12±0.05	0.20±0.03	0.24±0.00
2	1.96±0.24	0.27±0.01	0.11±0.03	0.12±0.00	0.10±0.00
3	2.88±0.020	0.12±0.11	0.08±0.00	0.13±0.06	0.07±0.00
4	2.94±0.01	0.24±0.00	0.36±0.27	0.32 ± 0.00^{bc}	0.12 ± 0.01^{a}
5	6.02 ± 0.50^{abc}	6.308±0E-7	0.08±0.01	$0.04\pm0E7^{a}$	0.02 ± 0.01^{abc}
6	3.180 ± 0.00^{b}	0.234±0.04	0.116±0.014	0.39 ± 0.04^{abc}	0.21 ± 0.00^{abc}

Grp 1: Normal control, Grp 2: Negative control, Grp 3: Positive control, Grps 4, 5 and 6 received 250,500 and 1000mg/kg of extract, respectively. Total chol = Total cholesterol. Values are presented as mean ± standard error of mean. ^{a} = significantly different ($p<0.05$) from the Normal control, ^{b} = significantly different ($p<0.05$) from the Negative control, ^{c} = significantly different ($p<0.05$) from Positive control.

Table 4.16: Effect of oral administration of aqueous fraction of leaf extract of *Vitex simplicifolia* on the activity of SOD and level of vitamin A, C, E on alloxan induced diabetic rats.

Groups	SOD (µmole/min/mg protein)×10^5	VIT-A (mg/dl)	VIT-C (mg/dl)	VIT-E (mg/dl)
1	1.57±0.28	632.40±34.50	0.53±0.24	20.60±0.88
2	0.41±0.16	499.67±113.92	0.28±0.14	22.96±1.73
3	0.44±0.39	46.000±1.00	0.92±0.08	23.52±3.49
4	-5942±0.86	18.75±8.67[ab]	0.60±0.11	17.22±2.00
5	-3990±0.93	451.33±47.16[c]	0.28±0.42	16.54±7.35
6	-3915±0.28	295.00±97.00	0.38±0.04	18.69±0.43

Grp 1: Normal control, Grp 2: Negative control, Grp 3: Positive control, Grp 4, 5 and 6 received 250,500 and 1000mg/kg of extract, respectively. Values are presented as mean ± standard error of mean. [a] = significantly different ($p<0.05$) from the Normal control, [b] = significantly different ($p<0.05$) from the Negative control, [c] = significantly different ($p<0.05$) from Positive control.

Table 4.17: Effect of oral administration of methanolic fraction of leaf extract of *Vitex simplicifolia* on the activity of SOD and level of vitamin A, C, E on alloxan induced diabetic rats.

Groups	SOD (µmole/min/mg protein)×10^5	VIT-A (mg/dl)	VIT-C (mg/dl)	VIT-E (mg/dl)
1	1.57±0.28	632.40±34.50	0.53±0.24	20.60±0.88
2	0.40±0.32	499.67±113.92	0.28±14	22.69±1.73
3	0.44±0.09	46.00±21.79	0.92±0.13	23.52±3.49
4	0.79±0.089[a]	79.67±21.79[ab]	0.64±0.13	23.45±1.85
5	0.50±0.10	124.00±1.00[b]	0.58±0.01	27.45±1.00
6	0.100±0E-7[a]	313.50±59.50	0.59±0.10	19.085±0.18

Grp 1: Normal control, Grp 2: Negative control, Grp 3: Positive control, Grp 4, 5 and 6 received 250,500 and 1000mg/kg of extract, respectively. Values are presented as mean ± standard error of mean. [a] = significantly different ($p<0.05$) from the Normal control, [b] = significantly different ($p<0.05$) from the Negative control, [c] = significantly different ($p<0.05$) from Positive control.

Table 4.18: Effect of oral administration of ethyl acetate fraction of leaf extract of *Vitex simplicifolia* on the activity of SOD and level of vitamin A, C, E on alloxan induced diabetic rats.

Groups	SOD (µmole/min/mg protein)×10^5	VIT-A (mg/dl)	VIT-C (mg/dl)	VIT-E (mg/dl)
1	1.57±0.28	632.40±34.50	0.53±0.24	20.60±0.88
2	0.41±0.16	499.67±113.92	0.28±0.14	22.69±1.73
3	0.44±0.32	46.00±1.00	0.92±0.79	23.52±3.49
4	0.74±0.27	144.67±19.88[ab]	0.65±0.17	24.12±1.10
5	0.80±0.20	249.00±2.00[a]	0.40±0.10	26.40±1.10
6	0.90±0.40	240.00±5.00[a]	0.55±0.10	20.60±0.88

Grp 1: Normal control, Grp 2: Negative control, Grp 3: Positive control, Grp 4, 5 and 6 received 250,500 and 1000mg/kg of extract, respectively. Values are presented as mean ± standard error of mean. [a] = significantly different ($p<0.05$) from the Normal control, [b] = significantly different ($p<0.05$) from the Negative control, [c] = significantly different ($p<0.05$) from Positive control.

Table 4.19: Effect of oral administration of N hexane fraction of leaf extract of *Vitex simplicifolia* on the activity of SOD and level of vitamin A, C, E on alloxan induced diabetic rats.

Group	SOD (µmole/min/mg protein)×10^5	VIT-A (mg/dl)	VIT-C (mg/dl)	VIT-E (mg/dl)
1	1.57±0.28	632.40±72.00	0.53±0.23	20.60±0.88
2	0.41±1.16	499.67±113.92	0.28±0.14	22.69±1.73
3	0.44±0.32	46.00±1.00	0.92±0.08	23.52±3.49
4	0.90±0.58	27.33±6.06[ab]	0.97±0.03	21.67±0.60
5	0.23±0.23[a]	771.00±0.00[b]	1.00±0.57	20.00±0.00
6	1.22±0.29	72.00±26.00[ab]	0.50±0.50	19.45±0.45

Grp 1: Normal control, Grp 2: Negative control, Grp 3: Positive control, Grp 4, 5 and 6 received 250,500 and 1000mg/kg of extract, respectively. Values are presented as mean ± standard error of mean. [a] = significantly different ($p<0.05$) from the Normal control, [b] = significantly different ($p<0.05$) from the Negative control, [c] = significantly different ($p<0.05$) from Positive control.

Table 4.20: Effect of oral administration of aqueous fraction of leaf extract of *Vitex simplicifolia* on liver enzymes on alloxan induced - diabetic rats.

GROUP	ALP/ (U/l)	AST/ (U/l)	ALT/ (U/l)	U.Bil (µmol/l)
1	200.33±7.40	84.67±4.70	39.00±3.06	1.148±0.325
2	1233.5±76.30[a]	25.82±0.61[a]	26.50±0.96	0.427±0.109
3	692.33±108.2[a]	43.81±1.74[a]	44.00±7.50	1.253±0.553
4	326.00±14.00[a]	38.50±2.50[a]	57.00±2.00	0.920±0.170

Grp 1: Normal control, Grps 2, 3 and 4 received 250, 500 and 1000mg/kg of extract, respectively. Values are presented as mean ± standard error of mean. [a] = significantly different ($p<0.05$) from the Normal control.

Table 4.21: Effect of oral administration of methanolic fraction of leaf extract of *Vitex simplicifolia* on liver enzymes alloxan induced diabetic rats.

GROUP	ALP/ (U/l)	AST/ (U/l)	ALT/ (U/l)	U.Bil (µmol/l)
1	200.33±7.84	84.667±4.70	39.00±3.05	1.148±0.325
2	354.0±67.95[a]	27.68±1.03[a]	33.00±1.73	0.633±0.064
3	939.00±0.00[a]	67.00 ±1.00[a]	36.00±1.00	0.720±0.110
4	1119.0±17.00[a]	26.86±0.44[a]	45.50±3.50	0.585±0.055

Grp 1: Normal control, Grps 2, 3 and 4 received 250, 500 and 1000mg/kg of extract, respectively. Values are presented as mean ± standard error of mean. [a] = significantly different ($p<0.05$) from the Normal control.

Table 4.22: Effect of oral administration of ethyl acetate fraction of leaf extract of *Vitex simplicifolia* on liver enzymes alloxan induced diabetic rats.

GROUP	ALP/ (U/l)	AST/ (U/l)	ALT/ (U/l)	U.Bil (µmol/l)
1	200.33±7.84	84.667±4.70	39.00±3.05	1.148±0.325
2	575.00±16.86[a]	74.13±1.03	53.00±0.57[a]	2.367±0.3
3	171.00±1.00	23.00±1.00[a]	41.00±1.00	0.84±0.09
4	647.00±6.00[a]	0.424±0.30[a]	50.50±1.50[a]	0.53±0.070

Grp 1: Normal control, Grps 2, 3 and 4 received 250, 500 and 1000mg/kg of extract, respectively. Values are presented as mean ± standard error of mean. [a] = significantly different ($p<0.05$) from the Normal control.

Table 4.23: Effect of oral administration of N hexane fraction of leaf extract of *Vitex simplicifolia* on liver enzymes alloxan induced diabetic rats.

GROUP	ALP/ (U/l)	AST/ (U/l)	ALT/ (U/l)	U.Bil (µmol/l)
1	200.33±7.84	84.667±4.70	39.00±3.05	1.148±0.325
2	369.67±7.79	108.89±12.03	27.67±0.57	0.28±0.115
3	137.00±10.00[a]	24.00 ±2.00[a]	21.00±1.00[a]	0.89±0.79
4	786.50±237.50[a]	107.00±13.00	30.00±0.00	2.28±0.550

Grp 1: Normal control, Grps 2, 3 and 4 received 250, 500 and 1000mg/kg of extract, respectively. Values are presented as mean ± standard error of mean. [a] = significantly different ($P < 0.05$) from the Normal control.

Table 4.24: Effect of oral administration of aqueous fraction of leaf extract of *Vitex simplicifolia* on urea, creatinine and electrolytes in alloxan induced diabetic rats.

Groups	Urea (mg/dl)	Creatinine (µmol/l)	Na^+ (mEq/L)	K^+ (mEq/L)	Cl^- (mEq/L)	HCO_3^- (mEq/L)
1	25.72± 0.83	186.33±3.18	219.48±2.31	5.86 ±0.80	75.71±1.04	44.33±1.45
2	100.3± 1.38[a]	249.25±2.93[a]	207.39±9.89	5.29 ±0.05	89.09±0.92	25.25±1.11[a]
3	142.53±2.03[a]	278.00±17.32[a]	240.39±8.15	5.59±0.1	88.1±4.23	38.33±2.03
4	99.52 ± 7.10[a]	113.50±4.50[a]	191.08±8.00	4.60±0.91	84.5±7.97	30.00±2.00[a]

Grp 1: Normal control, Grps 2, 3 and 4 received 250, 500 and 1000mg/kg of extract, respectively. Values are presented as mean ± standard error of mean. [a] = significantly different ($p<0.05$) from the Normal control.

Table 4.25: Effect of oral administration of methanolic fraction of leaf extract of *Vitex simplicifolia* on urea, creatinine and electrolytes in alloxan induced diabetic rats.

Groups	Urea (mg/dl)	Creatinine (µmol/l)	Na^+ (mEq/L)	K^+ (mEq/L)	Cl^- (mEq/L)	HCO_3^- (mEq/L)
1	25.72± 0.83	186.33±3.18	219.48±2.31	5.86 ±0.80	75.71±1.04	44.33±1.45
2	67.52±2.56[a]	118.67±23.68[a]	227.75±8.02	5.69±0.43	87.38±2.99	33.00±1.55[a]
3	112.38± 2.00[a]	185.00±1.00	221.98±0.00	5.70±0.10	78.150±2.00	36.00±2.00
4	85.57± 2.46[a]	149.00±2.00	249.33±5.91[a]	4.44±0.20[a]	89.38±2.24[a]	38.00±2.00[a]

Grp 1: Normal control, Grps 2, 3 and 4 received 250, 500 and 1000mg/kg of extract, respectively. Values are presented as mean ± standard error of mean. [a] = significantly different ($p<0.05$) from the Normal control.

Table 4.26: Effect of oral administration of ethyl acetate fraction of leaf extract of *Vitex simplicifolia* on urea, creatinine and electrolytes in alloxan induced diabetic rats.

Groups	Urea (mg/dl)	Creatinine (µmol/l)	Na^+ (mEq/L)	K^+ (mEq/L)	Cl^- (mEq/L)	HCO_3^- (mEq/L)
1	25.72± 0.83	186.33±3.18	219.48±2.31	5.86 ±0.80	75.71±1.04	44.33±1.45
2	156.62±3.10[a]	158.61±3.10[a]	190.43±3.31[a]	3.73±0.15	88.37±1.52[a]	24.33±1.20[a]
3	122.61±2.00[a]	125.00±2.00[a]	243.61±3.00[a]	3.54±0.10	80.78±1.00	42.00±2.00
4	77.27±1.29[a]	147.00±2.00[a]	244.94±2.15[a]	5.18 ±2.1	88.30±0.64[a]	42.00±1.00

Grp 1: Normal control, Grps 2, 3 and 4 received 250, 500 and 1000mg/kg of extract, respectively. Values are presented as mean ± standard error of mean. [a] = significantly different ($p<0.05$) from the Normal control.

Table 4.27: Effect of oral administration of n- hexane fraction of leaf extract of *Vitex simplicifolia* on urea, creatinine and electrolytes in alloxan induced diabetic rats.

Groups	Urea (mg/dl)	Creatinine (µmol/l)	Na^+ (mEq/L)	K^+ (mEq/L)	Cl^- (mEq/L)	HCO_3^- (mEq/L)
1	25.72± 0.83	186.33±3.18	219.48±2.31	5.86 ±0.80	75.71±1.04	44.33±1.45
2	56.19± 0.74[a]	236.00±2.301[a]	211.34±2.89[a]	5.00±E0-7	92.91±1.07	61.00±1.15[a]
3	70.00± 0E-7[a]	25.00±1.00[a]	191.00±1.00[a]	5.00 ±E0-7	74.00±E0-7	90.00±1.00[a]
4	91.50± 0.50[a]	259.50±63.50[a]	190.50±15.50[a]	5.00 ±E0-7	68.50±12.50	51.50±1.50

Grp 1: Normal control, Grps 2, 3 and 4 received 250, 500 and 1000mg/kg of extract, respectively. Values are presented as mean ± standard error of mean. [a] = significantly different ($p<0.05$) from the Normal control.

4.3 Histopathology Result

4.3.1 Liver

Histopathological examination of the rats liver from normal group 1 (Plate 1) showed normal hepatocytes arrange as radiating cord forming hexagonal units containing a central venules and that of the aqeous fraction (Plate 2) where as that of the methanol fraction of the extract (Plate 3) shows mild sinusoidal,lymphocytosis and mild expansion. The liver section of the ethyl acetate fraction (Plate 4) and that of the N hexane fraction (Plate 5) showed moderate sinoidal lymphocytosis, prominent kupfer cells and focal portal inflammation indicating liver damage, but the section of the liver of the positive control (Plate 6) is normal after 3 weeks of administering glibenclamide**.**

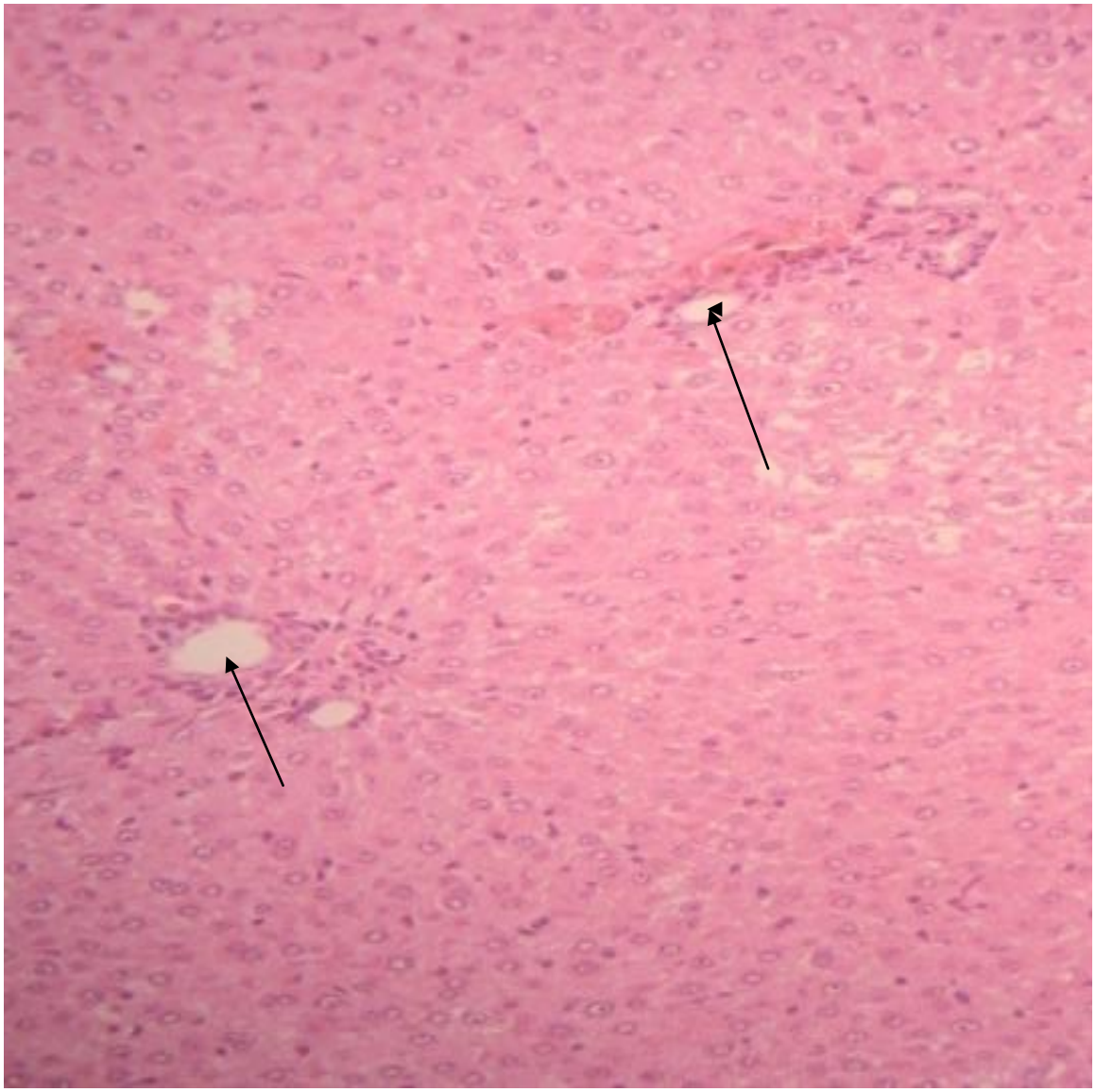


Plate 11:Photomicrograph of a section of liver from untreated Wistar rat showing the portal tract area (black arrows), with no pathological changes (H and E Stain, ×250).

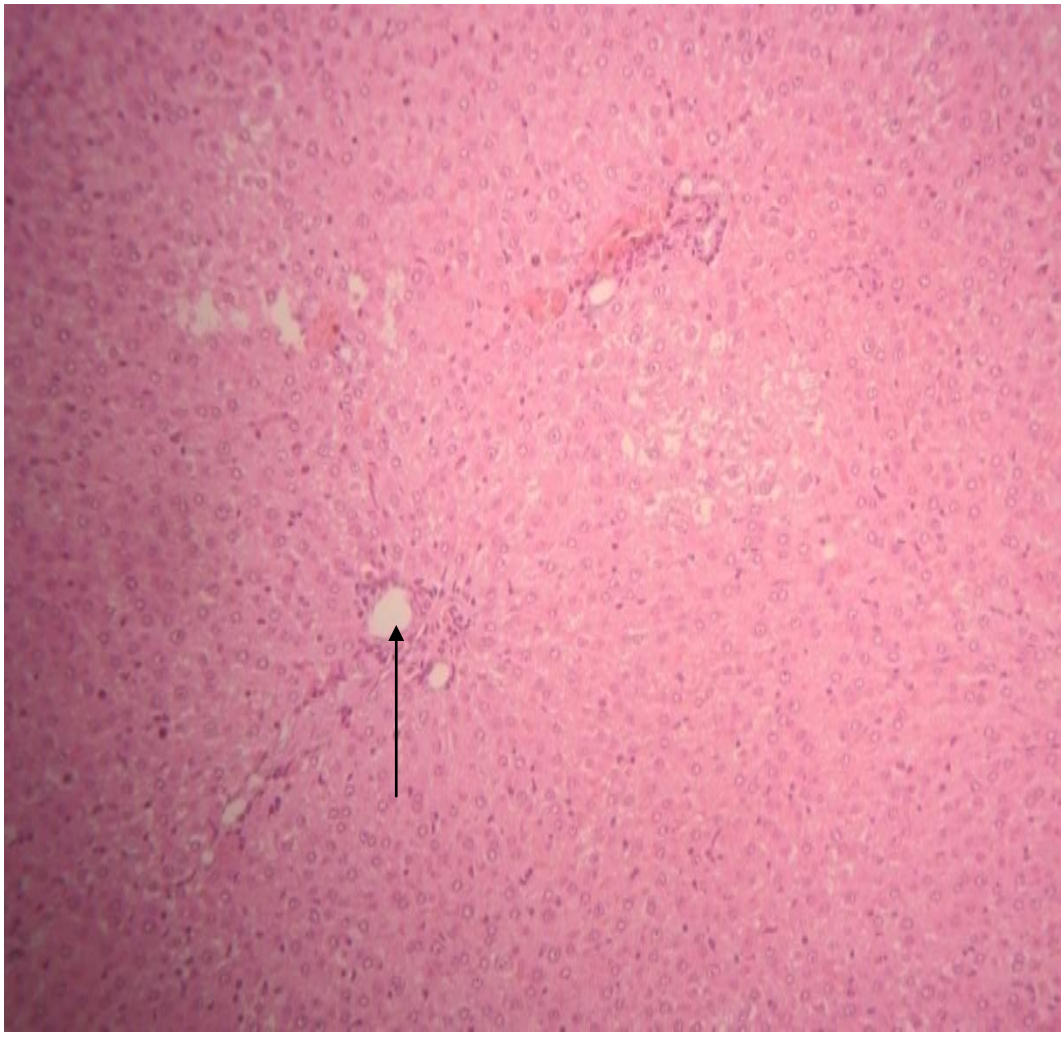


Plate III: Photomicrograph of section of liver of alloxan induced wistar rats that were administered 1000 mg/kg of aqueous fraction of *Vitex simplicifolia* leaf extract for twenty one days showing portal tract area(black arrows), with no pathological changes (H and E stain x250).

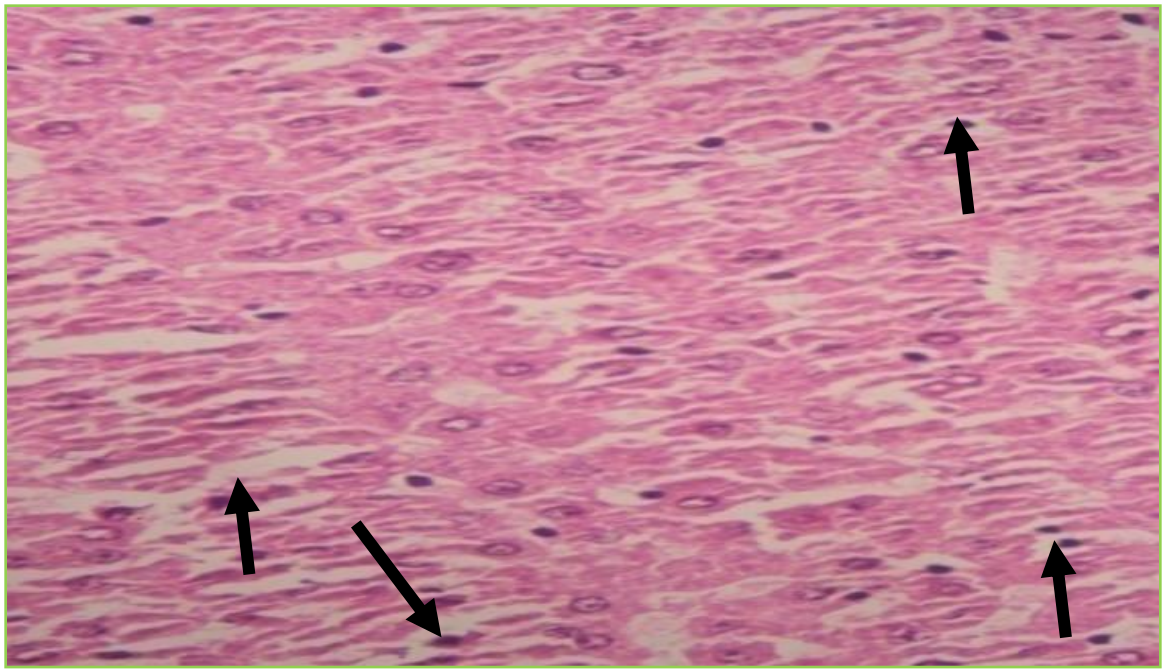


Plate IV: III:Photomicrograph of a section of liver from rats that were administered 1000 mg/kg methanolic fraction of *Vitex simplicifolia* leaf extract showing the portal tract area (black arrows), with mild sinusoidal lymphocytosis and mild expansion (H and E stain x250).

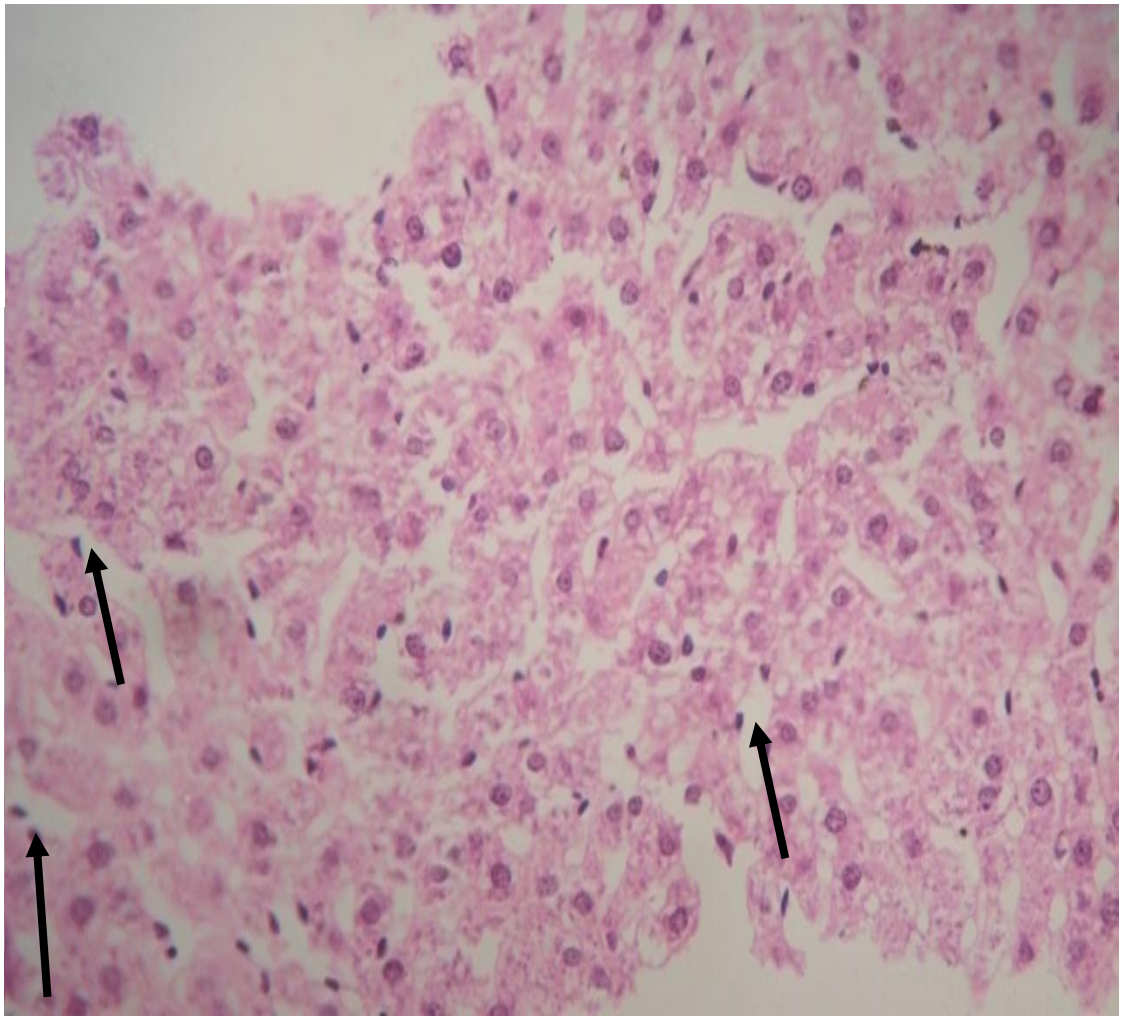


Plate V: Photomicrograph of a section of liver from wistar rats administered ethyl acetate fraction of *Vitex simplicifolia* leaf extract for twenty one days, showing the portal tract area(black arrows), with mild fatty change, moderate sinusoidal lymphocytosis, with prominent kupfer cells (H and E stain x250).

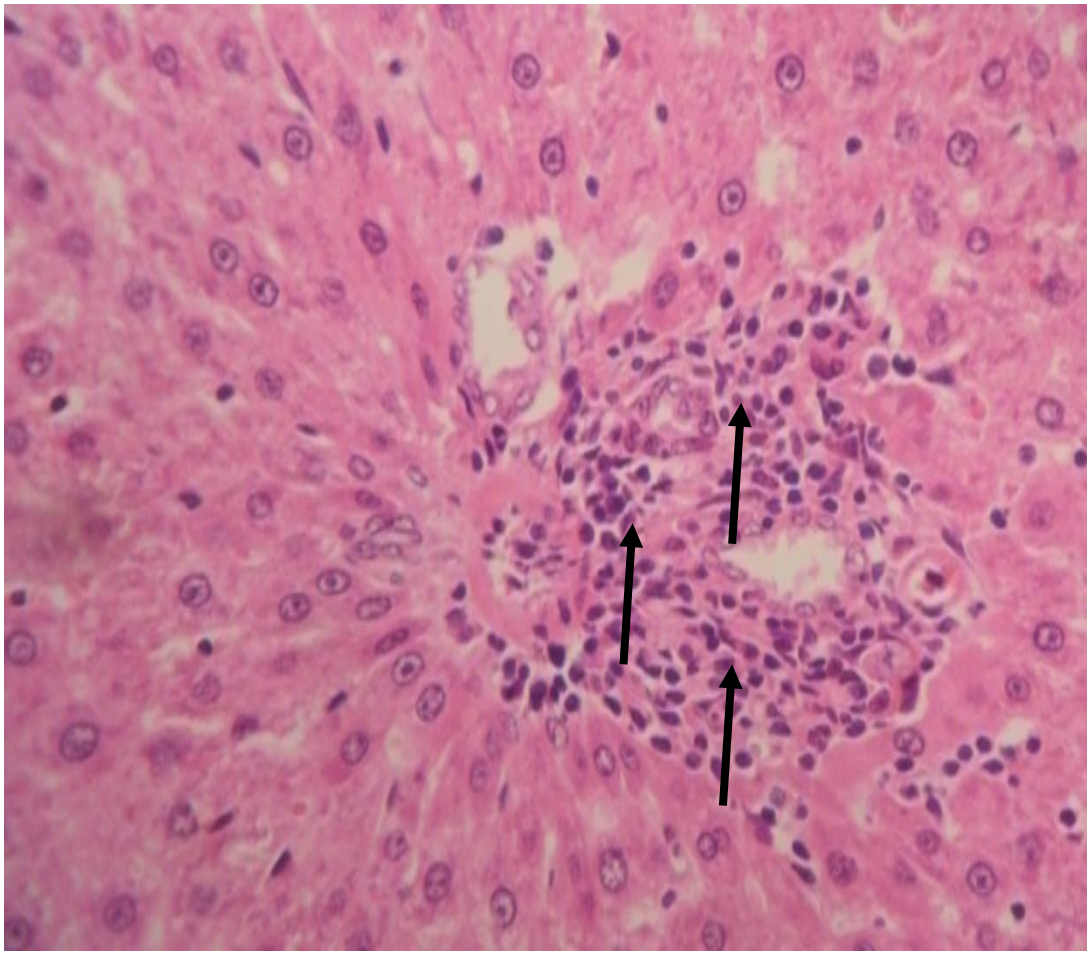


Plate VI: Photomicrograph of a section from a liver of the wistar rats administered 1000 mg/kg of N- hexane fraction of *Vitex simplicifolia* leaf extract,showing the portal tract area (black arrows), with mild increase in kupfer cells, focal portal inflammation (H and E stain x250).

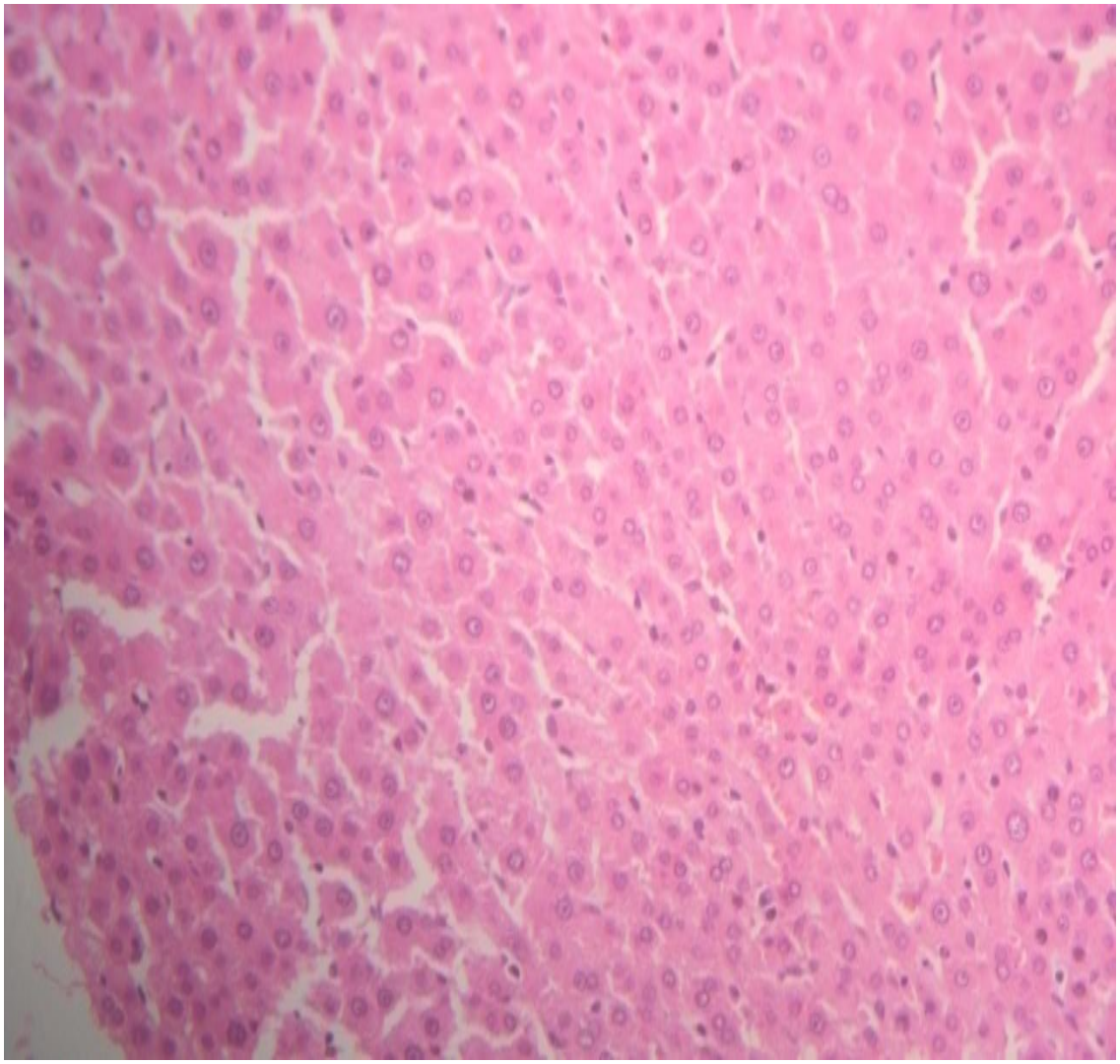


Plate VII: Photomicrograph of a section of liver from alloxan induced diabetic wistar rats administered with glibenclamide, showing the portal tract area, with no pathological changes (H and E stain x250).

4.3.2 The Kidney

Histopathology of the rats in the normal group 1(Plate 7) showed normal kidney architecture with cortex containing the glomerulus and the medulla while that of the rats administered with the aqueous fraction of *Vitex simplicifolia* leaf extract (Plate 8) showed mild damage to the distal tubules and that of ethyl acetate fraction (Plate 10) showed moderate distal tubular epithelial cell edema and the section the kidney of the rats administered with N-hexane fraction (Plate 11) showed mild interstitial inflammation, where as that of the rats administered with methanol fraction (Plate 9) and the positive control rats administered with glibenclamide (Plate 12) are normal.

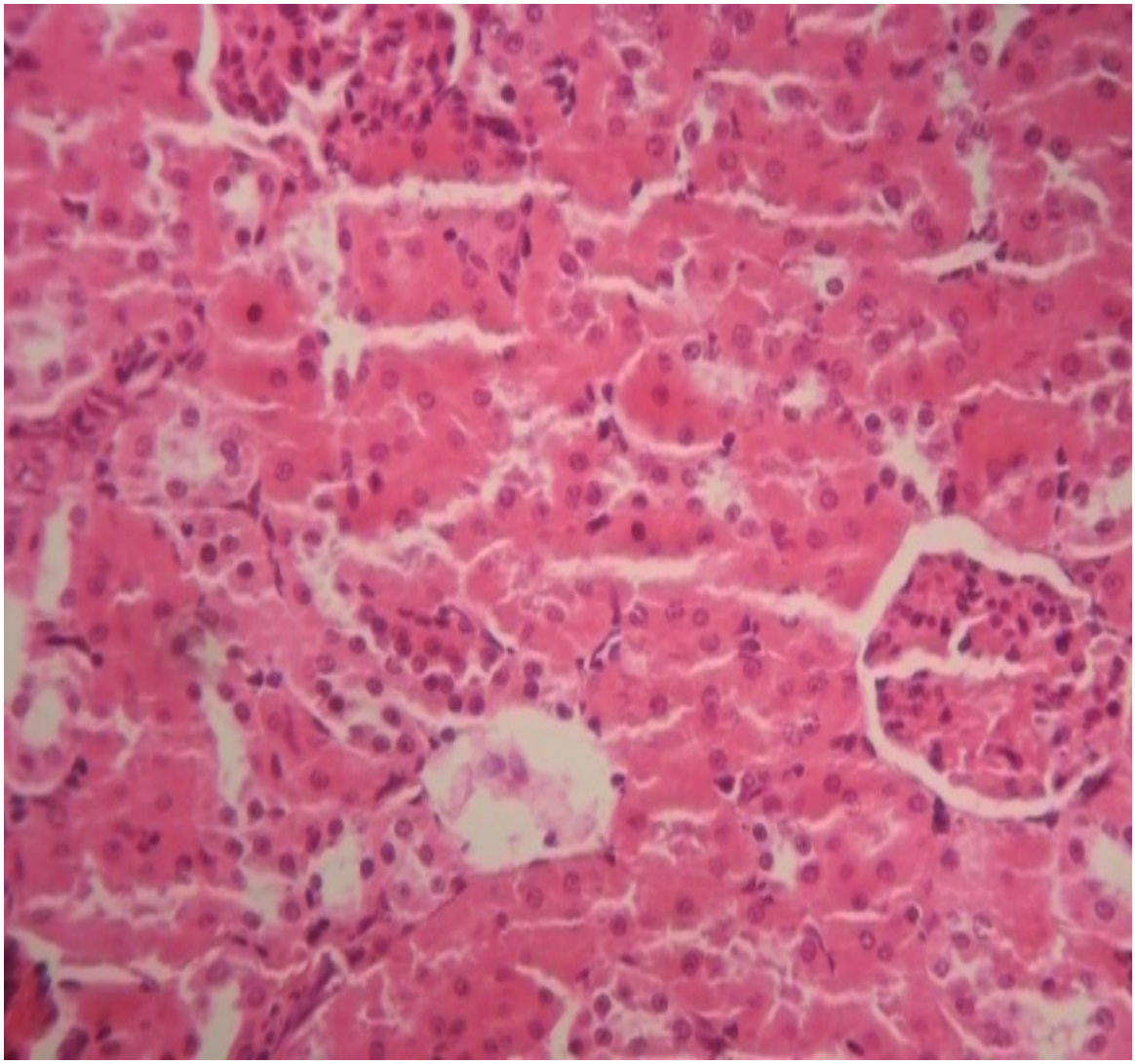


Plate VIII: Photomicrograph of a section of kidney from wistar that were not administered extracts, showing the medullary area, with no pathological changes (H and E stain x250).

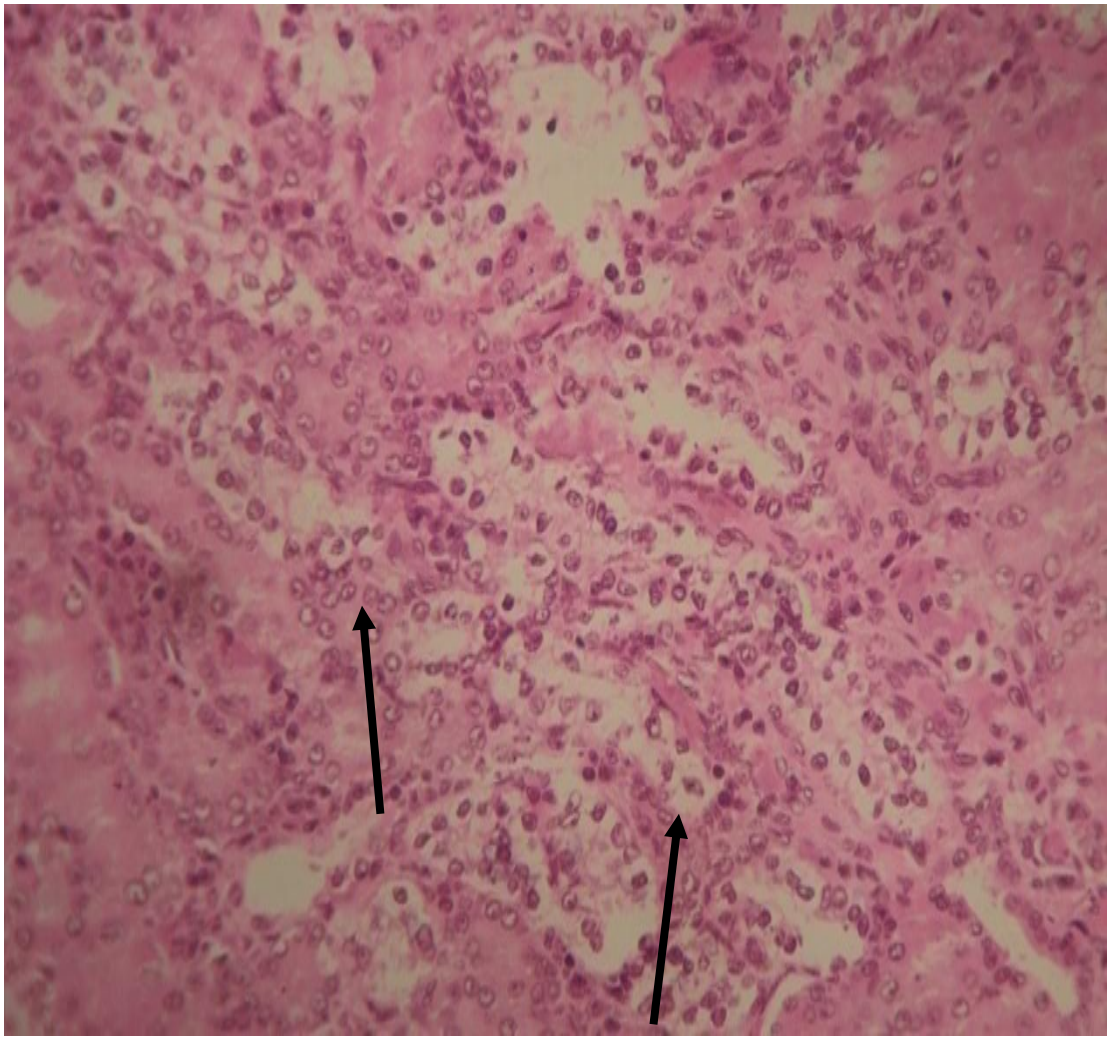


Plate IX: Photomicrograph of cross section of kidney of alloxan induced diabetic wistar rat administered 1000mg/kg of aqueous fraction of *Vitex simplicifolia* leaf extract for twenty one days, showing (black arrows) distortion of the tubules and the separation of the lining epithelium (H and E stain x250).

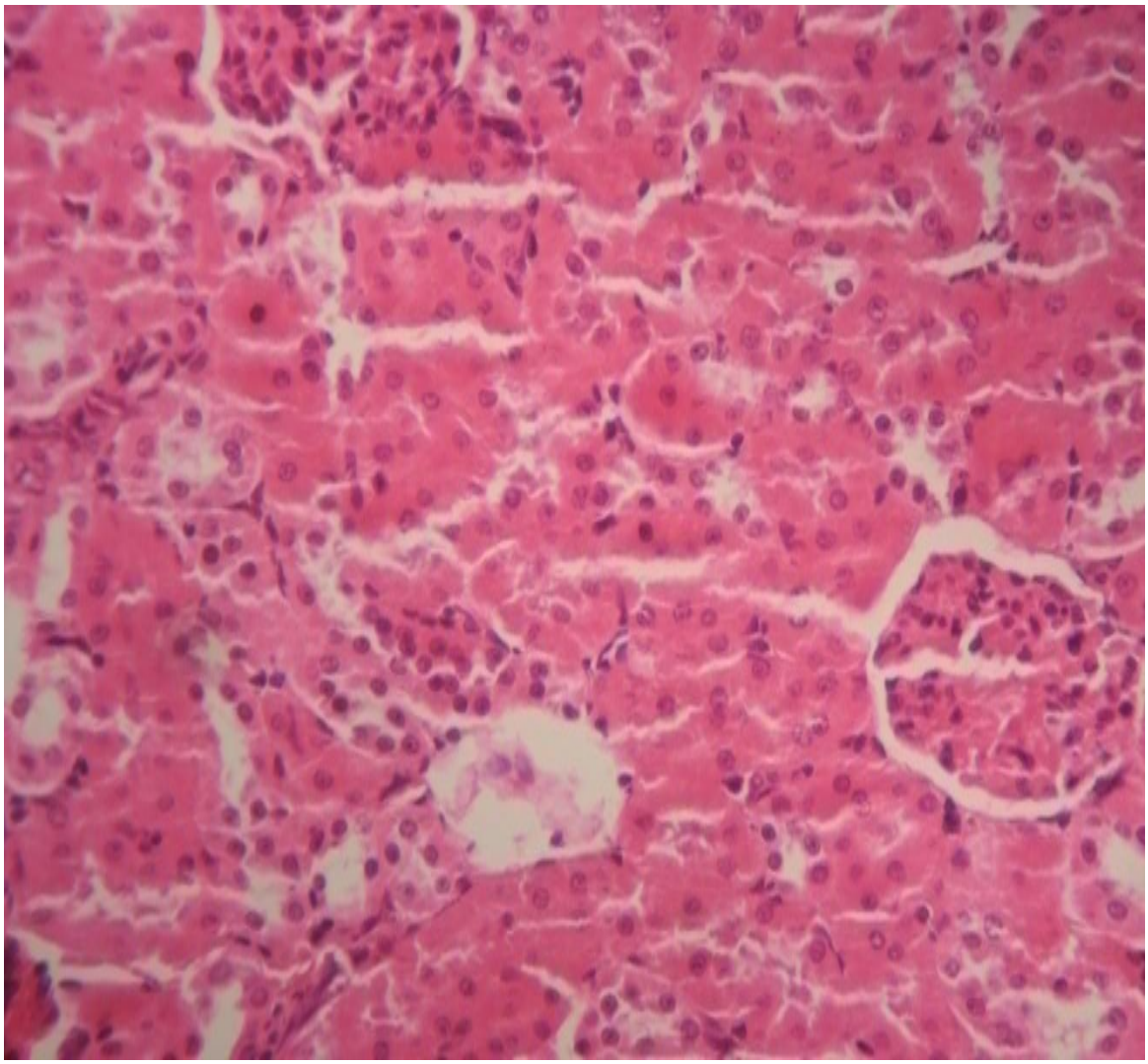


Plate X: Photomicrograph of a section of kidney from diabetic rats that were administered 1000 mg/kg of methanol fraction of *Vitex simplicifolia* extract showing no pathological changes (normal) (H and E Stain ×250)

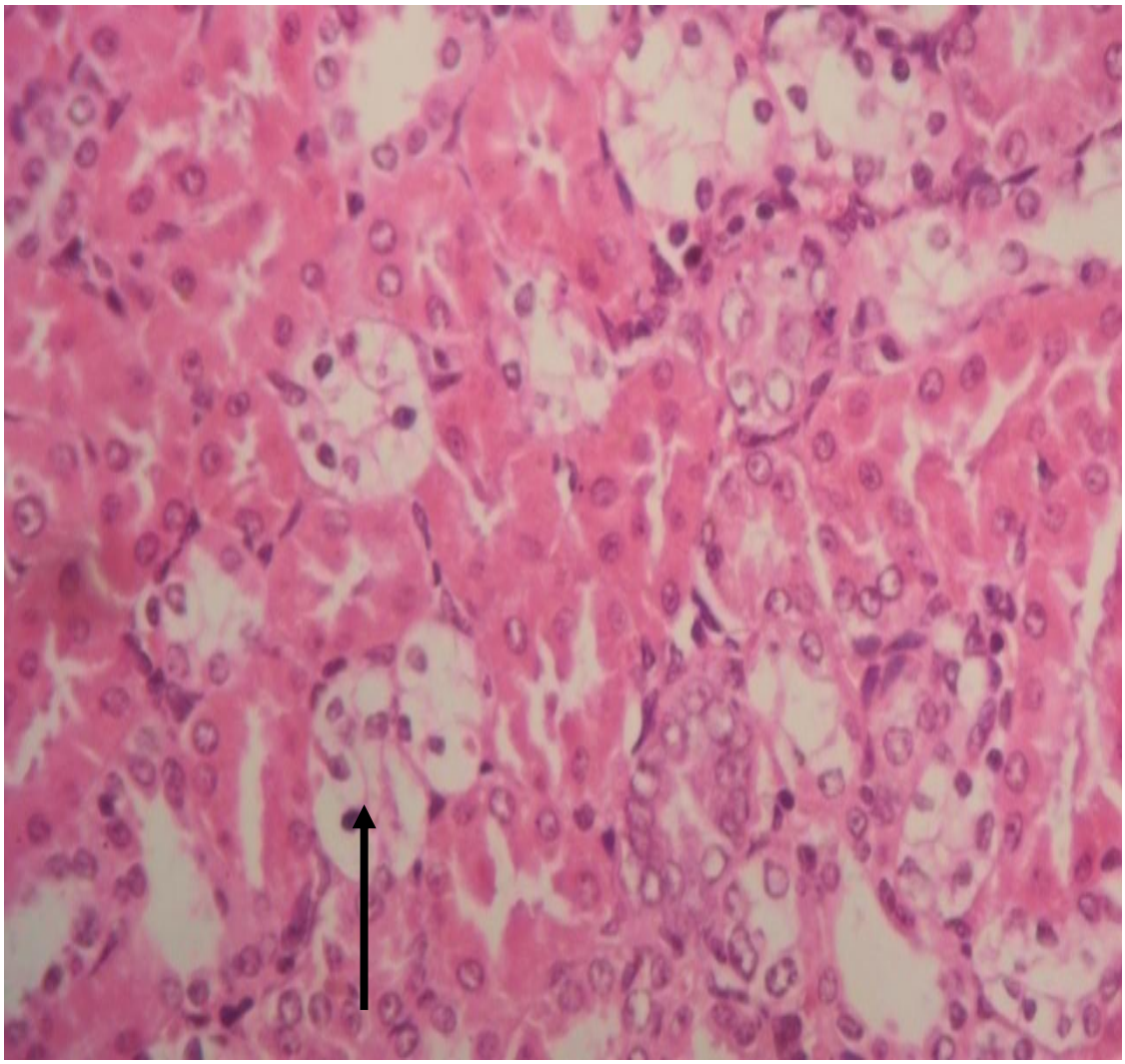


Plate XI: Photomicrograph cross section of kidney from diabetic wistar rat administered 1000 mg/kg of ethyl acetate fraction of *Vitex simplicifolia* leaf extract for twenty one days, showing medulla of the kidney (black arrows) with several tubules and lining epithelia demonstrating hydropic swelling (H and E stain x250).

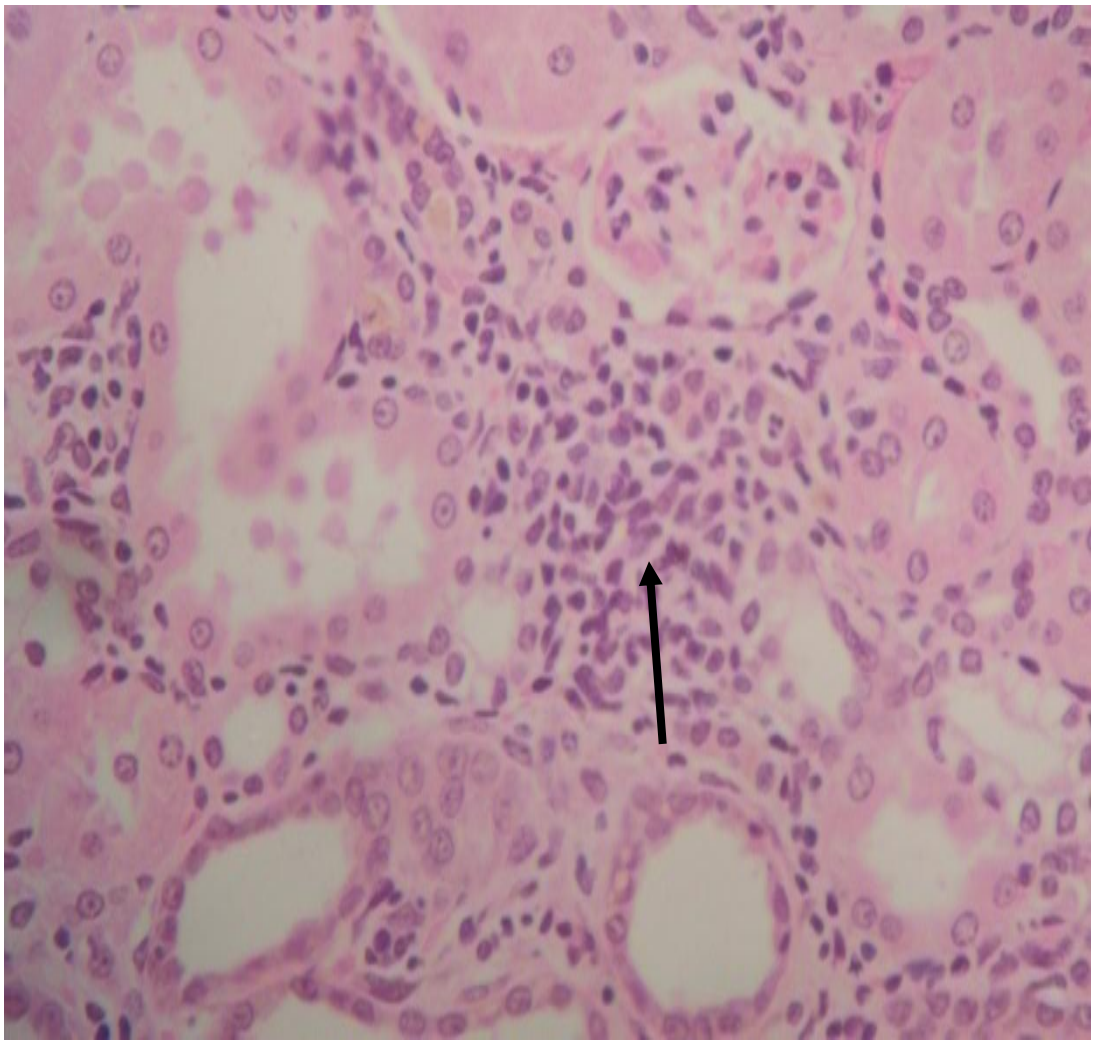


Plate XII: Photomicrograph cross section of kidney of alloxan induced diabetic wistar rat administered 1000mg/kg of n- hexane fraction of *Vitex simplicifolia* leaf extract for twenty one days,showing medulla of the kidney(black arrows) with multifocal expansion of the interstitium by aggregates of lymphocytes and histiocytes (H and E stain x250).

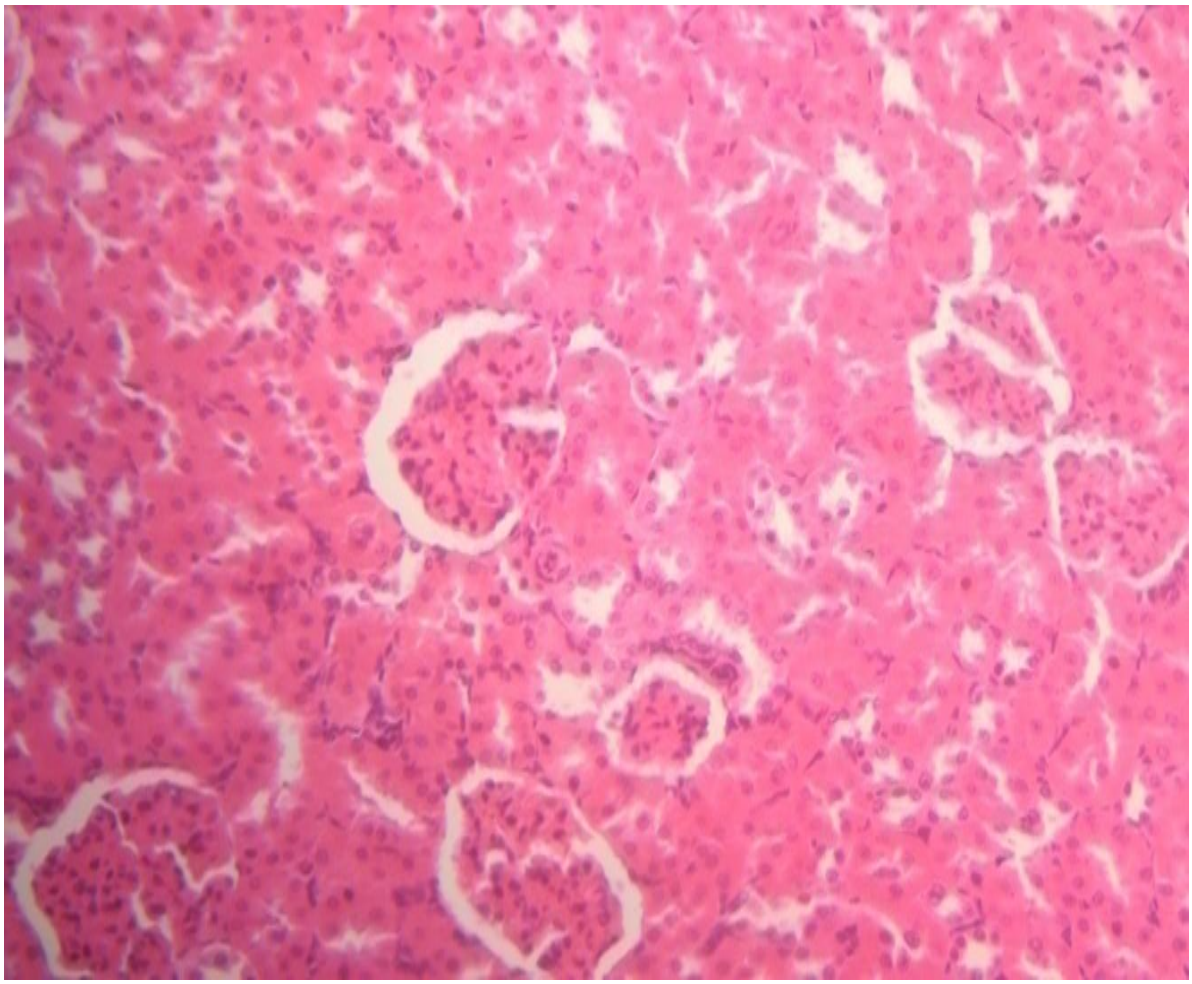


Plate XIII: Photomicrograph cross section of kidney of diabetic wistar rat administered 10 mg/kg of glibenclimade for twenty one days, showing medulla of the kidney no pathological changes (H and E stain x250).

CHAPTER FIVE

5.0 DISCUSSION

The result of acute toxicity study indicated that the LD_{50} of the leaf extract of *Vitex simplicifolia* is greater that 5000mg/kg body weight. Thus, the non-lethal effects produced with the high dose of this extract are an indication that the leaf extracts of *Vitex simplicifolia* is relatively safe on acute oral exposure. It can therefore be concluded that *Vitex simplicifolia* leaf extract is non-toxic, which is in agreement the reported study by Abdelmajid (2014) on essential oil of the leaves of *Vitex simplicifolia* and with Bruce (1987), American Society for Testing and Materials (1987), Aditya and Ravi (2014),Kingsley *et al* (2014) and Ravichandra *et al* (2014), that any chemical substance with LD_{50} estimate greater than 3000-5000mg/kg (oral route) could be considered of low toxicity and safe.

The use of plants in the treatment of disease and in particular diabetes mellitus is as old as man (Sofowora, 1993; Muhammad *et al.*, 2015). This is because plants have shown to contain some potent bioactive compounds with antidiabetic properties (Tanko *et al.*, 2013). In this study, diabetes established on the basis of fasting blood glucose concentration in the alloxan treated rats on 5^{th} day of the experimental period formed the baseline values (Table 4.10, 4.11 and 4.12). The result indicated that daily oral administration of reference drug and the fractionated extracts (aqueous, methanolic, ethylacetate and N-hexane) of *Vitex simplicifolia* for 21 days shows significant reduction in fasting blood glucose with aqueous fraction showing 72, 75 and 81%, n-hexane fraction 58, 71 and 77% reduction, ethyl acetate 54, 70 and 71, while the reference drug

(glibenclamide) 65% and methanol, 8, 16 and 17%. All the fractions with the exception of methanol fraction showed more potency then glibenclamide (reference drug). The observed anti diabetic effect of the fractionated extracts of *Vitex simplicifolia* is an indication that the extracts contain bioactive phytochemicals with potent antidiabetic property. Antidiabetic activity of *Vitex simplicifolia* has never been reported to our knowledge

The hypoglycemic action of *Vitex simplicifolia* may be of the following mechanism; inhibition of renal glucose reabsorption, enhanced secretion of insulin from existing B-cells of the pancreas, increased tissue uptake of glucose by enhancement of insulin sensitivity, as reported for flavonoids and saponins (Srinivasan et al,2008; Amy et al, 2011;Okpe *et al.,* 2012). Flavonoids and saponins, among other secondary metabolites were found to be present in the leaves of *Vitex simplicifolia* in this study (Table 4.9)

Oral administrations of fractionated extracts (aqueous, methanolic, ethylacete and n-hexane) of *Vitex simplicifolia* for 21 days significantly increase liver glycogen content. The liver glycogen content is significantly lower in the diabetic control compared to the normal control. This finding does not appear to be related to dietary conditions, since all experimental animals were feed the same type of feed. It is possible that diabetic subjects have defects in liver and muscle glycogen synthesis (Shulman *et al*, 1990). The increase in liver glycogen content is by possible reversal of the earlier mentioned (defects in liver and glycogen synthesis).

In this study all the fractionated extracts of *Vitex simplicifolia* (aqueous, methanol, ethylacetate, n-hexane) showed no significant effect on lipid profile of the experimental

animals. Hypolipidemic activity of *Vitex simplicifolia* has never been reported to best of our knowledge. The fractionated extracts (aqueous, methanolic, ethylacetate and N-hexane) of *Vitex simplicifolia* have no significant effect on the level of total cholesterol and low density lipoprotein. These observations may be attributed to the gut intra – luminal interactive effect of saponin. Saponins are known anti nutritional factors which reduce the uptake of certain nutrients including glucose and lipid especially cholesterol at the gut through intra-lumena physicochemical interaction. Hence saponins have been reported to have hypocholestrolemic effect (Price *et al.,* 1987). Saponin, among other secondary metabolites is found to be present in the leaves of *Vitex simplicifolia* in this study (Table 4.9) the low concentration of cholesterol may have contributed to the observed non – significant high serum HDL – cholesterol in the experimental animals. About 30% of blood cholesterol is carried in the form of HDL and it is hypothesized that HDL – cholesterol can remove cholesterol from antheroma within arteries and transport it back to the liver. HDL – cholesterol protect against cardiovascular disease (Okpe *et al.,* 2012). The observed non – significant ($P>0.05$) increase in HDL – cholesterol concentration after administration of the fractionated extracts (250, 500 and 1000 mg/kg bw) indicates that the fractions (aqueous, methanolic, ethylacetate and n-hexane) does not have HDL – cholesterol boosting effect and it does not also have significant ($P>0.05$) LDL – cholesterol lowering effect at these concentrations in induced diabetic experimental animals.

The administration of aqueous, methanolic and ethyl acetate fractionated extracts of *Vitex simplicifolia* at 250, 500 and 1000 mg/kg doses for 21 days orally was observed to significantly increase ALP ($P>0.05$) and decrease significantly ($P>0.05$) AST and had no

significant change in ALT and Unconjugated billirubin. The elevation of levels of Alkaline Phosphatase (ALP) as observed in the present study may be an indication of either liver or bone disease, since the two main sources of ALP are liver and bone. However the experimental animals treated with n- hexane fraction of *Vitex simplicifolia* showed significant increase in ALP, AST and Unconjugated billirubin. The indicators of liver function were all increased indicating possible liver damage. ALT is a cystosolic enzyme more specific to the liver, so a rise only occurs with liver diseases (Almdal *et al.,* 2008).

Thus a high level of bilirubin is used as indices for liver function and bile excretion status (Abdurrahman *et al.,* 2007). In this study the n- hexane fraction of *Vitex simplicifolia* showed a significant increase in unconjugated bilirubin, an indication of liver disease.

The level of these marker enzymes particularly the n-hexane treated group is a strong index of liver toxicity.There is paucity of data on the toxic effect of *Vitex simplicifolia*.

The administrations of aqueous extract of *Vitex simplicifolia* to alloxan induce diabetic rats showed no significant change in sodium, potassium, chloride but showed significant increase in urea and decrease in carbonate, cretinine levels. The elevation of serum urea level observed in this study may have resulted from kidney damage from exposure to the extract. It is an established fact that a wide variety of renal diseases with different permutation of glomerular, tubular, interstitial or vascular damage can cause an increase in serum urea concentration (Roct *et al.,* 1998). Urea is a byproduct of protein metabolism that is excreted through urine (Abdulrahman *et al.,* 2007).

The observed decrease in serum level of both carbonate and cretinine is non dose dependent

However, the oral administration of n-hexane fraction of *Vitex simplicifolia* for 21 days to experimental animals showed no significant change in sodium and chloride but a significant increase was observed in potassium, carbonate and cretinine. (Table 4.27)

The effect of methanolic and ethyl acetate fractions of *Vitex simplicifolia* extracts in this study shows a significant increase in sodium, chloride, urea and carbonate with no significant change in potassium and creatinin serum levels (Table 4.25 and 4.26). The increase in blood level of sodium ion concentration could be an indication of decreased sodium excretion or dehydration (Odutulla, 1992 and Abdulrahman *et al*, 2007) but in this study the rats were not diarrheic, hence dehydration may not explain the presence of high sodium level in the blood (Abdulrahman *et al*, 2007).

Therefore, the elevated serum level of sodium, chloride, urea and carbonate in this study may have resulted from kidney damage from exposure to the extract. However the observed increase in both creatinin and carbonate were non dose dependent. Increase in serum urea as mentioned earlier, is an indication of kidney damage. The result corroborated by the histophathology report in this study, indicating moderate distal tubular epithelial cell edema and mild interstial inflammation.

The administration of n-hexane fraction of *Vitex simplicifolia* extract for 21 days to experimental animals showed significant increase in carbonate, urea and creatinin. But no significant change observed in sodium, chloride and potassium ions. However the significant increase observed in carbonate and creatinin was not dose dependant. The

significant change observe in urea is dose dependent. This may be due to kidney damage. The histophathology report showed mild insterstial inflammation. It is an established fact that a wide variety of renal diseases with different permutation of glumerular, tubular, interstitial or vascular damage can cause an increase in serum urea concentration (Roct *et al.*, 1998).

Oral administration of fractionated extracts (aqueous, methanolic, ethyl acetate and n-hexane) of *Vitex simplicifolia* in this study showed a significant decrease in vitamin A and superoxide dismutase (SOD) and no significant change in vitamin C and E. A decrease in SOD and vitamin A is an indication of lack of antioxidant activity (Giugliano, 1995). Anti oxidant defense mechanism involves both enzymatic and non enzymatic strategies. Common antioxidants including vitamins A, C and E and superoxide dismutase etc work in synergy with each to neutralize different types of free radicals (Halliwell and Gulteridge, 1989). There is paucity of data on the antioxidant activities of *Vitex simplicifolia*. The organ morphological changes in the liver are mild characterised by preservation of the native architecture but mild expansion of the sinusoids with lymphocytosis.This is not unexpected becouse, liver cells being stable cells heal by regeneration and mild sub lethal injury to the liver cells may heal within a short time. However there is a moderate morphologic change in the kidneys as evidenced by interstitial inflammation and cell swelling.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In conclusion, four fractionated extract of *Vitex simplicifolia* leaf were observed to possess a potent hypoglyceamic activity but have no antioxidant and hypolipidemic potential. However, it was also observed to possess moderate hepato-renal toxicity.

6.2 Recommendations

Considering the findings and importance of the study, the following recommendations could be made:

1. In view of the potent hypoglycemic action of *Vitex simplicifolia* leaf extract, further bioactivity guided studies are needed to isolate the bioactive component(s) and characterize them.
2. The intake of Vitex *simplicifolia* leaf could be recommended as dietary supplements or food additives for diabetic patients.
3. Caution must be exercised in its usage, in view of the moderate hepato - renal toxicity observed in this study.

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APPENDIX

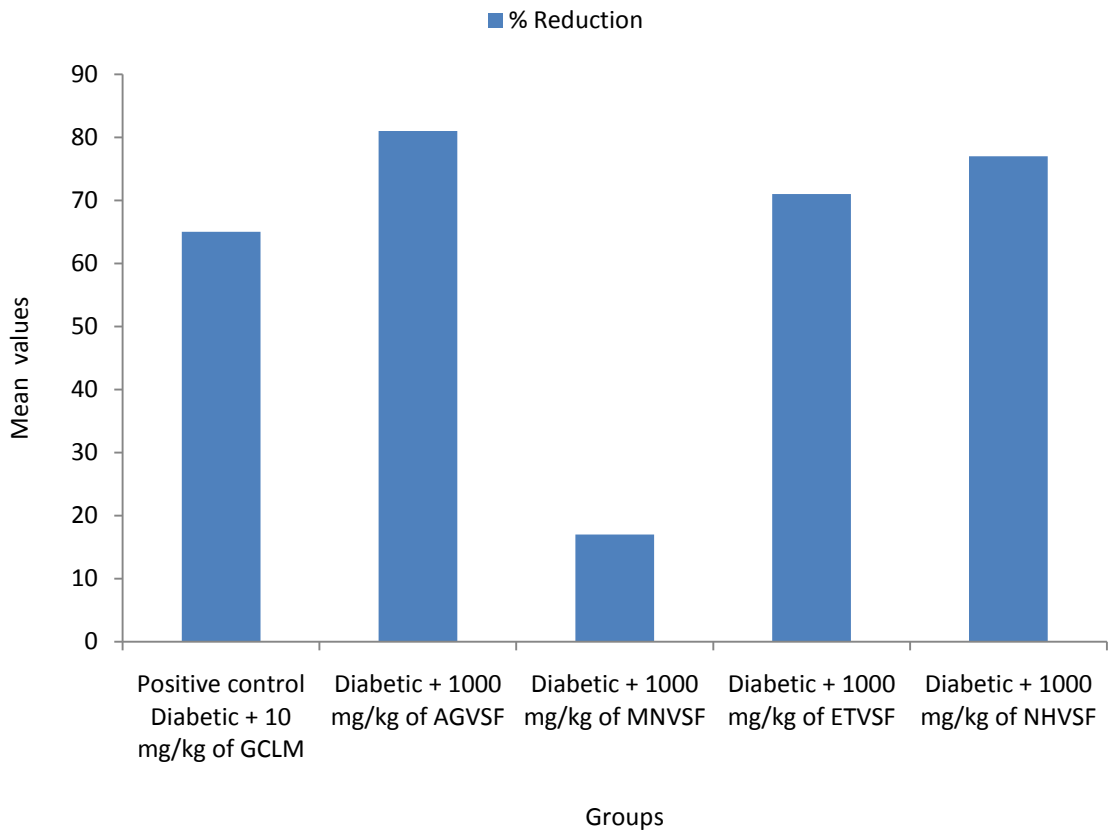


Figure 1: Percentage changes in fasting blood glucose of alloxan- induced diabetic rats treated with aqueous, methanolic, ethyl acetate and N hexane fractionated extracts of *Vitex Simplicifolia* leaf.

Key

GCLM – Glibenclimade

AGVSF- Aqueos fraction of *Vitex simplicifolia* leaf

MNVSF-Methanolic fraction of *Vitex simplicifolia* leaf

ETVSF-Ethyl acetate fraction of *Vitex simplicifolia* leaf

NHVSF-N-hexane fraction of *Vitex simplicifolia* leaf

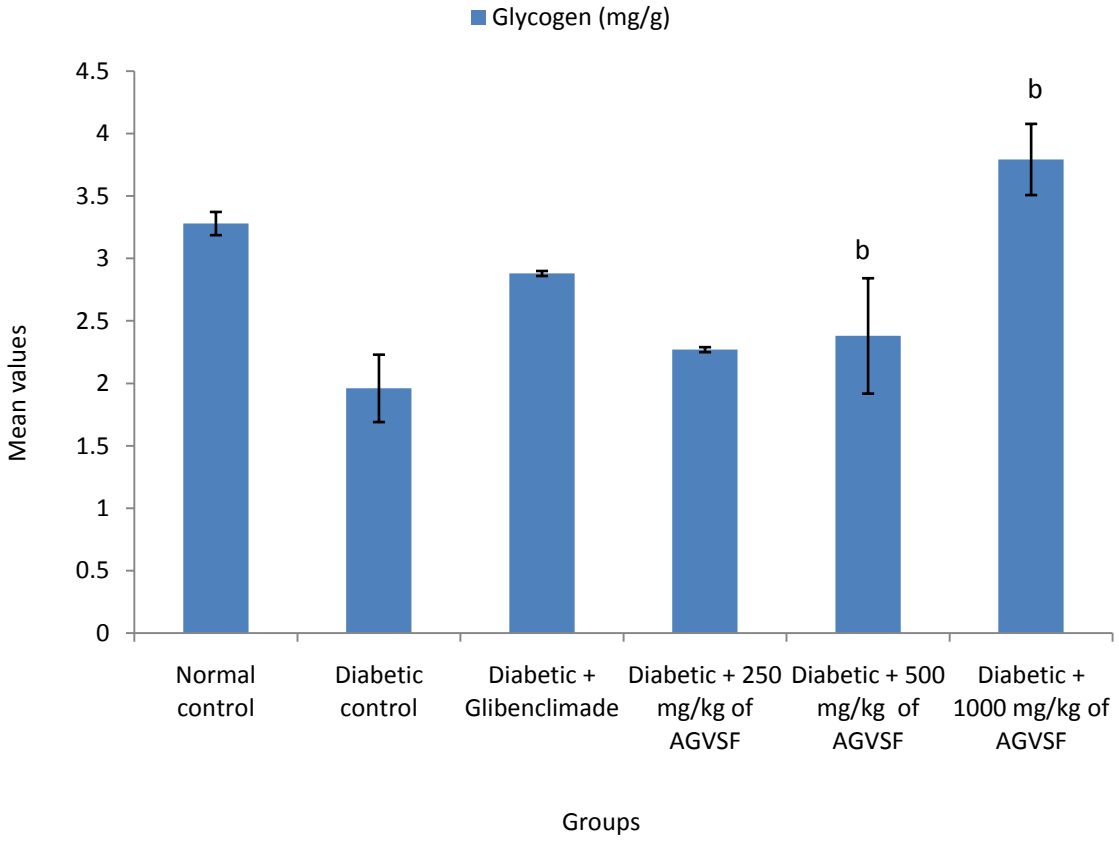


Figure 2: Effect of aqueous fraction of leaf extract of *Vitex Simplicifolia* on liver glycogen content in alloxan- induced diabetic rats.

[b] = significant compared to diabetic control.

AGVSF- Aqueos fraction of *Vitex simplicifolia* leaf

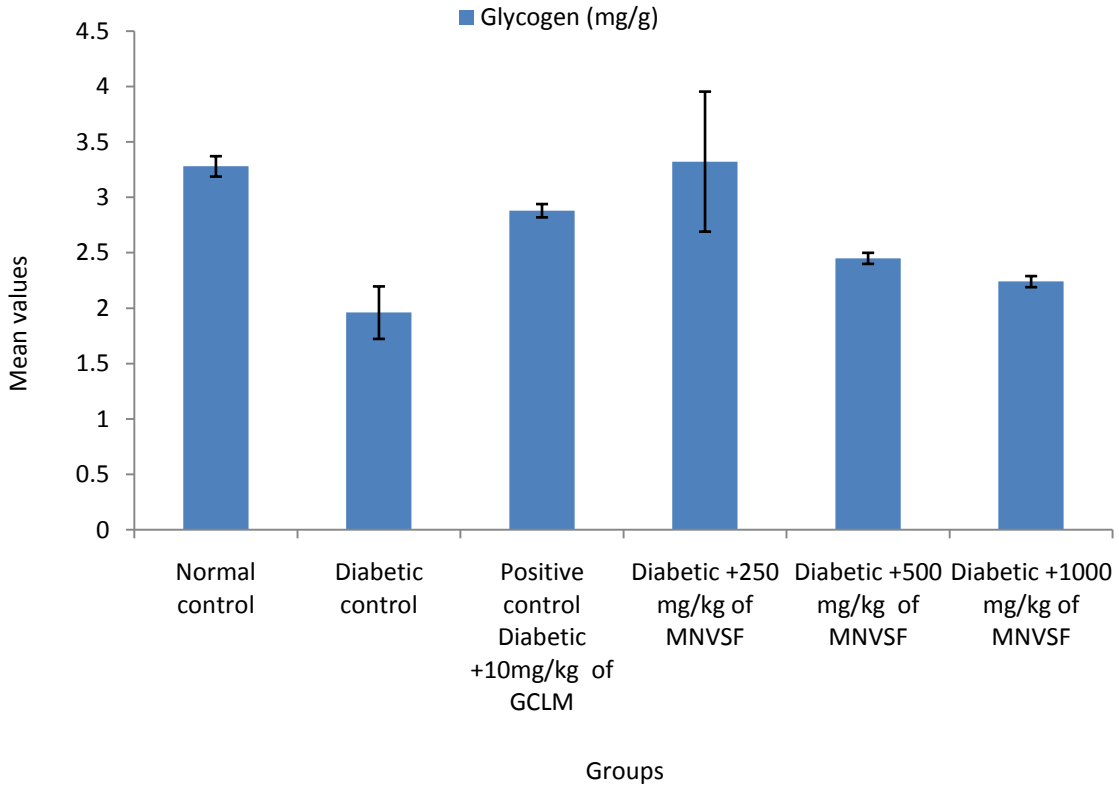


Figure 3: Effect of methanolic fraction of leaf extract of *Vitex Simplicifolia* on liver glycogen content on alloxan- induced diabetic rats.

GCLM- Glibenclimade

MNVSF-Methanolic fraction of *Vitex simplicifolia* leaf

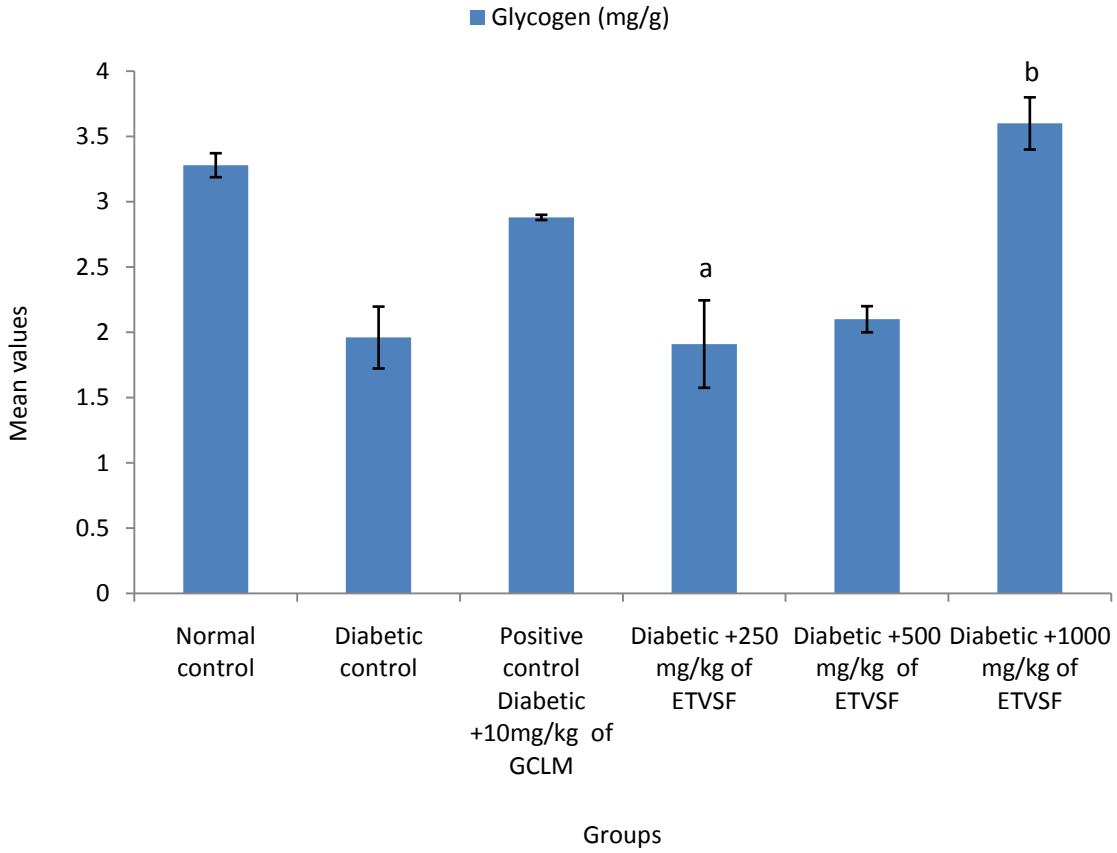


Figure 4: Effect of ethyl acetate fraction of leaf extract of *Vitex Simplicifolia* on liver glycogen content in alloxan- induced diabetic rats.

[a] = significant compared to normal control.

[b] = significant compared to diabetic control.

GCLM- Glibenclimade

ETVSF-Ethyl acetate fraction of *Vitex simplicifolia* leaf

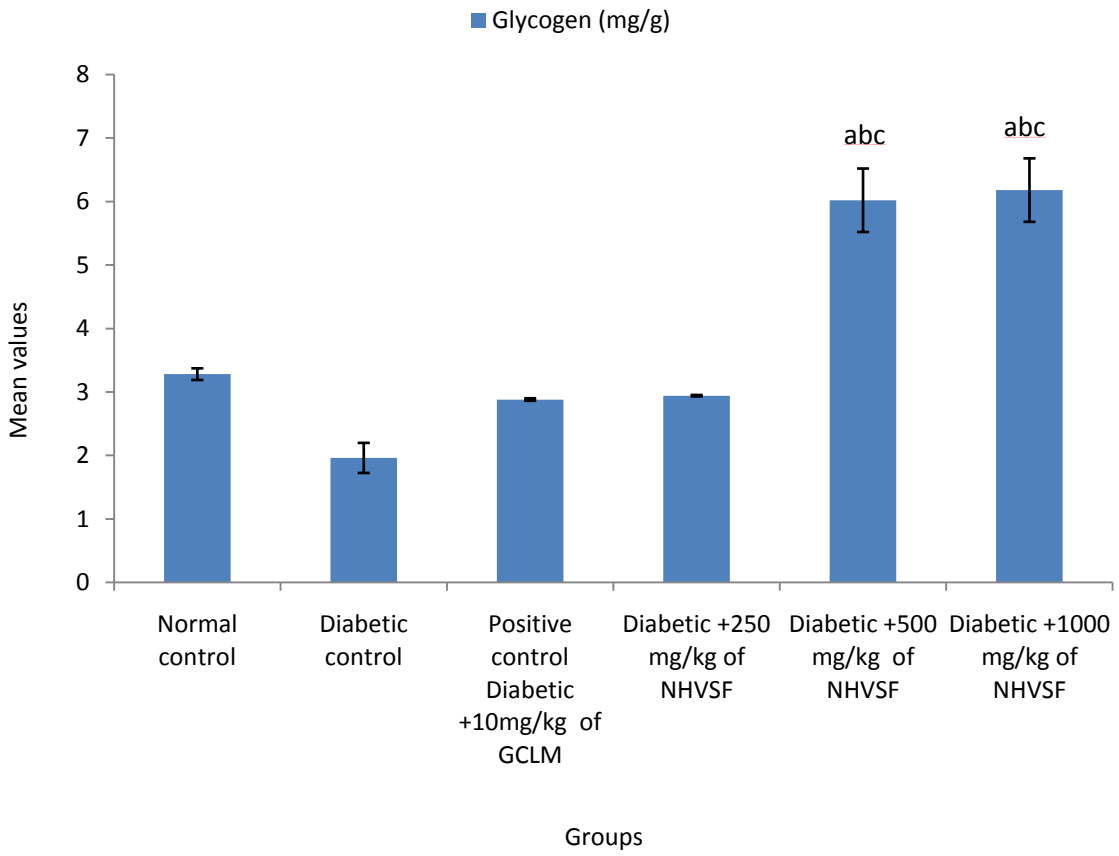


Figure 5: Effect of n-hexane fraction of leaf extract of *Vitex Simplicifolia* on liver glycogen content in Alloxan- induced diabetic rats.

[a] = significant compared to normal control.

[b] = significant compared to diabetic control.

[c]= significant compared to positive control.

GCLM- Glibenclimade

NHVSF-N-hexane fraction of *Vitex simplicifolia* leaf

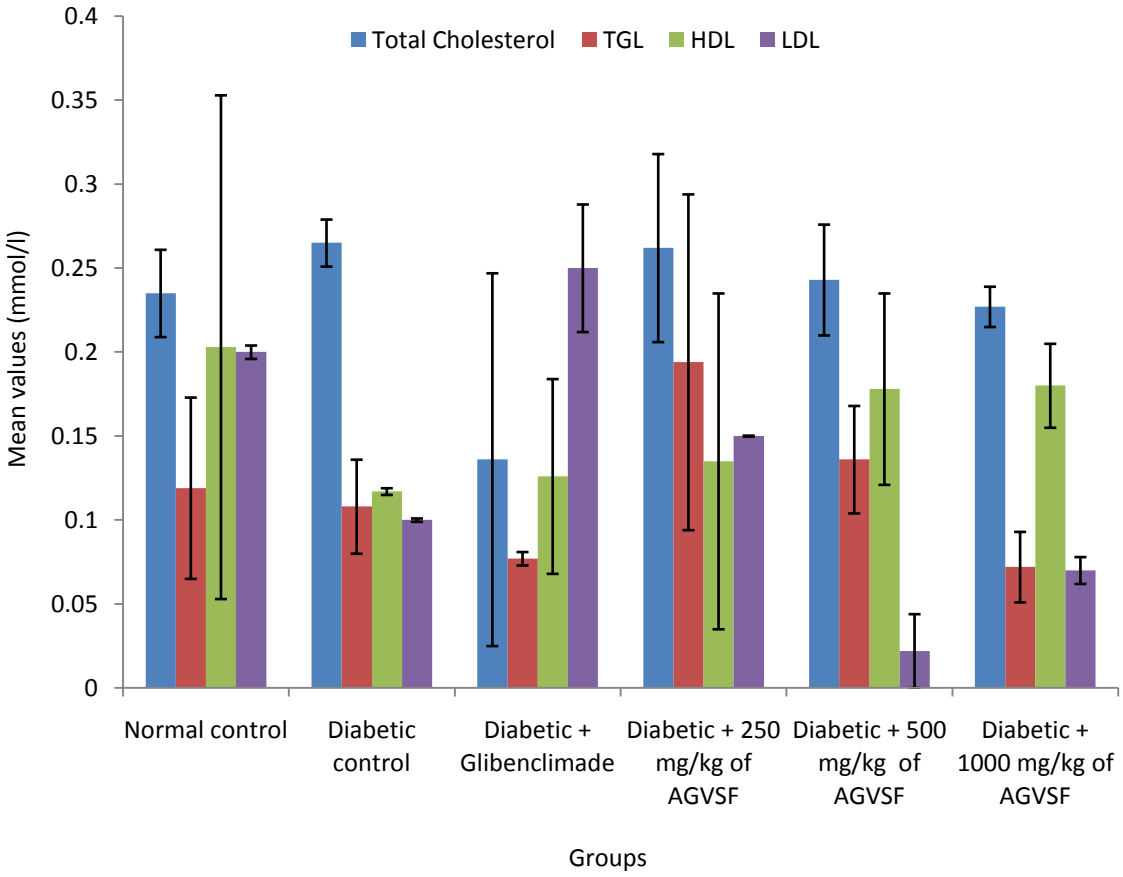


Figure 6: Effect of aqueous fraction of leaf extract of *Vitex Simplicifolia* on lipid profile in Alloxan- induced diabetic rats.

AGVSF-Aqueos fraction of *Vitex simplicifolia* leaf

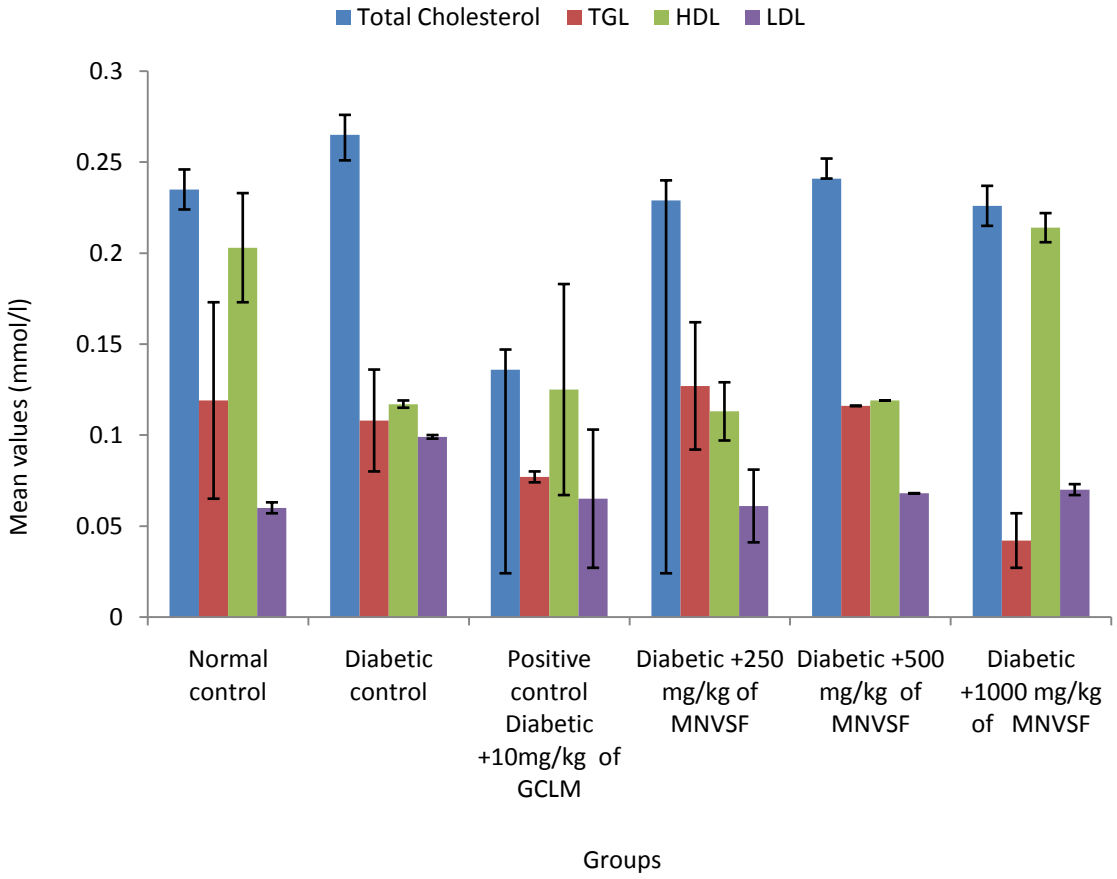


Figure 7: Effect of methanolic fraction of leaf extract of *Vitex Simplicifolia* on lipid profile in alloxan- induced diabetic rats.

GCLM- Glibenclimade

MNVSF-Methanolic fraction of *Vitex simplicifolia* leaf

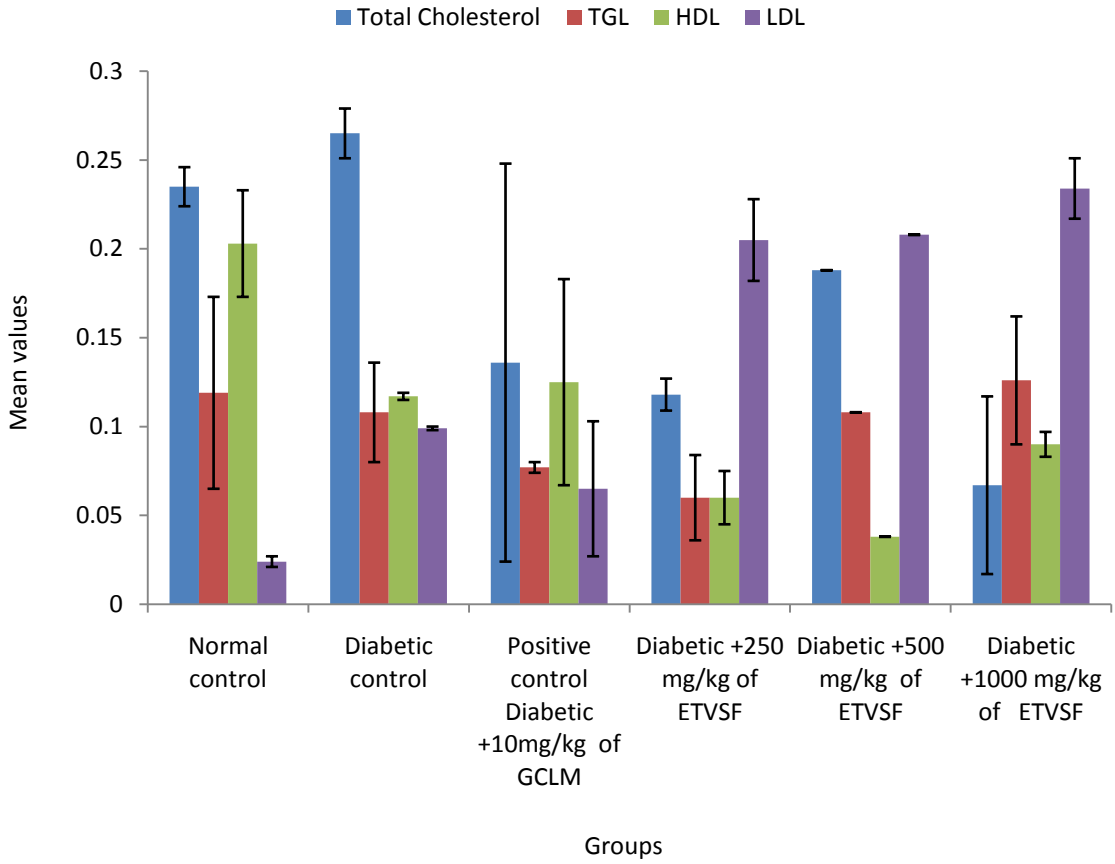


Figure 8: Effect of ethyl acetate fraction of leaf extract of *Vitex Simplicifolia* on lipid profile in alloxan- induced diabetic rats.

GCLM- Glibenclimade

ETVSF-Ethyl acetate fraction of *Vitex simplicifolia* leaf

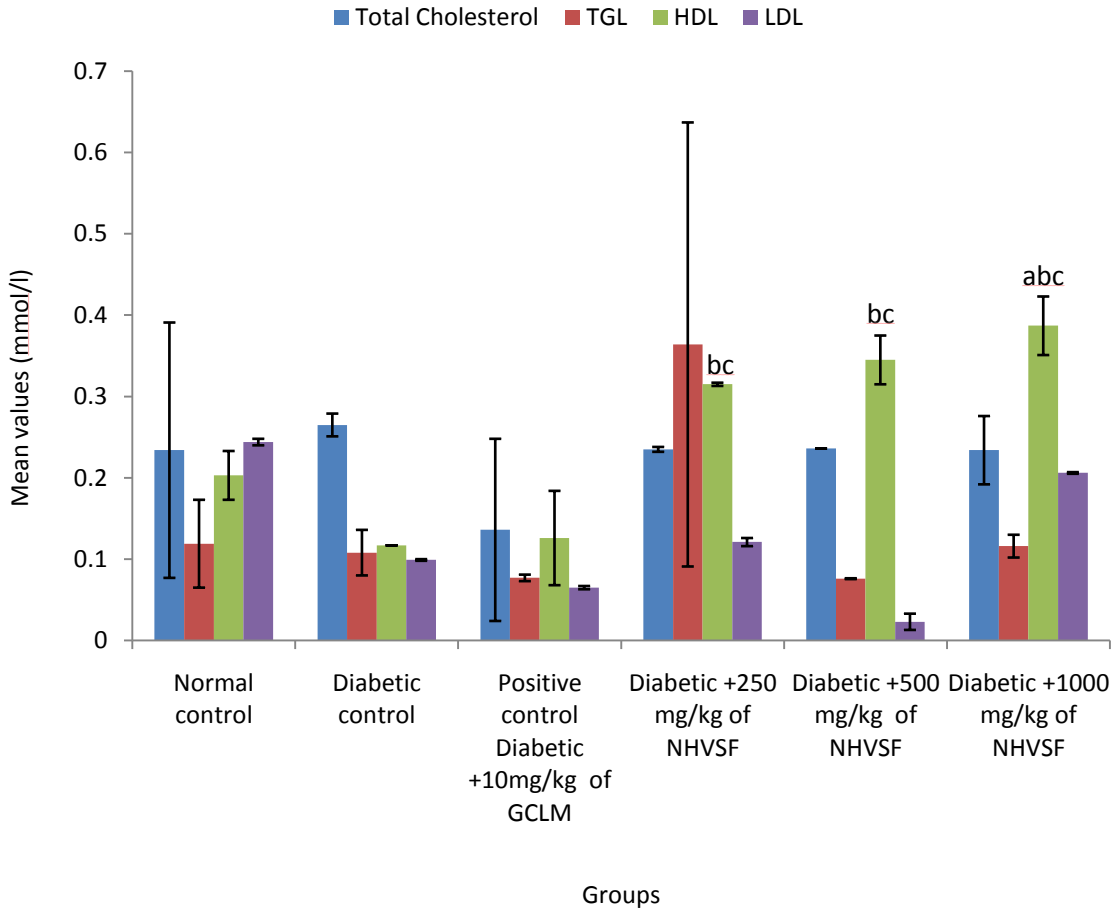


Figure 9: Effect of N hexane fraction of leaf extract of *Vitex Simplicifolia* on lipid profile in alloxan- induced diabetic rats.

[a] = significant compared to normal control.

[b] = significant compared to diabetic control.

[c]= significant compared to positive control.

GCLM- Glibenclimade

NHVSF-N-hexane fraction of *Vitex simplicifolia* leaf

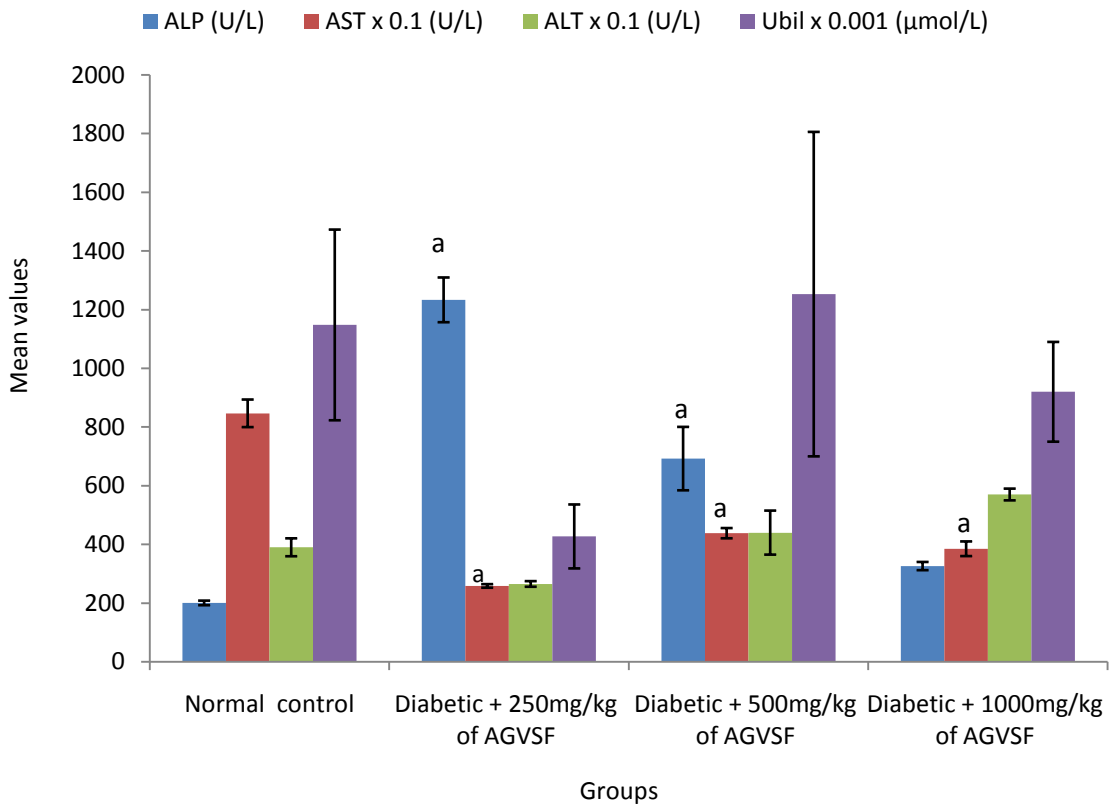


Figure 10: Shows the effect of aqueous leaf extract of *Vitex Simplicifolia* on ALP, AST, ALT and unconjugated billirubin in alloxan- induced diabetic rats.

[a] = significant compared to control.

AGVSF-Aqueos fraction of *Vitex simplicifolia* leaf

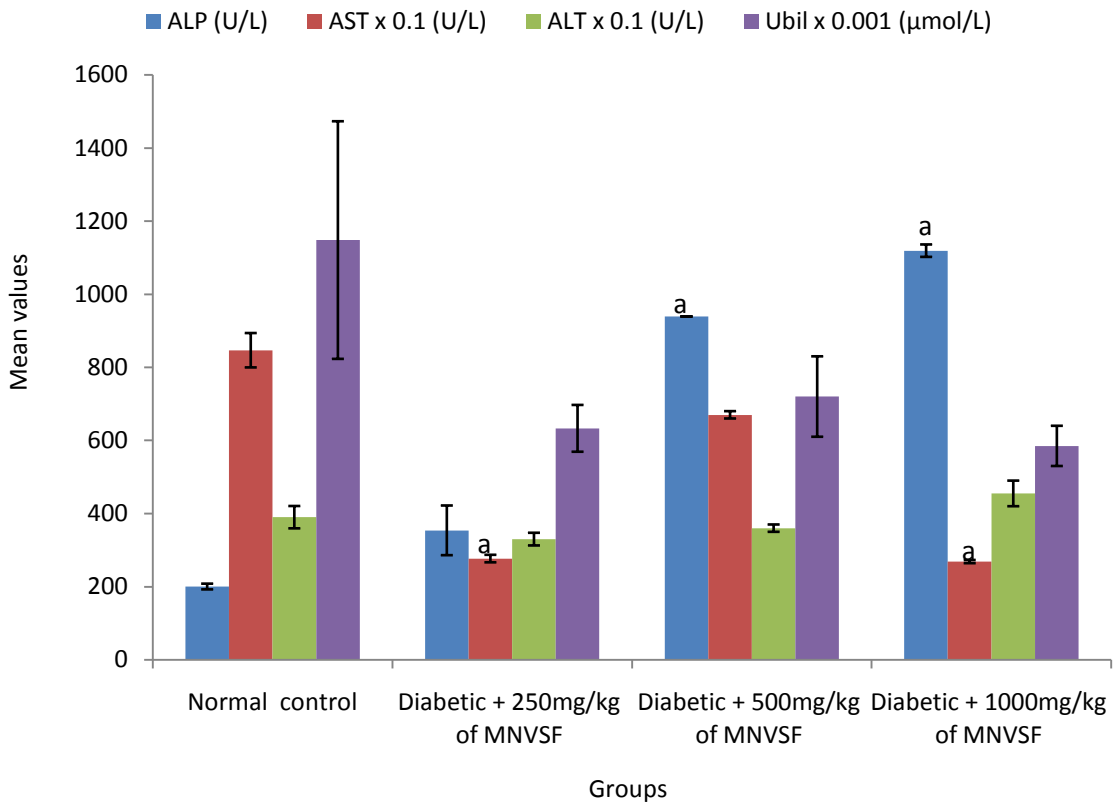


Figure 11: Shows the effect of methanolic leaf extract of *Vitex Simplicifolia* on ALP, AST, ALT and unconjugated billirubin in alloxan- induced diabetic rats.

[a] = significant compared to control.

MNVSF-Methanolic fraction of *Vitex simplicifolia* leaf

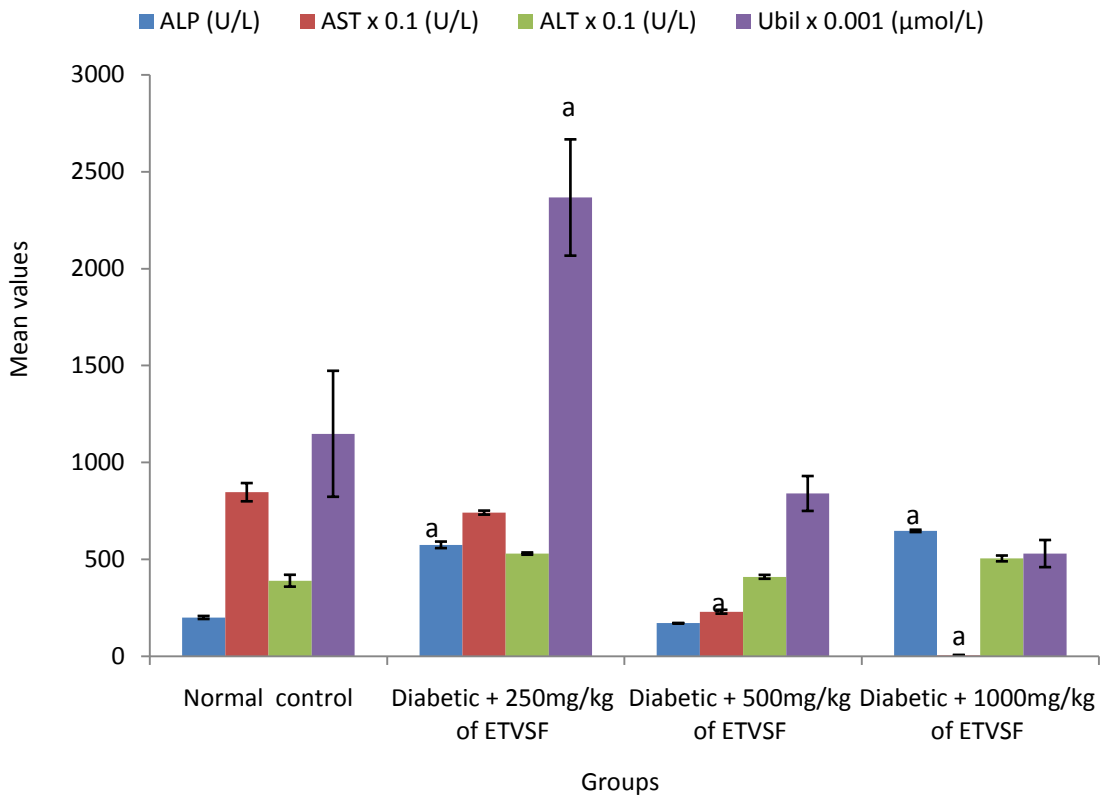


Figure 12: Shows the effect of ethyl acetate leaf extract of *Vitex Simplicifolia* on ALP,AST,ALT and unconjugated billirubin on alloxan- induced diabetic rats.

[a] = significant compared to control

ETVSF-Ethyl acetate fraction of *Vitex simplicifolia* leaf

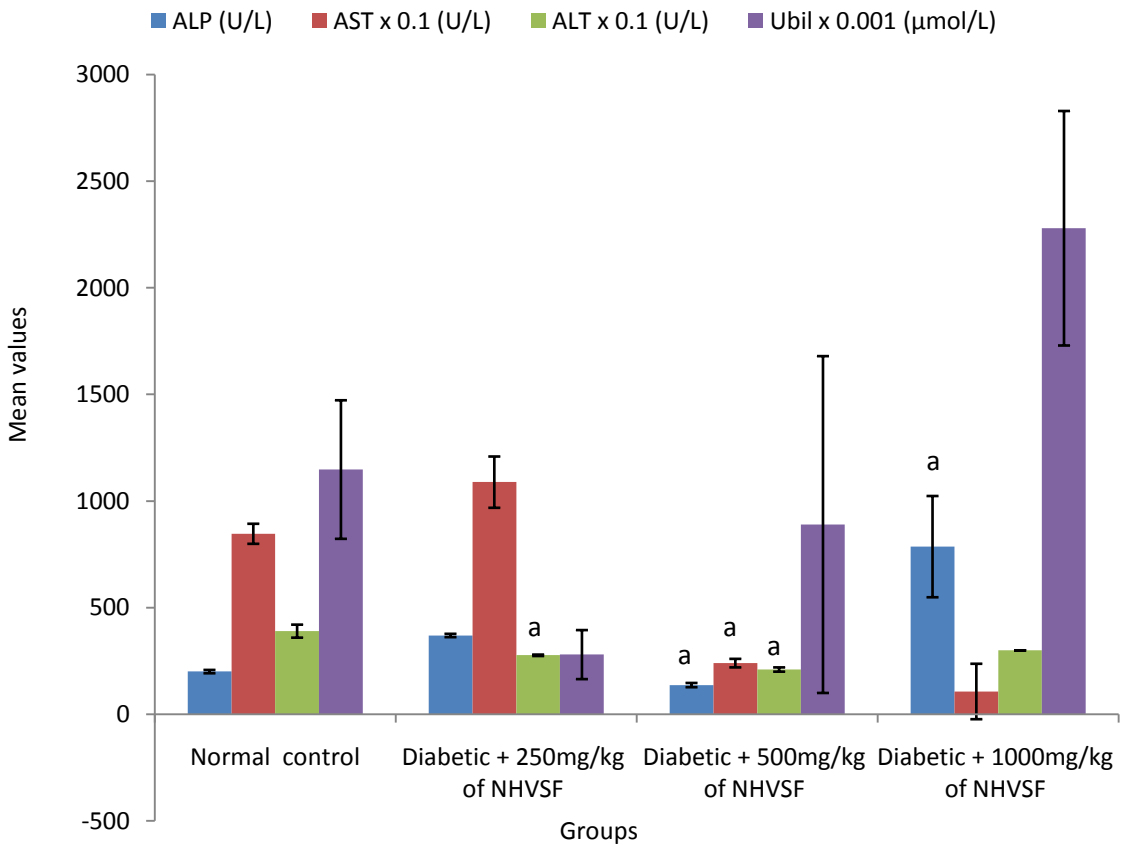


Figure 13: Shows the effect of N hexane leaf extract of *Vitex Simplicifolia* on ALP,AST,ALT and unconjugated billirubin on alloxan- induced diabetic rats.

[a] = significant compared to control.

NHVSF-N-hexane fraction of *Vitex simplicifolia* leaf

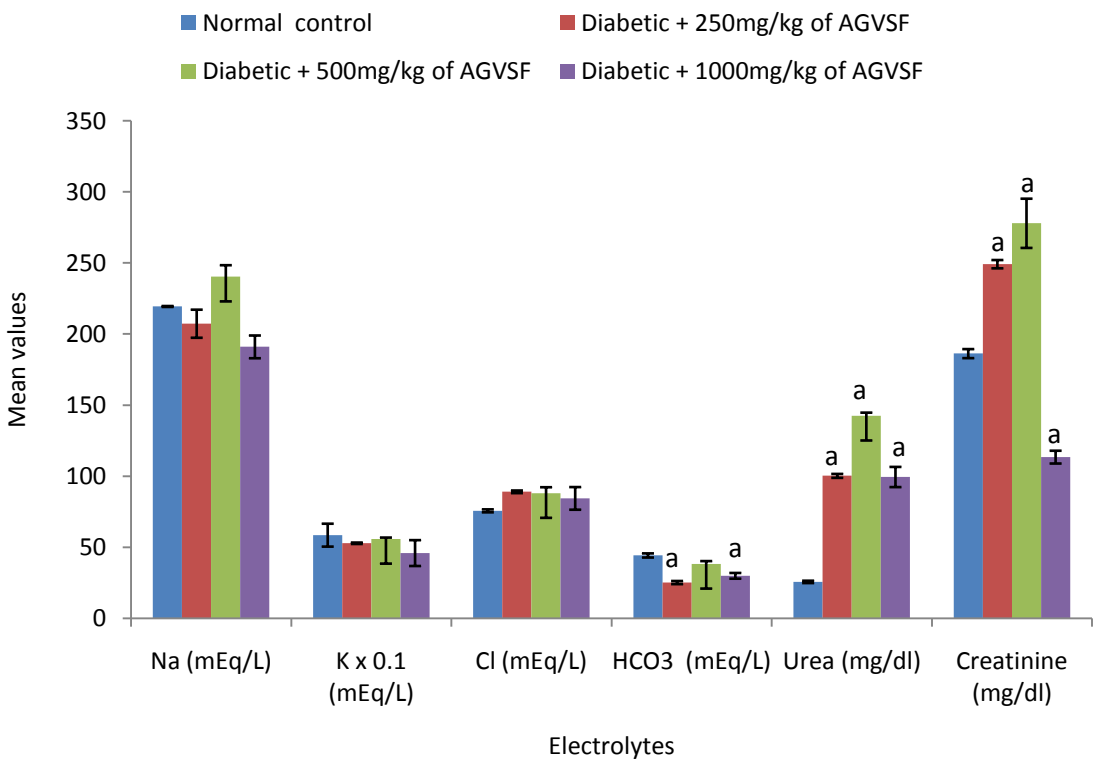


Figure 14: Shows the effect of aqueous leaf extract of *Vitex Simplicifolia* on electrolytes in alloxan- induced diabetic rats.

[a] = significant compared to control.

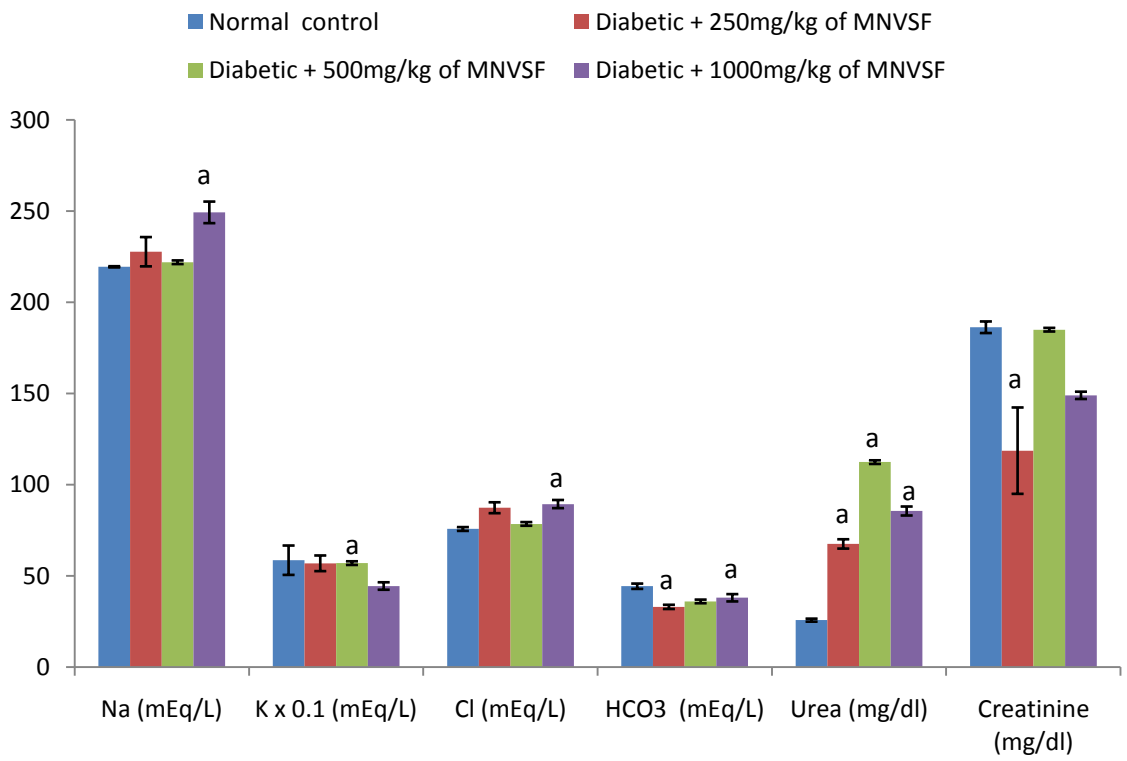


Figure 15: Shows the effect of methanolic leaf extract of *Vitex Simplicifolia* on electrolytes in alloxan- induced diabetic rats.

[a] = significant compared to control.

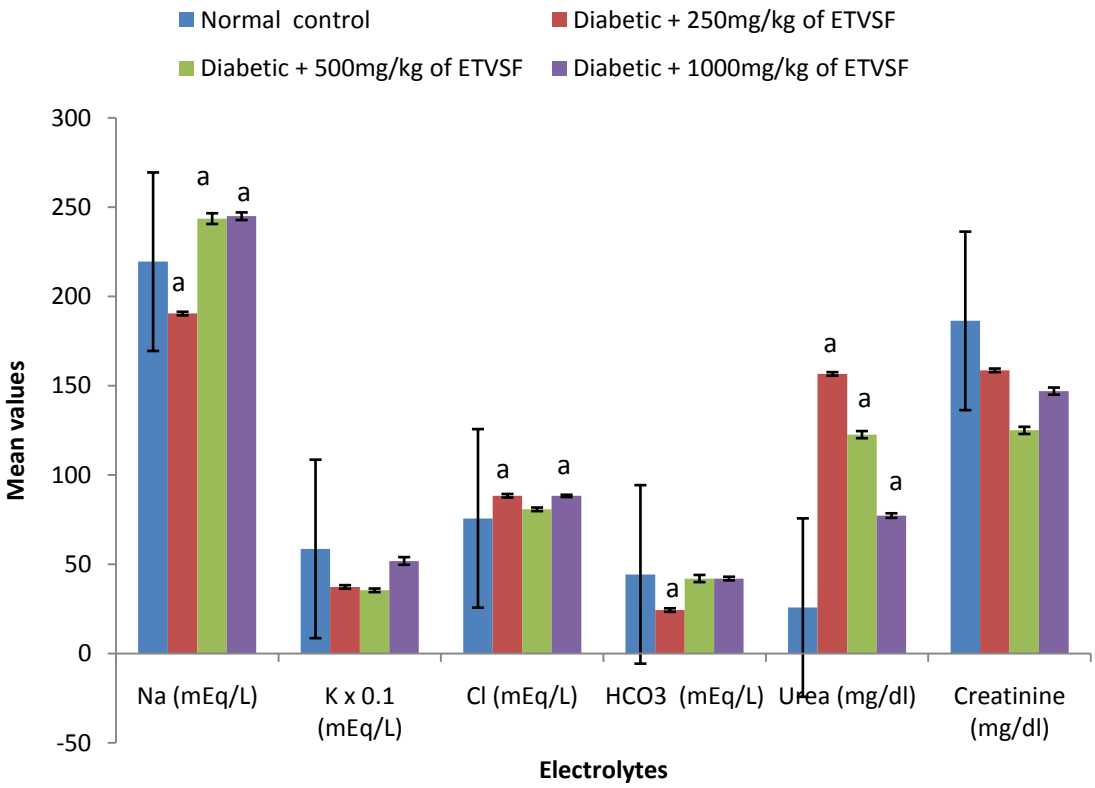


Figure 16: Shows the effect of ethyl acetate leaf extract of *Vitex Simplicifolia* on electrolytes in alloxan- induced diabetic rats.

[a] = significant compared to control.

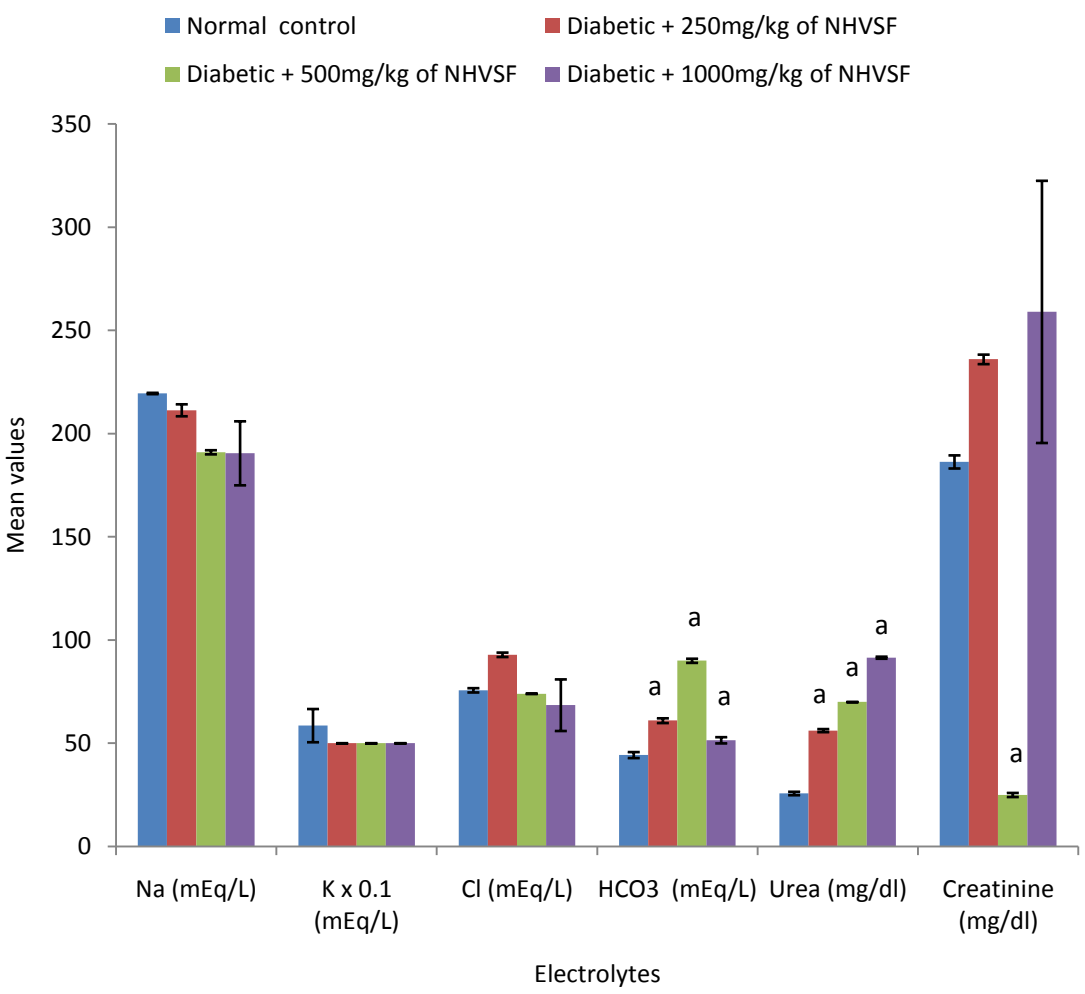


Figure 17: Shows the effect of N hexane leaf extract of *Vitex Simplicifolia* on electrolytes in alloxan- induced diabetic rats.

[a] = significant compared to control.