EFFECT OF METHANOL EXTRACT OF GINGER RHIZOME (*Zingiber officinale*) ON FASTING BLOOD GLUCOSE, LIPID PROFILE AND SOME OXIDATIVE STRESS MARKERS IN ALLOXAN-INDUCED DIABETIC RATS.

BY

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A DISSERTATION SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY, FACULTY OF BASIC MEDICAL SCIENCES, BAYERO UNIVERSITY, KANO IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN BIOCHEMISTRY

JANUARY, 2020

DECLARATION

I hereby declare that this research work titled "EFFECT OF METHANOL EXTRACT OF GINGER RHIZOME (*Zingiber officinale*) ON FASTING BLOOD GLUCOSE, LIPID PROFILE AND SOME OXIDATIVE STRESS MARKERS IN ALLOXAN-INDUCED DIABETIC RATS" is the product of my own research efforts; undertaken under the supervision of Dr. Aisha Muhammad Gadanya and has not been presented and will not be presented elsewhere for the award degree of Master of science or certificate. All sources of literature have been duly acknowledged.

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SAIFULLAHI BALA (SPS/16/MBC/00089) Date

CERTIFICATION

This is to certify that the research work titled"EFFECT OF METHANOL EXTRACT OF GINGER RHIZOME (*Zingiber officinale*) ON FASTING BLOOD GLUCOSE, LIPID PROFILE AND SOME OXIDATIVE STRESS MARKERS IN ALLOXAN-INDUCED DIABETIC RATS" for the project and the subsequent preparation of this report by Saifullahi Bala (SPS/16/MBC/00089) was carried out under my supervision.

_____ _____

Dr. Aisha Muhammad Gadanya Date
(Supervisor)

DEDICATION

This research work is dedicated in memory of my beloved mother Hajiya Hafsat Usman Dandashire may her soul rest in perfect peace and may Allah (S.W.A) make Jannatul Firdaus to be her final abode.

APPROVAL

This dissertation has been examined and approved for the award of Master of Science Degree in Biochemistry.

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ABBREVIATIONS

5-HMF	5-hydroxymethylfurfural
AGE	Advanced glycation end products
ATP	Adenosine triphosphate
CHD	Coronary heart disease
CVD	Cardiovascular diseases
DF	Dietary fibers
DHAA	Dehydroascorbic acid
DKA	Diabetic keto –acidosis
DM	Diabetes mellitus
DN	Diabetic neuropathy
DR	Diabetic retinopathy
eNOS	Endothelial nitric oxide synthatase
Glu	Glucose
GLUT	Glucose transporter
GPX	Glutathione Peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HbA_{1c}	Glycated hemoglobin
HDL-C	High density lipoprotein - cholesterol
HNS	Hyperosmolar nonketotic state
IDF	International Diabetes Federation
GSSG	Oxidized glutathione
IR	Insulin resistance
NADH	Nicotinamide adenine dinucleotide

PKC	Protein kinase C
PN	Peripheral neuropathy
PUFA	Polyunsaturated fatty acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TAG	Triacylglyceride
TC	Total cholesterol
TAG	Triacyglycerol
VLDL-C	Very low density lipoprotein – cholesterol
WHO	World Health Organization
DPPH	1,1-diphenyl-2-picryl hydrazyl

TABLE OF CONTENTS

ABSTRACT

Diabetes mellitus is a complex metabolic disorder with high socioeconomic costs that is considered a worldwide epidemic and is associated with the development of many complications. The present study was carried out to evaluate the antidiabetic effect of gradeddoses (1500, 1000, and 500mg/kg) ofmethanol extractof ginger rhizome in alloxan-induced diabetic rats. Rats were randomly divided into six groups of six rats each: Alloxan treated group as positive control (PC) was administered with 120mg/kg of alloxan and no treatment, normal control group (NC), metformin treated group (MC) received 500mg/kg of metfomin, group1 (GR1) received 1500mg/kg of methanol extract of ginger rhizome, group2 (GR2) received 1000mg/kg of methanol extract of ginger rhizome, and group3 (G3) received 500mg/kg of methanol extract of ginger rhizome.The rats were treated with their respective doses once daily by intubation for six weeks.The findings of this study showed a decrease of 51.4% in fasting blood glucose, 51.2% in glycated heamoglobin, 21.3% in total cholesterol, 24.9% in triacylglycerol, 57.6% in low density lipoprotein cholesterol and 35.9% in malondialdehyde among the ginger treated groups. These decreases is statistically significant ($p<0.05$) in contrast to positive control group (PC). In the same vein, the result showed an increase of 43.2% in high density lipoprotein cholesterol, 51.2% in superoxide dismutase, 28.3% in reduced glutathione and 24.2% in catalase among the ginger treated groups.Also theseincreases is statistically significant ($p>0.05$) when compared with positive control group.Daily administration of graded doses of methanol extract of ginger rhizome for sixweeks ameliorated the effect of the alloxan, and improved the changes in the serum glucose, glycated haemoglobin (HbA_{1c}), lipid profile, and oxidative stress markers. The results of the current study showed that methanol extract of ginger rhizome may be a promising intervention in the management of diabetes.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion and insulin action or both. The chronic hyperglycemia is associated with long-term damage, dysfunction, and failure of normal functioning of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels renal failure, and nerve damage(American Diabetes Association, 2012; Paneni. *et al.,* 2013).

There are three major types of diabetes (Stryer, 2000). Type 1 diabetes is usually diagnosed in childhood, hence called juvenile onset diabetes. In this diabetic type, the body makes little or no insulin and daily injection of insulin is needed. The exact cause is unknownhowever, genetics, viruses, and autoimmune problems may play a role (Dyck, 2003). Symptoms of type 1 diabetes include; fatigue, increased thirst, increased urination, nausea, vomiting and weight loss in spite of increased appetite (Eisenberth*et al.,* 2008).Type 2 diabetes, the commonest type of diabetes, occurs in adulthood, but young people are increasingly being diagnosed with this disease. In this case, the pancreas does not produce enough insulin to keep the blood glucose levels normal, most often because the body does not respond well to insulin (Alemzadeh & Wyatt, 2010). This type of diabetes is exacerbated by increasing occurrence of obesity, sedentary life style and failure to exercise (Atkins & Brice, 1955) and its often associated with symptoms such as blurred vision, fatigue, increased appetite, increased thirst and increased urination (Alemzadeh & Wyatt, 2010). Gestational diabetes which is the third type of diabetes is as a result of high blood glucose condition that develops at any time during pregnancy

in non-diabetic individuals. Women with this condition are at high risk of type 2 diabetes and cardiovascular disease later in life (Dyck, 2003).

The World Health Organization (WHO) has predicted that the number of patients with diabetes worldwide will double by the year 2025, from the current number of approximately 150 million to 300 million (Coskun *et al.,* 2005). Diabetes mellitus (DM) is associated with the production of reactive oxygen species (ROS) and consequently oxidative stress, which promotes not only an alteration in the cellular redox state (Coskun *et al.,* 2005) in the presence of chronic hyperglycaemia, but also reduces the ability of tissues to utilize carbohydrates, leading to disturbances in the metabolism of fat and protein (Je *et al.,* 2001). Moreover, this etiology is accompanied by an imbalance between the oxidant and antioxidant status, i.e., increased production of ROS and/or decline in antioxidant defense systems (Young *et al.,* 1995;Baydas *et al.,* 2002; Fakher *et al.,* 2007). Chronic high blood glucose levels may contribute to the formation of ROS, through several mechanisms such as glucose autoxidation, the oxidation of protein (Bonnefont Rousselot *et al.,* 2000; Maritim *et al.,* 2002) and non-enzymatic glycation of protein (Szaleczky *et al.,* 1998), thus exacerbating oxidative stress.

Diabetes mellitus has been associated with an increased risk of mortality and prevalence of cardiovascular disease. Atherosclerotic cardiovascular disease is the main source of morbidity and mortality in patients with diabetes (Bray, 2000). In addition, oxidative stress may occur as a consequence of abnormalities in glucose and lipid metabolism, which favour hyperglycaemia and dyslipidaemia. These phenomena are associated with the development of atherosclerosis and cardiovascular complications in diabetic patients (Bray, 2000; Chertow & Edwards, 2004).Since numerous studies have indicated that hyperglycaemia in diabetes contributes to oxidative stress, it is suggested that the nutritional supplementation of antioxidants might reduce the oxidative

stress, and hence protect tissues from ROS damage (Sharma *et al.,* 2000; Coskun *et al.,* 2005; Ramkumar *et al.,* 2008). Such supplementation mayplay a protective role and has been correlated with a decrease in the incidence of various degenerative diseases, such as diabetes and its complications (Sharma *et al.,* 2000; Ramkumar *et al.,* 2008).

The prevalence of diabetes is rising all over the world due to population growth, aging, urbanization, lifestyle and the increase of obesity as a result of physical inactivity. Unlike in the olden days, where the older are most affected, diabetes nowadays is comparatively high in young to middle-aged people. All these complications have long-lasting adverse effects on a nation's health and economy, especially for developing countries.Hyperglycaemia generates reactive oxygen species (ROS), which in turn cause damage to the cells inmany ways. Damage to the cells ultimately results in secondary complications in diabetes mellitus (Hunt*et al.,* 1988; Jaganjac*et al.,* 2013).Diabetes is a major source of morbidity, mortality, and economic cost to the society. People with diabetes exhibit the risk of development of acute metabolic complications such as diabetic ketoacidosis, hyperglycaemic hyperosmolar non-ketotic coma, and hypoglycaemia (Umpierrez *et al.,* 2002; English& Williams, 2004). In addition to this, diabetics are also at risk of experiencing chronic complications such as coronary heart diseases, retinopathy, nephropathy and neuropathy, and foot ulceration. Since food intake influences the amount of insulin required to meet blood glucose target goals, the food especially carbohydrate intake could contribute to the pathology of diabetes. Dietary carbohydrate influences postprandial blood glucose levels the most and is the major determinant of meal-related insulin requirements.

Diabetes remedy that is gaining popularity today is herbal treatment, with a variety of plant- derived preparations being promoted as capable of controlling blood sugar levels, in fact,

herbal treatment for diabetes is not known. Plants and plant extracts were used to combat the disease as early as 1550 B. C., with as many as 400 (prescribed) before the development earlier this century of effective medications to control diabetes. Phytochemicals identified from traditional medicinal plants are presenting an exciting opportunity for the development of new types of therapeutics (Zimmet *et al.,* 1999). It is a known fact that nutrition and health care are interrelated. Thus, many plants are consumed as food and for health benefits (Pieroni *et al.,* 2005). The nutraceutical value and the antioxidant activity of semi-cultivated or neglected vegetables are regarded worldwide as an important area of the nutritional and phytotherapic research (Eastwood, 1992).The use of herbs as medicines has played an important role in nearly every culture on earth, including Asia, Africa, Europe and the Americas (Wargovich *et al.,* 2001). Herbal medicine is based on the premise that plants contain natural substances that can promote health and alleviate illness. Several herbs can help to reduce blood sugar, high blood cholesterol concentrations, provide some protection against cancer and stimulate the immune system. Furthermore, a diet in which culinary herbs are used generously to flavor food provides a variety of active phytochemicals that promote health and protection against chronic diseases.

Ginger (*Zingiber officinale*) belonging to the family Zingiberaceae and it is one of the most commonly consumed spices worldwide. It has a long history of use as herbal medicine to treat a variety of diseases including nausea and vomiting, constipation, indigestion (dyspepsia), pain, and cold induced syndromes. More recently, it was reported to also possess anti-cancer, anti-clotting, anti-inflammatory, and anti-oxidative characteristics, since it can scavenge superoxide anion and hydroxyl radicals(Baliga *et al.,* 2013).Ginger is known to contain a number of potentially bioactive compounds, mainly gingerols and their related dehydration products, the shogaols, as well as volatile oils including sesquiterpenes, (such as beta-bisabolene and

zingiberene, and monoterpenes, mainly geranial and neral). In addition, phytochemical reports have shown that the main constituents of ginger are gingerol, shogaol, zingerone and paradol. It was reported that 6-gingerol and 6-shogaol are the major gingerol and shogaol present in the rhizome (Li*et al.,* 2012).The myriad beneficial effects of ginger are supposed to be due to the presence of bioactive phytochemicals like gingerols, shogaols, paradols, gingerdiols, and zingerone (Baliga *et al.,* 2013).

Ginger has been shown to possess anti-diabetic activity in a variety of studies. Akhani *et al.* (2004) reported that ginger pretreatment inhibited the induced hyperglycemia and hypoinsulinemia. Other investigators have showed the hypolipidemic effect of ginger. Some experimental studies published on anti-diabetic, hypolipidemic and anti-oxidative properties of ginger are controversial and more investigations are needed to clarify its potency in the protection and treatment of metabolic disorders (Al-Azhary, 2011).

Zingerone scavenges superoxide anion. 6-gingerol and zingerone are reported to be good scavengers of peroxyl radicals. 6-shogoal also inhibited the production of nitrogen oxide (NO). 6-Gingerol is the major bioactive constituent responsible for the anti-inflammatory, anti-tumour and anti-oxidant activities of ginger (Nagendra *et al.,* 2013). Ginger and its constituents are reported to have antiemetic, antithrombotic, anti-hepatotoxic, anti-inflammatory stimulant, cholagogue, androgenic and antioxidant effects (Khaki *et al.,* 2009). Ginger is a strong anti-oxidant substance and may either mitigate or prevent generation of free radicals. It is considered a safe herbal medicine with only few and insignificant adverse/side effects (Ali *et al.,* 2008). Ginger extracts have been extensively studied for a broad range of biological activities, especially antioxidant activities (Miller *et al.,* 1993). Ahmed *et al.* (2000) found that ginger

significantly lowered lipid peroxidation by maintaining the activities of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase in rats.

Several works have reported the effects of ginger in animals with experimentally induced type 1 diabetes mellitus with relatively little reports on experimentally induced type 2 diabetes mellitus using the insulin resistance mechanism. There have been variable reports on glycaemic properties of ginger with some reporting a small but significant blood glucose-lowering effect of ginger juice in diabetic and non-diabetic animals (Sharma & Shukla, 1977). Likewise Akhani *et al*. (2004) also observed that ginger juice exhibits hypoglycaemic activity in both normal and streptozotocin-incuded diabetic rats. Other authors like Weidner and Sigwart (2000) reported that an ethanol extract of ginger had no effect on blood glucose levels in normal rats.

1.2:STATEMENT OF THE PROBLEM

Epidemiological studies show that the incidence of diabetes mellitus will double by 2030, affecting mostly the developing countries like Nigeria where adequate treatment is often very expensive (Sarah,2004; WHO, 2011). In addition to various health problem posed by diabetes, it also has severe economic implication in both developed and developing countries. For example, United States estimated that diabetic related conditions, leads to health costs of about hundreds billions of dollars yearly (Reaven, 1998). This is frightening as such an economic burden cannot be borne by developing countries like Nigeria.Management of diabetes mellitus by insulin therapy has several draw backs such as insulin resistance (Piedrola, 2001). Also, anorexia nervosa, brain atrophy, and fatty liver are encountered in chronic treatment with insulin (Tobias, 2001). For oral hypoglycemic drugs, sulphonylureas and biguanide are commonly used in controlling hyperglycaemia (Evans, 1999).However, it has been reported that their use could exert some side effects such as hepatotoxicity, abdominal pain, flatulence, diarrhoea and

hypoglycaemia (Fujisawa *et al*., 2005). Drug resistance to these medicines has also been reported after prolonged period of treatment of diabetes with such drugs (King *et al*., 1998; Shaw *et al.,* 2010).Even with this therapeutics, diabetes remains an exceedingly difficult disease to control.

1.3: JUSTIFICATION

Considering the devastating complication posed by diabetes mellitus, and high cost of treatment as well as the side effect associated with the current medication, there is urgent need to develop new medications or strategies to counter the huge increase in prevalence and incidence of diabetes mellitus.Management of diabetes mellitus by insulin therapy has several draw backs such as insulin resistance. Also, anorexia nervosa, brain atrophy, and fatty liver are encountered in chronic treatment with insulin. Fororal hypoglycemic drugs, sulphonylureas and biguanide are being used effectively in controlling hyperglycaemia, however, it has been reported that their use could exert some side effects such as hepatotoxicity, abdominal pain, flatulence, diarrhoea and hypoglycaemia. Drug resistance to these medicines has also been reported after prolonged period of treatment of diabetes with such drugs.Even with this therapeutics,diabetes remains an exceedingly difficult disease to control.Ginger rhizome isreadily available and affordable in Nigerian communities, and it is also widely used as spices and as medicinal plants around the world to manage body weight, insulin resistance and lipid profiles. Hence, exploration of potential antidiabetic property of ginger rhizome for possible development of antidiabetic nutraceutical formulation is critical for future management of diabetes mellitus.

1.4: AIM AND OBJECTIVES

1.4.1: AIM

To evaluate the effect ofmethanol extract of ginger rhizome(*Zingiber officinale*) on fasting blood sugar, glycated haemoglobin, lipid profile and some oxidative stress markers in alloxan induced diabetic rats.

1.4.2: OBJECTIVES

i. To determine the *in-vitro* antioxidant activityof different fractions of *Z. officinale* extracts.

ii. To determine the effect of methanol extractof *Z. officinale* on fasting blood glucose (FBS) and glycated haemoglobin in alloxan-induced diabetic rats

iii. To determine theeffect of methanol extractof *Z. officinale* on lipid profile; total cholesterol, triacylglycerols (TAG), high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, very low density lipoprotein (VLDL) cholesterol in alloxan-induced diabetic rats.

iv. To determine theeffect of methanol extractof *Z. officinale* on some oxidative stress markers;superoxide dismutase (SOD), catalase (CAT), malondealdehyde (MDA), and reduced glutathione (GSH) peroxidase in alloxan-induced diabetic rats.

CHAPTER TWO

LITERATURE REVIEW

2.1. DIABETES MELLITUS

The term diabetes mellitus describes a metabolic disorder of multiple aetiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The effect of diabetes mellitus includes long-term damage, dysfuntion and failure of various organs. Diabetes mellitus may present with characteristics symptoms such as thirst, polyuria, blurring vision, and weight loss. In its most severe forms, ketoacidosis or a non-ketoacitotic hyperosmolar state may develop and lead to stupor, coma and in the absence of effective treatment, death. Often symptoms are not severe, or may be absent, and consequently hyperglycemia sufficient to cause pathological and functional changes may be present for a long time before the diagnosis is made. The long-term effect of diabetes mellitus include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation, charcot joints, and features of autonomic dysfunction, including sexual dysfunction. People with diabetes are at increased risk of cardiovascular, peripheral vascular and cerebrovascular diseases.

Several pathogenetic processes are involved in the development of diabetes. These include processes which destroy the beta cells of the pancreas with consequent insulin deficiency, and others that result in resistance to insulin action. The abnormalities of carbohydrate, fat and protein metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin. Metabolic abnormalities in carbohydrates, lipids, and proteins result from the importance of insulin as an anabolic hormone. Low levels of insulin

to achieve adequate response and/or insulin resistance of target tissues, mainly skeletal muscles, adipose tissue, and to a lesser extent, liver, at the level of insulin receptors, signal transduction system, and/or effector enzymes or genes are responsible for these metabolic abnormalities. The severity of symptoms is due to the type and duration of diabetes. Some of the diabetes patients are asymptomatic especially those with type 2 diabetes during the early years of the disease, others with marked hyperglycemia and especially in children with absolute insulin deficiency may suffer from polyuria, polydipsia, polyphagia, weight loss, and blurred vision. Uncontrolled diabetes may lead to stupor, coma and if not treated death, due to ketoacidosis or the rare form non-ketotic hyperosmolar syndrome (Craig*et al.,* 2009;Galtier, 2010; American Diabetes Association, 2014).

2.2.0. Classification of diabetes mellitus

Although classification of diabetes is important and has implications for the treatment strategies, this is not an easy task and many patients do not easily fit into a single class especially younger adults and 10% of those initially classified may require revisiting. The classical classification of diabetes as proposed by the American Diabetes Association (ADA) in 1997 as type 1, type 2, other types, and gestational diabetes mellitus (GDM) is still the most accepted classification which is adopted by ADA (American Diabetes Association, 2014).

There are several forms of diabetes. Scientists are still defining and categorizing some of these variations and establishing their prevalence in the population. Types of diabetes include:

2.2.1. Type 1 diabetes mellitus

2.2.1.1. Autoimmune type 1 diabetes

This type of diabetes constitutes 5%-10% of subjects diagnosed with diabetes and is due to destruction of β cells of the pancreas. Type 1 diabetes accounts for 80%-90% of diabetes in

children and adolescents (Craig*et al.,* 2009; Dabelea*et al.,* 2014). According to the International Diabetes Federation (IDF), the number of youth (0-14 years) diagnosed with type 1 diabetes worldwide in 2013 was 497100 and the number of newly diagnosed cases per year was 78900. These figures do not represent the total number of type 1 diabetes patients because of the high prevalence of type 1 diabetes in adolescence and adults above 14 years of age. Type 1 diabetes is mainly due to an autoimmune destruction of the pancreatic β cells through T-cell mediated inflammatory response (insulitis) as well as a humoral (B cell) response (Devendra*et al.,* 2004). The presence of autoantibodies against the pancreatic islet cells is the hallmark of type 1 diabetes, even though the role of these antibodies in the pathogenesis of the disease is not clear. These autoantibodies include islet cell autoantibodies, and autoantibodies to insulin (IAA), glutamic acid decarboxylase (GAD, GAD65), protein tyrosine phosphatase (IA2 and IA2β) and zinc transporter protein (ZnT8A) (Vermeulen*et al.,* 2011). These pancreatic autoantibodies are characteristics of type 1 diabetes and could be detected in the serum of these patients' months or years before the onset of the disease (Couper& Donaghue, 2009).

Autoimmune type1 diabetes has strong HLA associations, with linkage to *DR* and *DQ* genes. HLA-DR/DQ alleles can be either predisposing or protective (American Diabetes Association, 2014). This autoimmune type 1 diabetes is characterized by the absence of insulin secretion and is more dominant in children and adolescents. In addition to the importance of genetic predisposition in type 1 diabetes, several environmental factors have been implicated in the etiology of the disease. Viral factors include congenital rubella, viral infection with enterovirus, rotavirus, herpes virus, cytomegalovirus, endogenous retrovirus and Ljungan virus (Stene*et al.,* 2010). Other factors include low vitamin D levels, prenatal exposure to pollutants, improved hygiene and living conditions decreased childhood infections in countries with high

socioeconomic status leading to increased autoimmune diseases (hygiene hypothesis), early infant nutrition such as using cow's milk formula instead of breast feeding in addition to insulin resistance in early childhood due to obesity or increased height growth velocity. The role of environmental factors remains controversial (Forlenza& Rewers, 2011).

Type1 diabetes often develops suddenly and can produce symptoms such as polydipsia, polyuria, lack of energy, extreme tiredness, polyphagia, sudden weight loss, slow-healing wounds, recurrent infections and blurred vision with severe dehydration and diabetic ketoacidosis in children and adolescents (International Diabetes Federation, 2013). The symptoms are more severe in children compared to adults. These autoimmune type 1 diabetes patients are also prone to other autoimmune disorders such as Graves' disease, Hashimoto's thyroiditis, Addison's disease, vitiligo, celiac sprue, autoimmune hepatitis, myasthenia gravis, and pernicious anemia (American Diabetes Association, 2014). In some children, the requirement for insulin therapy may drop to a point where insulin therapy could be withdrawn temporarily without detectable hyperglycemia (Lombardo*et al.,* 2002).

2.2.1.2. Idiopathic type 1 diabetes

A rare form of type 1 diabetes of unknown origin (idiopathic) has been reported,it is less severe than autoimmune type 1 diabetes and is not due to autoimmunity. Most patients with this type of diabetes are of African or Asian descent and suffer from varying degrees of insulin deficiency and episodic ketoacidosis (Abiru*et al.,* 2002).

2.2.1.3. Fulminant type 1 diabetes

This is a distinct form of type 1 diabetes, first described in the year 2000, and has some common features with idiopathic type 1 diabetes being non-immune mediated (Imagawa*et al.,* 2000). It is characterized by ketoacidosis soon after the onset of hyperglycemia, with

undetectable levels of serum C-peptide, an indicator of endogenous insulin secretion. It has been described mainly in East Asian countries and accounted for approximately 20% of acute-onset type 1 diabetes patients in Japan (5000-7000 cases) with an extremely rapid and almost complete beta-cell destruction resulting in nearly no residual insulin secretion. Both genetic and environmental factors, especially viral infection, have been implicated in the disease. Anti-viral immune response may trigger the destruction of pancreatic beta cells through the accelerated immune reaction with no detectable autoantibodies against pancreatic beta cells (Imagawa& Hanafusa, 2006;Imagawa& Hanafusa, 2011).

Autoimmune polyglandular syndrome (APS):Group of autoimmune endocrine diseases. Two of the three forms of APS feature type 1 diabetes.Unstable diabetes, also known as brittle or labile diabetes, is a term that may be used to describe any case of poorly controlled diabetes regardless of the type. All of these conditions involve diabetes mellitus ("sugar diabetes"). Diabetes insipidus ("water diabetes") is an unrelated endocrine system disorder in which the kidneys release too much water (Frank, 2004; Jawa *et al.,* 2004).

2.2.2. Type 2 diabetes mellitus

Type 2 diabetes mellitus (formerly known as non-insulin dependent diabetes mellitus) is the most common form of diabetes mellitus characterized by hyperglycemia, insulin resistance, and relative insulin deficiency. Type 2 diabetes mellitus results from interaction between genetic, environmental and behavioral risk factors (Chen*et al.,* 2011) People living with type 2 diabetes mellitus are more vulnerable to various forms of both short- and long-term complications, which often lead to their premature death. This is due the late onset and recognition, especially in resource-poor developingcountries like Africa (Azevedo&Alla, 2008).

The global prevalence of diabetes in adults (20-79 years old) according to a report published in 2013 by the International Diabetes Federation (IDF) was 8.3% (382 million people), with 14 million more men than women (198 million men and 184 million women), the majority between the ages of 40 and 59 years.The number is expected to rise beyond 592 million by 2035 with a 10.1% global prevalence. With 175 million cases still undiagnosed, the number of people currently suffering from diabetes exceeds half a billion. The Middle East and North Africa region has the highest prevalence of diabetes (10.9%), while, Western Pacific region has the highest number of adults diagnosed with diabetes (138.2 millions) and has also countries with the highest prevalence (International Diabetes Federation, 2013). Low- and middle income countries encompass 80% of the cases, "where the epidemic is gathering pace at alarming rates" (International Diabetes Federation, 2013).

Despite the fact that adult diabetes patients are mainly type 2 patients, it is not clear whether the reported 382 million adults diagnosed with diabetes also include type 1 diabetes patients. More than 90%-95% of diabetes patients belong to this type and most of these patients are adults. The number of youth (less than 20 years) with type 2 diabetes in the United States in the year 2009 was 0.46 in 1000 and accounted for approximately 20% of type 2 diabetes in youth (Dabelea*et al.,* 2014). The increased incidence of type 2 diabetes in youth is mainly due to the change in the lifestyle. Obesity is the major reason behind insulin resistance which is mainly responsible for type2 diabetes (Kraemer*et al.,* 2014). The prevalence of obesity in children is on the rise and this may account for the increased incidence of type 2 diabetes in the young (30.3% overall increase in type 2 diabetes in children and adolescence between 2001 and 2009) (Dabelea*et al.,* 2014).

Insulin resistance in type 2 diabetes patients increases the demand for insulin in insulin-target tissues. In addition to insulin resistance, the increased demand for insulin may not be met by the pancreatic β cells due to defects in the function of these cells (Halban*et al.,* 2014). On the contrary, insulin secretion decreases with the increased demand for insulin with time due to the gradual destruction of β cells that could transform some of the type 2 diabetes patients from being independent to become dependent on insulin. Most type 2 diabetes patients are not dependent on insulin where insulin secretion continues and insulin depletion rarely occurs. Dependence on insulin is one of the major differences from type 1 diabetes. Other differences include the absence of ketoacidosis in most patients of type 2 diabetes and autoimmune destruction of β cells does not occur. Both type 1 and type 2 diabetes have genetic predisposition, however, it is stronger in type 2 but the genes are more characterized in type 1 (the *TCF7L2* gene is strongly associated with type 2 diabetes) (Saadi*et al.,* 2008).

Due to the mild symptoms of type 2 diabetes in the beginning, its diagnosis is usually delayed for years especially in countries where regular checkup without symptoms is not part of the culture. This delay in diagnosis could increase the incidence of long-term complications in type 2 diabetes patients since hyperglycemia is not treated during this undiagnosed period. In addition to diabetes, insulin resistance has many manifestations that include obesity, nephropathy, essential hypertension, dyslipidemia (hypertriglyceridemia, low HDL, decreased LDL particle diameter, enhanced postprandial lipemia and remnant lipoprotein accumulation), ovarian hyperandrogenism and premature adrenarche, non-alcoholic fatty liver disease and systemic inflammation(Rosenbloom*et al.,* 2009; Kraemer*et al.,* 2014). The presence of type2 diabetes in children and adolescence that are not obese, the occasional severe dehydration and

the presence of ketoacidosis in some pediatric patients with type 2 diabetes had led to the misclassification of type 2 to type1 diabetes.

Some patients with many features of type 2 diabetes have some type 1 characteristics including the presence of islet cell autoantibodies or autoantibodies to GAD65 are classified as a distinct type of diabetes called latent autoimmune diabetes in adults (LADA) (Pozzilli& Di Mario, 2001). People diagnosed with LADA do not require insulin treatment. In a recent study, Hawa *et al.*(2014) reported 7.1% of European patients with type2 diabetes with a mean age of 62 years, tested positive for GAD autoantibodies and the prevalence of LADA was higher in patients diagnosed with diabetes at a younger age.

Defects in the insulin-dependent substrate proteins (IRS-1 and IRS-2) mediated signaling pathway are implicated in the development of metabolic disorders, mainly diabetes. This pathway mediates the cellular response to insulin and involves a large array of insulin-stimulated protein kinases including the serine/threonine kinase (AKT) and protein kinase C (PKC) that phosphorylate a large number of Ser/Thr residues in the insulin receptor substrate (IRS) proteins involved in the metabolic response to insulin (Copps& White, 2012). In addition, other non-insulin dependent kinases including the AMP-activated protein kinase, c-Jun N-terminal protein kinase and G protein-coupled receptor kinase 2 that are activated under various conditions can phosphorylate the two insulin responsive substrates (Copps& White, 2012;Boura-Halfon& Zick, 2009;White, 2003). Disruption in the AKT and PKC kinases is central to the development of diabetes and is associated with all major features of the disease including hyperinsulinemia, dyslipidemia and insulin resistance (Farese*et al.,* 2007). Replacing the wild type IRS-1 with a mutant version of the protein having alanine instead of tyrosine in three locations using genetic

knock-in approach provided evidence to the central role of IRS-1 phosphorylation in the development of insulin resistance (Morino*et al.,* 2008).

On the other hand, using a similar approach to generate IRS-1 mutant with a single mutation involving a specific tyrosine residue, confirmed the role of IRS-1 phosphorylation in the development of insulin resistance pathogenesis (Copps*et al.,* 2010). The large cumulative evidence indicates a complex array of factors including environmental factors and a wide range of cellular disturbances in glucose and lipid metabolism in various tissues contributes to the development of insulin resistance. This condition generates complex cellular metabolic changes in a variety of tissues, mainly liver and muscles that include the inability of the liver to transport and dispose glucose, control glucose production *via* gluconeogenesis, impaired storage of glucose as glycogen, *de novo* lipogenesis and hypertriglyceridemia (Otero*et al.,* 2014). Among the factors implicated in the development of insulin resistance, obesity is the most predominant risk factor leading to insulin insensitivity and diabetes which involves several mechanisms that participate in the pathogenesis of the disease. Obesity-induced insulin resistance is directly linked to increased nutrient flux and energy accumulation in tissues that directly affect cell responsiveness to insulin(Otero*et al.,* 2014). However, it seems that other insulin-independent mechanisms are involved in the overall metabolic disturbances of glucose homeostasis and diabetes including activities in extra-hepatic tissues in addition to the central role of the liver(Copps*et al.,* 2010).

2.2.3. Gestational diabetes mellitus

Gestational diabetes mellitus is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. Hormonal changes contribute to this condition which can develop in any previously non-diabetic woman during pregnancy, especially those who are

overweight.Hyperglycemia in pregnancy whether in the form of type 2 diabetes diagnosed before or during pregnancy or in other form has an increased risk of adverse maternal, fetal and neonatal outcome.

Mothers with gestational diabetes and babies born to such mothers have increased risk of developing diabetes later in life. Hyperglycemia in pregnancy is responsible for the increased risk for macrosomia (birth weight ≥ 4.5 kg), large for gestational age births, preeclampsia, preterm birth and cesarean delivery due to large babies (HAPO Study Cooperative Research Group, 2008). Risk factors for gestational diabetes include obesity, personal history of gestational diabetes, and family history of diabetes, maternal age, polycystic ovary syndrome, sedentary life, and exposure to toxic factors (Galtier, 2010).

2.2.3. Other types of diabetes mellitus

2.2.3.1. Monogenic diabetes mellitus

Monogenic diabetes is due to a genetic defect in single genes in pancreatic β cells which results in disruption of β cell function or a reduction in the number of β cells. Conventionally, monogenic diabetes is classified according to the age of onset as neonatal diabetes before the age of six months or maturity onset diabetes of the young (MODY) before the age of 25 years. However, certain familial defects are manifested in neonatal diabetes, MODY or adult onset diabetes (Craig*et al.,* 2009;Canivell& Gomis, 2014;Schwitzgebel, 2014). Others believe that classification of diabetes as MODY and neonatal diabetes is obsolete and monogenic diabetes is currently used relating specific genetic etiologies with their specific treatment implications. Beta cell differentiation depends on the expression of the homeodomain transcription factor (PDX1) where mutation in the gene results in early onset diabetes (MODY) and its expression decreases before the onset of diabetes (Kushner*et al.,* 2002).

The angiopoietin-like protein 8 (ANGPTL8) may represent a potential "betatrophin" that acts to promote the proliferation of beta cells.However, Gusarova*et al.* (2014) shows that mice lacking the ANGPTL8 active gene or overexpressed protein indicated that it did not seem to play a role in beta cells proliferation. Mitochondrial diabetes is due to a point mutation in the mitochondrial DNA associated with deafness and maternal transmission of the mutant DNA can result in maternally-inherited diabetes (Reardon*et al.,* 1992; American Diabetes Association, 2014). Mutations that result in mutant insulin or the inability to convert proinsulin to insulin may result in glucose intolerance in some of these cases. Genetic defects in the insulin receptor or in the signal transduction pathway of insulin have been demonstrated to result in hyperinsulinemia and modest hyperglycemia to severe diabetes (American Diabetes Association, 2014).

2.2.3.2. Disease of the exocrine pancreas

Damage of the β cells of the pancreas due to diffused injury of the pancreas can cause diabetes. This damage could be due to pancreatic carcinoma, pancreatitis, infection, pancreatectomy, and trauma (American Diabetes Association, 2014). Atrophy of the exocrine pancreas may lead to progressive loss of the β cells (Chen*et al.,* 2011). Accumulation of fat in the pancreas or pancreatic steatosis could lead to diabetes due to decreased insulin secretion but may require a long time before the damage to β cells occurs (Pezzilli& Calculli, 2014). In most cases, extensive damage of the pancreas is required before diabetes occurs and the exocrine function of the pancreas is decreased in these patients (Larger*et al.,* 2012). Cirrhosis in cystic fibrosis may contribute to insulin resistance and diabetes (Craig*et al.,* 2009).

2.2.3.3. Hormones and drugs

Diabetes has been found in patients with endocrine diseases that secrete excess hormones like growth hormone, glucocorticoids, glucagon and epinephrine in certain endocrinopathies like

acromegaly, Cushing's syndrome, glucagonoma, and pheochromocytoma, respectively (American Diabetes Association, 2014). Some of these hormones are used as drugs such as glucocorticoids to suppress the immune system and in chemotherapy and growth hormone to treat children with stunted growth.

2.2.3.4. Genetic syndromes

Diabetes has been detected in patients with various genetic syndromes such as Down syndrome, Klinefelter syndrome, Turner syndrome and Wolfram syndrome (American Diabetes Association, 2014).

2.3. Glycated Hemoglobin.

Glycated or glycosylated hemoglobin (HbA_{1c}) refers to the glucose derived products of normal adult hemoglobin (HbA). Glycation is a post-translational, non-enzymatic addition of sugar residue to amino acids of proteins (Jain, 1989). During diabetes, the excess glucose present in the blood reacts with hemoglobin to form glycosylated hemoglobin (HbA_{1c}). This is produced by the condensation of glucose with N-terminal valine of each β-chain of HbA. It has been reported that various proteins, including hemoglobin, albumin, collagen, low density lipoprotein, or crystalline proteins undergo non-enzymatic glycation in diabetes. Among the glycated hemoglobins, the most abundant form is HbA_{1c} (Klein, 1995). The rate of glycation is proportional to the concentration of blood glucose (Sheela & Augusti, 1992). Glycosylated hemoglobin has been found to be increased over a long period of time in diabetes (Bunn *et al*., 1978). Thus, the concentration of HbA_{1c} serves as an indication of the blood glucose concentration over a period, approximating to the half-life of RBC (hemoglobin) i.e. 6-8 weeks. A close correlation between the blood glucose and HbA_{1c} concentrations have been observed when simultaneously monitored for several months.

Normally, HbA_{1c} concentration is about 4.5-6.5% of the total haemoglobin, but in diabetic patients, HbA_{1c} value can be 2-3 times higher (Mallya & Pattabiraman,2001). Glycated hemoglobin reflects the mean blood glucose level for over 2 months period prior to its measurement. In the routine clinical practice, if the HbA_{1c} concentration is between 8.0 - 9.0 %, the diabetic patient is considered to be in good control. The control is said to be fair if the HbA_{1c} concentration is between 9.0 – 10.0 % while the patient is said to be at poor control if the HbA_{1c} concentration is greater than 10% (Schifreen *et al*., 1980). There is an evidence that glycation itself may induce the formation of oxygen-derived free radicals in diabetic condition (Gupta, 1997). Therefore, the measurement of glycosylated hemoglobin is a very sensitive index for glycemic control (Jain, 1989).

2.4.0. Complications of Diabetes Mellitus

People with diabetes have an increased risk of developing a number of serious health problems (Nathan *et al*., 2005). Long-term complications of diabetes develop gradually. Consistent high blood glucose levels can lead to serious diseases that eventually affect the heart and blood vessels, eyes, kidneys, nerves and teeth (cardiovascular disease, blindness, kidney failure, and lower limb amputation). In addition, people with diabetes also have a higher risk of developing infections. In general, diabetes complications may be disabling or even life-threatening (DCCTRG, 1995; Ahmed *et al.,* 2008). Complications of diabetes mellitus could be broadly divided into two classes, viz: acute and chronic complications. Acute complications include ketoacidosis while chronic complications include atherosclerosis, hematological abnormalities, kidney, eye, neuropathic diseases and foot alceration (WHO, 1994)

2.4.1. Acute Complications of Diabetes Mellitus

2.4.1.1: Diabetic Ketoacidosis (DKA)

Diabetic ketoacidosis (DKA) is an acute and dangerous complication that is always a medical emergency and requires prompt medical attention (Rosenstock *et al.,* 2008). Low insulin levels can cause the liver to turn fatty acid to ketone for fuel (i.e., ketosis); ketone bodies are intermediate substrates in the metabolic sequence. This is normal when periodic, but can become a serious problem if sustained. Elevated levels of ketone bodies in the blood decrease the blood's pH, leading to DKA. The patient in DKA is typically dehydrated and breathing rapidly and deeply. The level of consciousness is typically normal until late in the process, when lethargy may progress to coma (Rosenstock *et al.,* 2008). Ketoacidosis can easily become severe enough to cause hypotension, shock; and death can result from inadequate or delayed treatment, or from complications (e.g., brain edema). Ketoacidosis is much more common in type 1 diabetes than type 2diabetes (Sacks *et al.,* 2002).

2.4.1.2: Hyperosmolar Nonketotic State (HNS)

Hyperosmolar nonketotic state (HNS**)** is an acute complication sharing many symptoms with diabetic ketoacidosis (DKA), but it is entirely of different origin and treatment (Rosenstock *et al.,* 2008). In an individual with very high [usually considered to be above 300 mg/dL (16 mmol/L)] blood glucose levels, water is osmotically drawn out of cells into the blood and the kidneys eventually begin to dump glucose into the urine. This results in loss of water and an increase in blood osmolarity. If fluid is not replaced (by mouth or intravenously), the osmotic effect of high glucose levels, combined with the loss of water, will eventually lead to dehydration. The body's cells become progressively dehydrated as water is taken from them and excreted (Sacks *et al.,* 2002). Electrolyte imbalances are also common and are always dangerous.

As with DKA, urgent medical treatment is necessary, commonly beginning with fluid volume replacement. Lethargy may ultimately progress to a coma, though this is more common in type 2 diabetes than type 1 diabetes (Sacks *et al.,* 2002).

2.4.1.3: Hypoglycemia

Hypoglycemia, or abnormally low blood glucose, is an acute complication of several diabetes treatments. It is rare otherwise, either in diabetic or non-diabetic patients (Rosenstock *et al.,* 2008). The patient may become agitated, sweaty, weak, and have many symptoms of sympathetic activation of the autonomic nervous system resulting in feelings akin to dread and immobilized pain. Consciousness can be altered or even lost in extreme cases, leading to coma, seizures, or even brain damage and death in patients with diabetes. This may be caused by several factors, such as too much or incorrectly timed insulin, too much or incorrectly timed exercise (exercise decreases insulin requirements) or not enough food (specifically glucose containing carbohydrates)(Wold *et al*., 2005). It is more accurate to note that iatrogenic hypoglycemia is typically the result of the interplay of absolute (or relative) insulin excess and compromised glucose counterregulation in type 1 and advanced type 2 diabetes (Sacks *et al.,* 2002; Wold *et al*; 2005). In most cases, hypoglycemia is treated with sugary drinks or food. In severe cases, an injection of glucagon (a hormone with effects largely opposite to those of insulin) or an intravenous infusion of dextrose is used for treatment, but usually only if the person is unconscious. In any given incident, glucagon will only work once as it uses stored liver glycogen as a glucose source; in the absence of such stores, glucagon is largely ineffective. In hospitals, intravenous dextrose is often used (Soyers *et al*., 2001).

2.4.1.4: Diabetic Coma

Diabetic coma is a medical emergency in which a person with diabetes mellitus is comatose (unconscious) because of one of the acute complications of diabetes such as:

1. Severe diabetic hypoglycemia

2. Diabetic ketoacidosis advanced enough to result in unconsciousness from a combination of severe hyperglycemia, dehydration and shock, and exhaustion.

3. Hyperosmolar nonketotic coma in which extreme hyperglycemia and dehydration alone are sufficient to cause unconsciousness (Eriksson *et al.,* 1989).

2.4.1.5: Respiratory Infections

The immune response is impaired in individuals with diabetes mellitus (Ahmed *et al.,* 2008). Cellular studies have shown that hyperglycemia both reduces the function of immune cells and increases inflammation. The vascular effects of diabetes also tend to alter lung function, all of which leads to an increase in susceptibility to respiratory infections such as pneumonia and influenza among individuals with diabetes. Several studies also show diabetes associated with a worse disease course and slower recovery from respiratory infections (Ahmed *et al.,* 2008)

2.4.1.6: Periodontal Disease

Diabetes associated with periodontal disease (gum disease) may make diabetes more difficult to treat. Gum disease is frequently related to bacterial infection by organisms such as *Porphyromonas gingivalis* and *Actinobacillus Actinomycet*e (Lakschevitz *et al*., 2011) A number of trials have found improved blood sugar levels in type 2 diabetics who have undergone periodontal treatment (Mombelli, 2012).

2.4.2. Chronic Complication of Diabetes Mellitus

Chronic complication of diabetes mellitus could be grouped under microvascular and macrovascular diseases. Microvascular disease (due to damage to small blood vessels, such as capillaries) could results in retinopathy, nephropathy, and neuropathy while macrovascular disease (due to damage to large vessels, such as arteries and veins) could results in ischemic heart disease, peripheral vascular disease, and cerebrovascular diseases (Deshpande *et al.,* 2008). Examples of chronic complications are damage to small blood vessels leading to microangiopathy, which can cause one or more of the following:

2.4.2.1: Diabetic Cardiomyopathy

Damage to the heart muscle, leading to impaired relaxation and filling of the heart with blood (diastolic dysfunction) and eventually heart failure; this condition can occur independent of damage done to the blood vessels over time from high levels of blood glucose. (Kobayashi *et al*., 2014).

2.4.2.2: Diabetic Nephropathy

Diabetic nephropathy (DN) is a serious and progressive complication of both type 1 DM and type 2 DM. The first manifestation of DN is typically microalbuminuria, which progresses to overt albuminuria (i.e, increased albumin levels in the urine, indicating more severe renal dysfunction) and damage to the kidney which can lead to chronic renal failure, eventually requiring dialysis. (Drummond & Mauer, 2002). Diabetes mellitus is the most common cause of adult kidney failure in the developed world and is the leading cause of end-stage renal disease (ESRD) (Brenner, 2001). Approximately one fourth of people with type 2 diabetes have microalbuminuria or a more advanced stage of DN that worsens at a rate of 2% to 3% per year (Adler, 2003).

2.4.2.3: Diabetic Neuropathy (DN)

Abnormal and decreased sensation, usually in a 'glove and stocking' distribution starting with the feet but potentially in other nerves, later often fingers and hands; when combined with damaged blood vessels canlead to diabetic neuropathy. Other forms of diabetic neuropathy may present as mononeuritis or autonomic neuropathy. Approximately one half of people with diabetes have some form of peripheral neuropathy (PN), either polydiabetic or monodiabetic neuropathy (Dyck, 1993). Individuals with diabetes also frequently have autonomic neuropathy, including cardiovascular autonomic dysfunction, which is manifested as abnormal heart rate and vascular control (Vinik, 2003). Physical therapists commonly encounter diabetes- associated PN in the evaluation and treatment of balance and movement disorders because these disorders frequently affect lower-extremity sensation and can cause lower-extremity pain in people with diabetes. Loss of lower-extremity sensation coupled with impaired peripheral vascular function can contribute to lower-extremity (commonly foot) ulceration (Boulton, 1997).

2.4.2.4: Diabetic Foot Ulceration

Diabetic foot, often due to a combination of sensory neuropathy (numbness or insensitivity) and vascular damage, increases rates of skin ulcers (diabetic foot ulcers) and infection and, in serious cases, necrosis and gangrene. This is why diabetics are prone to leg and foot infections and why it takes longer for them to heal from leg and foot wounds. It is the most common cause of non-traumatic adult amputation, usually of toes and or feet, in the developed world. (Scott, 2013).

2.4.2.5: Diabetic Retinopathy

Diabetic retinopathy (DR) is a microvascular complication that can affect the peripheral retina, the macula, or both. It is characterized by growth of friable and poor-quality new blood

vessels in the retina as well as macular edema (swelling of the macula), which can lead to severe vision loss or blindness (Nathan *et al*., 2005). Retinal damage (from microangiopathy) is a leading cause of visual disability and blindness in people with diabetes. Also, it is the most common cause of blindness among non-elderly adults in the US (Nathan *et al*., 2005). The severity of DR ranges from non-proliferative and pre proliferative to more severely proliferative DR, in which the abnormal growth of new vessels occurs (Harding, 2003). Total or partial vision loss can occur through a vitreous hemorrhage or retinal detachment, and central vision loss can occur through retinal vessel leakage and subsequent macular edema (Sheetz, 2002).

The prevalence of DR increases with prolonged duration of diabetes (Orchard, 1990). In studies including people with both type 1 diabetes and type 2 diabetes, after 30 years of diabetes, most patients had some form of DR, and over half had proliferative DR; people with type 1 diabetes and taking insulin had the highest prevalence of DR, and people with type 2 diabetes diagnosed after age 30 had the lowest prevalence of DR (Klein *et al.,* 1984; Kempen *et al.,* 2004; Tyrberg *et al.,* 2007). Diabetic retinopathy also recently was seen in approximately 10% of people with insulin resistance (prediabetes) and was associated with the presence of hypertension and a higher body mass index (Tyrberg *et al*., 2007).

2.5. Molecular genetic basis of diabetes mellitus

Diabetes is a complex disease that involves a wide range of genetic and environmental factors. Over the past several years, many studies have focused on the elucidation of the wide spectrum of genes that played a role in the molecular mechanism of diabetes development. However, despite the vast flow of genetic information including the identification of many gene mutations and a large array of single nucleotide polymorphisms (SNPs) in many genes involved in the metabolic pathways that affect blood glucose levels, the exact genetic mechanism of

diabetes remains elusive (Doria*et al.,* 2008;Ahlqvist*et al.,* 2011). Evidently, a major complication is the fact that a single gene mutation or polymorphism will not impose the same effect among different individuals within a population or different populations.

This variation is directly or indirectly affected by the overall genetic background of the individual, family or population levels that are potentially further complicated by interaction with highly variable environmental modifier factors (Staiger*et al.,* 2009).

2.5.1. Molecular genetics and type 2 diabetes

Genome wide association studies (GWAS) in various populations identified 70 loci associated with type 2 diabetes and revealed positive linkage of many mutations and SNPs that influence the expression and physiological impact of the related proteins and risk to develop type 2 diabetes. One study involved several thousand type 2 diabetes patients and control subjects from the United Kingdom allowed the identification of several diabetes putative loci positioned in and around the *CDKAL1*, *CDKN2A/B*, *HHEX/IDE* and *SLC30A8* genes in addition to the contribution of a large number of other genetic variants that are involved in the development of the disease (Zegginі*et al.,* 2007). Two similar studies from the Finns and Swedish populations and the United States resulted in the identification of similar single nucleotide variants that are linked to the risk of acquiring type2 diabetes (Scott*et al.,* 2007).

Other GWAS analysis studies were performed in the Chinese, Malays, and Asian-Indian populations which are distinct from the European and United States populations in addition to meta-analysis of data from other populations in the region revealed relevant findings among patients with European ancestry (Hwang*et al*., 2014). The results of the combined analysis showed significant association of SNPs in the *CDKAL1*, *CDKN2A/B*, *HHEX*, *KCNQ1* and *SLC30A8* genes after adjustment with gender and body mass index. More recently, meta-analysis

of GWAS data involving African-American type 2 diabetes patients identified similar loci to the previous studies with the addition of two novel loci, HLA-B and INS-IGF (Ng*et al.,* 2014). These results provide strong evidence of common genetic determinants including common specific genes that are linked to diabetes. A small list of specific genetic markers seem strongly associated with the risk of developing type 2 diabetes including the *TCF7L2*, and *CAPN10* genes, which also play a significant role in the risk and pathogenesis of the disease. The association of *TCF7L2* gene variants with type2 diabetes and its mechanism of action, received special attention by several investigators (Cauchi*et al.,* 2006).

Over expression of the protein was shown to decrease the sensitivity of beta islet cells to secrete insulin and was more precisely involved in the regulation of secretary granule fusion that constitute a late event in insulin secretion pathway(da Silva Xavier*et al.,* 2009). The role of TCF7L2 in insulin secretion was partially clarified that involves modifying the effect of incretins on insulin secretion by lowering the sensitivity of beta cells to incretins. Several other genes have been found to be significantly associated with the risk of developing type 2 diabetes including a specific SNP in a hematopoietically-expressed homeobox (*HHEX*) gene (Li, *et al.,* 2012). The islet zinc transporter protein (SLC30A8) showed positive correlation with the risk of developing type 2 diabetes where variant mutations in this gene seem protective against the disease which provides a potential tool for therapy (Flannick*et al.,* 2014).

More recently, a low frequency variant of the HNF1A identified by whole exome sequencing was associated with the risk of developing type 2 diabetes among the Latino population and potentially may serve as a screening tool. Genetic variants and specific combined polymorphisms in the interleukin and related genes including interlukin-6 (*IL-6*), tumor necrosis factor-α and *IL-10* genes were found to be associated with greater risk of developing type 2

diabetes, in addition to genetic variants in the genes for *IL12B*, *IL23R* and *IL23A* genes (Eiríse*t al.,* 2014). In a study involving the hormone sensitive lipase responsible for lipolysis in adipose tissues, a deletion null mutation, which resulted in the absence of the protein from adipocytes, was reported to be associated with diabetes (Alberte*t al.,* 2014).

Nine specific rare variants in the peroxisome proliferator-activated receptor gamma (*PPARG*) gene that resulted in loss of the function of the protein in adipocytes differentiation, were significantly associated with the risk of developing type 2 diabetes (Majithiae*t al.,* 2014). In addition, certain SNPs in the alpha 2A adrenergic receptor (*ADRA2A*) gene, involved in the sympathetic nervous system control of insulin secretion and lipolysis, were found to be associated with obesity and type 2 diabetes (Långberge*t al.,* 2013). Link analysis between the melatonin MT2 receptor (*MTNR1B*) gene, a G-protein coupled receptor, identified 14 mutant variants from 40 known variants revealed by exome sequencing, to be positively linked with type 2 diabetes (Karamitrie*t al.,* 2013). The authors suggested that mutations in the *MT2* gene could provide a tool with other related genes in modifying therapy for type 2 diabetes patients based on their specific genetic background to formulate personalized therapies which potentially may ensures the optimum response. Interestingly, mutations in the clock and *Bmal1* transcription factor genes which are involved in beta cells biological clock affecting growth, survival and synaptic vesicle assembly in these cells, resulted in reduced insulin secretion and diabetes.

Evidently, prominent metabolic functions involve the production of specific reactive metabolites, leading to oxidative stress, which affect lipids, proteins and other biological compounds leading to serious damage in various tissues and organs. Mutations and SNPs in the antioxidant genes, including superoxide dismutase, catalase and glutathione peroxidase, that

decrease their activity are implicated in the risk and pathogenesis of type 2 diabetes (Banerjee& Vats, 2014).

2.5.2. Molecular genetics and type 1 diabetes

Even though type1 diabetes is basically described as an autoimmune disease that results in the destruction of pancreatic beta cells, however, single gene mutations and SNPs have been found to be associated with the susceptibility to this type of diabetes. Initially, two gene mutations were linked to the development of type 1 diabetes including the autoimmune regulator (*AIRE*) gene which affect the immune tolerance to self-antigens leading to autoimmunity and the *FOXP3* gene which results in defective regulatory T cells (Bennett*et al.,* 2001).

On the other hand, a mutation in the histone deacetylase *SIRTI* gene predominantly expressed in beta cells involved in the regulation of insulin secretion and played a role in modulating the sensitivity of peripheral tissues to insulin was detected in type 1 diabetes patients (Biason-Lauber*et al.,* 2013). Recently, additional mutations and SNPs in the *CTLA-4 +49A/G* and HLA-DQB1 and *INS* gene VNTR alleles were found to be associated with type 1 diabetes, which have the advantage of differentiating between Latent autoimmune type 1 diabetes and type 2 diabetes(Haller*et al.,* 2007). The HLA-DQB1, in combination with HLA-DR alleles and a polymorphism in *PTPN22* gene seem to be associated with the age onset of late type1 diabetes. Two specific polymorphisms in the promoter region of a transmembrane protein (DCSIGN) gene expressed in macrophages and played an important role of T- cell activation and inflammation were found to be protective against type 1 diabetes (da Silva*et al.,* 2014). An innovative non-parametric SNP enrichment tool using summary GWAS DATA allowed the identification of association between several transcription factors and type1 diabetes and is located in a type 1 diabetes susceptibility region (Burren*et al.,* 2014). Nine SNP variants in several genes associated

with type1 diabetes, not including the major histocompatibility gene region, were identified using extensive GWAS analysis (Evangelou*et al.,* 2014). Furthermore, several novel SNPs in a region in chromosome 16 located in the *CLEC16A* gene were shown to be associated with type1 diabetes and seem to function through the reduced expression of DEX1 in B lymphoblastoid cells (Tomlinson*et al.,* 2014). Since more than 40 regions in the human genome were identified to be associated with the susceptibility to type1 diabetes, a weighted risk model was developed utilizing selected genes SNPs could be used for testing infants for these genetic markers that could provide insights in the susceptibility to type1 diabetes development or safe prevention of the disease among young children (Winkler*et al.,* 2014).

2.6. Nutrition and diabetes

The principal step in the management of Diabetes mellitus is to train the patients in self-management care to prevent the early onset of diabetes complications. Dietary management is considered as a corner stone in the management of type 2 Diabetes mellitus.

2.6.1. Impact of carbohydrate intake on diabetes

It is known that a high carbohydrate intake increases the requirement for insulin secretion in order to maintain glucose homeostasis. Insulin secretion by beta-cells is glucose sensitive and a high intake of carbohydrate in relation to energy intake produces higher post-prandial insulin levels. It is possible that repeated stimulation of a high insulin output by a high carbohydrate diet could speed up an age-related decline in insulin secretion and lead to an earlier onset of type2 diabetes(Grundy,1999). The quality as well as the quantity of carbohydrate may hasten this response (Grundy,1999). The most recent American dietary guidelines recommend intake of a variety of grain products (including whole grains) equating to six or more servings a day (Krauss *et al.,* 2000). The FAO/WHO recommend that carbohydrate in the diet should comprise at least

55% of total energy intake in 'normal' healthy individuals (Carbohydrates in Human Nutrition, 1998). There is, however, no specific carbohydrate guideline, which is aimed at the prevention of type2 diabetes. Therefore, a wide range of carbohydrate intakes may be acceptable in terms of achieving a low risk of type2diabetes with type and source of carbohydrate being more important than quantity.

A randomized controlled trial carried out by Foster *et al.* (2003) stated that diabetics consuming a low carbohydrate diet had a significant weight loss as compared to diabetics who were on a conventional diet. A study conducted in women reported that carbohydrates like rice and barley had harmful effects on HbA1c. Another study conducted on type2 Diabetes mellitus patients revealed that low carbohydrate and low fat hypo-caloric diets led to an improvement in HbA1c level. Shadman *et al.* (2013) suggested that type 2 diabetics on low carbohydrate & low saturated fat diet had better glycaemic control. Hajime *et al.* (2009) reported that restricting carbohydrate diet to 45 per cent led to a greater reduction in HbA1c as compared to diets high in carbohydrates. Study conducted by Arora (2005), concluded that during 5 weeks dietary intervention HbA1c decreased significantly on limiting the carbohydrates consumption between 40–55 per cent.

2.6.2. Impact of protein (meat and fish) intake on diabetes

A systematic review of 12 cohort studies stated that consuming red meat more than three times a week significantly increases the risk of type 2 Diabetes mellitus. A longitudinal study conducted in men by Van Dam *et al.* (2002) reported that increased consumption of processed meat was related with a higher risk for type 2 Diabetes mellitus (relative risk=1.46, for more than five times a week and less than once per month, $p<0.0001$). Gross *et al.* (2002) suggested that limiting the amount of red meat as compared to chicken had a significant effect on HbA1c,

fasting blood glucose (FBG), low density lipoprotein – cholesterol (LDL-C), and high density lipoprotein – cholesterol (HDL-C). In women with type 2 Diabetes mellitus, frequent intake of red meat as compared to chicken was related with high risk of coronary heart disease (CHD) (p<0.001). From previous studies, it has been reported that the eating of fish, especially long chain omega 3 fatty acids have several beneficial metabolic effects in diabetics. Wallin *et al.* (2012) concluded from his study conducted on type 2 Diabetes mellitus patients that consuming fish had positive effects on their glycaemic control, glucose tolerance and microalbuminuria.

2.6.3. Impact of fatty acids on diabetes mellitus

Higher proportions of saturated fatty acids in serum lipids/muscle phospholipids have been associated with higher fasting insulin levels, lower insulin sensitivity and higher risk of developing type 2 diabetes (Vessby*et al.,* 1994). Higher vegetable fat (unsaturated fat) and PUFA intake have in turn been associated with a lower risk of type 2 diabetes, as well as lower fasting and 2-hr glucose concentrations (Mooy*et al.,* 1995). Furthermore, higher proportions of long-chain polyunsaturated fatty acids in skeletal muscle phospholipids have been associated with better insulin sensitivity in humans. With respect to monounsaturated fatty acids, the epidemiological data are inconsistent.

In the light of the present knowledge regarding the relationships between type2 diabetes and nature and quantity of dietary fat, as well as the absence of definitive data regarding precise percentage of fat to total energy, it seems reasonable to suggest that quantitative recommendations should follow those suggested for reduction of cardiovascular risk(Mooy *et al.,* 1995).

2.6.4. Impact of high fibre intake on diabetes mellitus

Various studies have reported that high intake of fibre has a positive effect on diabetes mellitus. Fujii *et al.* (2013) conducted a study in Japanese type2 diabetics and stated that diabetics should be encouraged to consume diets high in fibre on a daily basis because of their beneficial effect on HbA1c; and it also lowers the risk of cardiovascular diseases. Another study conducted by Chandalia *et al.* (2000) in type2 diabetics concluded that consumption of soluble dietary fibre, as recommended by the American Diabetes Association (ADA), improves glycaemic control, and decreases hyperinsulinemia. Mcintosh (2001) conducted a study in a group of type 2 diabetic patients and reported that decline in HbA1c values and lipid profile was observed in patients on a high fibre diet. Patients on high fibre diets also had a significantly lower risk of cardiovascular diseases (CVD), microvascular and macrovascular complication of diabetes mellitus.

2.6.5. Impact of dairy products on diabetes mellitus

Studies have reported that intake of dairy products leads to positive effects on weight, glycemic control and cardiovascular diseases (Sanchez-Lugo*et al.,* 1997;Imai*et al.,* 2013). However, there is a scarcity of literature relating frequency of dairy intake and control of diabetes. A study conducted by Mizoue *et al.* (2006) found that low fat dairy products had beneficial effect on glycemic control. Calcium and vitamin D deficiencies have harmful effect on glycaemia, whereas the combined effect of these supplementations is beneficial in enhancing glucose metabolism. According to Liu *et al.* (2006) men and women who consume low fat dairy products have a lower risk of developing type2 Diabetes mellitus. A meta-analysis conducted on dairy products and the risk of type 2 diabetes showed that there was a significant inverse

relationship between intake of low fat dairy products, and cheese with the risk of type2 Diabetes mellitus.

2.6.6. Impact of fruits and vegetables on diabetes mellitus

Various studies have reported that consuming fruits and vegetables are helpful in decreasing the risk of diabetes mellitus. Moreover in type2 diabetes mellitus, fruits and vegetables are also beneficial in maintaining good glycaemic control. Study conducted by Franz *et al.* (2002) reported that increased intake of vegetables and fruits significantly decreased HbA1c level. However, the same study also reported that vegetable intake significantly decreased Triacylglycerol (TAG) levels ($p<0.001$). A systematic review and meta-analysis conducted by Carter *et al.* (2010) concluded that consuming vegetables and fruits in an increased amount had a significant effect on body mass index (BMI), Triacylglycerol (TAG) and HbA1c. A study done by Hamer (2007) on diabetic patients reported a significant effect of a semi-vegetarian and full vegetarian diet on glycaemic control. Patients on a semi-vegetarian diet had lower fasting plasma glucose levels; whereas, patients on a full vegetarian diet had significant lower HbA1c levels. More studies that investigated the association between vegetables and fruits intake and diabetes mellitus concluded that the onset of diabetes can be prevented by increasing the intake of vegetables and fruits (Harding*et al.,* 2008).

2.6.7. Impact of legumes on diabetes mellitus

Low glycemic index (LGI) foods (beans, lentils and chickpeas) have been shown to improve glycemic control in patients with type2 diabetes mellitus. Legumes have also been included in the International Diabetes Guidelines, 2013. Legumes comprise of various nutrients including dietary fiber, oligosaccharides, vegetable protein, complex carbohydrates and minerals. Shoff *et al.* (1993) stated that foods with low glycemic index (LGI) such as legumes are

beneficial for diabetic patients. Another study by Wolever *et al.* (1992) also reported a beneficial effect of a low glycemic index (LGI) diet on HbA1c in treatment of type 2 diabetes mellitus.

2.6.8. Impact of drinks on diabetes mellitus

Consumption of sweetened beverages (soft drinks, fruit drinks, iced drinks, energy drinks and vitamin water drinks) has risen across the globe. Consuming soft drinks and other sweetened beverages can significantly increase the risk of obesity and type2 diabetes mellitus (Malik*et al.,* 2010). A prospective study conducted in a Chinese population reported that consuming soft drinks more than two times per day has a relative risk of 1.42 for type2 diabetes mellitus (Odegaard*et al.,* 2010). It further concluded that consuming 200 ml of soft drinks had a significant effect on blood sugar levels. The results from meta-analysis based on eight cohort studies revealed that persons who consume soft drinks more than three times per day had 26 per cent more chance to develop type2 diabetes mellitus as compared to those who consume soft drinks less than once a day (Malik*et al.,* 2010).

Another study by Seifert*et al.* (2011) reported the relation between coffee consumption and risk of type 2 diabetes mellitus and, the findings revealed that long term coffee consumption significantly lowers the risk of type 2 diabetes mellitus. Over the past few decades, globally the consumption of energy drinks (red bull, power horse etc.) has increased dramatically. Several studies have reported adverse effect of energy drinks on health especially in adolescents. Moreover, it increases the risk of obesity, type 2 diabetes mellitus and has a direct effect on blood sugar levels (Alsunni & Badar, 2011).

2.7.0. Role of oxidative stress in 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 condition of imbalance between ROS and their protection occurs which leads to domination of the condition of oxidative stress. 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.,* 2012). ROS are produced by neutrophils and macrophages during the process of respiratory burst in order to eliminate antigens, they also serve as stimulating signals of several genes which encode transcription factors, differentiation, and development as well as stimulating cell-cell adhesion, cell signalling, involvement in vasoregulation, fibroblast proliferation, and increased expression of antioxidant enzymes (Gomes*et al.,* 2012 & Sen, 2001). However 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 wellas in the myocardium (Giacco & Brownlee, 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.

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 & Yorek, 2010).

Oxidative stress in diabetes mellitus causes several adverse effects on the cellular physiology. This is particularly relevant and dangerous for the islet, which is among those tissues that have the lowest levels of intrinsic antioxidant defences. Multiple biochemical pathways and mechanisms of action have been implicated in the deleterious effects of chronic hyperglycemia and oxidative stress on the function of vascular, retinal, and renal tissues (Folli*et al.,* 2011; Fiorentino *et al.,* 2013). The oxidative stress-induced alterations in the major biomolecules in the cell during type 2 diabetes mellitus are described below.

2.7.1. 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& Chirico, 1993). Lipids peroxidation produces highly reactive aldehydes, such as malondialdehyde (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 (Lobo*et al.,* 2010). MDA has

been documented as a primary biomarker of free radical mediated lipid damage and oxidative stress (Shodehinde&Oboh, 2013).

Significant changes in lipid metabolism and structure have been reported in diabetes, particularly in patients with vascular complications. 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 non-enzymatic 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 & Rizvi, 2011). Baynes in 1991, followed by Ramesh *et al.* in 2012, reported that lipid peroxidation in diabetes induced many secondary chronic complications including atherosclerosis and neural disorders. Yang *et al.* (2009) observed 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 infraction through NADPH oxidase activation.

Lipid peroxidation of cellular structures is thought to play an essential 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& Shin, 2009;Varashree& Bhat, 2011). Increased lipid peroxidation presents a close relationship with the high glycemic levels and oxidative stress in diabetes mellitus (Salgueiro*et al.,* 2013). A clinical study performed by Bandeira and coworkers (2012) 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.

2.7.2. Protein Oxidation

Proteins are the most important vital biomoleculesof the cell. They are involved in many physiologicalfunctions such as cell signalling and transport across thecells. Proteins are another potential target of ROS, whosestructure and function can be affected by modification. Thereare many side chain targets for protein oxidation includingcysteine, methionine, and tyrosine. Carbonyls are the oxidationproduct of proteins and are reported as the potent biomarkerof oxidative stress (Suzuki&Miyata, 1999).They represent the stable endproduct generated upon formation of transient radical species,such as chloramines and nitrogen/carbon radicals,which are induced by oxidant stimuli. Glycation has beenreported to induce the formation of protein carbonyls, such asketoamine derivatives, thus generating reactive radicals andperpetuating a vicious cycle. Damage of proteins followed by accumulation of their oxidation products affects cellular physiology adversely. Increased glycol- and lipooxidation are reported as one of the major factors in the accumulation of non-functional damaged proteins (Sakul*et al.,* 2013).

Gradinaru *et al.* (2013) have reported the significance of the oxidative and glycoxidative protein damage in subjects with prediabetes and type 2 diabetes mellitus. Advanced glycated end products (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. AGEs are formed through non-enzymatic amino-carbonyl interactions between reducing sugars or oxidized lipids and proteins, amino phospholipids, or nucleic acids. The generation of AGEs may lead to intracellular modifications of proteins, including those involved in the regulation of gene

expression (Barbosa*et al.,* 2008). AGEs are capable of modifying the circulating proteins in the blood that have receptors for AGEs, activating them followed by inducing the production of inflammatory cytokines and growth factors in endothelial cells (de*et al.,* 2013).

2.7.3. Glutathione concentration

Glutathione (GSH), a tripeptide, γ-Lglutamyl- L-cysteinylglycine, is present in all mammalian tissues at 1–10mM concentrations (highest concentration in liver) as the most abundant non-protein thiol that defends against oxidative stress. GSH can maintain SH groups of proteins in a reduced state, participate in amino acid transport, detoxify foreign radicals, act as coenzyme in several enzymatic reactions, and also prevent tissue damage. It is an efficient antioxidant present in almost all living cells and is also considered as a biomarker of redox imbalance at cellular level (Rizvi& Chakravarty, 2011). There are several reports that claim reduced level of GSH in diabetes mellitus, Dincer *et al.* (2002) shows that decreased GSH level may be one of the factors in the oxidative DNA damage in type 2 diabetic's patients.

As a consequence of increased oxidative status, GSH showed the frequent alteration in its concentration. Plasma GSH/GSSG showed a significant decrease in type 2 diabetes as compared to normal (Calabrese*et al.,* 2012). Hyperlipidemia, inflammation, and altered antioxidant profiles are the usual complications in diabetes mellitus as a result of decreased GSH/GSSG ratio (Das*et al.,* 2012). Abnormal GSH status is involved in β-cell dysfunction and in the pathogenesis of long-term complications of diabetes. The dysregulation is widely implicated in disease states (Livingstone& Davis, 2007).

Glutathione reductase (GSR) plays an important role through the reduction of GSSG to GSH and oxidation of NADPH to NAD+. GSSG is unable to perform antioxidant functions; however, GSH can be reclaimed from GSSG through the use of glutathione reductase (GSR) by

the use of NADPH as a cofactor. Unfortunately, this GSH system can be overwhelmed if ROS are produced in excess (Morris*et al.,* 2013). Uncontrolled type 2 diabetes has severely deficient synthesis of GSH attributed to limited precursor availability. Dietary supplementation with GSH precursor amino acids can restore GSH synthesis and lower oxidative stress and oxidant damage in the face of persistent hyperglycemia (Sekhar*et al.,* 2011).

It has been observed that GSH deficiency in diabetics increased their susceptibility to melioidosis. It is hypothesized that maintenance of GSH redox state may be a new therapeutic avenue to protect diabetic patients against some intracellular bacterial pathogens (Tan*et al.,* 2012).

2.7.4. Catalase

Catalase is an antioxidative enzyme present nearly in all living organisms. It plays an important role against oxidative stress-generated complications such as diabetes and cardiovascular diseases (Chelikani*et al.,* 2004). Catalase acts as main regulator of hydrogen peroxide metabolism. Hydrogen peroxide is a highly reactive small molecule formed as natural by-product of energy metabolism. Excessive concentration of hydrogen peroxide may cause significant damages to proteins, DNA, RNA, and lipids (Takemoto*et al.,* 2009).Catalase enzymatically processes hydrogen peroxide into oxygen and water and thus neutralizes it. Increased risk of diabetes has been documented in patients with catalase deficiency. The deficiency of this enzyme leads, in the β-cell, to an increase in oxidative stress and ultimately to a failure of this cell type. β-cells are rich in mitochondria, and thus this organelle might be a source of ROS (Goth& Eaton, 2000).m

Catalase protects pancreatic β-cells from damage by hydrogen peroxide. Low catalase activities, which have been reported in patients with schizophrenia and atherosclerosis, are

consistent with the hypothesis that long-term oxidative stress may contribute to the development of a variety of late-onset disorders, such as type 2 diabetes (Goth, 2000). Deficiency of catalase increases mitochondrial ROS and fibronectin expression in response to free fatty acids, which were effectively restored by catalase overexpression or N-acetyl cysteine (Hwang*et al.,* 2012). Low catalase activities can cause methemoglobinaemia and hemolytic anemia which may be attributed either to deficiency of glucose-6-phosphate dehydrogenase or to other unknown circumstances and also may damage heme proteins, cause cell death, and, together with redox active metal ions, produce highly toxic hydroxyl radicals (Goth& Bigler, 2007; Goth*et al.,* 2005).

Patel *et al.* (2013), during investigation of hyperglycemia- induced functional changes, superoxide, hydrogen peroxide production, mitochondrial membrane polarization, and gene expression fingerprints of related enzymes in endothelial cells, have reported that hyperglycemia increased hydrogen peroxide production, hyperpolarized mitochondrial membrane, and down regulated CAT gene expression.

2.7.5. Superoxide Dismutase

Superoxide dismutase (SOD) is the antioxidant enzyme that catalyses the dismutation of superoxide anion (O^{2-}) into hydrogen peroxide and molecular oxygen. 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 catalase, it is converted into oxygen and water. All mammalian tissues contain three forms of SOD: Cu-Zn-SOD, Mn-SOD, and extracellular EC-SOD, and each of them is a product of a distinct gene (Beyer*et al.,* 1991; Li*et al.,* 2012). 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.

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 downregulation of renal SOD (SOD 1 and SOD 3) may play a key role in the pathogenesis of diabetic nephropathy (Fujita*et al.,* 2009). Overexpression of SOD or the supplements of antioxidants including SOD mimetics, targeted to overcome oxidative stress, reduce ROS, and I ncrease 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 (Fattman*et al.,* 2003). 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.,* 2003). 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 and colleagues (2013) to observe the oxidative balance of diabetic rats reported diminished activity of SOD and other antioxidative enzymes in the liver tissue.

2.8.0. GINGER

2.8.1. General description

Ginger is a tropical plant, grows well in hot and humid climates. The plant is cultivated in China, Nepal, US, India, Bangladesh, Taiwan, Jamaica, Nigeria and Indonesia. India is the biggest producer of *Z. officinale*. In Indonesia, *Z. officinale* is one of the export commodities, with the development area in 2010 reaching 6,053 ha and the requirement of ginger seed is rhizome 12.106 tons/year. In accordance with the requirements of growing ginger, seed production sites can be selected on land with climate type A, B, or C, with altitude of 300-900 dpl, average temperature 25-30^{o}c, wet month number 7-9 months, rainfall 2,500-4,000 mm/year, with light intensity 70-100% (Sukarman, 2013).

Ginger is herbaceous rhizomatous perennial, reaching up to 90 cm in height under cultivation. Rhizomes are aromatic, thick lobed, pale yellowish, bearing simple alternate distichous narrow oblong lanceolate leaves. The herb develops several lateral shoots in clumps, which begin to dry when the plant matures. Leaves are long and 2 - 3 cm broad with sheathing bases, the blade gradually tapering to a point. Inflorescence solitary, lateral radical pedunculate oblongcylindrical spikes. Flowers are rare, rather small, calyx superior, gamosepalous, three toothed; open splitting on one side, corolla of three sub equal oblong to lanceolate connate greenish segments, stems erect (Mishra *et al.,* 2012). The leaves are often clear 2 rows with stem hugging, and tongue between the borders and leaf blades. Zygomorph flowers are bandaged. Petals forms as tube, with a tip hammered, often split in a midrib. Rhizome slightly flat, bagin tip short and flat branches, at each end of the branch there is a grooved curve inside. The outer pieces are yellowish brown, grooved lengthwise (Kepmenkes, 2011).

2.8.2. Botanical description

Kingdom : Plantae

Division : Magnoliophyta

Class : Liliopsida

Order : Zingiberales

Family : Zingiberaceae

Genus : Zingiber

Species : *Zingiber officinale*

2.8.3. History and Traditional uses

Ginger is native to Southeastern Asia (Wagner, 1980). It is mentioned in ancient Chinese, Indian, and Middle Eastern periodicals and has long been valued for its aromatic, culinary, and medicinal properties (Langner, 1998). Confucius wrote about ginger in his Analects and the Greek physician Dioscorides listed ginger as an antidote to poisoning, as a digestive, and as being warming to the stomach in De Materia Medica (Langner, 1998). Many religious holy books the Quran, the Talmud, the Bible, Ayurveda, CharakSushutra, Vagbhatta and CharakDutta have mentioned ginger. The medicinal properties of ginger were known in ninth century inGermany and France and in tenth century inEngland**.** Records suggest that ginger was highly valued as an article of trade during the 13th and 14th century in England; one pound of ginger had the same worth as that of sheep. Ginger migrated westward to Europe by Greek and Roman times. History shows that ancient Romans imported ginger from China almost two thousand years ago. By the middleAges it was a very popular spice in the Mediterranean region and had spread throughout other countries. Medieval writing from many European countries indicates that ginger was a standard ingredient in recipes for the kitchen and the apothecary (Widmaier,1986). In an attempt to make it more available, Spanish explorers introduced ginger

to the West Indies, Mexico, and South America in the 16th century and these areas began exporting this precious herb back to Europe.

Ginger plants grown in pots were carried abroad onlong sea voyages to prevent scurvy. The Eclectic physicians of the 19th century relied on ginger to induce sweating, improve the appetite, and curb nausea and as a topical counterirritant. Ginger is an integral part of Ayurveda, the traditional medicine of India, and is known as sunthi in Ayurveda (Hrdayam of Srimadvagbhatt, 1999). It was used to block excessive clotting of blood in arteries and veins, to reduce cholesterol, and to fight against arthritis. In Traditional Chinese Medicine (TCM) ginger is considered a pungent, dry, warming herb to be used for ailments triggered by cold and damp weather. It was also used as a digestive aid and antinausea remedy and to treat bleeding disorders, rheumatism, baldness, toothache, snakebite, and respiratory conditions. The Romans added ginger to the oil in lamps to render an aroma in the air. Meanwhile in England ginger was added to spice up beer. The Greeks wrapped ginger in bread and ate it after meals as digestive aid. Subsequently, ginger was incorporated directly into bread and confectionaries such as gingerbread. As ginger resembles fingers, pregnant women in China are advised to avoid ginger during pregnancy, as they might give birth to babies with more than five fingers. But after birth a woman may take it for strength, to clean out all poison from her body, and to protect the newborn (Wong, 2001). In Malaysia and Indonesia, ginger soup is given to new mothers for 30 days after their delivery to help them sweat out impurities. In Arabian medicine, ginger is considered an aphrodisiac. Some Africans believe that eating ginger regularly will help repel mosquitoes and women of central Africa make belts of ginger roots to attract the attention of their husbands. Ginger flowers are traditionally worn by Hawaiian dancers (Gilani, 2005).

2.8.4. Culinary Use

Ginger is consumed worldwide as spice, flavoring agent, garnish, medicine, and food preservative and is used either fresh, in a fresh paste, or dry, in a dry powder. Fresh ginger can be substituted for dried ground ginger, although the flavors of fresh and dried ginger are somewhat different. Powdered dry ginger is typically used as a flavoring for recipes such as gingerbread, cookies, crackers and cakes, ginger ale, and beer. The fragrance of ginger is penetrating and aromatic. It tastes spicy, hot, and biting and is an integral part of almost all the cuisines of the world. The pungent, spicy sweetness of ginger adds a unique taste to many recipes ranging from sweet to savory.

In the subcontinents (India and Pakistan) ginger is called *Adrak* (local name) and is an essential ingredient of many dishes. Fresh ginger is one of the main spices used for making pulse, vegetablecurries and meat preparations. Fresh as well as dried ginger is used to spice tea and coffee, especially in winter. In Burma, it is consumed as a salad dish called *Gyin-thot*, which consists of shredded ginger preserved in oil, and a variety of nuts and seeds. In Indonesia, a beverage called *wedangjahe* is made from ginger and palm sugar. In the Philippines, it is brewed into a tea called *salabat*. In Vietnam, the fresh finely chopped leaves can also be added to shrimp-and-yam soup (*canhkhoaimỡ*) as a top garnish and spice to add a much subtler flavor of ginger than the chopped root. In China, sliced or whole ginger is often paired with savory dishes such as fish, and chopped ginger root is commonly paired with meat, when it is cooked. In Japan, ginger is pickled to make *BeniShoga* and gari or grated and used raw on tofu or noodles. It is also used to make candy called *Shoga no satozuke*. In the traditional Korean *Kimchi*, ginger is finely minced and added to the ingredients of the spicy paste just before the fermenting process (Kim *et al.,* 2005).

In the Caribbean, ginger is a popular spice for cooking and making drinks such as *sorrel*, a seasonal drink made during the Christmas season. Jamaicans make ginger beer both as a carbonated beverage and also fresh in their homes. Ginger tea is often made from fresh ginger, as well as the famous regional specialty Jamaican ginger cake.

In Arabic, ginger is called *Zanjabil*, and in some parts of the Middle East, ginger powder is used as a spice for coffee and for milk. In the Ivory Coast, ginger is ground and mixed with orange, pineapple, and lemon to produce a juice called *Nyamanku*. Yemenite Jews add ginger powder in *Hawaij*, a spice mixture used mostly for soups and coffee (Roden, 1996).

2.8.5. Chemical Composition

Ginger contains approximately 50% carbohydrates, 9% protein and free amino acids, 6-8 % fatty acids and triglycerides, 3-6% ash, and 3-6% crude fiber (on dry matter basis) depending on variety, geography, and climatic conditions (Leung, 1984; Tang, 1992). Some African ginger varieties contain 5.98 and3.72g /100 proteins and fat (Shrin, 2010). Soluble and insoluble fibers are also found in ginger. Ginger is a good source of essential micronutrients such as potassium, magnesium, copper, manganese and silicon. Potassium and manganese help to build resistance to disease and protect the lining of heart, blood vessels and urinary passages. Silicon promotes healthy skin, hair, teeth, and nails and helps to assimilate calcium. Small amount of vitamins A, E and some amounts of B- vitamins and Vitamin C are also found in ginger rhizome.

2.8.6. Phytochemical composition

Ginger is a complex substance consisting of more than 60 compounds (Srivastava *et al.,* 2000). The ginger rhizome contains an essential oil and resin known collectively as oleoresin. The composition of the essential oil varies according to the geographical origin, but the chief constituents are sesquiterpene hydrocarbons, which are responsible for the characteristic aroma.

Gingerole is the main phenolic compond and once degraded gives shogaols, zingerone, and paradol. Zingerone and shogaols are found in small amounts in fresh ginger and in larger amounts in dried or extracted products. Zingerone is also produced from gingerols during this process; this compound is less pungent and has a spicy-sweet aroma. Smaller amounts of other sesquiterpenoidsbisabolene, geranyl acetate, terpeneol, terpanes, geraniol, alpha pinene, limonene, zigerbene, batabeasabolene, alpha paradol, farnesene, and monoterpenoid fraction (β-phelladrene, cineol, and citral) have also been identified. Ginger contains a special group of compounds called diasyleheptanoids, which includesgingerenone. A very small amount of curcumin is also found in ginger. In addition to that it also contains small amounts of alkaloids, tannins, carotenoids, saponins, flavonoids, steroids, and cardinolides (Shrin, 2010).

The composition of fresh ginger oil contains more oxygenated compounds compared to dry ginger oil, making it more potent than dry ginger oil. There are more hydrocarbon compounds in dry ginger oil compared to fresh ginger oil. Monoterpene compounds are more active than sesquiterpene compounds. Dry ginger oil also has higher content of sesquiterpene hydrocarbons and they are reported to have less activity compared to oxygenated compounds (Srivastava*et al.,* 2000; Sinha*et al.,*1990). Ginger oil (GEO) has been characterized to have a high content of sesquiterpene hydrocarbons, including β-sesquiphellandrene (27.16%), caryophyllene (15.29%), zingiberene (13.97%), *α*-farnesene (10.52%) and *ar*-curcumin (6.62%) (El-Baroty *et al.,* 2010).

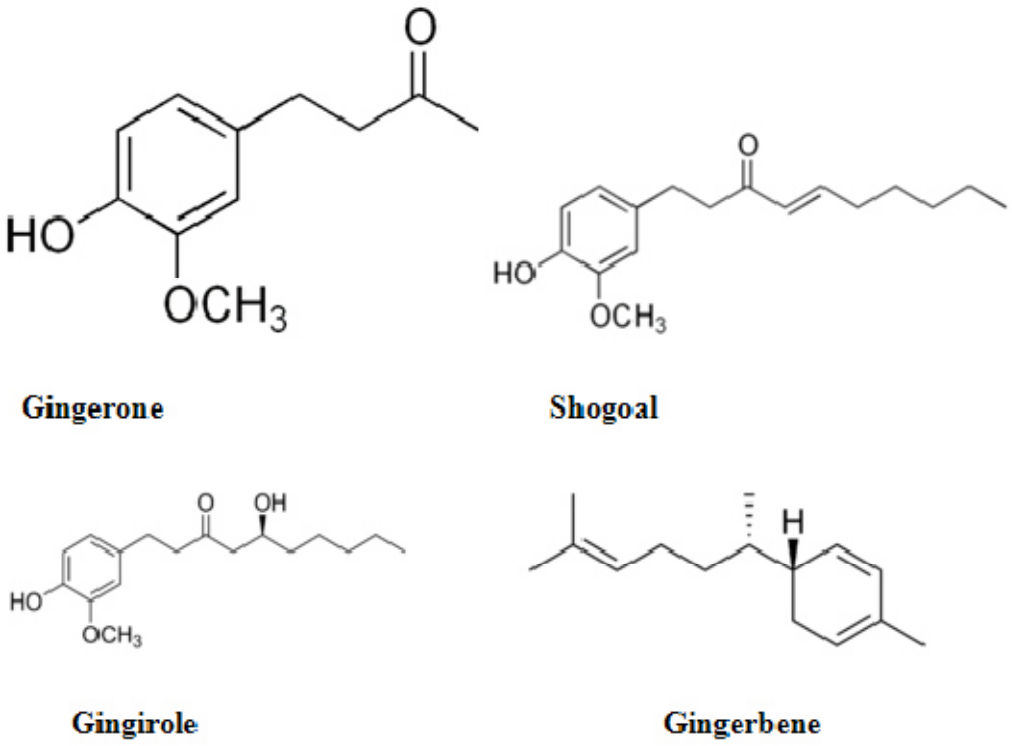


Figure2.1. Chemical structure of components of ginger

2.8.7. Pharmacological activity

Ginger has been used as Ayurvedic medicine from Vedic period and is called "mahaaushadhi", means the great medicine. In traditional medicine, it was used as a carminative or anti-flatulent. The Greek physician Galen used ginger as a purificant of body (Polasa, 2003). Recent study showed that it has antioxidant, anticancer, anti-inflammatory, antiapoptotic, anti-hyperglycemic, antihyperlipidemic and anti-emetic actions (Rehman*et al.,* 2010; Kumar*et al.,* 2013). Owing to its different active ingredients, ginger is considered as a safe medicinal plant with only few and insignificant adverse effects (Toader, 2014).

The major pharmacological activities of ginger are summarized as follows.

2.8.7.1. Lipolytic or Cholesterol-lowering properties

Ginger may reduce the rate of weight gain and hence adjust the Body Mass Index (BMI). It can improve body composition by decreasing body fat levels and increasing Soft Lean Mass (SLM). In addition, some enzymes such as Acetyl-coenzyme A, acyltransferase1 and enoyl-CoA hydratase, which participate in the β-oxidation of fatty acids, have been increased by consumption of Ginger. Moreover, ginger extract prevents high-fat diet-induced obesity. The aqueous extract of *Z. officinale* called Roscoe might inhibit the intestinal absorption of dietary fat by inhibiting its hydrolysis. Therefore, ginger seems to improve body composition via its effects on liver enzymes, by reducing fat absorption, by increasing beta-oxidation of fats and energy expenditure (Aryaeian& Tavakkoli, 2015).

2.8.7.2. Anti-inflammatory and analgesic actions

Ginger suppresses prostaglandin synthesis through inhibition of cyclooxygenase-1 and cyclooxygenase-2. It also suppresses leukotriene biosynthesis by inhibiting 5-lipoxygenase. This pharmacological property distinguishes ginger from NSAID (Non-Steroidal Anti-Inflammatory Drugs). Dual inhibitors of cyclooxygenase and 5-lipoxygenase may have a better therapeutic profile and have fewer side effects than NSAID (Yadav*et al.,* 2016). For the human being, the consumption of fresh ginger demonstrated promising results for the decrease of arthritis-induced pain. However, more studies are necessary before concluding on a real effect of the consumption of ginger for the prevention and treatment of pain caused by chronic inflammatory disorders (Tchombe*et al.,* 2012).

Ginger has a strong analgesic action which is many cases act by cyclooxygenase-1 (COX-1) inhibition. Gingerol and their derivatives, especially paradol, have been reported to be

more potent anti-platelet and cyclo-oxygenase-1 (COX-1) inhibitors than aspirin (Ghosh*et al.,* 2011).

2.8.7.3. As antioxidant

Antioxidants are the chemical substances that reduce or prevent oxidation stress and have the ability to counteract the damaging effects of free radicals in tissues. According to Chakraborty *et al.* (2012) gingerols are known to ease oxidative stress due to stimulation of superoxide dismutase, catalase, glutathione peroxidase and GSH activities. They are believed to protect against cancer, arteriosclerosis, heart disease and several other diseases (Semwal*et al.,* 2015).

According to Kikuzaki and Nakatani (1996) about 40 antioxidant compounds have been discovered in ginger. Because of these chemicals, ginger has shown a protective role to toxicity and lethality against some agent like carbon-tetra chloride. It can also protect DNA from lipopolysaccharide-induced oxidation damage in rats (Tchombe*et al.,* 2012). Ginger oil can act as a scavenger of oxygen radical and might be used as an antioxidant (Yadav*et al.,* 2016).

2.8.7.4. Antiemetic effects

Ginger is one of the herbs most commonly used to treat nausea and vomiting in pregnancy (Allaire*et al.,* 2000). Although, the exact mechanism of action of ginger on nausea and vomiting remains uncertain, the components in ginger that are responsible for the antiemetic effect are thought to be the gingerols, shogaols, and galanolactone of ginger (Yadav*et al.,* 2016). A 5% solution of the essential oil of ginger in grape seed carrier oil, when applied nasocutaneously, can be administered safely for the effective prevention and therapeutic management of nausea in general anesthesia for patients at high risk for post-operative nausea and vomiting (Tchombe *et al.,* 2012). The possible mechanism of action of ginger is thought to

be a gastric effect, i.e., it increases tone and peristalsis due to anti-cholinergic and anti-serotonin action (Bryer, 2005).

2.8.7.5. Anti-microbial activity

Red ginger had strong antibacterial and to some extent antifungal properties because of gingerol, paradol, shogaols, zingerone (Malu*et al.,* 2008;Rahmani*et al.,* 2014). Ingenol and shogaols, isolated from ginger rhizome, also demonstrated antiviral activity. In vitro studies had shown that active constituents of ginger inhibited multiplication of gastroenteric bacteria including Helicobacter pylori. The higher the concentration of the red ginger extracts, the more antimicrobial properties of the red ginger extracts, and the larger the diameter of the bacterial growth inhibition zones obtained (Poeloengan, 2011). Gingerol has been reported as active inhibitor of Mycobacterium avium and M. tuberculosis in vitro (Semwal*et al.,* 2015;Mishra&Kumar, 2012). Ginger has shown antimicrobial activity against E. coli , Salmonella typhi and Bacillus subtilis and ethanolic extract of ginger showed widest zone of inhibition against Salmonella typhi (Rahmani*et al.,* 2014; Azu& Onyeagba, 2007). According to Teimoory *et al.* (2013), the best antibacterial effect were obtained for the dried ginger at 42.6 mg punch with the zone of 15.8 millimeter on Staphylococcus aureus and Listeria monocytogenes.

2.8.7.6. Anti-parasitic effect

Ginger is said to be anti-parasite plant in Iranian herbal medicine. A study conducted by Bahmani et al. showed a maximum anti-leech effect (33.33 ± 11.40 min) of ginger that causes paralysis and death of the leeches in short time (Bahmani*et al.,* 2013). In this study, the lethal effect of methanolic extract of ginger against Limnatis nilotica (the common leech) was equal to levamisole and more than triclabendazole and methanolic extract of onion. So it can be

concluded that ginger is a good natural medicine that can be used as anti-leech in order to decrease the leech pathological effects.

Gingerol and shogaols were also proved to be lethal to anisakis larvae at a minimal effective dose of 62.5 and 250 lg/mL, respectively. Therefore these compounds could have potential role to treat anisakiasis, a human parasitic infection of the gastrointestinal tract (Semwal*et al.,* 2015).

2.8.7.7. Gastro-protective effect

Ginger is the most proven herbal treatment for ulcers in human and horses, perhaps in other animals too. Ginger is a strong gastro-protectant that works by increasing mucin secretion, reducing the numbers of inflammatory cytokines, small proteins that signal to the immune system to begin an inflammatory response, in the stomach (Wang*et al.,* 2011). As a treatment for ulcers, when properly administered, ginger has shown to be as effective or better as cimetidine for treating ulcers (Kumar& Pal, 2011). Ginger does help repair existing damage to the stomach lining as well, but it has no direct effect on controlling inflammation, mucosal prostaglandin E2 (PGE2) content or reducing acidity in the stomach. According to Wang et al. the protective effect of ginger against gastric ulcers may be attributable to both gingerol and shogaol (Wang*et al.,* 2011). Moreover, some active components of ginger, including gingerol and shogaol, are reported to stimulate digestion, absorption, relieve constipation and flatulence by increasing muscular activity in the digestive tract (Ghosh*et al.,* 2011).

2.8.7.8. Anti-tumor activity

Ginger and its constituents show a vital effect in the control of tumor development through up-regulation of tumor suppressor gene, induction of apoptosis and inactivation of VEGF pathways. Angiogenic factors such as VEGF (Vascular Endothelial Growth Factor) and

FGF (fribroblast growth factor) play a significant role in the development and progression of tumor. Therefore, inhibition of VEGF and FGF is an important step in the prevention of tumor development/management (Kim*et al.,* 2005). The active ingredient 6-gingerol has considerable role in the suppression of neoplastic transformation, hyperproliferation, and inflammatory processes that involve in various steps of carcinogenesis, angiogenesis and metastasis (Rahmani*et al.,* 2014;Lee*et al.,* 2008). Several studies have shown that ginger has promising effect for liver cancer, breast cancer, prostate cancer and colorectal carcinomas through its diverse pharmaceutical mechanisms (Pour*et al.,* 2014).

2.8.7.9. Effects on cardiovascular system

Ginger is used to improve the flow of body fluids including blood. It stimulates blood circulation throughout the body by powerful stimulatory effect on the heart muscle and by diluting blood. A Japanese study showed that active constituents in ginger reduced the blood pressure and decreased cardiac workload (Zadeh&Kor, 2014). Ginger has been shown to inhibit platelet aggregation and to decrease platelet thromboxane production in vitro (Tchombe*et al.,* 2012;Mishra& Kumar, 2012). Ginger reduced the formation of pro inflammatory prostaglandins and thromboxane thus lowering the clotting ability of the blood. Ginger can also prevent the increase in cholesterol levels following intake of cholesterol-rich diet (Zadeh&Kor, 2014).

2.8.7.10. Anti-diabetic activity of ginger

Ginger and its constituents showed pivotal role in the control of diabetes and the associated complications via its antihyperglycemic effect. Studies on animal models showed that ginger extract and ginger juice have significant blood glucose lowering effect or hypoglycemic activity both in diabetic and non-diabetic groups (Ahmed&Sharma, 1997).

Although the exact mechanism of action of ginger in diabetes control is not still fully understood inhibition of oxidative stress and anti- inflammatory process could be another possible mechanism (Rahmani*et al.,* 2014). Ginger shows antagonistic activity against serotonin receptors. Moreover, it inhibits the activity of intestinal glucosidase and amylase, resulting in the reduction of glucose absorption (Aryaeian& Tavakkoli, 2015). Animal based studies further indicated the ability of 6-GN to increase plasma insulin levels and to improve diabetes-induced myocardial diastolic dysfunction and enhance the relaxation and the Ca^{2+} transient decay rate (Semwal*et al.,* 2015;Namekata*et al.,* 2013).

2.8.7.11. Renoprotective and kidney function

Gingerol fraction from Zingiber officinale prevents gentamicin-induced nephrotoxicity. It improves kidney functions, reduces lipid peroxidation, and enhancing the levels of reduced glutathione, superoxide dismutase and catalase activities (Semwal*et al.,* 2015). In addition, Ginger extract diminishes chronic fructose consumption-induced kidney injury by suppression of renal over expression of pro-inflammatory cytokines in rats (Aryaeian & Tavakkoli, 2015). The nephron protection of ginger is mediated by preventing the Doxorubicin induced decline of renal antioxidant status, and also by increasing the activity of Glutathione -S- transferse (Singh*et al.,* 2014).

2.8.7.12. Neuro-protective effect

Ginger plays an important role in the improvement of symptoms in patients who suffer from Alzheimer and other neurological diseases. The neuro-protective effect is partly attributable to an antagonistic action of ginger root extracts on monosodium glutamate effect, so the monoamines content was increased. From these results, we can say that the ginger extract has a neuro-protective role against monosodium glutamate toxicity effect (Singh*et al.,*

2014).Experimental trials on animal models further indicate that gingerol and other constituents of ginger were found to be effective in alleviating neuropathic pain (Gauthier*et al.,* 2013), to block prion peptide-mediated neurotoxicity and to be strong antidepressants (Ittiyavirah&Paul, 2013).

2.9.0. Metformin

2.9.1. General overview

Metformin (dimethylbiguanide) has become the preferred first-line oral blood glucose-lowering agent to managetype 2 diabetes. Its history is linked to *Galega officinalis*(also known as goat's rue), a traditional herbal medicine inEurope, found to be rich in guanidine, which, in 1918, was shown to lower blood glucose. Guanidine derivatives, includingmetformin, were synthesized and some (not metformin) were used to treat diabetes in the 1920s and 1930s but were discontinued due to toxicity and the increased availability of insulin (Hadden, 2005). Metformin was rediscovered in the search for antimalarial agents in the 1940s and, during clinical tests, proved useful to treat influenza when it sometimes lowered blood glucose (Bailey &Day, 2004). This property was pursued by the French physician Jean Sterne, who first reported the use of metformin to treat diabetes in 1957. However, metformin received limited attention as it was less potent than other glucose-lowering biguanides (phenformin and buformin), which were generally discontinued in the late 1970s due to high risk of lactic acidosis.

Metformin's future was precarious, its reputation tarnished by association with other biguanides despite evident differences. The ability of metformin to counter insulin resistance and address adult-onset hyperglycaemia without weight gainor increased risk of hypoglycaemia gradually gathered credencein Europe, and after intensive scrutiny metformin was introduced into the USA in 1995. Long-term cardiovascular benefits of metformin were identified by the

UK ProspectiveDiabetes Study (UKPDS) in 1998, providing a new rationale to adopt metformin as initial therapy to managehyperglycaemia in type 2 diabetes (Lalau, 2010). Sixty years after its introduction in diabetes treatment, metformin has become the mostprescribed glucose-lowering medicine worldwide with the potentialfor further therapeutic applications.

Metformin is one of the most effective antihyperglycemic agents, possessing the capability to lowerglycosylated hemoglobin A1c (HbA1c) levels, it has been reported that metformin treatment counteracts insulin resistance, reduces hyperinsulinemia, reduces body mass index and improves lipid profile, especially by reducing triacylglycerol and low density lipoproteins (LDL)-cholesterol levels and increasing high density lipoproteins (HDL)-cholesterol levels (DeFronzo & Goodman, 1995). Other study also showed that metformin treatment in people at risk for diabetes improves weight, lipid profiles, and insulin resistance, and reduces new-onset diabetes by 40% compared with placebo or no treatment (Guntone*t al.,* 2002).

2.9.2. The antihyperglycemic actionof metformin

The antihyperglycemic effect of metformin relays in its ability to suppress gluconeogenesis and enhance glucose uptake and insulin sensitivity in peripheral tissues (Kirpichnikvo*et al.,* 2002). Therefore, this antidiabetic drug is capable to ameliorate insulin resistance and to reduce plasma glucose levels, which are crucial factors in the development of type 2 diabetes and associated complications. Indeed, several studies demonstrated that metformin reduces glucose production mainly due to an inhibitory effect on gluconeogenesis (Jenge*t al.,* 1994). Radziuk *et al.* (1997) reported a decreased gluconeogenesis in perfused livers, essentially through inhibition of lactate uptake, by metformin. Furthermore, *in vitro* studies using isolated rat hepatocytes showed that metformin lowers intracellular levels of ATP, an inhibitor of pyruvate kinase (Large &Beylot, 1999). Moreover, this antidiabetic drug also inhibits pyruvate

carboxylase and phosphoenol-pyruvate carboxykinase (PEPCK) activity and activates the pyruvate to alanine conversion (Large &Beylot, 1999). Despite the metformin's mechanisms of action in hepatocytes remain uncertain, the primary site of action of this drug appears to be mitochondria, since metformin inhibits mitochondrial respiratory chain particularly at the complex I level, impairing mitochondrial function and, consequently, cell function. The inhibition ofcellular respiration decreases gluconeogenesis and enhancesthe expression of glucose transporters, stimulating glucose uptake (Ebert*et al.,* 1995).

Further, the insulin receptor and the glucose transporters seem to be potential sites of action of metformin. A study performed in human hepatocytes showed that metformin quickly increases insulin receptor activation and signaling, essentially through insulin-receptor substrate-2 (IRS-2), and improves glucose transport through increased GLUT-1 translocation (Guntone*t al.,* 2002). Besides the effect of metformin in gluconeogenesis,some studies also indicate that metformin reduces glycogenolysis. Evidence from the literature also demonstrates that metformin enhances insulin-mediated glucose uptake. It was observed that metformin normalizes insulin-mediated glucose disposal and muscle glycogen synthesis in diabetic rats (Rossetti*et al.,* 1990). Furthermore*, in-vitro* studies also demonstrated the ability of metformin to increase glucose uptake in skeletal muscle. This finding has been associated with increased insulin receptor tyrosine kinase activity, enhanced glycogen synthesis, and increased GLUT-4 transporter number and activity (Klip & Leiter, 1990). Although the mechanism that leads to GLUT-4 translocation is unclear, studies in different cell types demonstrated that this antihyperglycemic drug increases insulin receptorbinding tyrosine kinase activity, and insulin receptorinternalization (Rossetti*et al.,* 1990). It has also observed that metformin improves

abnormal insulin receptor tyrosine kinase activity inmuscle from streptozotocin-induced diabetic rodents (Rossetti*et al.,* 1990).

Elevated plasma free fatty acids (FFA) levels play an important role in the establishment of insulin resistance. Chronic elevation in plasma FFA levels is commonly associated with impaired insulin-mediated glucose uptake in skeletal muscle and often coexists with obesity and type 2 diabetes (McGarry, 2002).Furthermore, increased plasma FFA concentration exerts a lipotoxic effect on the β-cell. In type 2 diabetic patients, metformin leads to the suppression of FFA and lipid oxidation. Metformin induces the activation of AMP-activated protein kinase (AMPK), therefore it would be expected a stimulation of fatty acid oxidation instead of suppression. It has also been reported that chronic metformin treatment results in the lowering of lipids in human skeletal muscle (Mathieu-Costello*et al.,* 2002).

Moreover, metformin treatment is frequently associated witha reduction in circulating triacylglycerols as a consequence of decreased synthesis and increased clearance of very low-density lipoproteins (VLDL). It has also been reported that metformin has a significanteffect on the digestive tract by inducing a decrease in intestinal absorption of glucose, which could reduce postprandialblood glucose levels (McGarry, 2002).In summary, metformin ameliorates hyperglycemia andinsulin resistance through the suppression of gluconeogenesis, glycogenolysis and intestinal glucose absorption, reduction of FFA, and by the improvement in glucose uptake as shown in figure (2.2).

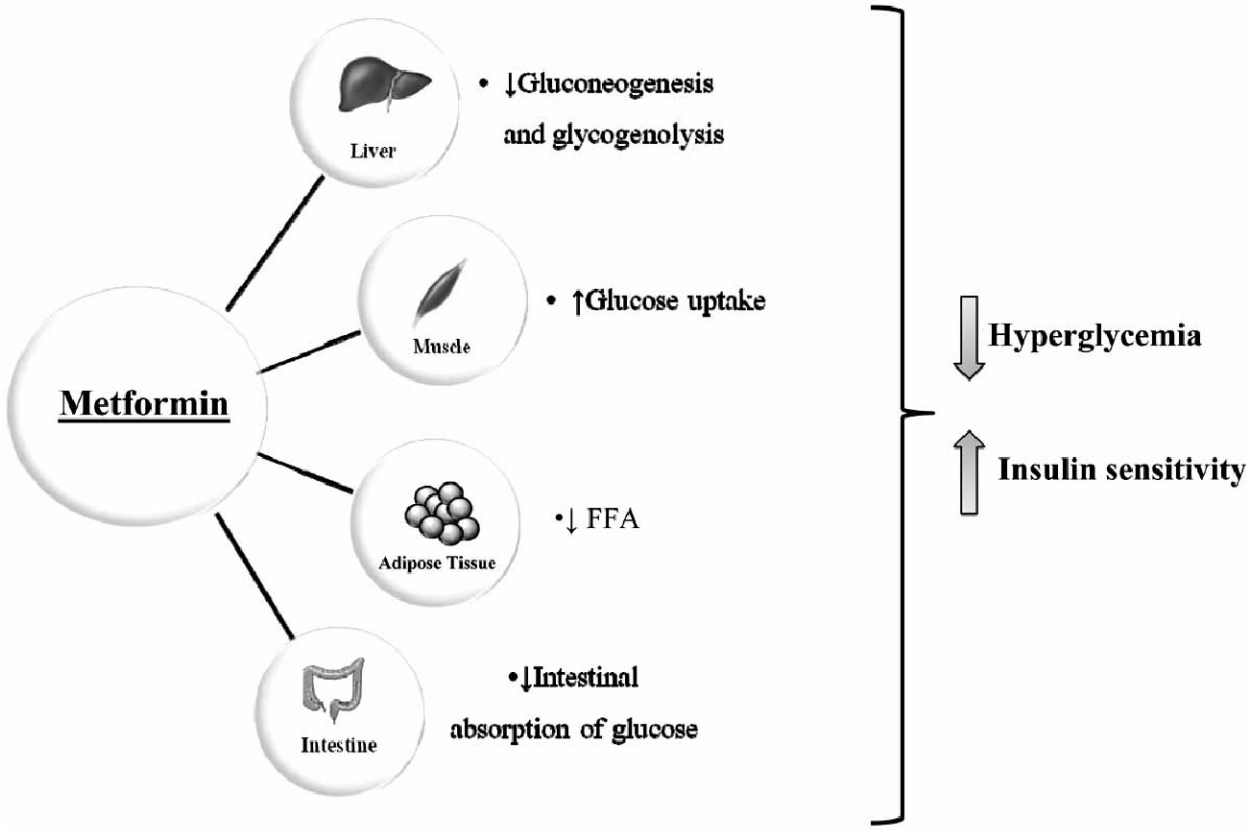


Figure 2.3: Antihyperglycemic action of metformin.

2.9.3. Molecularmechanismsofmetforminaction

Although the molecular mechanism underlying metformin action remains unclear, it has been suggested that this drug activates AMPK, a major regulator of cell and body energy homeostasis, by increasing its phosphorylation state but without any changes in AMP/ ATP ratio (Woods*et al.,* 2003).Recent studies demonstrated that serine-threonine kinase 11(STK11/LKB1), which phosphorylates AMPK, is also a targetof metformin. Activation of AMPK leads to theinhibition of ATP consuming pathways and enhance ATP production pathways (Hardie, 2007). Indeed, the increase in AMPKactivity is associated with the translocation of GLUT-4 to the membrane, the stimulation of glucose uptake in muscle andliver, glycolysis, fatty acid oxidation, and suppression ofgluconeogenesis, glycogen, fatty acid and cholesterol synthesis. It was showed that activation of AMPKby metformin is crucial for the decrease in glucose

productionand the increase in fatty acid oxidation in hepatocytesand for the increase in glucose uptake in muscle (Winder&Hardie, 1999).

The main biological effects of AMPK are the phosphorylationand inactivation of acetyl-CoA carboxylase (ACC), which plays a pivotal role in hepatic lipid metabolism. It was observed in cultured human hepatoma HepG2 cells that the stimulation of ACC phosphorylation by metformin induces the reduction in triacylglycerol levels, which can be supported with increased fatty acid oxidation and/or decreased fatty acid synthesis (Zang, 2004).Activation of AMPK by metformin reduces the expression of sterol response element binding protein-1 (SREBP-1), a transcription factor which induces the expression of lipogenic genes, including fatty acid synthase (FAS) and Spot-14 (S14) as shown in figure (2.3). It has been postulated that SREBP-1 is a crucial mediator of insulin resistance in type 2 diabetes and associated metabolic disorders (Shimomura*et al.,* 2000). Metformin's effects to modulate circulating lipids and to reduce hepatic lipidsynthesis and fatty liver may be promoted by the reducedexpression of SREP-1 induced by this antidiabetic drug. Further, AMPK inhibits protein synthesis in many cells through the inhibition of TOR pathway (Inoki *et al.,* 2003).

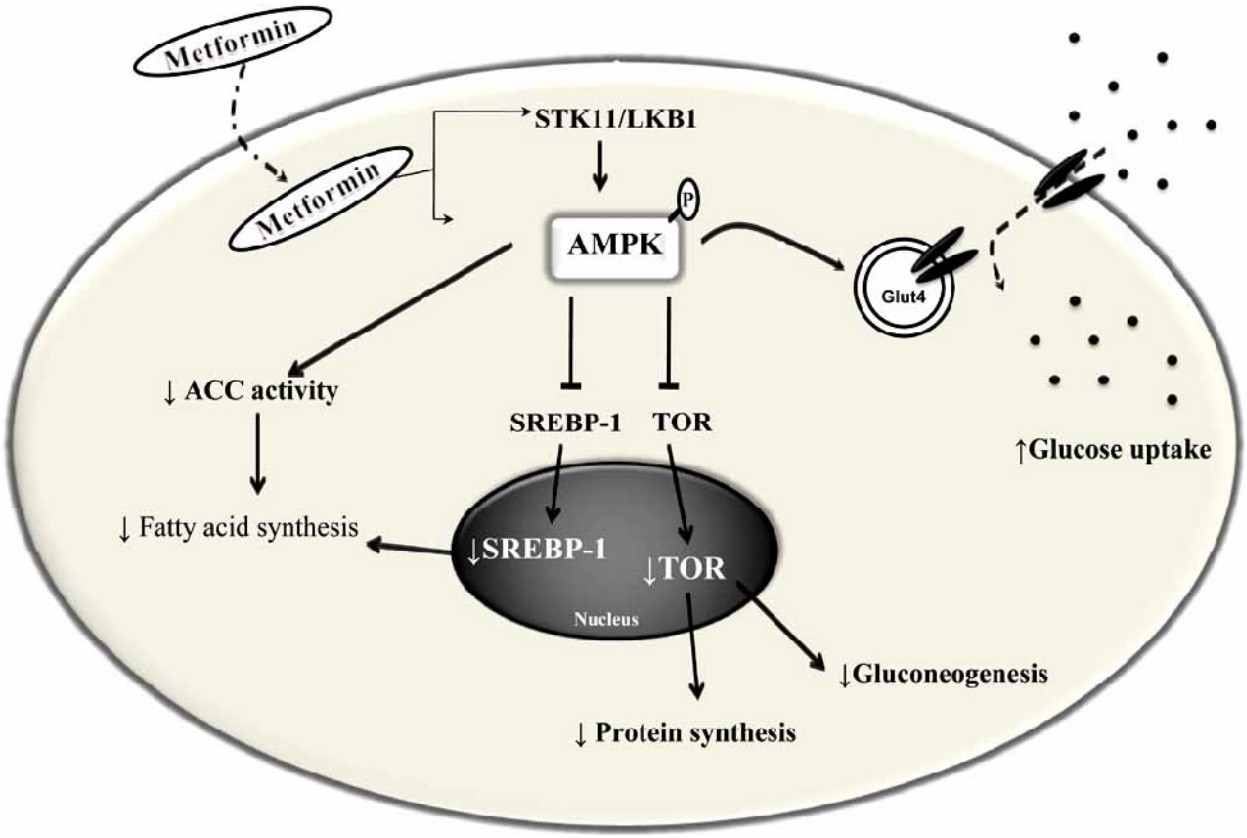


Figure 2.3: molecular mechanisms of metformin action.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals and Reagents

All the chemicals and reagents used for this work are of analytical grade. Go to appendix for detail.

3.1.2. Equipments

Standard laboratory equipments were used for this work.Go to appendix for detail.

3.1.3. Ginger Sample

Fresh ginger rhizome was obtained from Malumfashi market at Malumfashi local government, Katsina State, Nigeria.The sample was botanically identified and authenticated by the Taxonomist in the Department of Plants Science, Faculty of Biological Sciences, Bayero University, Kano. A voucher specimen number BUKHAN 296 was assigned to the sample.A specimen of the sample was deposited in the Herbarium of the same department.

3.1.4. Experimental Animals

Thirty (36) healthy young Wistar albino rats of both sexes weighing between 100 – 200 g were used in this study. The rats were kept at the animals' house under normal environmental conditions and maintained with free access to pelletized growers feed, and access to water *ad libitum.* The animals were allowed to acclimatize for two weeks (14 days)before the commencement of the study.

3.2. Methods

3.2.1. Sample Preparation

Fresh ginger rhizome was thoroughly washed with distilled water several times to remove dust, sand and stones. It was then cut into smaller pieces using a table knife and air-dried under shed for a period of one week. The dried ginger was then grind into powder using a domestic grinder (mortar and pestle).

Powder of ginger (40 g) was soaked in 400mL of methanol and stored in a dark place at room temperature for 48 hours, after which the extract was filtered using a whatman filter paper number 1. The filterate was then concentrated under a reduced pressure using Rotove-flash evaporator at a temperature of 45^{o}C and a pressure of 700mmHg

3.2.2. Fractionation method

The process of fractionation of the extract is summarized in Figure 3.1. Briefly, the concentrated methanol extract of ginger was further subjected to partial fractionation with solvents of increasing polarity viz; hexane: chloroform: ethyl acetate and methanol.DPPH analysis was carried out on different fractions in order to determine the most active fraction in terms of free radical scavenging ability.

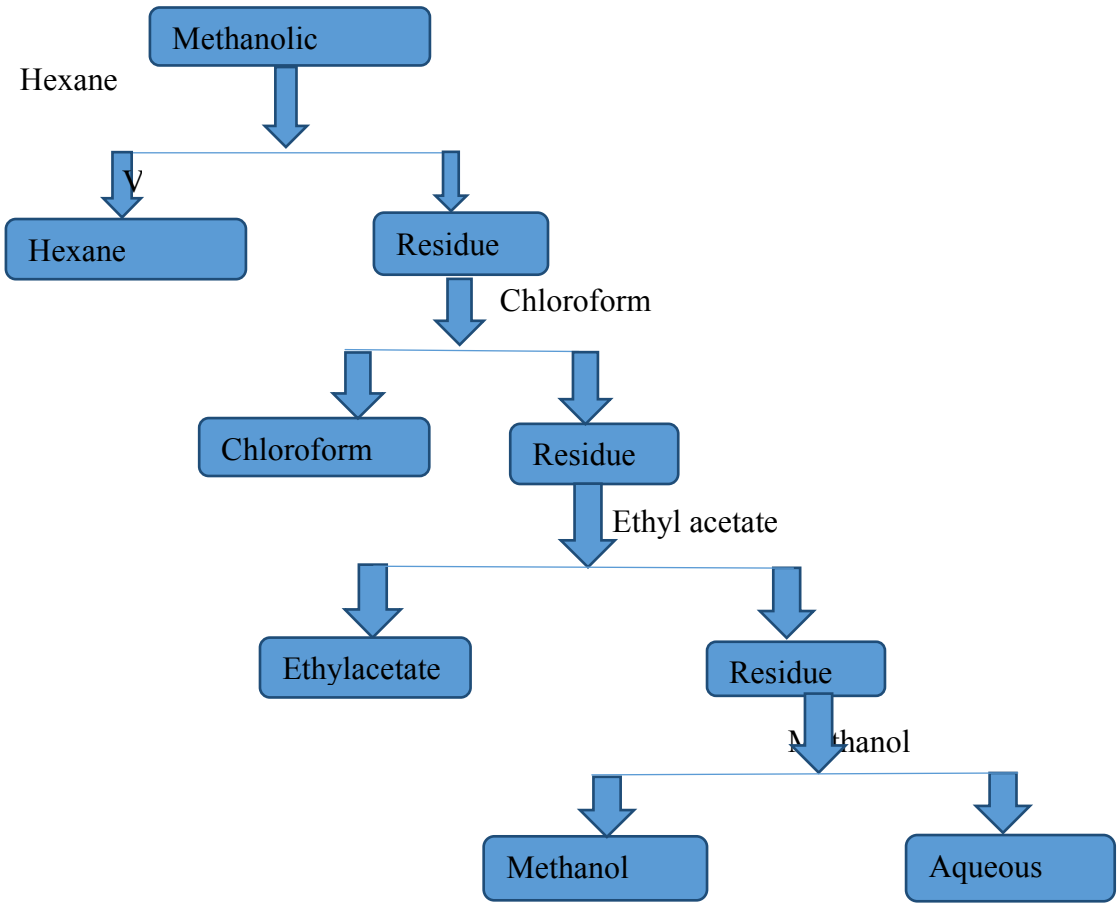


Figure 3.1: Fractionation method

3.2.3. 1,1-diphenyl-2-picryl hydrazyl (DPPH) Analysis:

The free radical scavenging activity of different fractions of ginger rhizome extracts was determined by using DPPH assay according to Chang *et al.* (2001). The decrease in the absorption of the DPPH solution after the addition of an antioxidant was measured at 517 nm. Ascorbic acid (10mg/mLdimethyl sulfoxide (DMSO)) was used as reference standard (Merish*et al.,* 2015).**Principle**

1,1-diphenyl-2-picryl hydrazyl is a stable free radical with red color which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as,

DPPH + [H-A] →DPPH-H + (A)

Antioxidants react with DPPH and reduce it to DPPH-H and as a consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

Procedure

Different volumes (0.2-1.0mL) of different fractions of ginger rhizome extracts were made up to 40µL with DMSO and 2.00mL DPPH (0.1mM) solution was added. The reaction mixture was incubated in dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm. The control used was 3mL of DPPH solution.

Calculation

$$\% \text{ inhibition} = \frac{\text{Control} - \text{test}}{\text{Control}} \times 100$$

3.3.1. Induction of Diabetes in Rats.

The range of diabetogenic dose of alloxan is quite narrow even in the same species of albino rats. Therefore even slight overdosing may be generally toxic causing the loss of many animals (Lenzen *et al.*, 1996). To prevent the toxic side effects, ranges of 80 to 180 mg/kg of alloxan (20 mg interval) was tested and 120 mg/kg was selected as minimum and safest dose for induction of Alloxan-diabetes in this work. The Alloxan diabetic rat models were prepared by adopting the method of Kandur and Goyal (2005). All rats, except the normal control group were intraperitoneally injected with 120 mg/kg body weight of the prepared alloxan. After 6 hours of alloxan administration, rats in their cages were then allowed 10 % glucose solution for the next 24 hours in other to prevent alloxan- induced hypoglycemia. The animals were observed for polydipsia, polyuria, polyphagia as well as general reduction of body weight. Seventy two hours (three days) after alloxan administration, the animals were fasted overnight and diabetes was

confirmed from the rats by measuring their fasting blood glucose level with the aid of a single touch glucometer. Rats that have fasting blood glucose level >7.0 mmol/L (126mg/dL) were considered diabetic and included in the study (Kandur & Goyal, 2005).

3.3.2. Grouping of Experimental Rats and Treatments.

Simple random sampling technique was used in grouping the rats for this study. By applying this method, rats were randomly selected and divided in to six (6) groups: Alloxantreated group (PC), Normal control group (NC), group one (GR1=treated with 1500mg/kg body weight), group two (GR2 = treated with 1000mg/kg body weight), group three (GR3= treated with 500mg/kg body weight), and metformin treated group (MC) 500 mg/kg body weight metformin. Each group had a total of six (6) rats per group and the rats were housed in labeled cages as described above, fed with pelletized growers feed (Vital feed, Jos, Nigeria), and allowed access to water *ad libitum* throughout the period of the study.

3.3.3. Administration of Methanol Extract of Ginger rhizome to Rats

Methanol extract of ginger rhizome were administered orally every morning by intubation using intravenous cannula tube at doses of 1500, 1000 and 500 mg/kg body weight to the respective rats in their respective groups by single forced oral feeding once per day for a period of 42 days. The rats in the metformin group were administerd with 500 mg/kg body weight metformin using the same procedure.

3.4.1. Collection of Blood Samples and Preparation of Serum

24 hours after the last treatment, the animals were subjected to 12 hours fasting after which the animals were anaesthetised by dropping each individual animal in a plastic jar saturated with chloroform vapour. The animal were then removed from the jar and blood samples were collected through decapitation into labelled plastic sample bottles containing

EDTA (disodium ethylinediamine tetraacetate) for glycated hemoglobin assay; the remaining blood was collected into plastic centrifuge tubes without anticoagulant and were allowed to clot then centrifuged at 4000 g for ten minutes. The sera obtained were pipetted into labelled specimen test tubes for estimation of serum glucose, lipid profiles, catalase, reduced glutathione, superoxide dismutase and malondialdehyde (MDA) levels respectively.

3.4.2. Determination of Biochemical Parameters

3.4.2.1. Estimation of Serum Glucose Level

Serum glucose was estimated by glucose oxidase/ peroxidase method using Randox kit (Trinder, 1969).

Principle

Glucose was determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with 4-aminophenazone and phenol to produce a red-violet coloured quinoneimine complex that can be measured spectrophotometrically at 500nm.

The equation is:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\textit{glucose oxidase}} \text{Gluconic acid} + H_2O$$

$$2H_2O_2 + 4 - \text{aminophenazone} + \text{phenol} \xrightarrow{\textit{peroxidase}} \text{Quinoneimine} + 4H_20$$

Procedure

The procedure for the determination of serum glucose level is summerised in table (3.1). Briefly, Serum (10µL), standard (10µLof 5.5 mmol/L) and distilled water (10µL) were respectively pipetted in to the test tubes. Each test tube was then followed by 1000 µL of the reagent as shown below. The tubes were mixed properly, incubated at 37^{o}C for 10 minutes and the absorbance of standard and tests read against the blank at 500nm using spectrophotometer.

Test tubes were set up in triplicates and labelled blank, test and standard as follows:

Table 3.1: Determination of serum glucose level

	Blank	Test	Standard
Serum (µL)	-	10	-
Standard glucose(µL)	-	-	10
Distilled water (µL)	10	-	-
Reagent (µL)	1000	1000	1000

Calculation: The glucose concentration was calculated using the relation:

$$\text{Serum glucose (mmol/L)} = \frac{Absorbance\ of\ Test}{Absorbance\ of\ \text{Standard}} \times conc\ of standard$$

3.4.2.2. Estimation of Glycated Hemoglobin (HbA_{1c})Principle

The principle is based on heating a sample of hemolyzed blood at 100^{o}C for 5 hours in the presence of a weak acid (oxalic acid) to hydrolyze the hexose moiety off glycated haemoglobin and convert it to 5-hydroxymethylfurfural (5-HMF). This hydrolysis step is followed by adding 2-thiobarbituric acid, which couples with 5-HMF to produce a coloured complex that is measured spectrophotometrically at 443 nm and results are expresed as fructose equivalents (Michael *et al.,* 1981; Jim & Phillip, 1983).

Procedure

Sample Preparation: Whole blood anticoagulated with the EDTA was centrifuged (1000 x g for 10 minutes) the plasma was removed and the packed erythrocytes was washed three times with saline (sodium chloride 9 g/L).

Hydrolysis The red blood cells were hemolysed/hydrolised by adding 100 µL of the erythrocytes to 1.5 ml distilled water and vortexed. Then 1.0 mL of the hemolysate was taken in to new test tube and mixed with 0.5 mL oxalic acid incubated in boiling water at 100 ^{o}C for 5 hours. The

tubes were removed after incubation and cooled in cold water bath for 10 minutes; then 1.0 mL of the ice-cold trichioroacetic acid reagent was added and vortexed before centrifuging at 1000 x g for 10 minutes.

A seperate test tube containing 1.0 mL of oxalic acid, 0.5 mL of distilled water, and 1.0 mL of the trichloroacetic acid reagent was included as a zero standard (blank).

Colorimetric reaction

The supernatant (1.5mL) from the hydrolysis step were seperately collected in to labelled glass test tubes and each was mixed with 0.5 mL of the thiobarbituric acid reagent except for the assay blank, in which 0.5 mL of distilled water was substituted for the thiobarbituric acid reagent.

All tubes were incubated at 40 ^{o}C for 30 minutes. Afterwhich the spectrophotometer was adjusted to zero with the zero standards (blank) then the absorbance of each test solution was measured at 443 nm.

Calculations: The corresponding absorbance of 5- HMF concentration (fructose equivalent) of the unknown glycated hemoglobin was extrapolated from the graph of fructose standard curve (See Appendixes).

% HbA_{1c} is calculated as thus: $\frac{\text{fructose concentration (5-HMF) (g/dL)}}{\text{Total Hemoglobin concentration (g/dL)}} \times \frac{100}{1}$

3.4.2.3. Estimation of Serum Total Cholesterol

Serum total cholesterol (TC) was estimated by enzymatic method using Randox kit (Allain *et al.,* 1974).

Principle

The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4 – aminoantipyrine in the presence of phenol and peroxidase. The absorbance of the dye is measured spectrophotometrically at 500nm. The equations for the reaction are:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\textit{Cholesterol esterase}} \text{Cholesterol} + \text{fatty acids}$$

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

$$2H_2O_2 + \text{Phenol} + 4 - \text{aminoantipyrine} \xrightarrow{\textit{peroxidase}} \text{Quinoneimine} + 4H_2$$

Procedure

The procedure for the estimation of serum total cholesterol is summerised in table (3.2). Briefly,in to test tubes labelled test, standard and blank, 10 µL of serum, 10 µL of standard (200 mg/dL) and 10 µL of distilled water were respectively pipetted. Each test tube was followed by 1000 µL of the reagent as shown below.The test tubes were mixed, incubated at 37^{o}C for 5 minutes and the absorbance of the standard and test were read against the blank at 500 nm.

Three test tubes were set up and labelled blank, test and standard as follow:

Table 3.2: Estimation of serum total cholesterol

	Blank	**Test**	**Standard**
Serum (µL)	-	10	-
Standard cholesterol (µL)	-	-	10
Distilled water (µL)	10	-	-
Reagent (µL)	1000	1000	1000

Calculation

Cholesterol concentration was obtained using the relation:

$$\text{Serum total cholesterol (mg/dL)} = \frac{Absorbance\ of\ Test}{Absorbance\ of\ \text{Standard}} \times \text{Concentration of Standard}$$

3.4.2.4 Estimation of Serum High Density Lipoprotein Cholesterol (HDL-C)

This was done by free enzymatic method of Burstein *et al.* (1970) using Randox Kit

Principle

Low density lipoproteins, very low density lipoproteins and chylomicron fractions are precipitated quantitatively by the addition of phosphotungastic acid in the presence of magnesium ions. After centrifugation, the cholesterol concentration in the high density lipoprotein fraction, which remains in the supernatant, is determined spectrophotometrically at 500 nm.

Procedure

Into centrifuge tubes, 200 µL of serum and 500 µL of precipitant (0.55 mmol/L phosphotungastic acid and 25 mmol/L magnesium chloride) were added, mixed and allowed to stand for 10 minutes at room temperature. The tubes were centrifuged for 10 minutes at 4000 rpm. The supernatant was colected and used for the cholesterol analysis.

The procedure for the estimation of serum high density lipoprotein cholesterol is summerised in table (3.3). Three test tubes were set up and75labeled blank, standard and test, as follows:

Table 3.3: Estimation of serum high density lipoprotein cholesterol

	Blank	Standard	Test
Distilled water (µL)	100	-	-
Supernatant (µL)	-	-	100
Standard supernatant(µL)	-	100	-
Reagent (µL)	1000	1000	1000

The tubes were mixed and incubated for 5 minutes at 37^0 C and the absorbance of the samples and standard were measured against the reagent blank at 500nm.

Calculation

The HDL-C concentration was obtained from the relation:

$$\text{Serum HDL-C (mg/dL)} = \frac{\textit{Absorbance of Test}}{\textit{Absorbance of Standard}} \times \text{Concentration of Standard}$$

3.2.7.6 Estimation of Serum Triacylglycerol Concentration

This was assayed by the method of Tietz (1990), using Randox Kit.

Principle

The triacylgycerols were estimated after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from H_2O_2, 4- aminophenazone and 4 – chlorophenol under the catalytic influence of peroxidase (POD).

The equations for the reactions are:

$$\text{Triacylglycerol} + H_2O \xrightarrow{Lipase} \text{Glycerol + fatty acid}$$

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

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{Glycerol\ Phosphate\ Oxidase} \text{Dihdroxyacetone phosphate} + H_2O_2$$

$$H_2O_2 + \text{4 - aminophenazone + 4 - chlorophenol} \xrightarrow{Peroxidase} \text{Quinoneimine} + HCl + 4H_2O$$

Procedure

The procedure for the estimation of serum triacylglycerol concentration is summerised in table (3.4). Test tubes were set up in triplicates and labelled blank, test and standard as follows:

Table 3.4: Estimation of serum triacylglycerol concentration

	Blank	Test	Standard
Serum (µL)	-	10	-
Standard triglyceride (µL)	-	-	10
Distilled water (µL)	10	-	-
Reagent (µL)	1000	1000	1000

The tubes were mixed and incubated at 37^{o}C for 5 minutes and the absorbance of the standard and tests were read at 500nm against the blank.

Calculation: The triacylglycerol levels were calculated using the relation:

$$\text{Serum triacylglycerol (mg/dL)} = \frac{Absorbance\ of\ Test}{Absorbance\ of\ \text{Standard}} \times \text{Concentration of Standard}$$

3.4.2.5. Estimation of Serum Low Density Lipoprotein Cholesterol (LDL-C)

This was calculated using Friedewald formula (Friedewald *et al.,* 1972).

$$\text{LDL - C (mg/dl)} = \text{TC} - (\text{HDL - C}) + \left(\frac{TG}{5}\right)$$

3.4.2.6. Estimation of Serum Very Low Density Lipoprotein Cholesterol (VLDL-C)

This was calculated using Friedewald formula (Friedewald *et al.,* 1972).

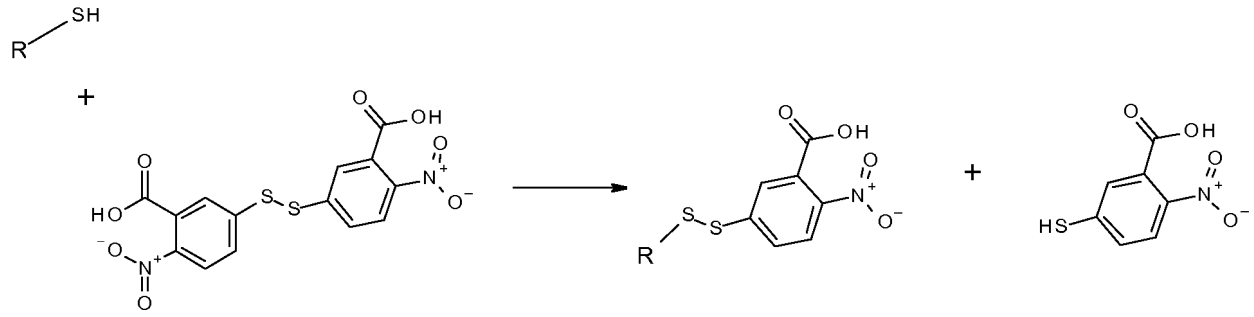
$$\text{VLDL - C (mg/dL)} = \frac{TG}{5}$$

3.4.2.7. Estimation of Reduced Glutathione (GSH) Concentration

The method of Beutler *et al*. (1963) was employed in estimating the concentration of reduced glutathione.

Principle

The reduced form of glutathione comprises in most instances the bulk of cellular non protein sulfhydryl groups. This method is therefore based upon the development of a relatively stable yellow colorwhen 5', 5'- dithios – (2-nitrobenzoic acid) (Ellman's reagent) is added to sulfhydryl compounds. The chromophoric product resulting from the reaction of Ellman reagent with the reduced GSH, 2-nitro 5-thiobenzoic acid possess a molar absorption at 412nm. Reduced GSH is proportional to the absorbance at 412nm.



Ellman's reagent Nitro-5-thiobenzoate

Figure 3.2: Mechanism of action of reduced GSH with Ellman's Reagent (Beutler *et al*., 1963)

Procedure

An aliquot of the homogenate was deproteinated by the addition of an equal volume of 4% sulfosalicyclic acid. This was centrifuged at 4,000 xg for 5 minutes. Therefafter, 0.5 mL of the supernatant was added to 4.5 mL of Ellman reagent. A blank was prepared with 0.5 mL of the diluted precipitating agent and 4.5mL of Ellman reagent. Reduced GSH level is proportional to the absorbance at 412 nm

3.4.2.8. Estimation of Lipid Peroxidation (LPO)

Lipid peroxidation was determined by measuring the levels of malondialdehyde (MDA) produced during lipid peroxidation according to the method described by Varshney and Kale (1990).

Principle

This method is based on the reaction between 2-thiobarbituric acid (TBA) and MDA: an end product of lipid peroxide during peroxidation. On heating in acidic pH, the product is a pink complex which absorbs maximally at 532nm and which is extractable into organic solvents such as butanol. Malondialdehyde is often used to calibrate this test and thus the results are expressed as the amount of the free MDA produced.

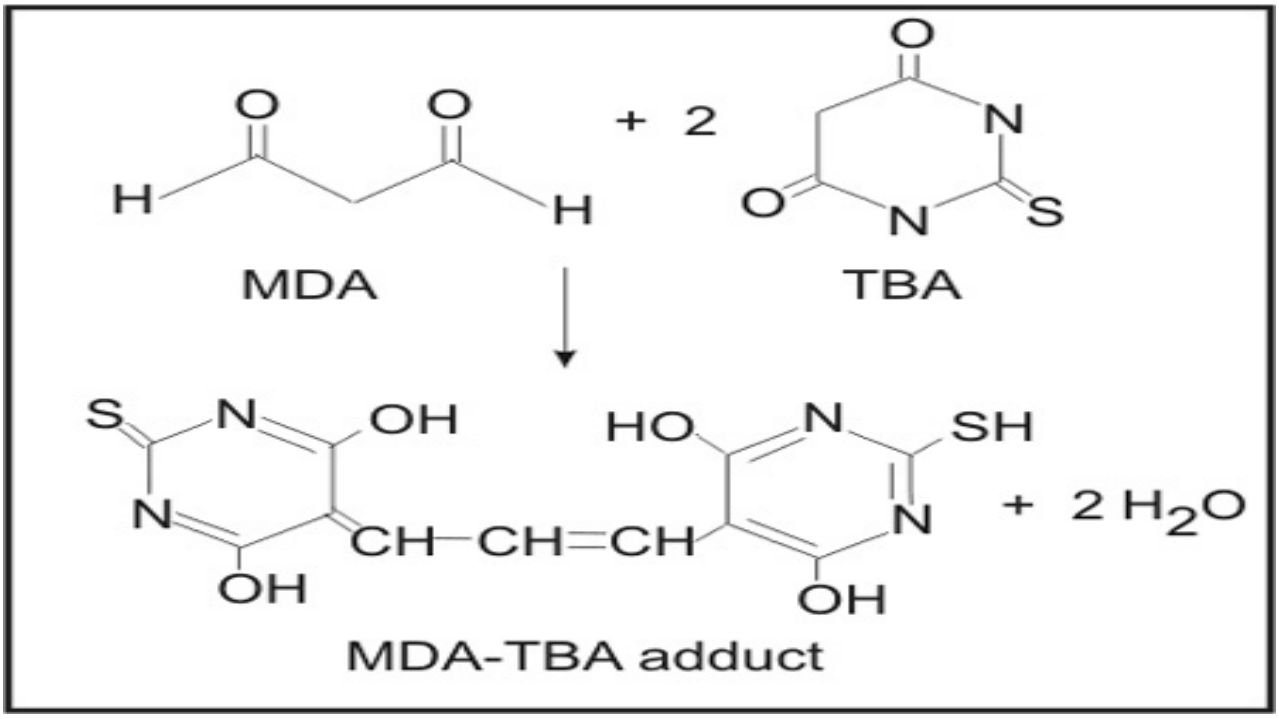


Figure 3.3: Structure of TBA + MDA ------ MDA-TBA (pink coloured complex)

Procedure

An aliquot(400µL) of the sample was mixed with 1.6mL of tris-KCl buffer to which 500 µL of 30% TCA was added. Then 500 µL of 0.75% TBA was added and placed in a water bath for 45 minutes at 80^0C. This was then cooled in ice and centrifuged at 3000 g for 5 minutes. The clear supernatant was collected and absorbance measured against a reference blank of distlled water at 532 nm. Lipid peroxidation (LPO) expressed as MDA formed/mg protein or gram tissue was computed with a molar extinction coefficient of $1.56 \times 10^5 M^{-1} Cm^{-1}$

$$\text{LPO (MDA formed/mg protein)} = \frac{\text{Absorbance x volume of mixture}}{E_{532nm} \text{ x volume of sample x mg protein}}$$

MDA formed =mmol/mg protein

3.4.2.9. Determination of Catalase Activity

Catalase activity was determined according to the method of Claiborne (1985)

Principle

This method is based on the loss of absorbance observed at 240 nm as catalase splits hydrogen peroxide. Despite the fact that hydrogen peroxide has no absorbance maximum at this wavelength, its absorbance correlates well enough with concentration to allow its use for a quantitative assay. An extinction coefficient of 0.0041 mM^{-1} cm^{-1} (Noble and Gibson, 1970) is employed.

Procedure

Hydrogen peroxide (2.95 mL of 19 mM solution) was pipetted into a 1 cm^3 quartz cuvette and 50 µL of sample added (as shown in the table below). This was done to reduce the dilution of the samples (done according to the other protocols whereby H_2O_2 was prepared separately in distilled water (100 mL) and the buffer was also prepared separately.

The mixture was rapidly inverted to mix and placed in a spectrophotometer. Change in absorbance was read at 240 nm every minute for 5 minutes.

Table 3.5: Determination of catalase activity

Test	Blank	Sample
Phosphate buffer	3 mL	2.95 mL
Sample	-	50 µL
Total	3 mL	3 Ml

Optical density was read at 240 nm at 1 min, 2, 3, 3:30, 4, 4:30, 5 mins

Calculation

Catalase activity = $\frac{(\Delta OD/min \times volume\ of\ assay\ system)}{(0.0041 \times Volume\ of\ Sample \times mg\ protein)}$ = IU/L

3.4.2.10. Determination of Superoxide Dismutase Activity

The level of SOD activity was determined by the method of Misra and Fridovich (1972).

Principle

The ability of superoxide dismutase to inhibit the autoxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for dismutase. Superoxide ($O_2\bullet^-$) radical generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per $O_2\bullet^-$ introduced increased with increasing pH (Valerino and McCormack, 1971) and also increased with increasing concentration of epinephrine. These results led to the proposal that autoxidation of epinephrine proceeds by at least two distinct pathways, only one of which is a free radical chain reaction involving superoxide ($O_2\bullet^-$) radical and hence inhabitable by superoxide dismutase.

Protocol

Sample (0.2 mL) was diluted in 0.8 mL of distilled water to make a 1 in 5 dilution. An aliquot (0.2 mL) of the diluted sample was added to 2.5 mL of 0.05 M carbonate buffer (pH

10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3 mL of freshly prepared 0.3 mM adrenaline to the mixture was quickly mixed by inversion. The reference cuvette contained 2.5 mL buffer, 0.3 ml of substrate (adrenaline) and 0.2 mL of distilled water. The increase in absorbance at 480 nm 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 30 seconds

A_3=absorbance after 150 seconds

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

1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. Results

4.1.1. Antioxidant potential of different fractions of ginger rhizomeextract

The result of the present study shows that all fractions of ginger rhizome extract showedanPLMN antioxidant capacity compared with ascorbic acid standard. The methanol fraction showed the highest DPPH activity (53.89%) at 1.00 mg/mL (Figure 4.1) followed by ethylacetate fraction (33.86%), then the chloroform fraction (20.00%) and least was the hexane fraction (15.47%).

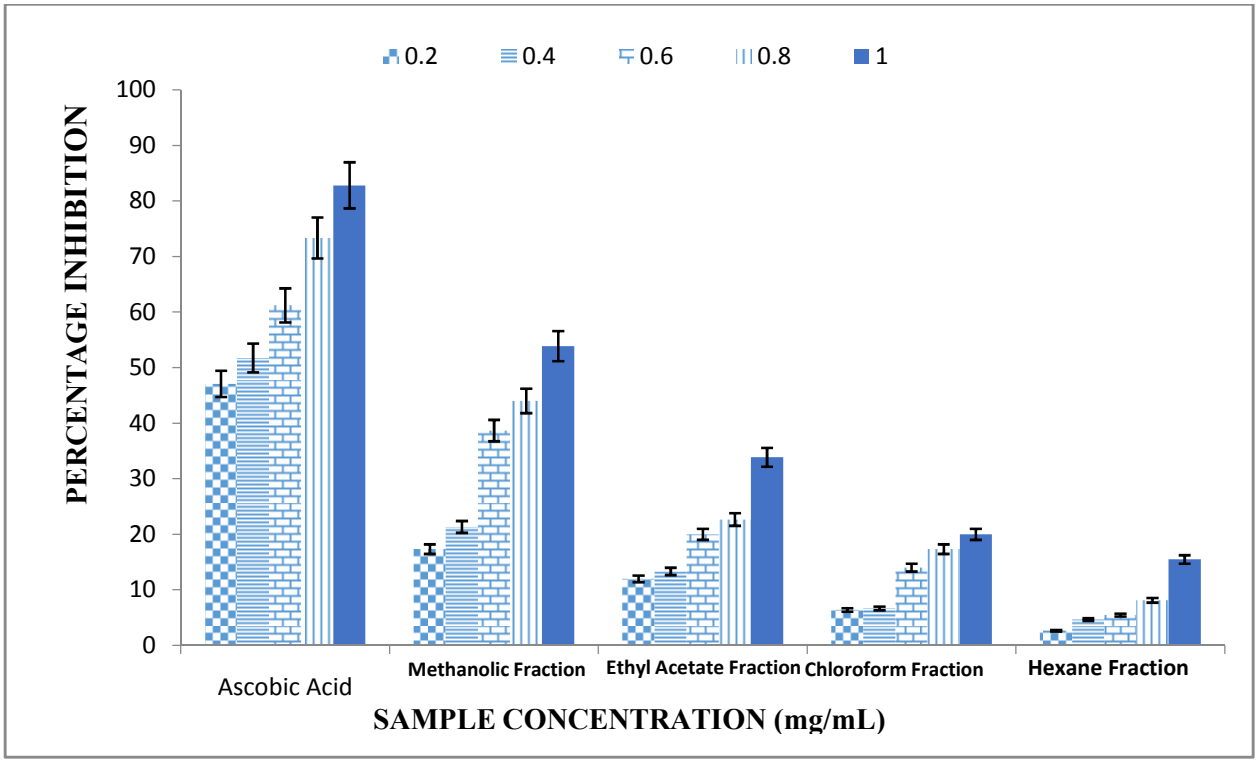


Figure 4.1: Antioxidant potential of different fractions of ginger rhizomeextract

4.1.2.Effect of Methanol Extract of Ginger Rhizome on Fasting Blood Glucose and Glycated Haemoglobin in Rats Treated for six Weeks

The results of the effect of treatment with graded concentrations ofmethanol extract ofginger rhizomeon fasting blood glucose and glycated heamoglobin are presented in Table 4.1. Generally, the results indicated significant increase (p<0.05) in the levels of fasting blood glucose and percentage glycated hemoglobin (HbA_{1c})levels in the alloxan (positive control) treated group (PC) when compared with the normal control group (NC). Results of the effect of treatments with methanol extract ofginger rhizome at graded concentrations (500, 1000 and 1500mg/kg body weight of the rats) for fourteen days showed significant (p<0.05) decrease in fasting blood glucose levels among the treated group in contrast to the positive control group (PC). Similarly, the result showed that, there was significant (p<0.05) difference in fasting blood glucose level between the normal control group and the Metformin treated group and all other treatment groups, but there was no significant (p>0.05) difference between the metformin control group and other treatment groups within the fourteen days of treatment.

The result of twenty eight days (28) of treatment shows a general decrease in fasting blood glucose level among the treated group as compared with positive control group. It was observed that, there is no significant difference (p>0.05) in the results of fasting blood glucose levels between the normal and metformin treated groups (NC and MC). However it has shown that there were a significant difference (p<0.05) between the normal control group and all the treatment groups. Also, it was observe with treatment group (GR1 and GR2) were statistically (p>0.05) the same as compared with that of the metformin treated group (MC). However, they were statistically differed (p>0.05) with the GR3 treated group.

The result of fasting blood glucose levels at the forty two days (42) of treatment shows a general decrease in the blood glucose levels among all the groups (NC,MC and the treatment groups) in contrast with the alloxan treated group (PC). GR1treated group showed the highest hypoglycaemic effect as compared with normal and metformin control groups. This trend was followed by GR2 with high hypoglycaemic effect. Lastly, the GR3 has the lowest hypoglycaemic effect among the treatment groups but nevertheless it has a promising result in lowering the blood glucose level.

The result of glycated haemoglobin shows reduction in the level of percentage glycated haemoglobin among the treatment group in contrast with alloxan treated group (PC). However it has been observed that there were no significant ($p>0.05$) difference between the normal control group (NC),the metformin treated group, and group1 treated groups. On the other hand, there was a significant($p<0.05$) difference between the normal control group and other treated groups GR2 and GR3, this difference happened to be statistically the same with the metformin and GR1 treated group. The result generally shows that MC and GR1 have the lowest level of glycated haemoglobin as compared with the positive control (PC) group.

Table 4.1:Fasting Blood Glucose and Glycated Heamoglobin levels in Rats Treated with Methanol Extract ofGinger RhizomeforSix Weeks.

GROUP	FBG for 14 Days (mg/dl)	FBG for 28 Days (mg/dl)	FBG for 42 Days (mg/dl)	HbA_{1c} (%)
NC	88.6±5.5^{a}	88.4±4.5^{a}	87.2±4.6^{b}	3.4±1.1^{a}
PC	309.0±30.2^{bc}	293.0±6.1^{b}	267.0±15.3^{a}	16.6±2.7^{b}
MC	162.2±23.5^{b}	108.0±9.9^{ac}	92.2±8.4^{b}	5.2±0.9^{a}
GR1	180.4±28.8^{b}	116.6±8.1c	95.8±7.3^{bd}	5.2±1.3^{a}
GR2	182.6±17.9^{b}	132.2±18.6^{c}	113.8±16.9^{cd}	9.2±1.9^{c}
GR3	171.0±21.9^{b}	157.8±17.9^{d}	125.8±20.1^{c}	10.0±2.2^{c}

Values are expressed as mean ± S.D., Mean values having different superscript letter in the same column are significantly different at (p<0.05).

Key:

NC: Normal Control, PC:Positive Control, MC: Metformin Control (500 mg/kg) body weight of rat; GR1; treatment group with high dose(1500mg/Kg), GR2; treatment group with medium dose (1000mg/Kg) and GR3; treatment group with low dose (500mg/Kg) body weight of rat.

4.1.3.Effect of Methanol Extract of Ginger Rhizome on Serum Lipid Profile in Rats Treated for Six Weeks

The results of the effect of treatment with graded concentrations (1500, 1000 and 500mg/kg) of methanol extract ofginger rhizome on serum lipid profile is presented in Table 4.2. The result indicated significant increase ($p<0.05$) in the levels of serum total cholesterol (TC), triacylglycerol (TAG), and low density lipoprotein (LDL-C) in the alloxantreated group (PC) in contrast with that of the normal control group (NC). In the same vein, there was significant decrease ($p<0.05$) in HDL-C in the alloxan-induced positive control group (PC) as compared with the normal control group (NC). The results showed significant ($p<0.05$) decrease in the levels of serum TC, TAG, and LDL-C while the serum HDL-C levels was significantly increased ($p<0.05$) in all the groups treated with the graded doses (1500, 1000 and 500mg/kg) of themethanol extract of ginger rhizome in contrast with the alloxan treated group (PC).

However, the serum VLDL-C levelwas found to be significantly ($p>0.05$) the same between the treatment groups and the alloxan treated (PC) group. It was observed that the level of serum TC in GR1 treated group, metformin treated and normal control groups were statistically ($p>0.05$) the same, and significantly ($p<0.05$) differed with GR2 and GR3 treated groups as compared with the normal control group. The treated groups (GR1, GR2, and GR3) show similar pattern in the levels of HDL-C and LDL-C and there were no significant ($p<0.05$) difference between them as compared with the normal control group.

Table 4.2:Serum Lipid Profile of Rats Treated with Methanol Extract ofGinger Rhizome for Six Weeks.

GROUP	TC (mg/dL)	HDL-C (mg/dL)	TG (mg/dL)	VLDL-C (mg/dL)	LDL-C (mg/dL)
NC	72.7±8.3[a]	53.3±9.3[a]	60.0±7.8[a]	13.9±4.1[a]	9.5±1.7[a]
PC	106.5±7.5[b]	23.5±4.2[b]	117.7±13.3[b]	19.6±2.3[a]	56.1±8.6[b]
MC	75.6±5.1[a]	51.3±6.7[a]	73.6±11.2[a]	13.8±3.8[a]	10.9±3.2[a]
GR1	76.6±11.2[a]	49.5±11.4[ac]	76.5±5.2[a]	16.1±1.6[a]	21.1±3.2[ac]
GR2	86.0±5.2[c]	37.2±3.6[c]	87.9±12.1[ac]	16.1±4.0[a]	23.5±6.7[c]
GR3	88.9±2.6[c]	37.5±3.1[c]	100.6±9.7[bc]	18.4±1.8[a]	26.7±8.6[c]

Values are expressed as mean ± S.D., Mean values having different superscript letter in the same column are significantly different at (p<0.05).

Key:

NC: Normal Control, PC: positive Control, MC: Metformin Control (500 mg/kg) body weight of rat Metformin; GR1; treatment group with high dose(1500mg/Kg), GR2; treatment group with medium dose (1000mg/Kg) and GR3; treatment group with low dose (500mg/Kg) body weight of rat.

4.1.4. Effect of Methanol Extract of Ginger Rhizome onSerum Catalase Activity, Reduced Glutathione (GSH), Superoxide Dismutase (SOD) and Malondialdehyde (MDA) Levels in Rats Treated for SixWeeks

The results of the effect of treatment with graded concentrations (1500, 1000, and 500mg/kg) of the methanol extract of ginger rhizomeon some antioxidant indices (catalase activity, reduced glutathione, superoxide dimutase and malondialdehyde levels) are presented in Table 4.3. The results indicated significant decrease ($p<0.05$) in serum levels of reduced glutathione (GSH), superoxide dismutase (SOD) and catalase activity in the alloxan treated group (PC) as compared with the normal control group (NC). It was also observed that there was significantly increased serum malondialdehyde (MDA) level in the alloxan treated group (PC) in contrast with that of the normal control group (NC). The effect of treatments with different concentrations (1500, 1000, and 500mg/kg) of the methanol extract ofginger rhizomefor six weeks resulted in significant ($p<0.05$) increase in serum levels of reduced glutathione (GSH), superoxide dismutase (SOD) and catalase activity as well as significant ($p<0.05$) decrease in serum level of malondialdehyde (MDA) when compared with the alloxan treated group (PC).

Table 4.3:Serum Catalase Activity, Reduced Glutathione (GSH), Superoxide Dismutase (SOD) and MDA Levels of Rats Treated withMethanol Extract ofGinger Rhizomefor Six Weeks.

GROUP	MDA (nmol/L)	SOD (µmolSOD/min/mg of protein)	GSH reduced (mg/dl)	CAT (IU/L)
NC	39.8 ± 4.4^{a}	15.4 ± 4.8^{a}	31.7 ± 3.1^{a}	38.6 ± 2.4^{a}
PC	101.8 ± 8.7^{b}	4.2 ± 0.5^{c}	19.4 ± 2.3^{b}	22.9 ± 2.1^{d}
MC	39.2 ± 7.3^{a}	13.8 ± 3.6^{ab}	30.8 ± 2.3^{a}	35.9 ± 5.3^{a}
GR1	54.8 ± 5.2^{c}	9.0 ± 1.3^{b}	28.3 ± 2.2^{ac}	31.4 ± 3.1^{c}
GR2	65.3 ± 5.5^{cd}	8.6 ± 2.4^{b}	25.1 ± 3.2^{cb}	31.1 ± 3.9^{c}
GR3	72.9 ± 4.3^{d}	8.2 ± 2.3^{b}	27.7 ± 4.0^{cb}	28.1 ± 2.2^{cd}

Values are expressed as mean ± S.D., Mean values having different superscript letter in the same column are significantly different at ($p<0.05$).

Key:

NC: Normal Control, PC: Positive Control, MC: Metformin Control (500 mg/kg) body weight of rat; GR1; treatment group with high dose(1500mg/Kg), GR2; treatment group with medium dose (1000mg/Kg) and GR3; treatment group with low dose (500mg/Kg) body weight of rat.

4.2. Discussion

Diabetes mellitus is a complex metabolic disorder characterized by high blood glucose levels due to the inability of the body cells to utilize glucose properly (King & Brownlee, 1996). The use of insulin, biguanides, sulphonylurea and other chemical therapies are valuable in the treatment of diabetes mellitus and can control many aspects of diabetes complications. But, their use is restricted by their limited action, pharmaco-kinetic properties and accompanying side effects (King *et al*., 1998; Shaw *et al.,* 2010). Moreover, these therapies only partially compensate for metabolic derangements seen in diabetes and do not necessarily correct the fundamental biochemical lesion (Taylor & Agius, 1988; Bailey *et al*., 1989).

In the present study, an attempt was made to elucidate the role of different concentrations (1500, 1000, and 500mg/kg) of methanol extract ofginger rhizomein controlling / managing diabetes mellitus in alloxan-induced diabetic rats and also, to study some of the possible mechanism of antidiabetic action of the ginger rhizome extract in controlling diabetes. Diabetes was induced by intraperitoneal administration of 120 mg/kg body weight of alloxan monohydrate to the Wistar albino rats.Although, the precise mechanism of alloxan-induced diabetes remains unclear, there is increasing evidence that it involves the degeneration of islet β-cells by accumulation of cytotoxic free radicals (Halliwell & Gutteridge, 1989). Following its administration, alloxan is concentrated in the islets and in the liver, where it is reduced to dialuric acid. This acid is unstable in aqueous solutions and undergoes oxidation back to alloxan, accompanied by generation of O^{2-} and hydroxyl radicals by Fenton type reaction (Halliwell & Gutteridge, 1989). The liver contains high super oxide dismutase (SOD), catalase and glutathione peroxidase activities, which can scavenge these free radicals. On the contrary, the

islet cells have low concentrations of these enzymes and are vulnerable to the cytotoxic effects of the free radicals (Halliwell & Gutteridge, 1989).

The selective toxicity on β-cell after the alloxan injection, leads to reduction in insulin level, causing alteration of glucose metabolism and utilization thereby resulting in hyperglycemia (Arumugam *et al.,* 2008). Generally, prolonged uncontrolled high blood glucose has been shown to results in elevated levels of serum glucose, glycated hemoglobin, oxidative stress indices as well as decreased levels of antioxidants defences and lipid abnormalities due to lipid peroxidation (Asayama *et al.,* 1986). Following injection with alloxan, the animals displayed theexpected symptoms of diabetes mellitus, such as hyperglycemia,polydipsia, polyuria, polyphagia, and depression of body weight as previously observed by Robert (2001).

Based on the results, the hypoglycemic activity of all the methanol extract of ginger rhizomeat various doses used for the treatment were comparable to that of the metformin control group. This might be connected with the role of the phytochemicals derived from ginger rhizome extract. Moreover, the result of the treatment withmethanolic extract of ginger rhizomeagrees with the findings of Ojewole (2006) who reported that oral intake of alcoholic extract of ginger (800 mg/Kg) significantly decreased the level of fasting blood glucose after 1 hour treatment in STZ-diabetic rats. Also Islam and Choi (2008), in nicotinamide and low dose STZ-diabetic ratmodel, noticed that oral administration of ginger powder at dose of 200 mg/kg resulted in alleviation of metabolic syndrome signs including blood glucose and serum lipids reduction and increasing total antioxidant capacity (TAC). However, Bordia*et al.* (1997) stated that the consumption of 4 g/day ginger powder in coronary artery disease (CAD) duration 3 months did not change the level of serum glucose and lipids.

The differences in my results with this study may be due to difference in chemical composition of administered ginger extract, preparation method, rhizome used, or storage time. Jafri*et al.* (2011) showed that oral administration of ginger extract with daily dose of 500 mg/kg for 6 weeks in Alloxan-diabetic rats caused decreased in blood glucose level at 21 and 42 days. Abdulrazaq*et al*. (2010) in a similar study found that daily administration of oral ginger aqueous at dose of 500 mg/Kg during 30days in STZ-diabetic rats caused 38% and 68% reduction in plasma glucose level, on the 15th and 30th day of study, respectively. Abdulrazaq *et al*. (2010) state that this solution (of ginger) have hypoglycemic effect possibly by increasing the activity of glycolytic enzymes (glucokinase, phosphofructokinase, pyruvate kinase) . Khadem *et al*.(2008) also found that blood glucose concentration have more decreased in STZ-diabetic rats treated with ginger powder (5% of daily dietary intake for 6 weeks) compared to control diabetic rats. He also added that the HbAlc level in the ginger-treated group was significantly lower than that in the non- treated diabetic group. It has been showed that HbAlc level is increased during diabetes and it is a marker which shows the degree of protein glycation. Administration of ginger to diabetic rats significantly decreased the level of glycosylated hemoglobin and this may be due to the decreased level of blood glucose. The present study is in agreement with these results. Glycated hemoglobin is formed through the nonenzymatic binding of circulating blood glucose to hemoglobin (Rohlfing *et al.*, 2000). Persistant hyperglycemia might have to contribute to non-enzymatic glycation of plasma proteinsleading to the production of more powerful oxidizing species (Hunt *et al.,* 1993). Thiscontributes to increased levels of glycated hemoglobin (Rohlfing *et al.,* 2000). HbA_{1c} concentration is associated with diabetic micro, macrovascular complications and risk of death (Khaw *et al.,* 2001). Many investigators reported that compounds of ginger such as 6-gingerol, tannins, polyphenolic compounds, flavonoids, and triterpenoids

possess hypoglycemic and other pharmacological properties (Shanmugam *et al.,* 2009). Rani *et al.* (2010) suggested that ginger, via it is major component, gingerol, by inhibition of key enzymes relevant to type 2 diabetes, *α*-glucosidase and α-amylase, are known to improve diabetes. Li *et al*. (2012) found that polar portion of ginger extract containing mainly gingerols, particularly (S)-[6]- and (S)-[8]- gingerol, promoted glucose uptake significantly in cultured rat skeletal muscle cells.

This action of gingerols was attributed to facilitation of insulin-independent glucose uptake by increasing translocation of glucose transporter GLUT4 to the muscle cell plasma membrane surface, together with small increases in total GLUT4 protein expression. Another mechanism for reducing blood glucose by ginger extract, is the inhibition of hepatic phosphorylase enzyme, hereby it prevents the breakdown of hepatic glycogen storages, also, can increases the activity of enzymes improving glycogen synthesis. The other possible effect is suppression the activity of hepatic glucose 6-phosphatase enzyme, that causes degradation of glucose 6-phosphate to glucose, and consequently increases blood glucose level (Zhang & Tan, 2003). *In-vitro* studies suggested that ethyl acetate extract of ginger have inhibitory effect on the two key enzymes of glucose metabolism (α-amylase and α-glucosidase); the function of ginger against these two enzymes was found to be correlated with phenolic content of gingerol and shogaol at these extracts. Ginger has been shown to modulate insulin release. Ginger promotes glucose clearances in insulin responsive peripheral tissues, which is crucial in maintaining blood glucose homeostasis (Rani *et al.,* 2010). Moreover, it is reported that 6-gingerol increases the glucose uptake at insulin responsive adipocytes (Sekiya *et al.,* 2004). Thus, at treated cells with gingerol, insulin responsive glucose uptake has increased and improved diabetes (Zhang & Tan, 2003).

The present study indicated that, alloxan-induced diabetic control rats (PC) had elevated levels of serum total cholesterol (TC), triglycerides (TG), and low density lipoprotein-C (LDL-C), but decreased level of high density lipoprotein-C (HDL-C). This is in line with the findings ofYadav *et al*. (2005) where they revealed an increase in serum, triglycerides and total cholesterol levels in alloxan-diabetic rats. Lebda *et al*. (2012) also suggested that intake of different forms of ginger (powder, warm or cold extract) in amount 2% of basal diet in rabbits resulted in significant decline in serum level of TG, TC, LDL-C, while it increased the level of blood glucose and HDL-C. Reduction of lipid peroxidation by ginger has been attributed to it is antioxidant activity, because ginger have many phenolic compounds, which have inhibitory effects on lipid peroxidation and preserve the antioxidant compounds. The increase in certain parameters (TC, TG, LDL-C and VLDL-C) in lipid profile may be a result of increased breakdown of lipids and mobilization of free fatty acids (FFA) from the peripheral deposits (Garg & Grundy, 1990). Also, due to the fact that insulin could inhibit the hormone-sensitive lipases and these become active in the absence of insulin.

Other hormones such as glucagon and catecholamines are known to increase during diabetes and stimulating lipolysis (Baquer *et al.,* 2011). Administration of different doses (1500, 1000 and 500 mg/kg) of the ginger rhizome extract showed reduction in TC, TG, LDL-C, and VLDL-C levels. Also, the HDL-C level was observed to increase in the treated groups. This also demonstrated the hypolipidemic effect of the ginger rhizome extract. There could be two possibilities for the normalisation of the altered parameters in the lipid profile: First, the rate of lipogenesis may be normalized by the micro nutrients and other antioxidant substances derived from the ginger rhizome extract which may work in a way similar to the effect of insulin on lipid metabolism which leads to change in the activity of cholesterol biosynthesis enzymes such as

hormone-sensitive lipases which may be inhibited and thereby deactivate the rate of lipolysis which is under the control of insulin (Raju *et al.,* 2001). Second, since the attainment of normoglycaemia in the animals was achieved by the ability of the ginger rhizome extract, this could lead to activation of lipogenesis and inhibition of lipolysis in the rats' adipose tissue (Muhammad *et al.,* 2006).

Diabetes mellitus is characterized by elevated levels of oxidative stress markers and decreased level of antioxidants leading to increased lipid peroxidation (Asayama *et al*., 1986). It has been suggested that oxidative stress plays an important role in many diseases, including diabetes mellitus (Wolff, 1993). In the current study, it was observed that there was increase in the level of oxidative stress markers in the alloxan-induced diabetic control rats (PC). The results indicated that the alloxan-induced diabetic control group (PC) have lower levels of serum reduced glutathaione (GSH), superoxide dismutase (SOD) and Catalase activity but havehigher level of serum MDA, (a marker of lipid peroxidation). The increase in lipid peroxidation as revealed by the high level of MDA formed in the alloxan-induced diabetic rats compared to the normal control rat suggests that the natural antioxidant defense mechanism to scavenge excessive free radical has been compromised in rats induced with diabetes (Pratibha *et al.,* 2004). Decrease in antioxidant enzyme activity as well as increased MDA as observed in diabetes mellitus might be due to an altered intracellular ratio between free radicals and antioxidant capacity because the reactive oxygen species (ROS), which are excessively produced in diabetes, are able to overwhelm the endogenous defense systems leading to oxidative stress (Mooradian *et al.,* 1996). ROS are considered important independent risk factors developed in diabetes mellitus via what is known as "auto-oxidative glycosylation", a process which is relevant at elevated blood glucose level. Hyperglycemia may also raise aldose reductase activity which depletes NADPH cell stores,

thus perturbing the defense system(Tames *et al.,* 1992). The elevated blood glucose level can also cause non-enzymatic glycation of plasma proteins leading to the production of more powerful oxidizing species which can bind with most normal cellular components to "pair up" its unpaired electrons; thus, they react with the unsaturated bonds of membrane lipids, denature the proteins, and attack nucleic acids, resulting in cellular oxidative damage (Chandra, 1994). This in turn may lead to the development of cardiovascular diseases (Fridovich, 1995). Results of the effect of treatments with different doses (1500, 1000, and 500mg/Kg) of themethanolic extract of ginger rhizomeafter six weeks of treatment showed elevation in the serum levels of reduced GSH, superoxide dismutase and CAT activity in comparison with that of the diabetic control (PC) group.

There are many reports available to support the multiple mechanisms for antidiabetic plants in their blood glucose lowering effect. This could include: Inhibition of carbohydrate metabolizing enzymes, Inhibiting glucose absorption from intestine, Enhancement of insulin sensitivity, Regeneration of damaged pancreatic islet and β-cells, Enhancement of insulin secretion and release, Inhibiting glucose production from hepatocytes, Enhancing glucose uptake by adipose and muscle tissues as well as plants that exhibits antioxidant effects etc. (Shokeen *et al*., 2008; El-Abhar *et al.,* 2014).

Oxidation of biological molecules induces a variety of pathological disease including atherosclerosis or cancer. These damages are caused due to the presence of free radicals. For that reason, the concept of pharmacological supplements to defend against free radicals with antioxidants has become an intense area of research (Gounder & Lingmallu, 2012). According to Atashak (2014), [6]-gingerol, [6]-shogaol have displayed strong antioxidant activity *in vitro.*

It is known that the antioxidant activity of plant extracts containing polyphenol components is due to their capacity to be donors of hydrogen atoms or electrons and to capture the free radicals. DPPH analysis is one of the tests used to prove the ability of the components of the ginger extract to act as donors of hydrogen atoms (Stoilova *et al.,* 2007). The result of the present study shows that both fractions of extracts of ginger rhizomehave an antioxidant capacity in reducing the DPPH reagent from a purple colour to yellowish coloration. It has been observed that the methanol fraction has the highest percentage inhibition on DPPH reagent a compared with the ascorbic acid standard. These results are in agreement with the findings of Stoilova *et al.* (2007), who studied the antioxidant activity of the alcohol extract of ginger from Vietnamand found that the DPPH radical inhibition reached up to 90.1%. Hinneburg *et al.* (2006) in Finland found high antioxidant action of the aqueous extracts of ginger, where the IC50 value for the inhibition of DPPH radical was ~9 mg/mL.

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1. Summary

The present study was carried out to evaluate the effect of methanol extract of ginger rhizome (*Zingiber officinale*) in alloxan-induced diabetic rats on fasting blood glucose (FBS) and glycated heamoglobin, lipid profile and some oxidative stress markers.

Thirty six (36) rats were randomly selected and divided in to six (6) groups of six rats each: Alloxan treated group (PC), Normal control group (NC), group one (GR1 = treated with 1500mg/kg body weight), group two (GR2 = treated with 1000mg/kg body weight), group three (GR3 = treated with 500mg/kg body weight), and metformin treated group (MC) 500 mg/kg body weight metformin. Methanol extract of ginger rhizome was given to the treatment groups (GR1, GR2, and GR3) at graded doses once daily for 48 days by intubation.

The results of the study are summerised below:

1. The findings of this study showed a significant ($p<0.05$) decrease in fasting blood glucose levels and percentage glycated heamoglobin among the treated groups (GR1, GR2 and GR3) in contrast to alloxan treated group (PC).
2. In the same vein, there is also a significant ($p<0.05$) decrease in the levels of serum total cholesterol, triacylglycerol, and low density lipoprotein cholesterol and significant ($p>0.05$) increase in the levels of serum high density lipoprotein cholesterol in all the groups treated with graded doses of methanol extract of ginger rhizome as compared with alloxan treated group.
3. Similarly, there is a significant ($p<0.05$) increase in serum levels of reduced glutathione (GSH), superoxide dismutase (SOD) and catalase activity as well as significant ($p<0.05$)

decrease in serum level of MDA in ginger treated groups when compared with the alloxan treated group (PC).

4. Generally, the findings of this study show that methanol extract of ginger rhizome may be a potential therapeutic agent in the management of diabetes and its associated complications.

5.2. Conclusion

The results of this study suggest that ginger rhizome (*Zingiber officinale*) extract possessed significant hypoglycemic activity and also have additional advantage of possessing significant hypolipidemic and antioxidant effect. This conclusion is drawn from the fact that all the graded doses (1500, 1000, and 500mg/kg) of themethanolic extract of ginger rhizomeshowed improvement in the reduction of fasting blood glucose and also glycated haemoglobin in rats,which is not only dose dependent but also time dependent. Methanolic extract of ginger rhizomealso showed the potentiality to decrease serum levels of TAG, TC, LDL-C, VLDL-C and MDA but improves the serum levels of HDL-C, GSH, SOD and CAT activity. This suggests that ginger may be a good remedy for diabetic patients to diminish the risk of some secondary chronic complications.

5.3. Recommendations

Based on the findings of this study the following recommendations are made:

1. Further studies on the effect of methanol extract of ginger rhizomeon the activities and the level of expression of gluconeogenic enzymes such as glucose -6- phosphatase, fructose -1, 6- bisphosphatase, phosphoenoyl pyruvate carboxy kinase and other gluconeogenic enzymes are recommended. This might be useful in further elucidation of the antidiabetic mechanism of action of theginger rhizome.

2. This study should also be extended to human subjects on the use ofginger rhizomeextract in the management of diabetes mellitus.

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APPENDICES

Appendix I:

Preparation of Working Reagentreagents for Glycated Haemoglobin Estimation

1. Oxalic acid, 0.5mol/L: 6.3 g of oxalic acid was dissolved in distilled water and then, diluted to 100 ml.
2. Thiobarbituric acid, 0.05mol/L: 0.721 g of 2-thiobarbituric acid was dissolved in distilled water and then, diluted to 100 ml.
3. Trichloroacetic acid, 400g/L: 40 g of trichioroacetic acid was diluted to 100 ml with distilled water.
4. Saline, 0.15 mol/L: 8.76 g of sodium chloride was dissolved in distilled water and then, diluted to 1 L with the distilled water.
5. Standard Curve for Fructose:

   Fructose standard (stock), 1g/dl: exactly1g of fructose was dissolved in little volume of saline and then diluted to 100ml with the saline. This is further diluted with saline to prepare 0.1, 0.2, 0.3, 0.4, and 0.5 g/dl fructose working standards in separate test tubes (A to E).

   1 ml each of the fructose working standard was mixed with 0.5 ml distilled water and 1.0 ml of the trichloroacetic acid reagent. A seperate test tube containing 1.0 ml of Oxalic acid, 0.5 ml of distilled water, and 1.0 ml of the trichloroacetic acid reagent was included as a zero standard (blank). Then, all test tubes were vortexed before centrifuging at 1000 x g for 10 minutes.

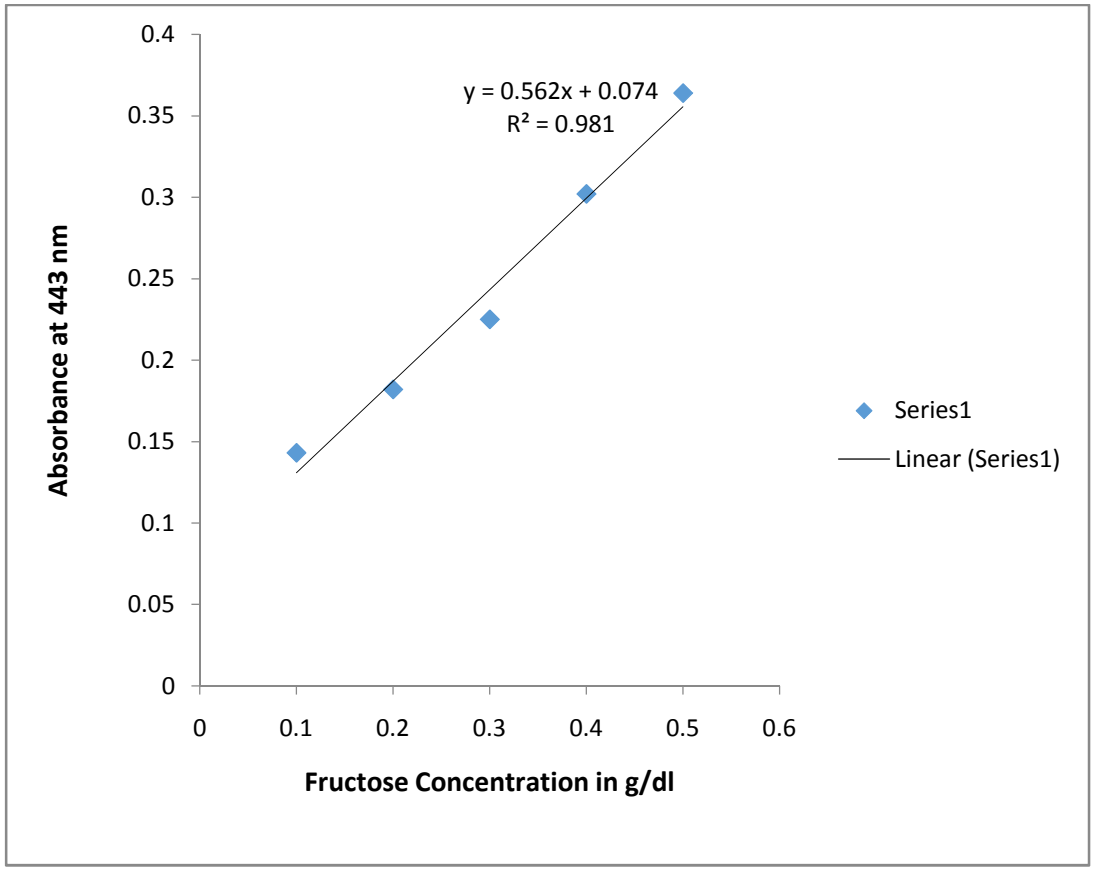
   Colorimetric reaction:

   1.5 ml each of the of the supernatant from the step above were seperately collected in to labelled glass test tubes and each was mixed with 0.5 ml of the thiobarbituric acid reagent except for the assay blank, in which 0.5 ml of distilled water was substituted for the thiobarbituric acid reagent.

   All tubes were incubated at 40 ^{o}C for 30 minutes. Afterwhich the spectrophotometer was adjusted to zero with the zero standards (blank) then the absorbance of each test solution was read spectrophotometrically at 443 nm and the graph of fructose absorbance aginst its coresponding concentration was plotted.

Tubes	A	B	C	D	E	F
Fructose Conc. (g/dl)	0	0.1	0.2	0.3	0.4	0.5
Absorbance at (443)	0	0.011	0.014	0.019	0.022	0.025

Standard Curve for Fructose



Appendix II:

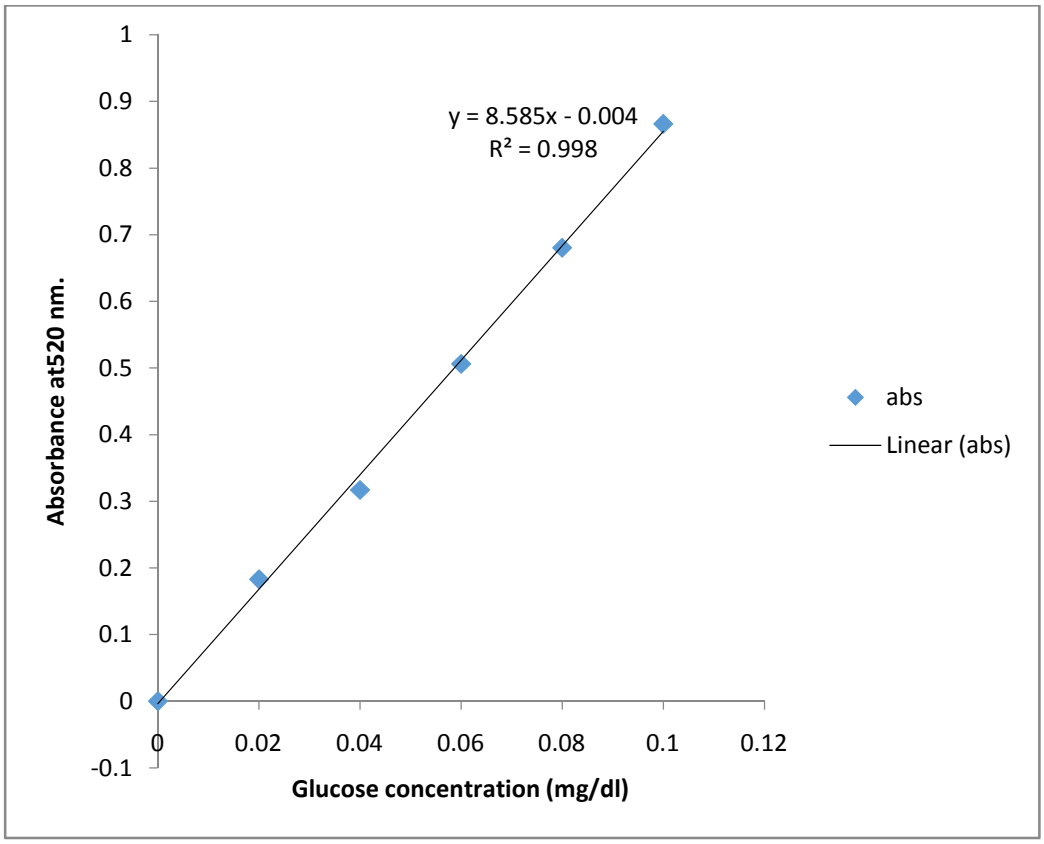
Preparation of Standard Curve for Glucose

Glucose standard (stock), 1.0 mg/dl: exactly 1.0 mg of glucose was weighed and dissolved in distilled water and then, diluted to 100 ml. This is further diluted with distilled water to prepare 0.02, 0.04, 0.06, 0.08 and 0.1 mg/dl standards of glucose solution in separate testubes (A to E). the absorbance

By the uses glucose oxidase/ peroxidase method using Randox kit reagents, 10 µl of each of the prepared glucose standard solution and distilled water were respectively pipetted in to labelled test tubes. Each test tube was then followed by 1000 µl of the glucose oxidase reagent. The tubes were mixed properly, incubated at 37^{o}C for 10 minutes and the absorbance were read against the blank at 500 nm using spectrophotometer. The graph glucose concentration was plotted against the corresponding absorbance.

Tubes	A	B	C	D	E	F
Glucose Conc. (mg/dl)	0	0.02	0.04	0.06	0.08	0.10
Absorbance at 520 nm	0.00	0.183	0.317	0.506	0.680	0.866

Standard Curve for Glucose



Appendix III:

PREPARATON OF REAGENTS FOR REDUCED GLUTHIONE

1. **GSH WORKING STANDARD**

40 mgGSH (Sigma Chemical Co., London, Mol. Wt 307.3g) was dissolved in 100 ml of 0.1M phosphate buffer, pH 7.4, and then stored in the refrigerator.

2. Phosphate buffer (0.1M, pH 7.4)

a. 7.16 g of K_2HPO_4 $12H_2O$ (Hopkins and Williams, Ltd, Mol. Wt. 358.22) was dissolved in 200ml of distilled water.

b. 1.56 g of KH_2PO_4. $2H_2O$ (MW. 156.03) was dissolved in 100 ml of distilled water.

Finally, solution (a) and (b) were added together and the pH adjusted to 7.4

3. Ellman Reagent [5', 5'-Dithiobis- (2-nitrobenzoate) DTNB]

40 mg of DTNB was dissolved in 0.1M phosphate buffer of pH 7.4 and made up to 100 ml

4. Precipitating Agent

4% sulphosalicyclic acid (C_7H_6S. $2H_20$;n Mol. Wt. 254.22) was prepared by dissolving 4g of sulphosalicyclic acid in 100ml of distilled water. This is stable for approximately three weeks at 4ºC.

Serial dilutions of GSH working standard were prepared as shown in the table below:

Preparation of GSH standard curve

Table 3: Preparation of serial dilutions of the GSH working Standard

Stock ml	Phosphate buffer	Ellman's reagent	Abs (412nm)	GSH conc (µg/ml)
0.01	0.24	2.25	0.04	8
0.025	0.225	2.25	0.101	20
0.05	0.20	2.25	0.194	40
0.10	0.15	2.25	0.38	80
0.15	0.10	2.25	0.572	120
0.20	0.05	2.25	0.749	160

Total reaction mixture: 2.25ml

GSH is proportional to absorbance at 412nm. All readings were taken within 5 minutes, as colour developed is not stable after that duration, following addition of Ellman's reagent

Determination of GSH concentration in the samples

An aliquot of the homogenate was deproteinated by the addition of an equal volume of 4% sulfosalicyclic acid. This was centrifuged at 4,000 xg for 5 minutes. Therefafter, 0.5 ml of the supernatant was added to 4.5 ml of Ellman reagent. A blank was prepared with 0.5 ml of the diluted precipitating agent and 4.5ml of Ellman reagent. Reduced GSH level is proportional to the absorbance at 412 nm

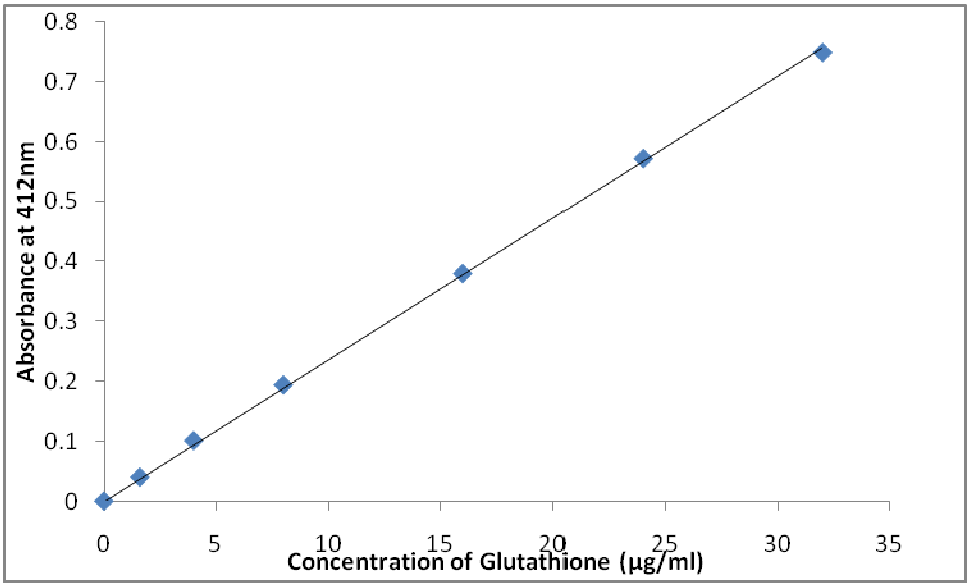


Figure 3: Standard curve for reduced glutathione

Appendix IV:

PREPARATION OF REAGENTS FOR MDA

1. 30% Trichloroacetic acid (TCA)

   4.5 g of TCA was dissolved in distilled water and made up to 15 ml with same

2. 0.75% Thiobarbituric acid (TBA)

   This was prepared by dissolving 0.1125 g of TBA in 0.1M HCl and made up to 15ml with same.

3. 0.15 M Tris-KCl buffer (pH 7.4)

   1.12 g of KCl and 1.817 g of Tris base were dissolved in 100 mls of distilled water and the pH was then adjusted to 7.4

4. 0.1M HCl

   0.124 ml conc HCl was diluted with 14.876 ml of distilled H_2O to make 15 mls.

Preparation of Reagents for catalase

1. Phosphate buffer (0.05 M, pH 7.0)

Dipotassium hydrogen phosphate (K_2HPO_4, 0.336 g) and potassium dihydrogen phosphate (KH_2PO_4, 0.417 g) were dissolved in 100 ml of distilled water, the pH adjusted to 7.0 and the volume made up to 100ml with distilled water.

2. Hydrogen peroxide (0.019M)

   109 µL of 59% $H_2O_2$was added to 50 ml of the 0.05M phosphate buffer, pH 7.0 and the volume was made up to 100 ml with the same.

Preparation of Reagents for superoxide dismutase

1. 0.05 M Carbonate buffer (pH 10.2)

3.58 g of $Na_2CO_3.10H_2O$ and 1.05 g of $NaHCO_3$ were dissolved in 200 ml of distilled water. The pH was adjusted to 10.2 and then made up to 250ml with distilled water.

2. 0.3 mM Adrenaline

0.01 g of adrenaline (epinephrine) was dissolved in 200 ml-distilled water, prepared fresh when needed.

Appendix V:

Table 1: List of Chemicals and Reagents

Chemicals	Manufacturer
Alloxan monohydrate	Sigma-Aldrich; Mumbai, India.
Glucose oxidase assay kit	Randox Laboratories Ltd. Antrim U.K.
Total cholesterol assay kit	Randox Laboratories Ltd. Antrim U.K.
Triglycerides assay kit	Randox Laboratories Ltd. Antrim U.K.
HDL- cholesterol assay kit	Randox Laboratories Ltd. Antrim U.K.
Potassium Chloride (KCl) MW: 74.55	Quali Chemicals Ltd.
Sodium Cloride	Avis Chemicals Ltd.
Sodium Hydrogen Carbonate	D. H Chemicals Ltd. Poole England.
Sodium Dihydrogen Orthoposphate	Hopkin and Williams.
Magnissium Chloride ($MgCl_2.5H_2O$)	Lab Tech Chemicals India.
Calcium chloride (CaCl)	Fishar Scientific Company.
Thiobarbituric acid	Sigma Chemicals Co., USA.
Fructose	J. T Baker Chemicals Co., Phillips.
Oxalic acid	Sigma Chemicals Co., USA.
Trichloroacetic acid	Sigma Chemicals Co., USA.
Nitric acid	Sigma Chemicals Co., USA.
Percloric acid	Fishar Scientific Company.
Sulphuric acid	Fishar Scientific Company.
Metformin	Product of Hovid compny .
Vitamin A Standard	Lab.Tech Chemicals; India.
Ascorbic acid Standard	Lab.Tech Chemicals; India.
Vitamin E Standard	Lab.Tech Chemicals; India.
α-α Dipyridyl Reagent	BDH Chemicals Ltd, England
cyanide ferricyanide reagent	Quail chemicals Ltd.

Appendix VI:

Table 2: List of Equipments /Materials and Glass wares

Equipments / Instruments	Model no.	Manufacturer/ Company
Spectrophotometer	SP 300	Opima, Germany
Centrifuge	800D	Shangai Med. Instr. Ltd, chaina
Refigerator	C1202	Thermocool Ltd.
Weighing balance	PC 440	Metter, Deltarange Ltd.
Water bath	GD 100	Grant Scientific Tech. Germny.
Glass wares	pyrex	USA
Glucometer	Fine touch	USA
Vortex mixer	SA 1	Great Britain
Water Distillator	B114	England
Micropipette	Diapette	TECO Diagnostic, USA