

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES



**Evaluation of Antidiabetic, Antihyperlipidemic and Antiglycation Effect
of *Moringa stenopetala* (Baker f) *Cufodontis* leaves**

By: Alemayehu Toma /B.Pharm, MSc/

A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements of Doctor of Philosophy Degree (Ph.D) in Pharmacology

March, 2016

Addis Ababa, Ethiopia

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Abbreviations

AAC- aminoglycoside acetyltransferases

ADP -Adenosine Diphosphate

ALP- Alkaline phosphatase

ALT -Alanine aminotransferase

ANT - aminoglycoside nucleotidyltransferases

APH - aminoglycoside phosphotransferases

AST- Aspartate aminotransferase

ATP -Adenosine Triphosphate

BSA -Bovine Serum Albumin

BW- Body Weight

cAMP -cyclic Adenosine Monophosphate

DM- Diabetes mellitus

DPP-4 -Dipeptidyl peptidase-4

DPPI-IV -Dipeptidyl peptidase Inhibitor- IV

FFAs- Free Fatty Acids

GIP- Gastric Inhibiting Polypeptides

GLP-1 -Glucagon –like Peptide -1

GLP-2- Glucagon –like Peptide -2

GLUT 2- Glucose transporter 2

GLUT4 -Glucose transporter-4

Grb2 -Growth factor receptor-bound protein 2

HDL -High Density Lipoprotein

IR -Insulin Receptor

IRS -Insulin receptor substrate

IRS-1 -Insulin Receptor Substrate -1

KATP- Adenosine Triphosphate Dependent Potassium Channel

LDH - Lactate Dehydrogenase

LDL-C -Low Density Lipoprotein cholesterol

MAPK -Mitogen-activated protein kinase

Ms -Moringa stenopetala

NF-kB- Necrosis Factor

PC -Protein Kinase

PDK-1 3-phosphoinositide-dependent protein kinase-1

PI3 -Phosphatidylinositol 3

PKC -Protein kinase C

PPAR- Peroxisome Proliferator-Activated Receptors

STZ -Streptozotocin

SUR1- Sulfonylurea receptor -1

T1DM -Type one diabetes Mellitus

T2DM -Type two Diabetes Mellitus

TNF- α -Tumor Necrosis Factor- α

TZD -Thiazolidinediones

VIP -Vasoactive Inhibitory Polypeptides

WHO -World Health Organization

Abstract

Background: *Moringa stenopetala* has been used in traditional health systems to treat diabetes mellitus. One of the successful methods to prevent onset of diabetes is to control postprandial hyperglycemia by inhibition of α -glucosidase and pancreatic α -amylase activities, resulting in the aggressive delay of the carbohydrate digestion of absorbable monosaccharides. The aim of the present study was to investigate the effect of the extract of the leaves of *Moringa stenopetala* on glycation control, α -glucosidase, pancreatic α -amylase, pancreatic lipase, and pancreatic cholesterol esterase activities, and, therefore find out the relevance of the plant in controlling blood sugar and lipid levels.

Methods: The dried leaves of *Moringa stenopetala* were extracted with hydroalcoholic solvent and the resulting extract was dried using rotary vapor under reduced pressure. The dried extracts were determined for the total phenolic compounds, flavonoid content and condensed tannins content using Folin-Ciocateu's reagent, $AlCl_3$ and vanillin assay, respectively. The dried extract of plant-based food was further quantified with respect to intestinal α -glucosidase (maltase and sucrase) inhibition and pancreatic α -amylase inhibition by glucose oxidase method and dinitrosalicylic (DNS) reagent, respectively. Aqueous ethanol and n-butanol fraction of *Moringa stenopetala* leaves (500mg/kg body weight) and metformin (150 mg/kg body weight) were administered to diabetic rats. Blood glucose, lipid profiles, liver and kidney function were examined after 14 days of experiment. The antioxidant activity was determined using 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) assay. Antiglycation activity was determined using inhibition of formation of advanced glycation end products (AGE), level of N^ε-(carboxymethyl) lysine (CML), the level of fructosamine, and the formation of amyloid cross β -structure in bovine serum albumin after incubation with fructose. The protein oxidation was examined using the level of protein carbonyl content and thiol group.

Results The present phytochemical analysis indicated that the flavonoid, total phenol, and condensed tannin contents in the extract were 71.73 \pm 2.48 mg quercetin equivalent/g of crude extract, 79.81 \pm 2.85 mg of gallic acid equivalent/ g of crude extract, 8.82 \pm 0.77 mg catechin equivalent/g of crude extract, respectively. The extract inhibited intestinal sucrase more than intestinal maltase with IC₅₀ value of 1.47 \pm 0.19 mg/ml. It also slightly

inhibited pancreatic α -amylase, pancreatic lipase and pancreatic cholesterol esterase. Oral administration of aqueous ethanol and n-butanol extract of *Moringa stenopetala* leaves (500 mg/kg body weight) and metformin (150mg/Kg) significantly reduced blood glucose level ($P<0.05$), significantly improved serum lipid profiles, liver enzymes and kidney functions in diabetic rats after 14 days. The extract also increased in size of islet of Langerhans in diabetic rats. *Moringa stenopetala* leaves significantly inhibited the formation of AGEs by approximately $54.75\pm 0.94\%$ at a concentration of 2mg/ml. Furthermore, *Moringa stenopetala* leaves extract reduced the levels of fructosamine, amyloid cross β -structure and N^ε-(carboxymethyl) lysine (CML) . The leaves also prevented oxidative protein damage, including effects on protein carbonyl formation, thiol oxidation of BSA and antioxidant activity in DPPH assay

Conclusion: The present results demonstrated the beneficial biochemical effects of *Moringa stenopetala* leaves extract by inhibiting intestinal α -glucosidase, pancreatic cholesterol esterase and pancreatic lipase activities. A daily supplement intake of the leaves of *Moringa stenopetala* may help in reducing diabetes induced glycation, hyperglycemia and hyperlipidemia.

Keywords: *Antihyperglycemic, Antihyperlipidemic, antiglycation, Moringa stenopetala, enzyme inhibition*

1. INTRODUCTION

1.1. Diabetes mellitus

Diabetes mellitus (DM) is a chronic systemic metabolic disease characterized by hyperglycemia, hyperlipidemia, and hyperaminoacidemia, and associated with reduced insulin secretion, insulin action, or both (Atlan, 2003). The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues (ADA, 2014). Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action. The chronic hyperglycemia in diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Failure of these organs is frequently associated with development of micro and macrovascular diseases which include neuropathy, nephropathy and cardiovascular as well as cerebrovascular diseases (Feldman, 1988 and ADA, 2014). The disease is associated with reduced quality of life and increased risk factors for mortality and morbidity. The long-term hyperglycemia is an important factor in the development and progression of micro and macrovascular complications (Strojek, 2003).

The word “diabetes” stems from a Greek term for passing through, a reference to increased urination (polyuria), a common symptom of the disease. “Mellitus” is the Latin word for honeyed, a reference to glucose noted in the urine of diabetic patients. Diabetes mellitus is sometimes referred to as sugar diabetes but usually is simply called diabetes. There is also a rare disease called diabetes insipidus (water diabetes) in which the kidneys release too much water. Like diabetes mellitus, it has excessive urination as a symptom, but these two endocrine disorders are otherwise unrelated (Samreen R. 2009).

Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycemia. DM may present with characteristic symptoms such as polydipsia, polyuria, and blurred vision, excessive fatigue, sometimes with polyphagia, body irritation and weight loss. In its most severe forms, ketoacidosis or a non-ketotic hyperosmolar state may develop and lead to stupor, coma and, in absence of effective treatment, death. Often symptoms are not severe, or may be absent, and consequently hyperglycaemia sufficient to cause pathological and functional changes may be present for a long time before the diagnosis is made (WHO, 1999, ADA, 2014 and WHO, 2014). Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia.

DM is often associated with increased risk of pansystemic complications which include ischaemic heart disease, nephropathy, retinopathy, neuropathy, ulceration and gangrene of extremities. Long-term complications of diabetes include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputations, and Charcot joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction (Baynes, 2015). Patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral arterial and cerebrovascular disease. DM could occur alone but more often co-exists with other systemic diseases such as hypertension, dyslipidaemia, ischaemic heart disease and renal diseases. As a result, DM and its associated complications have significant impact on health, quality of life and life expectancy of its sufferers (IJARNP, 2009).

1.1.1 Pathophysiology of diabetes mellitus

Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the pancreatic beta-cells with consequent insulin deficiency to abnormalities that result in resistance to insulin action. Thus, the Pathophysiology of diabetes mellitus is related to the hormone insulin, which is secreted by the beta cells of the pancreas (Ozougwe *et al*, 2013). This hormone is responsible for maintaining glucose level in the blood. It allows the body cells to use glucose as a main energy source. However, in a diabetic person, due to abnormal insulin metabolism, the body cells and tissues do not make use of glucose from the blood, resulting in an elevated level of blood glucose or hyperglycemia. Over a period of time, high glucose level in the bloodstream can lead to severe complications, such as eye disorders, cardiovascular diseases, kidney damage and nerve problems (WHO, 1999; AACEDM, 2007).

Diabetes mellitus is a complex systemic disorder that is characterized by chronic hyperglycemia and disturbances of fat and protein metabolism associated with malfunction in insulin secretion and/or insulin action. The carbohydrate metabolism and utilization leads to accelerated lipolysis and results in hyperlipidemia (Kim *et al.*, 2006).

Type1 DM accounts for up to 5-10% of all diabetes cases. It generally develops in childhood or early adulthood and results from immune-mediated destruction of pancreatic β -cells, resulting in an absolute deficiency of insulin (ADA, 2014). There is a long preclinical period (up to 9 to 13 years) marked by the presence of immune markers when β -cell destruction is thought to occur. Markers of the immune destruction of the β -cell include islet cell autoantibodies, autoantibodies to insulin, autoantibodies to GAD (GAD65), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2 β . Hyperglycemia occurs when 80% to 90% of β - cells are destroyed. There is a transient remission followed by established disease with associated risks for complications and death. Factors that initiate the autoimmune process are unknown, but the process is mediated by macrophages and T lymphocytes with circulating autoantibodies to various β -cell antigens (e.g., islet cell antibody, insulin antibodies) (Expert committee, 2003). Autoimmune destruction of β -cells has multiple genetic predispositions and is also

related to environmental factors that are still poorly defined (Figure 1) (Ozougwe *et al*, 2013).

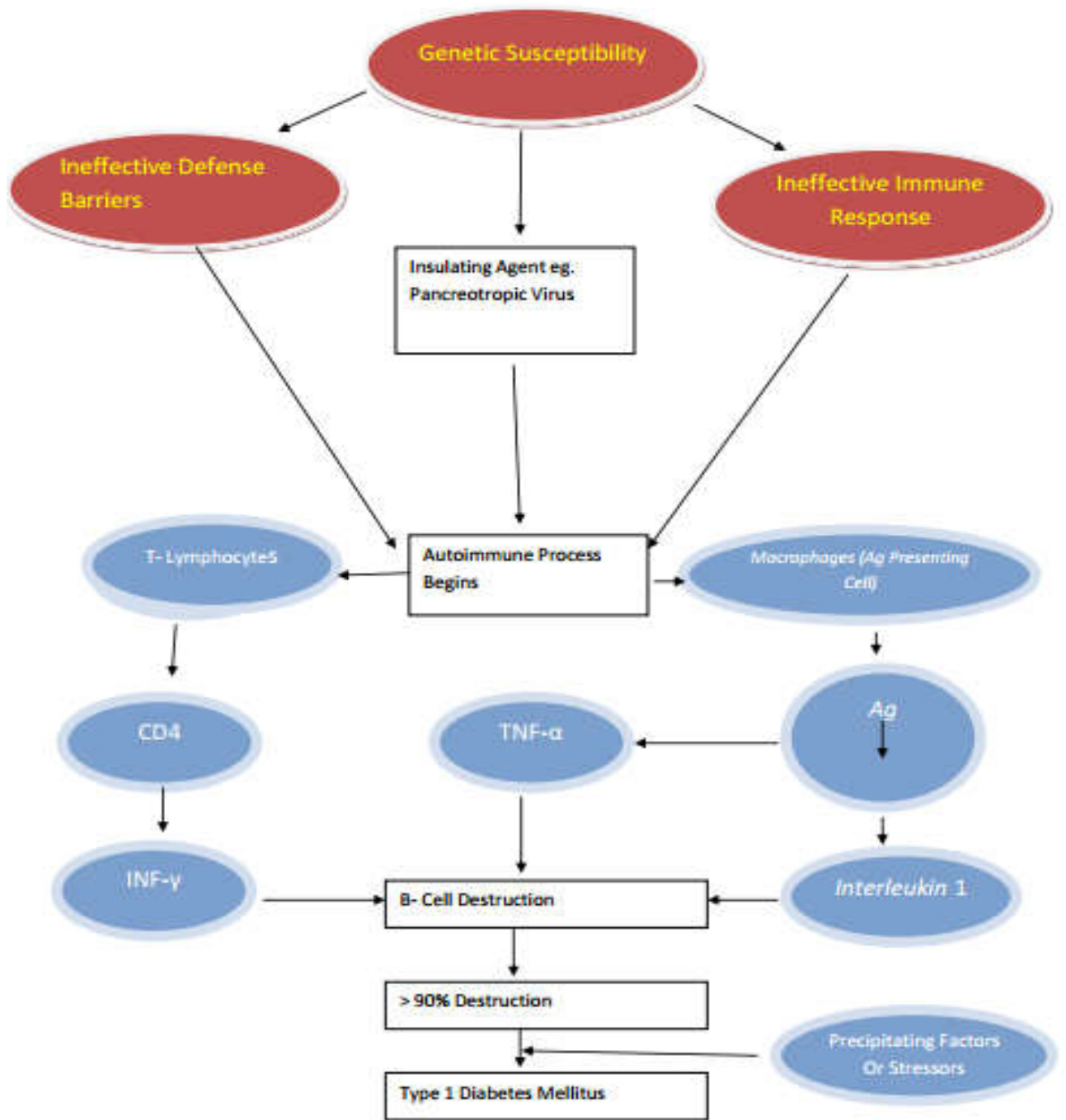


Figure 1: Pathogenesis of type 1 diabetes mellitus

Type 2 DM accounts for as many as 90% of DM cases and is usually characterized by the presence of both insulin resistance and relative insulin deficiency. Insulin resistance is manifested by increased lipolysis, free fatty acid production, increased hepatic glucose production, and decreased skeletal muscle uptake of glucose. β -Cell dysfunction is progressive and contributes to worsening blood glucose control over time. Type 2 DM occurs when a diabetogenic lifestyle (excessive calories, inadequate exercise, and obesity) is superimposed upon a susceptible genotype (Expert committee, 2003).

Patients with this form of diabetes may have insulin levels that appear normal or elevated; the higher blood glucose levels in these diabetic patients would be expected to result in even higher insulin values had their beta-cell function been normal. Thus, insulin secretion is defective in these patients and insufficient to compensate for insulin resistance. Insulin resistance may improve with weight reduction and/or pharmacological treatment of hyperglycemia but is seldom restored to normal. The risk of developing this form of diabetes increases with age, obesity, and lack of physical activity. It occurs more frequently in women with prior Gestational Diabetes Mellitus (GDM) and in individuals with hypertension or dyslipidemia, and its frequency varies in different racial/ethnic subgroups (Figure 2). It is often associated with a strong genetic predisposition, more so than is the autoimmune form of type 1 diabetes. However, the genetics of this form of diabetes are complex and not fully defined.

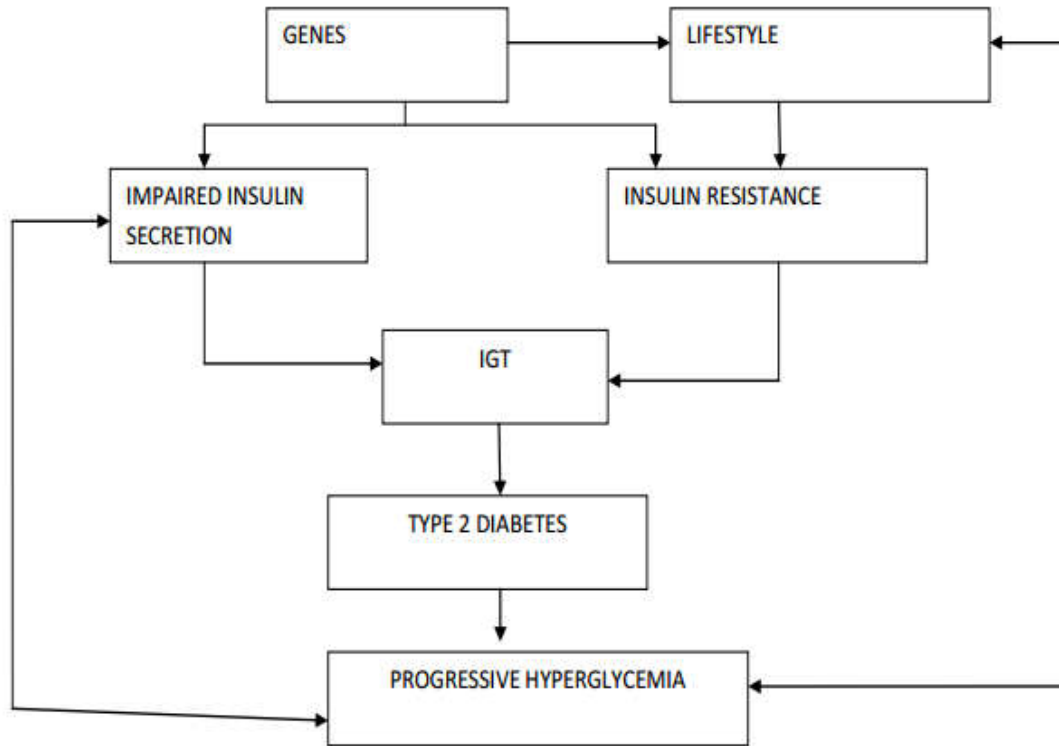


Figure 2: Pathogenesis of type 2 diabetes characterized by impaired insulin secretion and insulin resistance

Uncommon causes of diabetes (1% to 2% of cases) include endocrine disorders (e.g., acromegaly, Cushing's syndrome), gestational diabetes mellitus (GDM), diseases of the exocrine pancreas (e.g., pancreatitis), and medications (e.g., glucocorticoids, pentamidine, niacin, and β -interferon) (Expert committee, 2003). The pancreas plays a primary role in the metabolism of glucose by secreting the hormones insulin and glucagon (Figure 3). The islets of Langerhans secrete insulin and glucagon directly into the blood. Insulin is a peptide hormone that is essential for proper regulation of glucose and for maintenance of proper blood glucose levels (Worthley, 2003).

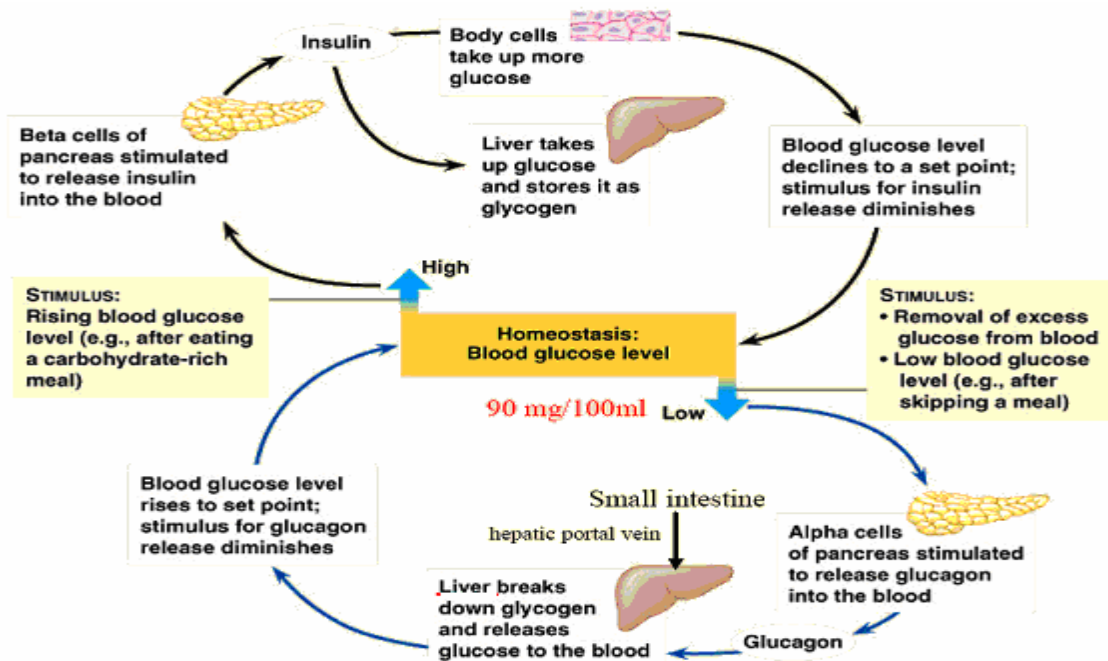


Figure 3: Role of pancreas in maintenance of normal blood glucose level

Glucagon is a hormone that opposes the action of insulin. It is secreted when blood glucose level falls. It increases blood glucose concentration partly by breaking down stored glycogen in the liver by a pathway known as glycogenolysis (Sowka *et al.*, 2001).

1.1.2 Diabetic complications

1.1.2.1 Glycations

Chronic hyperglycaemia causes the Maillard reaction in which reducing sugars, such as glucose and fructose, react non-enzymatically with amino groups of proteins through a series of reactions, ultimately forming advanced glycation end products (AGE), triggering several non-communicable diseases (Cohen *et al.*, 2007). It has been clearly demonstrated that the accumulation of AGEs in body tissue is the leading cause of several age-related degeneration, atherosclerosis and diabetic complications such as retinopathy, nephropathy and neuropathy (Khazaei *et al.*, 2010; Schmidt and Stern, 2000). Glycated albumin comprises about 6–15 % of the total albumin in normal individuals and rises to 32 – 40 % in hyperglycaemia. These modifications affect the properties of albumin in several ways, including altered conformation and consequently altered

binding. Diabetes mellitus, liver diseases and nephropathy are just a few disorders in which altered albumin functions have been described (Schmidt and Stern, 2000; Singh et al, 2001).

Advanced glycation end products (AGEs) are a heterogeneous, complex group of compounds that are formed mainly via the Maillard reaction. The Maillard reaction occurs when reducing sugar reacts in a non-enzymatic way with amino acids in proteins, lipids or DNA. This reaction has been studied for years in the food industry because its products add a desirable color and taste to foods. However, the study of the products of this reaction in vivo have received increasing attention in recent years due to association of AGEs with certain chronic diseases, such as diabetes mellitus, cardiovascular diseases, and Alzheimer's disease, as well as during the aging process (Ahmed, 2005).

The formation of AGEs through the Maillard reaction occurs in three phases (Figure 5). First, glucose attaches to a free amino acid (mainly lysine and arginine) of a protein, lipid or DNA, in a non-enzymatic way to form a Schiff base. A Schiff base is a compound that has a carbon to nitrogen double bond where the nitrogen is not connected to hydrogen. The initiation of this first step depends on glucose concentration and takes place within hours. If the concentration of glucose decreases, this reaction is reversible. During the second phase, the Schiff base undergoes chemical rearrangement over a period of days and form Amadori products (also known as early glycation products). The Amadori products are more stable compounds (hemoglobin A1c is the most well known), but the reaction is still reversible. If there is accumulation of Amadori products, they will undergo complicated chemical rearrangements (oxidations, reductions, and hydrations) and form crosslinked proteins. This process takes place in weeks or months and it is irreversible. The final brownish products are called AGEs and some of them have fluorescent properties. They are very stable, and accumulate inside and outside the cells and interfere with protein function (Uribarri and Tettle, 2006). Besides the Maillard reaction, other pathways can also form AGEs. For instance, the autoxidation of glucose and the peroxidation of lipids into dicarbonyls derivatives by an increase in

oxidative stress is another pathway described for the formation of AGEs (Sheetz and King, 2002). These dicarbonyl derivatives known as α -oxaldehydes (glyoxal, methylglyoxal (MG), and 3-deoxyglucosone) can interact with monoacids and form AGEs. The other well-studied mechanism for the formation of AGEs is the polyol pathway, where glucose is converted to sorbitol by the enzyme aldose reductase and then to fructose by the action of sorbitol dehydrogenase. Fructose metabolites (as fructose 3-phosphate) then are converted into α -oxaldehydes and interact with monoacids to form AGEs (Tessier, 2010). Thus, at least three pathways may form AGEs: The Maillard reaction; oxidation of glucose; and peroxidation of lipids and through the polyol pathway. Given these differing pathways, it is not surprising that AGEs are diverse in their chemical structure. Among the most widely studied AGEs are carboxymethyl-lysine (CML), pentosidine, and pyrraline, and, together with methylglyoxal (an α -oxaldehyde), they have been used as biomarkers for in vivo formation of AGEs (Figure 5) (Claudial and Karen, 2010). CML (not fluorescent, not cross-linked AGEs) has been consistently used also as a biomarker for long-term protein damage and can be formed by the Maillard reaction and by α -oxaldehydes. As well as CML, pentosidine (a fluorescent protein crosslink) is formed by the Maillard reaction and by the α -dicarbonyl glyoxal, while pyrraline (not fluorescent, not cross-linked AGEs) is formed by the Maillard reaction (Tessier, 2010 and Peppia et.al, 2009).

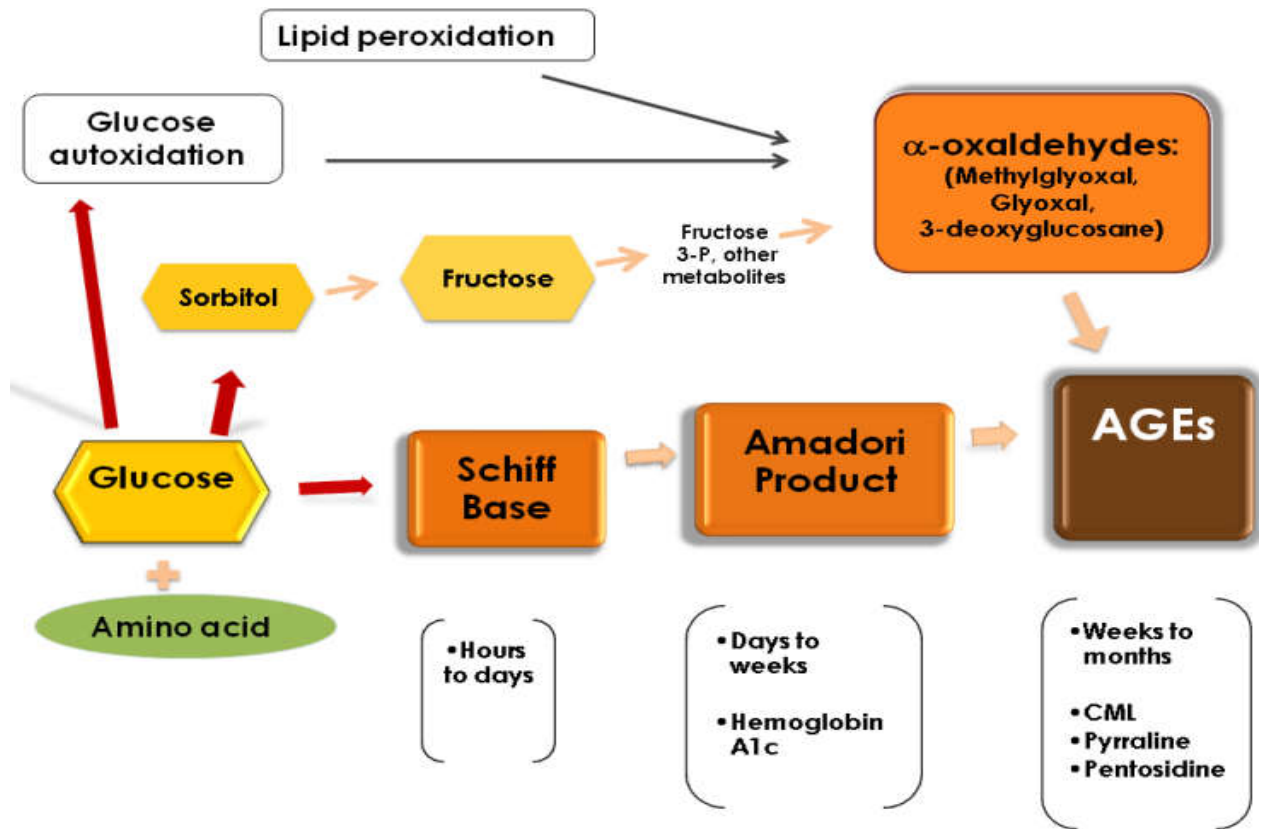


Figure 4: Formation of advanced glycation end products.

The deleterious effects of AGEs in different tissues are attributed to their chemical, pro-oxidant, and inflammatory actions. The biological effects of AGEs are exerted by two different mechanisms: One independent of the receptor (damage of protein structure and extracellular matrix metabolism); or one involving the receptor for advanced glycation end products (RAGE) (Figure 6). The interaction of AGEs with the receptor RAGE triggers the activation of the mitogen-activated protein kinases (MAPKs) and the phosphatidylinositol-3 kinase (PI3-K) pathways that will lead to the activation of the transcription factor NF- κ B (nuclear factor kappa B). After activation, NF- κ B translocates to the nucleus where it will activate the transcription of genes for cytokines, growth factors and adhesive molecules, such as tumor necrosis factor α (TNF α), interleukin 6 (Il-6), well known inflammation promoters, and vascular cell adhesion molecule 1 (VCAM1). NF- κ B activation increases RAGE expression, creating a positive feedback cycle that enhances the production of inflammation

promoters. In addition, AGE-RAGE interaction activates NAD(P)H oxidase (a complex of enzymes which produces superoxide) and when this complex is up-regulated, it increases intracellular oxidative stress (Figure 6). The sudden increase in oxidative stress by NAD(P)H oxidase in response to AGE-RAGE interaction will also activate NF- κ B (Claudial and Karen, 2010).

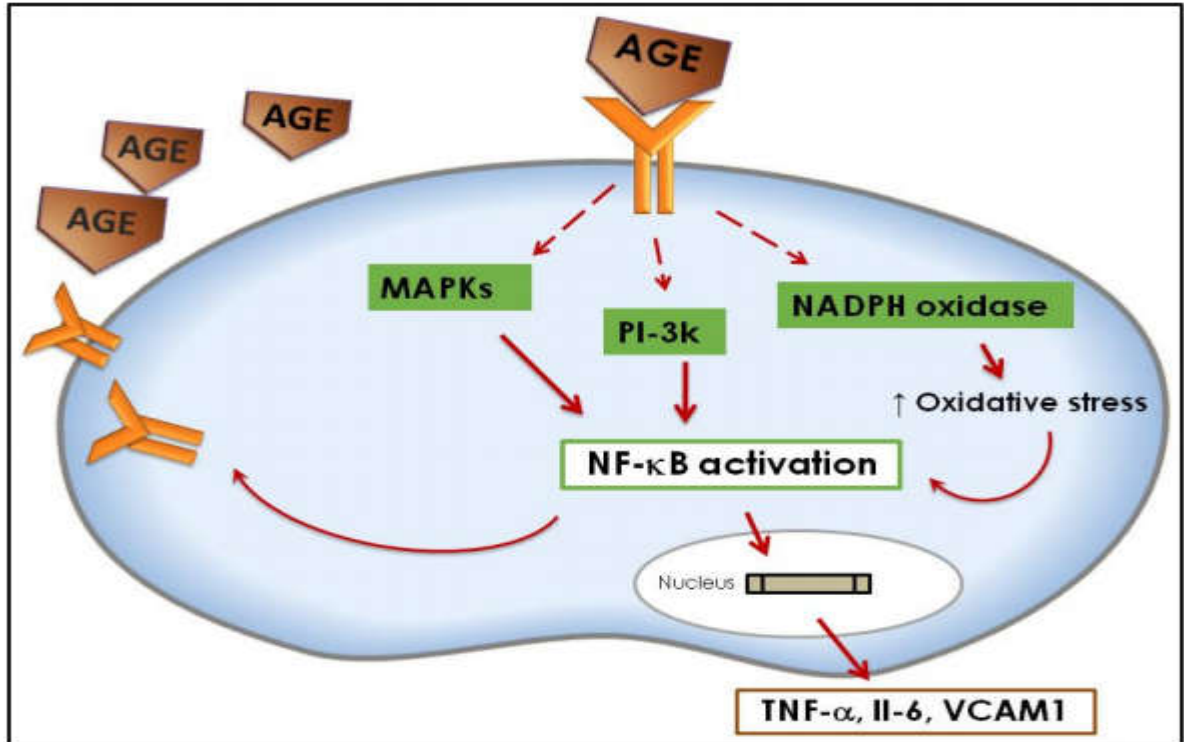


Figure 5: Cellular mechanism of advanced glycation end products effects in the body.

Hemoglobin A1c is the most widely recognized early glycation product, and is also used as an indicator of blood glucose management in those with diabetes. Hyperglycemia increases the glycation process, and is especially apparent in insulin independent tissues such as red blood cells, peripheral nerve tissue cells, endothelial cells, eye lens cells, and kidney cells. It is also hypothesized that glycation of proteolytic enzymes in diabetes reduces their efficiency, resulting in more build up of glycated end products. Not surprisingly, AGEs have also been

implicated in delayed wound healing associate with diabetes, presumably through vascular, neurological, or intermediary metabolic modifications (*Tessier, 2010, and Claudial and Karen, 2010*).

1.1.2.2 Dyslipidemia

Diabetes mellitus is known to cause hyperlipidemia through various metabolic derangements, which is found in about 40% of diabetic patients (Karimulla and Kumar, 2011). Hypercholesterolemia and hypertriglyceridemia are common complications of diabetes mellitus in addition to hyperglycemia. The frequency of hyperlipidemia in diabetes is indeed very high, depending on the type of diabetes and its degree of Control (Shah A., et al, 2007).

The dyslipidemia associated with insulin resistance (also referred to as Atherogenic dyslipidemia) is characterized by moderately increased triglyceride (TG) levels carried in very low density lipoprotein (VLDL) particles, low high density lipoprotein cholesterol (HDL-C) levels carried in small HDL particles, and LDL-C levels that do not differ substantially from those of individuals without type II diabetes (Figure3). In addition, TG-rich lipoproteins (after eating), remnant lipoproteins, apolipoprotein B 100 (ApoB) has also been shown to e increased in patients with type II diabetes. In addition, LDL-C particles are small and dense, carrying less cholesterol per particle; therefore, at any given LDL-C concentration, there are more LDL particles present in an individual with type II diabetes relative to an individual without the disease, which may make the LDL-C level a misleading measure of risk in patients with type II diabetes (Vijayaraghavan, 2010).

1.1.3 Types of diabetes mellitus

There are four types of DM based on their Pathophysiology (Expert Committee, 2003). Type 1 diabetes is DM where there is selective B-cell destruction resulting in *severe* or *absolute* insulin deficiency. Administration of insulin is essential in patients with type 1 diabetes. Type 1 diabetes is further subdivided into immune caused and idiopathic caused DM. The immune form is the most common form of type 1 diabetes. Although most patients are younger than 30 years of age at the time of diagnosis, the onset can occur at any age. Susceptibility appears to involve a multifactorial genetic linkage but only 10–15% of patients have a positive family history (WHO, 1999; AACEDM, 2007).

Type 1 diabetes is characterized by ketoacidosis which is caused by reduced insulin levels, decreased glucose use, and increased gluconeogenesis from elevated counter regulatory hormones, including catecholamines, glucagon, and cortisol. Diabetic ketoacidosis may also occur in patients with type 2 diabetes. Patients with diabetic ketoacidosis usually present with polyuria, polydypsia, polyphagia, and weakness (Trachtenbarg, 2005).

Type 2 diabetes is characterized by tissue resistance to the action of insulin combined with a *relative* deficiency in insulin secretion. A given individual may have more resistance or more B-cell deficiency, and the abnormalities may be mild or severe. Although insulin is produced by the B cells in these patients, it is inadequate to overcome the resistance, and the blood glucose rises. The impaired insulin action also affects fat metabolism, resulting in increased free fatty acid flux and triglyceride levels and reciprocally low levels of high-density lipoprotein (HDL). Individuals with type2 diabetes may not require insulin to survive, but 30% or more will benefit from insulin therapy to control the blood glucose. It is likely that 10–20% of individuals in whom type2 diabetes was initially diagnosed actually has both type 1 and type 2 or a slowly progressing type 1, and ultimately will require full insulin replacement. Although persons with type 2 diabetes ordinarily do not develop ketosis, ketoacidosis may occur as the

result of stress such as infection or use of medication that enhances resistance, eg, corticosteroids. Dehydration in untreated and poorly controlled individuals with type 2 diabetes can lead to a life-threatening condition called *nonketotic hyperosmolar coma*. In this condition, the blood glucose may rise to 6–20 times the normal range and an altered mental state develops or the person loses consciousness. Urgent medical care and rehydration is required (WHO, 1999; AACEDM, 2007).

The type 3 designation refers to multiple *other* specific causes of elevated blood glucose: non pancreatic diseases, drug therapy (Expert Committee, 2003). Other causes include genetic defects of the pancreatic β cell or in insulin action pathways (insulin receptor mutations or post-receptor defects) as well as disease of the exocrine pancreas (e.g., Pancreatitis, pancreatic reaction, or cystic fibrosis) are less common causes of DM. Endocrinopathies producing insulin counter regulatory hormones excess (e.g., Cushing's syndrome, acromegaly) may result in DM. Certain drugs like glucocorticoids, pentamidine, niacin, and α -interferon may also lead to DM (Salim B., 2005).

Gestational diabetes mellitus (GDM) is defined as any abnormality in glucose levels noted for the first time during pregnancy. Gestational diabetes is diagnosed in approximately 4% of all pregnancies in the USA (AACEDM, 2007). During pregnancy, the placenta and placental hormones create insulin resistance that is most pronounced in the last trimester. It is caused due to fluctuations of the hormonal level during pregnancy (Sugiyama, 2011). Usually, the blood glucose level returns to normal after the baby is born. Risk assessment for diabetes is suggested starting at the first prenatal visit. High-risk women should be screened immediately. Screening may be deferred in lower-risk women until the 24th to 28th week of gestation (WHO, 1999; AACEDM, 2007, Expert Committee, 2003).

1.1.4 Prevalence of diabetes mellitus

The number of people with diabetes and pre-diabetes is exponentially increasing mainly due to aging, urbanization, unhealthy eating habits, increasing prevalence of obesity and lack of physical activity (Wild et al, 2004). Diabetes mellitus is one of the leading causes of morbidity and mortality worldwide, with an estimated number of 382 million adults being affected and 5.1 million people killed in the year 2013 (IDF, 2013). The prevalence is expected to be 592 million in the year 2035, with the greatest increases expected in low- and middle-income developing countries of the African, Asian, and South American regions (WHO, 2014). Currently, 80% of the world's populations with diabetes live in low- and middle income countries (IDF, 2013 and WHO, 2015). Diabetes is also associated with a host of life threatening and potentially disabling macro- and micro-vascular complications (Levin and Pfeifer, 2009). Hence, there is a much larger burden in the form of loss of productivity as a result of restricted daily activity which results in high economic costs (Mathers and Loncar, 2006).

Diabetes is a common and very prevalent disease affecting the citizens of both developed and developing countries. The worldwide prevalence of diabetes for all age groups was estimated to be 2.8% in 2000, and it is projected to be 5.4% in 2025 (Upendra et, 2010). In 2005, an estimated 1.1 million people died from diabetes and almost 80% of diabetes deaths occurred in low and middle-income countries. WHO projects that diabetes death will increase by more than 50% in the next 10 years if no urgent action is taken. Most notably, diabetes deaths are projected to increase by over 80% in upper-middle income countries between 2006 and 2015 (WHO, 2008). According to WHO estimate, the number of diabetic cases in Ethiopia in the year 2000 were 800,000 and is expected to increase to 1.8 million by 2030 (Feleke and Enquessie, 2005).

Diabetes is a major threat to global public health that is rapidly getting worse and biggest impact in an adult of working age in developing countries (WHO, 2004). There are an

estimated 246 million people with diabetes in the world, of whom about 80% reside in developing countries (Sicree R., et al 2006). Although diabetes is often not recorded as the cause of death, globally, it is believed to be the fifth leading cause of death in 2000 after communicable diseases, cardiovascular disease, cancer and injuries (Roglic G., et al, 2005).

The number of cases of non -insulin dependent diabetes mellitus (Type-2) has increased dramatically due to the changes in lifestyle, increasing prevalence of obesity, and ageing of populations. The most disturbing trend is a shift in age of onset of diabetes to a younger age in recent years. This presents a serious challenge to the healthcare system because, at the peak of their working career, people with diabetes have an excess risk of mortality and morbidity compared with those without diabetes. Indeed, over two-thirds of deaths attributable to diabetes occur in developing countries (Roglic G., 2006).

1.1.5 Diagnosis of diabetes mellitus

Diagnostic Criteria for Diabetes Mellitus includes symptoms of diabetes (polyuria (increased urination), polydipsia (increased thirst), excessive fatigue, unexplained weight lose and body irritation) plus casual plasma glucose concentration $\geq 200\text{mg/dl}$, or fasting plasma glucose level $\geq 126\text{mg/dl}$,or 2-hour post challenge glucose concentration $\geq 200\text{mg/dl}$ during 75g oral glucose tolerance test (AACE DM, 2007). Based on these screening profiles of biochemical parameters the pharmacotherapeutic innervations should be addressed (Figure 4).

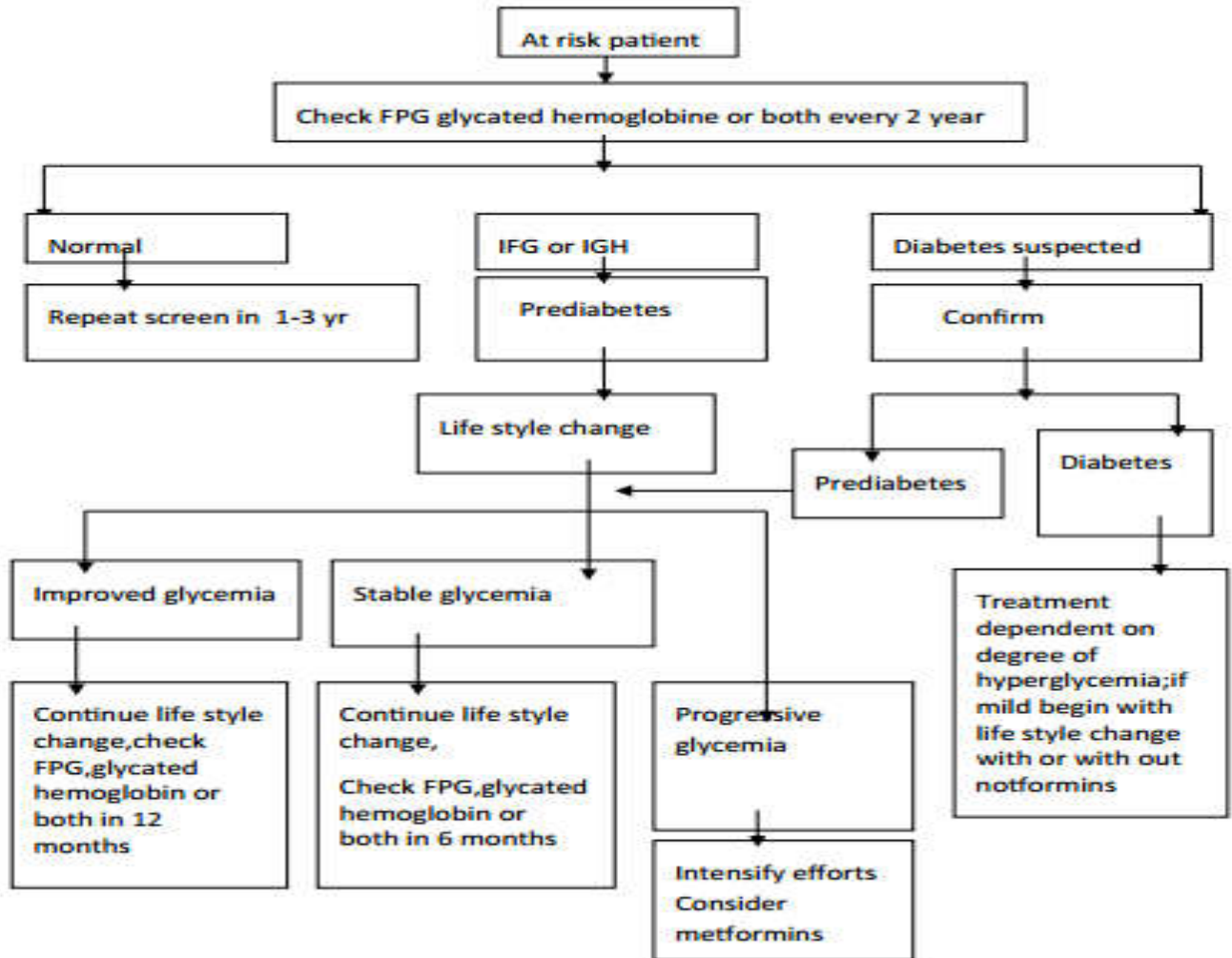


Figure 6: Suggested screening models for diabetes mellitus for high risk individuals and their interventions.

1.1.6 Risk factors of diabetes mellitus

The causes of diabetes are complex and only partly understood. This disease is generally considered multi-factorial, involving several predisposing conditions and risk factors. These factors can be categorized in to two; modifiable and non-modifiable factors. Among the modifiable risk factors, residence seems a major determinant, since urban residents have higher prevalence of diabetes compared to their rural counterparts. This is attributable to lifestyle changes associated with urbanization and westernization, diet, obesity and physical inactivity. Age, ethnicity, history of gestational diabetes and family

history of type II diabetes are the main non modifiable determinants of diabetes mellitus (Libman and Arslanian, 2007; Colagiuri et al., 2006).

1.1.7 Management of diabetes mellitus

Diet, exercise, modern drugs including insulin and oral administration of antihyperglycaemic drugs such as sulfonylureas and biguanides manage the pathogenesis of diabetes mellitus. Insulin plays a key role in glucose homeostasis along the side of a counter regulatory hormone, glucagon, which raises serum glucose level. Carrier proteins (GLUT 1- 5) are essential for glucose uptake into cells. In individuals with type II diabetes, a common sequence of therapy starts with diet treatment and exercise followed by oral antihyperglycemic agents.

Sulfonylureas (Glibenclimide, a prototype) and meglitinides produce hypoglycemia by stimulating insulin release from pancreatic β -cells. They bind to sulfonylurea (SUR) receptors on the β -cell plasma membrane, causing closure of adenosine triphosphate (ATP)-sensitive potassium channels, leading to depolarization of the cell membrane. This in turn opens voltage-gated channels, allowing influx of calcium ions and subsequent secretion of preformed insulin granules (Levine R, 1984 and Salim B., 2005).

In general, insulin therapy has been considered to be the last therapeutic option when diet, exercise and oral antihyperglycemic agent therapies have failed. Traditionally plants are also used for the treatment of diabetes throughout the world (Koski, 2006; Pari and Saravanan, 2004). Management of diabetes without any adverse effect is still a challenge. This leads to an increasing search for improved antidiabetic drugs. Currently available drugs for diabetes include insulin and various oral antidiabetic agents such as sulfonylureas, biguanides, α -glucosidase inhibitors and glinides, and these oral agents act through different mechanisms. In developing countries as products are expensive and not

easily accessible which makes wide spread use of complementary medicines (Upendra M, et al, 2010).

1.1.8 Plant medicines for treatment of diabetes mellitus

Presently, there is a growing interest in plant medicines in the treatment of diabetes mellitus due to the adverse effects associated with oral antidiabetic drugs. So plant medicines play important role in the management of diabetes mellitus (Patel, K et al, 1997). In recent years, plant medicines have started to gain importance as a source of antidiabetic agents. Marles and Farnsworth estimated that more than 1000 plant species are being used as folk medicine for diabetes (Marles R., et al, 1995). Biological actions of the plant products used as alternative medicines to treat diabetes are related to their chemical composition. Plant products are rich in phenolic compounds, flavonoids, terpenoids, coumarins, and other constituents which show reduction in blood glucose levels (Zhang Y, et al., 2009).

Several species of plant medicines have been described in the scientific and popular literature as having antidiabetic activity. Due to their perceived effectiveness, fewer adverse effects in clinical experience and relatively low costs, plant medicines are prescribed. Medicinal and herbal plant products are traditionally used from long ago in many countries for treatment of diabetes mellitus (Verspohl E., 2002).

Currently, there is growing evidence that plant polyphenols including flavonoids are unique nutraceuticals and supplementary treatments for various aspects of type 2 diabetes mellitus. Polyphenols can modulate carbohydrate and lipid metabolism, attenuate hyperglycemia, dyslipidemia, insulin resistance, alleviate oxidative stress and prevent the development of long-term diabetic complications associated with glycation (Bahadoran et al., 2013). Nowadays, there is a growing interest in the evaluation of antidiabetic and

antihyperglycemic activities of medicinal plants' phenolic constituents aiming at finding new sources for these compounds and to standardize their pharmacological activities.

Effective control of hyperglycaemia in diabetic patients is critical for reducing the risk of micro- and macrovascular complications associated with diabetes mellitus (Ismail *et al.*, 2010). Synthetic hypoglycemic agents that are capable of reducing blood sugar level possessed most worrying adverse effects such as weight gain and hypoglycemia, which have impeded their usefulness as antidiabetic agents (Gandhi and Sasikumar, 2012). Therefore, finding other anti-diabetic agents especially those made from natural sources is desired (Vishwakarma *et al.*, 2010). Plants have always been an exemplary source of drugs, to treat diabetes mellitus which represent a valuable alternative for the management of this disease. Amongst such plants reported to have beneficial effects in the treatment of diabetes, *Moringa stenopetala* is one which is traditionally employed.

In the last two decades, traditional systems of medicine and medicinal plant research have become topics of global interest and importance. In many developing countries, large number of peoples still rely heavily on traditional healers and medicinal plants to meet their daily primary health-care needs (Saganuwan, 2009). Complementary and alternative medicine involves the use of herbs and other dietary supplements as alternatives to mainstream western medical treatment. A recent study has estimated that up to 30% of patients with DM use complementary and alternative medicine (Ryan EA, et al. 2001).

According to the World Health Organization, more than 70% of the world's population must use traditional medicine to satisfy their principal health needs. A great number of medicinal plants used in the control of diabetes mellitus have been reported. There are various medicinal plants in the world, which are the potential sources of drugs. The discovery of the widely used antidiabetic drugs, metformin (N, N dimethylguanylguanidine) came from the traditional approach through the use of *Galega officinalis* (Khan1 M., 2010).

It is believed that, plant medicines have little adverse effects and are affordable. Now a days, researchers are looking for natural products through research all over the world and

a large number of evidence have shown the immense potential of medicinal plants used traditionally (Khan1 M., 2010).

In the last few years there has been an exponential growth in the field of plant medicine and these drugs are gaining popularity both in developing and developed countries because of their natural origin and less adverse effects (Modak, M., et al., 2007). Plant medicines continue to play an important role in diabetic therapy, particularly in developing countries where most people have limited resources and do not have access to modern treatment (Ali H., et al, 2006). World health organization has also authenticated the use of plant remedies for treatment of diabetes mellitus.. The use of plant materials as antidiabetics could be attributed to the belief that herbs do provide some benefits over and above allopathic medicine and allow the users to feel that they have some control in their choice of medication (Anthony J, et al, 2010).

Many efforts have been made to identify new antidiabetic agents from different sources, especially medicinal plants because of their effectiveness, fewer adverse effects and relatively low cost. The mechanism(s) of action of most plant medicines have not yet been determined (Bahman N. et al, 2009).

The local communities residing in the biodiversity-rich areas of the southern region of Ethiopia have traditionally used and relied on plants for treating various ailments. In many cases, local knowledge of medicinal plants remains poorly documented in scientific literature. These plants have found a prime place in the indigenous system of medicine and are in focus for evaluation of their active ingredients. *Moringa stenopetala* is one of these medicinal plants which is widely used for antidiabetic purpose in the area

1.1.9 *Moringa stenopetala*

Moringa stenopetala, family Moringaceae, is a deciduous plant which is widely distributed throughout Southern Ethiopia. The family comprises different species, such as *Moringa oleifera*, *Moringa concanensis*, *Moringa petrygosperma* and *Moringa stenopetala*. Most investigations so far have focused on the species *Moringa oleifera* and its varieties. There are also some reports, though limited on the chemistry and biological activity of *Moringa stenopetala*. The seeds of *Moringa stenopetala* and *oleifera* species

contain coagulant principles used to treat raw water to make it potable and both display antimicrobial activity (Mekonnen *et al.*, 1999).

The fresh leaves of *Moringa stenopetala* are cooked and eaten as a vegetable in Southern Ethiopia especially in Gammu Goffa and Wollayta areas with their traditional *kurkufa* (a cereal dish made with maize and sorghum). On the other hand, the people of Konso use the tree not only for food but also as medicine, and they cultivate it in large areas around their villages, the leaves are widely used as traditional herbal remedy by the local people. The decoction of the leaves is used to treat malaria, hypertension, diabetes, asthma, stomach problems and to expel retained placenta, while a decoction of the root is used to treat malaria (Mekonnen and Gessesse, 1998).

The ethanol extract of the fresh root wood and the acetone extract of dried leaves of *Moringa stenopetala* demonstrated activity against *Trypanosoma brucei* (Mekonnen and Gessesse, 1998), the parasite responsible for trypanosomiasis. The crude aqueous extract of the leaves demonstrated hypoglycemic activity (Makonnen et al, 1997,). The crude aqueous extract and n-butanol as well as chloroform fractions of the leaves of *Moringa stenopetala* have been reported to have both hypoglycemic and antihyperglycemic effect (Mussa, et.al, 2008). Furthermore the leaves crude ethanol extract and its solvent fractions were reported to have both hypoglycemic and antihyperglycemic effect (Nardos et al, 2011).The column chromatographic butanol fraction also showed antihyperglycemic effect in alloxan induced diabetic mice (Silesh et al, 2014).

There are limited number of reports on the chemistry and biological activity of *Moringa stenopetala*. Previous study on the chemical composition of the leaves of *Moringa stenopetala* revealed the presence of rutin, 4-(4'-O-acetyl-L-rhamnosyloxy)-benzylisothiocyanate and 4-(4'-O-acetyl-L-rhamnosyloxy)-benzaldehyde (Mekonnen and Gebrayesus, 2000) and O-(rhamnopyranosyloxy) benzyl glucosinolate (Mekonnen and Drager, 2003).The leaves have also been reported to have carbohydrates, crude fibers, vitamins (Vitamin C, β -carotene) and minerals (Potassium, iron, Zinc, Phosphorous and Calcium) are present in significant concentrations (Abuye et al., 2003).

Although there is a general agreement about the diverse biological activity of the different parts of plants belonging to the family Moringaceae (Figure 7), there are conflicting reports as to their safety. Mekonnen and coworkers (2005) showed that ethanol extract of the leaves and seeds of *Moringa stenopetala* contain toxic substances, while the aqueous extract found to be safe in the In-vitro cytotoxic test. Furthermore it was shown that there was a significant association between familial tendency of goiter and *Moringa stenopetala* consumption in Gammo goffa area (Abuye C, et al. 1999).

In the present study, phytochemical analysis and evaluations of antihyperglycemic, antihyperlipidemic and antiglycation activities of *Moringa stenopetalla* leaves extracts were carried out *in vivo* and *in vitro* models.



Figure 7: Photo of *Moringa stenopetala* tree by Alemayehu Toma

1.2 Statement of the problem

Management of diabetes with less adverse effect is still a challenge. This leads to an increasing search for improved antidiabetic drugs. Currently available therapies for diabetes include insulin and various oral antidiabetic agents such as sulfonylureas, biguanides, α -glucosidase inhibitors and glinides. As products are expensive and not easily accessible in developing countries, complementary medicines is spreading (Upendra M, et al, 2010).

Many efforts have been made to identify new antidiabetic agents from different sources, especially medicinal plants because of their effectiveness, fewer adverse effects and relatively low cost (Bahman N. et al, 2009).

The local communities residing in the biodiversity-rich areas of the southern region of Ethiopia have traditionally used and relied on plants for treating various ailments. In many cases, local knowledge of medicinal plants remains poorly documented in scientific literature. These plants have found a prime place in the indigenous system of medicine and are in focus for evaluation for their claimed uses. *Moringa stenopetala* is one of these medicinal plants which is widely used for antidiabetic purpose whose antidiabetic activities have been confirmed by Makonnen and coworkers (1997), Musa and coworkers (2008), Nardos and coworkers (2011), Tessema and coworkers (2014) and Toma and coworkers (2012) in animal models. More work, however, is needed to establish the mechanism(s) of action for its antihyperglycemic and antihyperlipidemic as well as antiglycation activities.

1.3 Significance of the study

According to the World Health Organization, more than 70% of the world's population use traditional medicine to satisfy their principal health needs. A great number of medicinal plants used in the control of diabetes mellitus have been reported. There are various medicinal plants in the world, which are the potential sources of drugs. The discovery of the widely used antidiabetic drugs, metformin (N, N dimethylguanylguanidine) came from the traditional approach through the use of *Galega officinalis* (Khan1 M., 2010). Therefore, this study could pave the way towards development of new drug from *Moringa stenopetala*.

Previous studies on the leaves of *Moringa stenopetala* demonstrated antihyperglycemic as well as hypoglycemic activities. The present study shall come up with the most possible mechanism(s) of action of the *Moringa stenopetala* leaves for its antihyperglycemic and antihyperlipidemic activities.

2 Objectives

2.1 General Objective

To evaluate the role of *Moringa stenopetala* leaves in diabetes mellitus and its associated complications in *in vitro* and *in vivo* models.

2.2 Specific objectives

- ❖ To carry out phytochemical analysis of *Moringa stenopetala* leaves for total phenolic compounds, flavonoid and condensed tannins.
- ❖ To determine effect of the plant material on intestinal alpha glucosidase and pancreatic alpha amylase activities
- ❖ To determine the effect of *Moringa stenopetala* leaves on pancreatic lipase and pancreatic cholesterol esterase activities
- ❖ To determine the effect of *Moringa stenopetala* on fasting blood glucose level
- ❖ To determine the effect of the plant material on lipid profile To determine pancreato-protective effect of the plant material in diabetic rats
- ❖ To assess advanced glycation end product inhibition activity of *Moringa stenopetala* leaves
- ❖ To determine the effect of *Moringa stenopetala* on the thiol group content after fructose incubation in bovine serum albumin
- ❖ To determine the effect of *Moringa stenopetala* leaves on fructosamine level in fructose bovine serum albumin system
- ❖ To determine the effect of *Moringa stenopetala* leaves on protein carbonyl content level in fructose bovine serum albumin system
- ❖ To determine the effect of *Moringa stenopetala* leaves on Congo red assay level in fructose bovine serum albumin system
- ❖ To determine the effect of *Moringa stenopetala* leaves on N^ε- (carboxymethyl) lysine level in fructose bovine serum albumin system
- ❖ To determine the antioxidant activity of *Moringa stenopetala* leaves extract in DPPH assay

3 Methods and Materials

3.1 Chemicals

Folin-Ciocalteu, quercetin, catechin, gallic acid, rat intestinal acetone powder, porcine pancreatic α -amylase, vanillin, 3,5-dinitrosalicylic acid, glucose oxidase kits, p-nitrophenylbutyrate (p-NPB), p-nitrophenylpalmitate (p-NPP), taurodeoxycholic acid, taurocholic acid, porcine cholesterol esterase, porcine pancreatic lipase were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Streptozotocin was purchased from (Sigma Aldrich, Germany), Metformin was purchased from (Sanofi winthrop industrie, France), SensoCard glucometer and strip was purchased from (77 Electronike Kft, Hungary). Congo red, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), nitroblue tetrazolium (NBT), 1-deoxy-1-morpholino-D-fructose (DMF), L-cysteine, and aminoguanidine (AG) were obtained from Sigma (St. Louis, MO, USA). 2, 4-dinitrophenylhydrazine (DNPH) was purchased from Ajax Finechem (Taren Point, Australia). Trichloroacetic acid (TCA) and guanidine hydrochloride were purchased from Merck (Darmstadt, F.R., Germany) and Fluka (Steinheim, Germany), respectively. OxiSelect™ N^c-(carboxymethyl) lysine (CML) ELISA kit was purchased from Cell Biolabs (San Diego, CA, USA).

3.2 Collections and preparation of plant materials

The leaves of *Moringa stenopetala* were collected from Gamo Gofa Zone, South Nation's Nationalities Peoples Region, 520 kilometer south of Addis Ababa. The plant was then identified and authenticated by a taxonomist, and deposited in herbarium of Ethiopian Public Health Institute (EPHI) with a voucher number AL-001. It was then dried under shade and crushed to powder for extraction.

3.3 Preparation of plant material extract

The powdered leaves (1.2 Kg) were extracted by percolation using 70 % (v/v) ethanol, and the mixture was then filtered using Whatmann filter paper no. 1. The extract was dried by evaporation using rotary vaporizers under reduced pressure at a temperature of 40-45°C. The residue filtrate obtained was then dried by steam bath at 40 °C and kept in

refrigerator at 8 °C for experimental usage. The yield of the extract was 20.1 % in weight in weight (w/w).

3.4 Solvent fractionation of the total ethanol extract

The procedure for solvent-solvent separation was adopted from Ranjan (2002) with minor modifications. Ten percent (w/v) of ethanol extract of the plant was prepared with mild hot distilled water. The dissolved aqueous ethanol extract was separated in a separatory funnel with 50 ml of n-hexane (3 times), and followed by 50 ml of dichloromethane extraction (3 times) and 50 ml of n-butanol extraction (3 times) successively until the extracting solvent became colorless. After completing the separation process, the solvents were removed by a rotary evaporator. The separated n-butanol separation were dried by steam bath at 40°C and kept in the refrigerator for the experiments. The percentage yield of n- butanol was 7.8 (w/w).

3.5 Animals

The Swiss albino rats weighing 180-250g were obtained from the animal department of Ethiopian Public Health Institute/EPHI/. They were kept under standard conditions (at a temperature of 22 ± 2 °C, and with 12 hr light/ 12 hr dark cycle) and provided with free access to standard pellet laboratory diet and water *ad libitum*. The experimental protocol was approved by the Institutional review board (IRB) of Addis Ababa University, School of Medicine with protocol number 001/13/pharma.

3.6 Determination of flavonoid content

Estimation of flavonoid content in the dried extracts was done according to method described by Adisakwattana et al (2011). The dried extract (0.5 mg) was dissolved in 80% ethanol (1 ml). The sample solution (50µl) was added to 10µl of AlCl₃ solution (10% w/v) and 10µl of 1 M sodium acetate in absolute ethanol (150µl). After incubation at 30°C for 30 min, the absorbance was measured immediately at 430 nm. The estimation of flavonoid content was calculated from a calibration curve using quercetin as a standard. The results were expressed as milligram quercetin equivalent/gram dry weight of extract based on the following standard curve (figure 8).

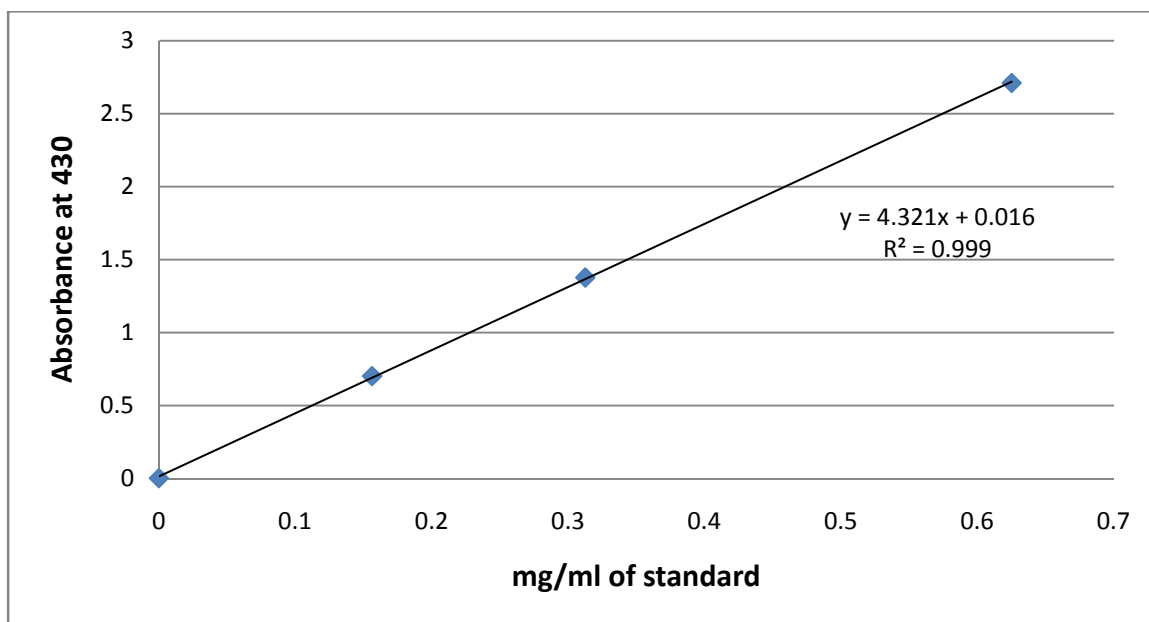


Figure 8: Standard curve for quarcetin

Flavonoid (mg/g) = $\frac{(\text{Abs test sample} - \text{Abs blank}) - \text{Intercept}}{\text{Slope} \times \text{amount of sample in gram}}$

Slope x amount of sample in gram

Where Abs test sample was the absorbance of extract with reagent, Abs blank was the absorbance of extract without reagent.

3.7 Determination of total phenolic content

Total phenolic content of the extract was performed according to the method described by Adisakwattana et al (2012). The dried extract (0.5 mg) was dissolved in distilled water (1 ml). The sample solution (50 μ l) was mixed with 50 μ l of Folin-Ciocateu's reagent followed by 50 μ l of Na₂CO₃ (10% w/v). After incubation at 30°C for 60 min, the absorbance was measured at 760 nm using a microplate reader. Total phenolic content was calculated from a calibration curve using gallic acid as a standard. The results were expressed as milligram gallic acid equivalent/gram dry weight of extract based on the following standard curve (figure 9).

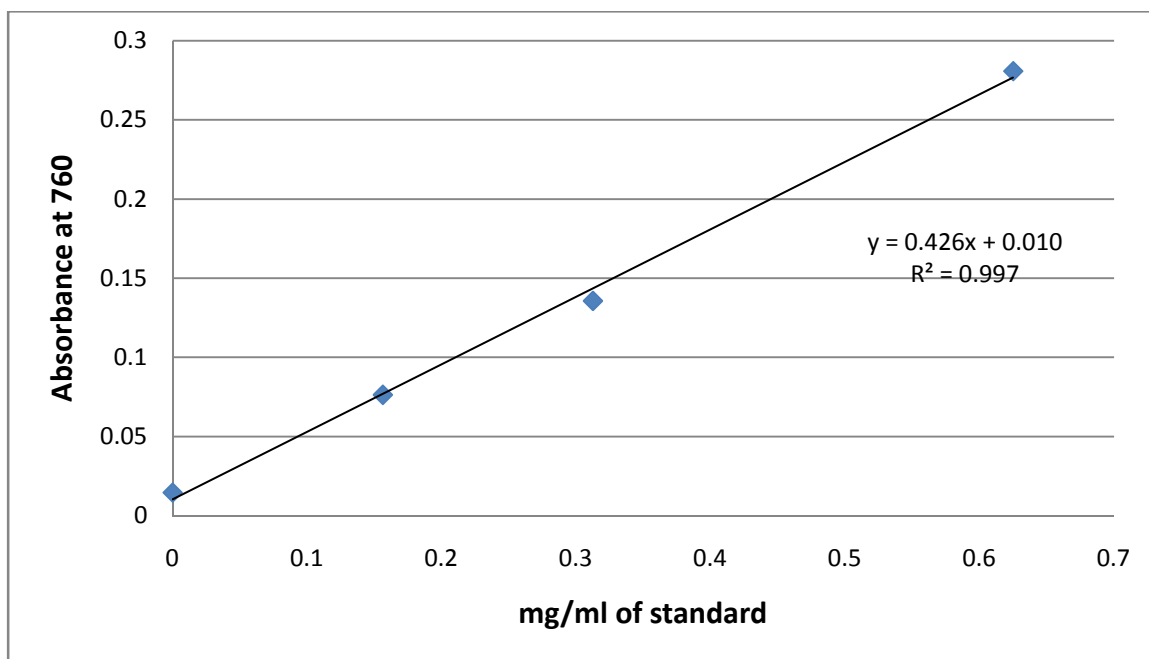


Figure 9: standard curve for gallic acid

Total phenolic compounds (mg/g) = $\frac{(\text{Abs test sample} - \text{Abs blank}) - \text{Intercept}}{\text{Slope} \times \text{amount of sample in gram}}$

Slope x amount of sample in gram

Where Abs test sample was the absorbance of extract with reagent, Abs blank was the absorbance of extract without reagent.

3.8 Determination of condensed tannin content

Estimation of condensed tannin content in the dried extracts was done according to the method described by Mishara et al (2009). The dried extract (5 mg) was dissolved in 80% ethanol (1 ml). The sample solution (50 μ l) was added to 100 μ l of vanillic acid solution (4% w/v) and 50 μ l of concentrated HCl. The absorbance was measured immediately at 500 nm. The estimation of condensed tannin content was calculated from a calibration curve using catechin as a standard. The results were expressed as milligram catechin equivalent/gram dry weight of extract (figure 10).

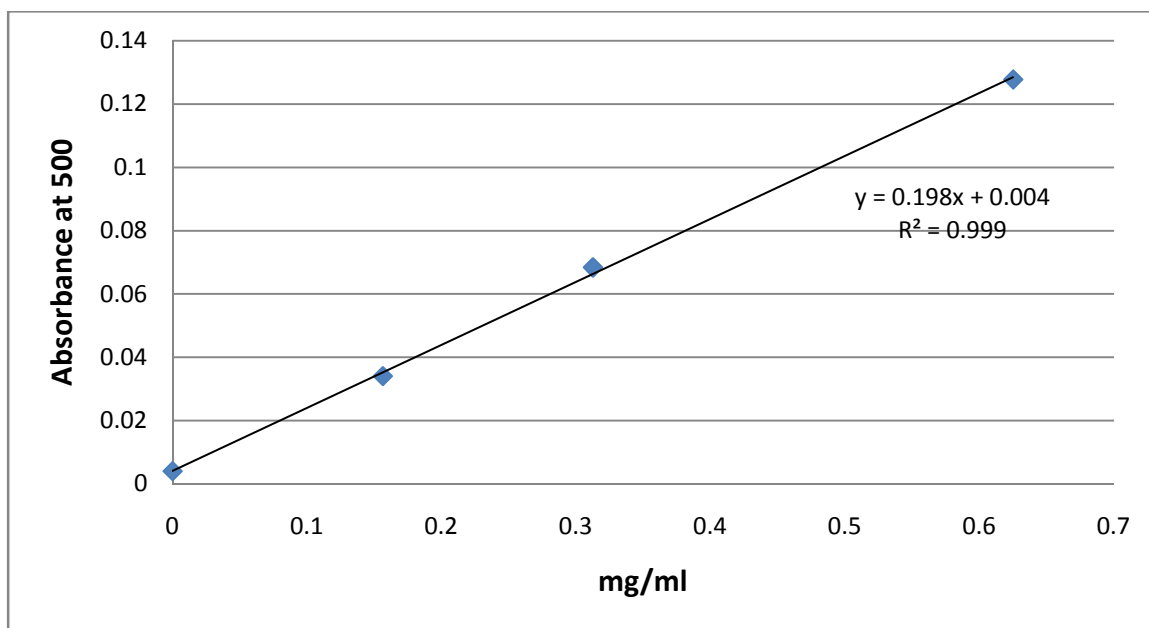


Figure 10: standard curve for catechin

$$\text{Condensed tannins (mg/g)} = \frac{(\text{Abs test sample} - \text{Abs blank}) - \text{Intercept}}{\text{Slope} \times \text{amount of sample in gram}}$$

Where Abs test sample was the absorbance of extract with reagent, Abs blank was the absorbance of extract without reagent.

3.9 Pancreatic α -amylase inhibitory activity

The pancreatic α -amylase inhibition assay was performed according to the method described by Adisakwattana et al (2012). Porcine pancreatic α -amylase (3units/ml) was dissolved in 0.1 M phosphate buffer saline, pH 6.9. The various concentrations of the extract (10 μ l) were added to a solution containing starch (1 g/l) and phosphate buffer (165 μ l). The reaction was initiated by adding enzyme solution (75 μ l) to the incubation medium. After 10 min incubation, the reaction was stopped by adding 250 μ l dinitrosalicylic (DNS) reagent (1% 3,5-dinitrosalicylic acid, 0.2% phenol, 0.05% Na₂SO₃ and 1% NaOH in aqueous solution) to the reaction mixture. The mixtures were

heated at 100°C for 10 min in order to stop the reaction. Thereafter, 250µl of 40% potassium sodium tartarate solution was added to the mixtures to stabilize the color. After cooling to room temperature in a cold water bath, the absorbance was recorded at 540 nm using a microplate reader.

$$\% \text{Inhibition} = \frac{\text{AbsControl} - \text{AbsSample}}{\text{AbsControl}} \times 100$$

Where Abs Control was the absorbance without sample, Abs samples was the absorbance of sample extract.

3.10 Intestinal α -glucosidase inhibitory activity

The assessment of intestinal α -glucosidase inhibitory activity was based on the modified method described by Adisakwattana et al (2012). Briefly, 100 mg of rat intestinal acetone powder was homogenized in 3 ml of 0.9% NaCl solution. The solution was centrifuged at 12,000g for 30min and then subjected to assay. The crude enzyme solution (as maltase assay, 10µl; as sucrase assay, 30 µl) was incubated with 30 µl maltose (86 mM) or 40 µl sucrose (400 mM), 10µl of the extract at various concentrations, followed by the addition of 0.1 M phosphate buffer, pH 6.9 to give a final volume of 100µl. The reaction was incubated at 37°C for 30 min (maltase assay) or 60 min (Sucrase assay). Thereafter, the mixtures were suspended in boiling water for 10 min to stop the reaction. The concentrations of glucose released from the reaction mixtures were determined by glucose oxidase method with absorbance at a wavelength of 450 nm. Intestinal α -glucosidase inhibitory activity was expressed as percentage inhibition using the following formula.

$$\% \text{Inhibition} = \frac{\text{AbsControl} - \text{AbsSample}}{\text{AbsControl}} \times 100$$

Where Abs Control was the absorbance without sample, Abs samples was the absorbance of sample extract.

3.11 Pancreatic Cholesterol Esterase Inhibition

The pancreatic cholesterol esterase inhibition was performed spectrophotometrically based on the method described by (Adisakwattana et al, 2011). The extract was incubated with mixtures containing 5.16 mM taurocholic acid, 0.2 mM p-NPB in 100 mM sodium phosphate buffer, 100 mM NaCl, pH 7.0. The reaction was initiated by adding porcine pancreatic cholesterol esterase (1µg/ml). After incubation for 5 min at 25°C, the absorbance of the mixtures was measured at 405 nm.

$$\% \text{Inhibition} = \frac{\text{AbsControl} - \text{AbsSample}}{\text{AbsControl}} \times 100$$

AbsControl

Where Abs Control was the absorbance without sample, Abs samples was the absorbance of sample extract.

3.12 Pancreatic Lipase Inhibition

The pancreatic lipase inhibition was performed spectrophotometrically based on the previous method with little modification by Lewis and Liu (2012). The extract was incubated with mixtures containing 5 mM deoxytaurocholic acid, 0.2 mM p-NPP in 50 mM sodium phosphate monobasic buffer, pH 8.0. The reaction was initiated by adding porcine pancreatic lipase (10mg/ml). After incubation for 5 min at 37°C, the absorbance of the mixtures was measured at 410 nm.

$$\% \text{Inhibition} = \frac{\text{AbsControl} - \text{AbsSample}}{\text{AbsControl}} \times 100$$

AbsControl

Where Abs Control was the absorbance without sample, Abs samples was the absorbance of sample extract.

3.14 Induction of experimental diabetes

Six rats were randomly selected as normal controls. Diabetic rats were induced by streptozotocin (STZ) 40mg/kg body weight, intraperitoneally (IP). STZ was dissolved in

citrate buffer solution (0.01M, pH=4.5). All the animals had free access to water and pellet diet after thirty minutes of administration. Five days thereafter the fasting blood glucose levels of rats were determined using glucose oxidase method with glucose analyzer. A blood glucose level greater than 126mg/dl was considered to be diabetes mellitus (DM). Streptozotocin induced diabetic rats were selected and divided in four groups; negative control, positive control, and test groups.

3.15 Sucrose tolerance test

The normal mice were divided into five groups and each group contained six animals. The control group was administered with distilled water. Other groups received three different doses of aqueous ethanol extract of *Moringa stenopetala* leaves (250, 500 and 750 mg/kg) by oral gavage. Acarbose (3 mg/kg) was used as a positive control. All treatments were administered to the mice 5 min before loading sucrose (3 g/kg). Blood samples were collected from a tail vein at 0, 30, 60, 90 and 180 min. The blood glucose levels were determined by glucose oxidase method.

3.16 Study on effect of aqueous ethanol extract and n-butanol fraction on blood glucose levels

Diabetic rats were administered with 500mg/kg body weight of ethanol extract and butanol fraction daily for 14 days via oral gavage. The diabetic and normal rats were given 10ml/kg of body weight of normal saline and positive control rats were given 150mg/kg of metformin by oral gavage. On days 0, 7, and 14 the blood samples were collected from tail vein following overnight fasting and blood glucose levels were measured. Body weight of each rat was determined at the same time.

3.17 Assay of serum lipid level, hepatic enzymes and renal function tests

On day 15, the rats were fasted overnight, blood samples were collected in a sterile tube by cardiac puncture under ether anesthesia and left to stand at room temperature for 2h, then centrifuged at 1500xg for 15 minutes at 4°C. The supernatant was immediately separated from the pellet to prepare serum samples in order to determine the level of triglyceride (TG), total cholesterol (TC), high density lipoprotein (HDL), aspartate

aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutathione transferase (GGT), bilirubin total (BLT), albumin, urea and creatinine using automated chemistry analyzer (humostar 80, Germany).

3.18 Histological sample preparation

For light microscopic preparation, the whole of the pancreas was dissected, fixed in 10% formalin for 24 hours, dehydrated in a graded ethanol series, cleared in xylene, infiltrated and embedded in paraffin. Thin sections (5 μm) were cut using a microtome (Leica RM 2125, Leica Microsystems Nussloch GmbH, Germany) and stained with hematoxylin and eosin. The preparation was examined under light microscope (Leitz, Wetzlar, Germany). Photomicrographs of selected samples of the pancreas tissue were then taken with TMX 200 film under x25 objective using a Leitz Dialux 20 Wild Photo Automat MPS 51. Following the evaluation of the control animals, the pancreas tissue in the remaining groups were evaluated blind to treatment.

3.19 *In Vitro* Glycation of Bovine Serum Albumin (BSA)

The formation of glycated BSA was done according to the previous method with minor modifications by Sharma et al (2002). BSA (10 mg/mL) was incubated with fructose (0.5 M) in 0.1 M phosphate buffer (PBS), pH 7.4 containing 0.02% sodium azide (NaN_3) at 37°C for 2 weeks in the absence or presence of *Moringa stenopetala* leaf extract (0.5, 1, and 2mg/ml) and aminoguanidine (1mg/ml). Dimethylsulfoxide (DMSO, 4%) was used as a solvent for this study. Aliquots of the reaction mixtures were then assayed for AGEs, fructosamine, protein carbonyl content, thiol group, amyloid cross β structure, and CML.

3.20 Determination of AGEs Formation

The formation of AGEs was determined with spectrofluorometer (Wallac 1420 Victor 3 V, PerkinElmer, Walham, MA, USA) at excitation and emission wavelengths of 355 and 460 nm, respectively. The inhibitory effect of extract and aminoguanidine was expressed as percentage inhibition compared with maximum glycation elicited by fructose.

The percentage of fluorescent AGE formation was calculated as follows:

Inhibition of fluorescent AGEs %

$$= [(FC-FCB) - (FS-FSB) / (FC-FCB)] \times 100$$

Where FC and FCB were the fluorescent intensity of control with fructose and blank of control without fructose, FS and FSB were the fluorescent intensity of sample with fructose and blank of sample without fructose.

3.21 Fructosamine Measurement

The concentration of the Amadori product fructosamine was measured by NBT assay (Ardestani and Yazdanparast, 2007). The glycated BSA (10 μ L) was incubated with 100 μ L of 0.5 mM NBT in 0.1 M carbonate buffer, pH 10.4 at 37 °C. The absorbance was measured at 530 nm at 10 and 15 min time points. The concentration of fructosamine was calculated compared to DMF, the standard (figure 11).

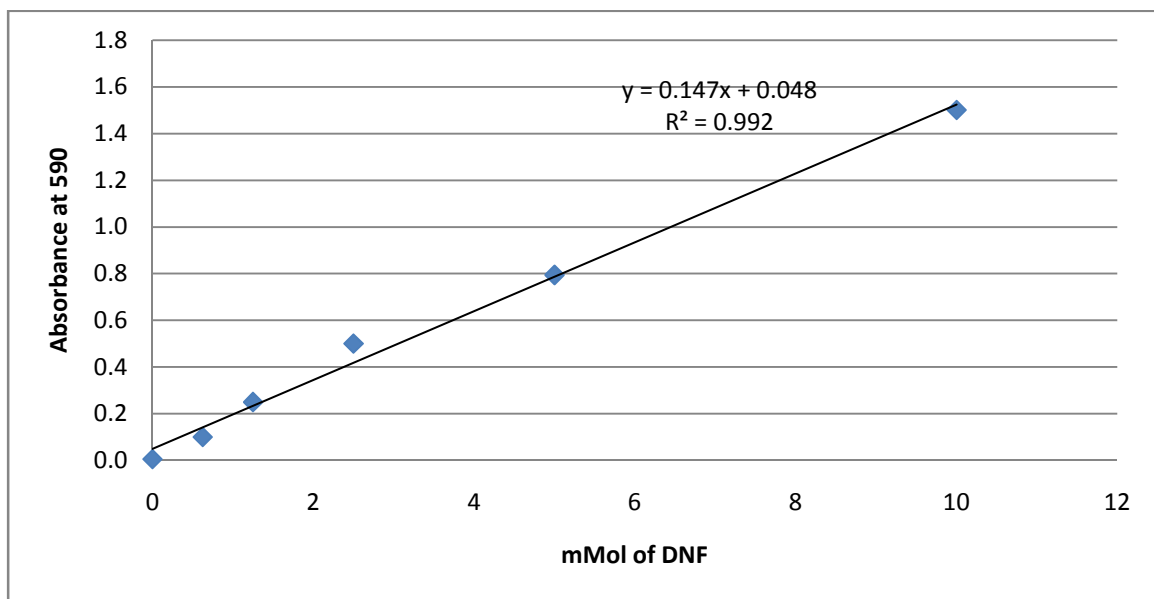


Figure 11: DNF standard curve

3.22 Determination of N^ε-(carboxymethyl) Lysine (CML)

After two weeks of incubation, N^ε-(carboxymethyl) lysine (CML), a major antigenic AGE structure, was determined using enzyme linked immunosorbant assay (ELISA) kit.

According to the manufacturer's protocol, the glycated samples were diluted to final concentration of 1 µg/ml (10,000-fold dilution) before used in the assay. Each diluted sample (100µl) was incubated in the 96-well protein binding plate at 37°C for at least 2 h. After washing with PBS, an assay diluent was added and further incubated for 2 h at room temperature on an orbital shaker. Three washes with wash buffer were needed before incubating for 1 h each with anti-CML antibody and with secondary antibody HRP conjugate. The substrate solution (100µl) was added for 20 min before adding stop solution in an equal volume. The absorbance of samples was measured immediately at 450 nm and compared with the absorbance of CML-BSA standard (figure 12) providing in the assay kit (Adisakwattana et al, 2012).

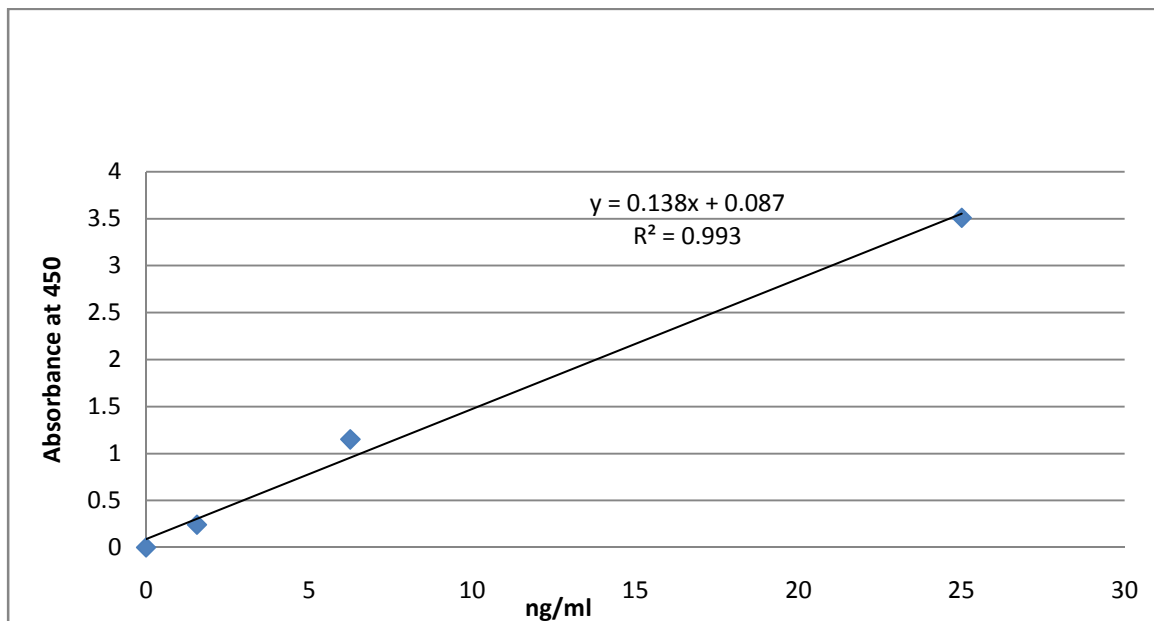


Figure 12: Nε-(Carboxymethyl) lysine standard curve

3.23 Determination of Protein Carbonyl Content

The carbonyl group in glycated BSA, a marker for protein oxidative damage, was assayed according to the method of Levine and colleagues with minor modifications (Wu et al, 2011). Four hundred µL of 10 mM DNPH in 2.5 M HCl was added to 100 µL of glycated samples. After 1 h incubation in the dark, 500 µL of 20% (w/v) TCA was used for protein precipitation (5 min on ice) and then centrifuged at 10,000 g for 10 min at 4 °C. The protein pellet was washed with 500 µL of ethanol/ethyl acetate (1:1) mixture

three times and re-suspended in 250 μL of 6 M guanidinehydrochloride. The absorbance was measured at 370 nm. The carbonyl content of each sample was calculated based on the extinction coefficient for DNPH ($\epsilon = 22,000 \text{ M}^{-1} \text{ cm}^{-1}$). The results were expressed as nmol carbonyl/mg protein.

3.24 Thiol Group Estimation

The free thiols in glycated samples were measured by Ellman's assay with minor modifications made by Adisakwattana et al (2012). Seventy μL of glycated samples were incubated with 5 mM DTNB in 0.1 M PBS, pH 7.4 at 25 $^{\circ}\text{C}$ for 15 min. The absorbance of samples was measured at 410 nm. The concentration of free thiols was calculated from L-cysteine standard (figure 13) and expressed as nmol/mg protein.

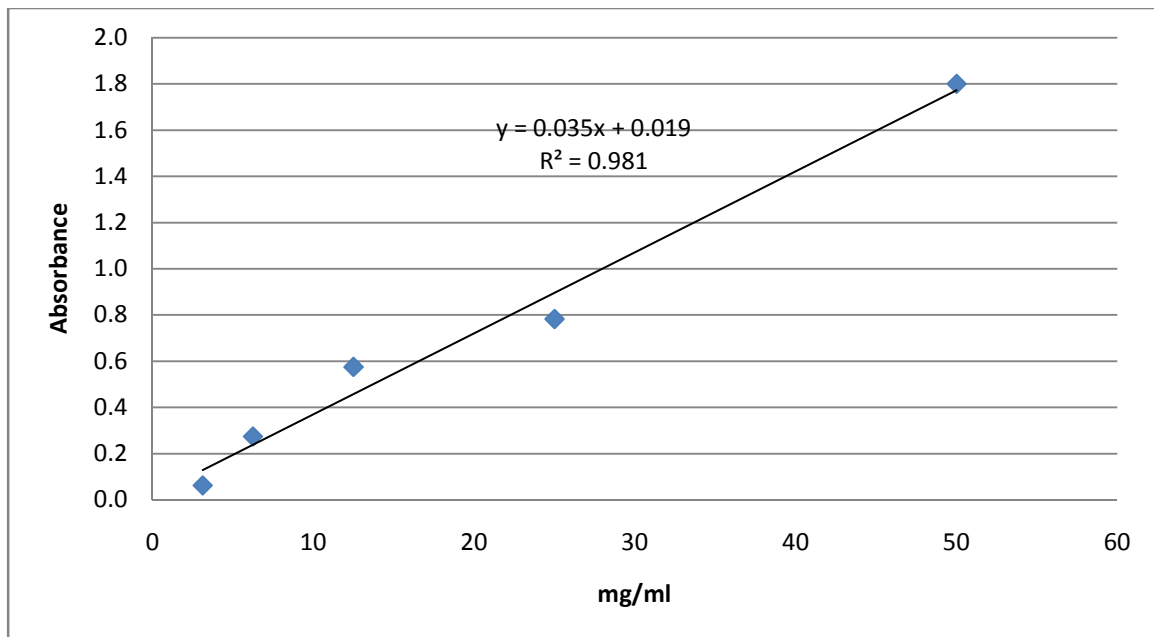


Figure 13: cysteine standard curve

3.25 Determination of protein aggregation

Amyloid cross β -structure, a common marker for protein aggregation was measured using a congo red assay according to the previous published method with minor modifications (Chiti and Dobson, 2006). Briefly, the glycated BSA (50 μL) was incubated with 50 μL of

100µM congo red in 10% (v/v) ethanol/PBS for 20 min at 25°C. The absorbance was measured at 530 nm.

3.26 DPPH Radical Scavenging Activity Assay

The free radical scavenging activity of the extract was measured in vitro by 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) assay according to the method described earlier. The stock solution was prepared by dissolving 24 mg DPPH with 100 ml methanol and stored at 20°C until required. The working solution was obtained by diluting DPPH solution with methanol to attain an absorbance of about 0.98±0.02 at 517 nm using the spectrophotometer. A 3 ml aliquot of this solution was mixed with 100 µl of the sample at various concentrations (10 - 500 µg/ml). The reaction mixture was shaken well and incubated in the dark for 15 min at room temperature. Then the absorbance was taken at 517 nm. The control was prepared as above without any sample. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as in the following equation:

$$\text{Scavenging effect (\%)} = [(A \text{ cont.} - A \text{ test}) / A \text{ cont.}] \times 100$$

Where, A cont is the absorbance of control reaction and A test is the absorbance in the presence of extract (Mishara et al, 2009).

3.27 Data analysis

The IC₅₀ values were calculated from plots of log concentration of inhibitor concentration versus percentage inhibition curves. The data were expressed as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was used to analyze the changes in plasma glucose and other parameters. Then Tukey post-hoc comparisons were used to determine the source of significant differences where appropriate. The level of statistical significance was set at p<0.05. Statistical analysis was done using graphpad Instat® software.

4 Results

4.1 Flavonoid, total phenolic, and condensed tannin contents of *Moringa stenopetala* leaves extract.

The amount of total flavonoids, total polyphenolic compounds, and condensed tannins contents determined in the extract are summarized in Table 1. The content of flavonoid in the extract was 71.73 ± 2.48 mg quercetin equivalent/g of crude extract. The total polyphenolic compound in the extract was 79.81 ± 2.85 mg of gallic acid equivalent/ g of crude extract. Furthermore, the content of condensed tannins in the extract was found to be 8.82 ± 0.77 mg catechin equivalent/g of crude extract.

Table 1: Flavonoid, total phenolic, and condensed tannin contents of *Moringa stenopetala* leaves extract.

	Phytochemical Analysis		
	Flavonoid (mg/g extract)	Total phenolics (mg/g extract)	Condensed tannins (mg/g extract)
<i>Moringa stenopetala</i>	71.73 ± 2.48	79.81 ± 2.85	8.82 ± 0.77

Results are expressed as means \pm S.E.M., n = 3.

4.2 The inhibitory effects of *Moringa stenopetala* leaf extract on pancreatic α -amylase, maltase, sucrase, pancreatic lipase, and pancreatic cholesterol esterase activities

As shown in Table 3, the IC₅₀ value of the extract was 1.47 ± 0.19 mg/ml for intestinal sucrase, where as the extract (5 mg/ml) inhibited intestinal maltase by $48.64 \pm 1.18\%$ (Table 2). The findings indicated that the extract was a more specific inhibitor of intestinal sucrase than intestinal maltase. The extract also inhibited pancreatic cholesterol esterase activity ($49.22 \pm 2.34\%$) more than pancreatic α -amylase ($6.06 \pm 0.75\%$). Moreover, it was found that the extract inhibited pancreatic lipase with IC₅₀ value of greater than 5mg/ml.

Table 2: The percent inhibitory effects of *Moringa stenopetala* leaf extract on pancreatic α -amylase, maltase, sucrase, pancreatic lipase, and pancreatic cholesterol esterase activities.

Concentration of Moringa stenopetala (mg/ml)	Percentage(%) inhibition				
	pancreatic α -Amylase	Maltase	Sucrase	Pancreatic lipase	Pancreatic Cholesterol esterase
0.625	0.61±0.01	5.11±0.19	8.91±0.22	10.15±0.21	20.91±0.27
1.250	0.96±0.11	30.12±0.17	38.12±0.35	28.11±0.28	26.03±1.11
2.500	1.91±0.18	45.01±0.22	71.25±1.71	46.03±1.11	38.81±0.91
5.000	6.06±0.75	48.64±1.18	84.35±1.11	50.26±0.75	49.22±2.34

Results are expressed as means \pm S.E.M., n = 3.

Table 3: The inhibitory concentration values of *Moringa stenopetala* leaf extract on pancreatic α -amylase, maltase, sucrase, pancreatic lipase, and pancreatic cholesterol esterase activities.

	IC ₅₀ values (mg/ml)				
	pancreatic		Pancreatic		Pancreatic
	α -Amylase	Maltase	Sucrase	lipase	Cholesterol esterase
<i>Moringa stenopetala</i>	> 5	> 5	1.47 \pm 0.19	>5	>5

Results are expressed as means \pm S.E.M., n = 3.

4.3 Effect of *Moringa stenopetala* leaf extract on postprandial glycemia in non-diabetic mice

A single oral administration of *Moringa stenopetala* extract was given to non-diabetic rats to confirm its antihyperglycemic activity by oral sucrose tolerance test. The results showed that blood glucose level reached the peak at 30min after sucrose administration in all groups (Table 4). In addition, the extract at the doses of 250 mg/kg, 500 mg/kg and 750 mg/kg significantly reduced the raised blood glucose level starting 30 minutes after administration comparable to that of acarbose (Table 4).

Table 4: Sucrose challenge test of hydroalcoholic extract of *Moringa stenopetala* in mice

Blood glucose(mg/dl) level in different time intervals (minutes)					
Groups	0	30	60	120	180
Control	203.2±1.75	301.5±0.79	274.2±1.01	243.1±1.11	195.6±0.53
250mg/kg MS extract	192.3±1.12	253.3±0.98*	226.2±0.78*	195.6±0.67*	152.3±0.78*
500mg/kg MS extract	189.3±1.23	249.3±0.91*	233.2±1.13*	189.3±0.57**	140.8±0.45**
750mg/kg MS extract	193.8±1.79	255.3±2.11*	226.1±1.10*	182.8±0.58**	133.4±0.23***
3mg/kg Acrabose	192.8±0.74	223.6±1.71***	171.0±0.79***	155.6±1.33***	124.8±0.37***

Results are expressed in Mean ± S.E.M, n = 6, * when p<0.05, ** when p<0.01, *** when p<0.001 verses control

4.4 Effects of *Moringa stenopetala* leaf extract on blood glucose level in normal and diabetic rats

Blood glucose levels were measured once weekly in diabetic and normal rats given *Moringa stenopetala* extract after the induction of diabetes. The results are summarized in Table 5. Before induction of diabetes, there was no significant difference in blood glucose levels between diabetic and non diabetic control groups (p>0.05). After induction, diabetic rats showed significant differences in blood glucose levels compared to normal rats (p<0.05). The groups treated with the aqueous ethanol extract and n-butanol fraction of *Moringa stenopetala* leaves showed significant reduction of blood glucose level on days 7 and 14 after administration comparable to metformin.

Table 5: Effects of aqueous ethanol and n-butanol fraction of *Moringa stenopetala* leaves on blood glucose level in diabetic rats

Treatment Groups	Fasting Blood Glucose level (mg/dl)			
	Before Induction	Day 0	Day 7	Day 14
<i>Normal control</i>	114.00±2.11	109.67±3.39 ^a	104.67±2.79 ^a	104.00±3.45 ^a
<i>Diabetic Control</i>	115.10±1.75	145.17±1.05 ^b	140.50±2.29 ^b	136.10±2.52 ^b
<i>DC + 500mg/Kg ethanol extract of MS</i>	111.67±1.19	141.56±1.50 ^b	123.83±1.99 ^{ab}	120.33±2.23 ^{ab}
<i>DC + 500mg/Kg butanol fraction of MS</i>	115.17±2.15	141.56±1.45 ^b	129.67±2.40 ^{ab}	123.00±1.94 ^{ab}
<i>DC + 150mg/Kg Metformin</i>	112.33±2.09	142.17±1.92 ^b	124.67±2.81 ^{ab}	117.83±3.05 ^{ab}

Results are expressed in Mean ± S.E.M, n = 6, ^a when p<0.05 verses Diabetic control and ^b when p<0.05 verses normal control

4.5 Effect of *Moringa stenopetala* leaves extract on body weight

The body weight of diabetic and normal rats given *Moringa stenopetala* extract after the induction of diabetes are given in Table 6. There were no significant differences in the initial body weights among the groups. After the induction of diabetes, significant differences in body weight of normal and diabetic rats were observed (p<0.01). Both the extract and fraction of *Moringa stenopetala* leaves improved the weight gain compared with the diabetic control rats. By the end of the experiment, the body weight of the normal control group significantly increased (p<0.001), while, the rats in the diabetic

control group increased body weight during the experimental period less significantly ($p < 0.05$).

Table 6: Effects of aqueous ethanol and n-butanol fraction of *Moringa stenopetala* leaves on body weight in diabetic rats.

Body Weight Change During Treatment (g)				
Treatment Groups	Before	Day 0	Day 7	Day 14
	Induction			
<i>Normal Control</i>	221.60±5.43	262.43±4.95 ^a	273.63±2.60 ^a	280.83±2.08 ^a
<i>Diabetic Control/DC/</i>	211.03±3.46 ^b	230.68±2.73 ^b	229.58±6.11 ^b	231.33±5.66 ^b
<i>DC + 500mg/Kg ethanol extract of MS</i>	212.52±2.96	233.65±4.21 ^b	244.08±2.42 ^b	250.57±2.43 ^{ab}
<i>DC + 500mg/Kg butanol fraction of MS</i>	212.70±3.12	230.03±2.87 ^b	241.50±3.78 ^b	249.67±5.55 ^b
<i>DC + 150mg/Kg Metformin</i>	220.32±5.41	236.67±6.04 ^b	248.67±5.67 ^b	249.50±6.31 ^b

Results are expressed in Mean ± S.E.M, n = 6, ^a when $p < 0.05$ versus Diabetic control and ^b when $p < 0.05$ versus normal control.

4.6 Effect of the aqueous ethanol extract and n-butanol fraction of *Moringa stenopetala* leaves on lipid profiles

After the induction of diabetes and subsequent treatment with either the aqueous ethanol extract, the n-butanol fraction or metformin, there was a significant increase of serum total cholesterol, triglycerides, and significant decrease in HDL cholesterol in diabetic rats when compared to normal rats. The results showed that administration of aqueous

ethanol extract and butanol fraction significantly decreased ($P < 0.05$) the levels of cholesterol and triglycerides (Table 7). HDL cholesterol level was also improved in diabetic rats after 14 days treatment (Table 7). In addition, metformin also improved the lipid profiles in diabetic rats.

Table 7: Effects of aqueous ethanol and n-butanol fraction of *Moringa stenopetala* leaves on lipid profiles in diabetic rats.

Groups	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)	LDL-C(mg/dL)
Normal control	84.0±1.7	81.5±1.7	29.3±1.8	43.76±2.7
Diabetic control/DC	189.5±2.3 ^a	158.50±3.1 ^a	19.8±0.2 ^a	141.63±2.1 ^a
<i>DC + 500mg/Kg</i>				
<i>ethanol</i>				
<i>extract of MS</i>	<i>127.5±1.1^{ab}</i>	<i>93.25±3.7^{ab}</i>	<i>25.54±1.4^{ab}</i>	<i>83.11±1.3^{ab}</i>
<i>DC + 500mg/Kg</i>				
<i>butanol</i>				
<i>fraction of MS</i>	<i>97.2±1.5^b</i>	<i>75.00±1.1^b</i>	<i>28.67±1.5^b</i>	<i>51.50±2.6^b</i>
<i>DC + 150mg/Kg</i>				
<i>Metformin</i>	<i>81.7±2.9^b</i>	<i>64.67±1.5^b</i>	<i>34.5±1.9^{ab}</i>	<i>38.03±0.5^b</i>

The results are expressed as Mean ± S.E.M (n=6). ^ap < 0.05 compared with normal control values and ^bp < 0.05 compared with diabetic control.

4.7 Effects of the aqueous ethanol extract and n-butanol fraction of *Moringa stenopetala* leaves on liver and kidney functions

The effects of *Moringa stenopetala* leaves on liver functions are shown in Table 8. The levels of ALT, AST, ALP, GGT and bilirubin were significantly elevated in diabetic rats. The rats treated with *Moringa stenopetala* extractions showed significant ($P < 0.05$) reduction in the elevated levels of liver enzymes (transaminase) and bilirubin as shown in Table 9. Diabetic rates showed a significant reduction in total protein levels whereas rats treated with the extraction of *Moringa stenopetala* leaves showed significantly increased levels ($P < 0.05$). The level of creatinine and urea were elevated in diabetic rats as compared with the normal rats (Table 9). Rats administered with *Moringa stenopetala*

extracts showed reduced levels of creatine and urea compared to untreated diabetic rats ($p < 0.05$, Table 6). For the n-butanol fraction, the level of creatine was not significantly different to that of non-diabetic rats (Table 9).

Table 8: Effects of the aqueous ethanol extract and n-butanol fraction of *Moringa stenopetala* leaves on liver and kidney functions

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)	BLT(mg/dl)	GGT (U/L)
Normal control	32.0±2.2	57.8±2.35	53.01±0.8	0.13±0.01	3.55±0.26
Diabetic control/DC	73.0±1.7 ^a	92.4±1.7 ^a	85.66±1.24 ^a	0.11±0.01	4.75±0.6
<i>DC + 500mg/Kg ethanol extract of MS</i>	53.2±1.88 ^{ab}	79.3±2.2 ^{ab}	81.34±1.5 ^{ab}	0.16±0.03	3.92±0.29
<i>DC + 500mg/Kg butanol fraction of MS</i>	49.2±1.9 ^{ab}	73.0±1.4 ^{ab}	72.00±2.5 ^{ab}	0.12±0.04	3.67±0.27
<i>DC + 150mg/Kg Metformin</i>	41.7±2.6 ^{ab}	68.5±0.5 ^b	61.23±1.4 ^{ab}	0.10±0.01	3.82±0.37

The results are expressed as Mean ± S.E.M (n=6). ^ap < 0.05 compared with normal control values and ^bp < 0.05 compared with diabetic control.

Table 9: Effect of *Moringa stenopetala* leaves extract and its butanol fraction on urea, creatinine, and total albumin STZ induced diabetic rats.

Groups	Urea (mg/dL)	Creatinine (mg/dL)	Total Albumin (mg/dL)
Normal control	40.2±1.6	0.85±0.13	6.1±0.12
Diabetic control/DC	79.0±1.5 ^a	1.87±0.18 ^a	4.57±0.11 ^a
<i>DC + 500mg/Kg ethanol extract of MS</i>	56.5±1.8 ^{ab}	1.35±0.23 ^{ab}	5.23±0.13 ^b
<i>DC + 500mg/Kg butanol fraction of MS</i>	53.15±1.12 ^{ab}	1.23±0.3 ^b	5.81±0.13 ^b
<i>DC + 150mg/Kg Metformin</i>	50.12±1.5 ^{ab}	1.15±0.2 ^b	5.79±0.11 ^b

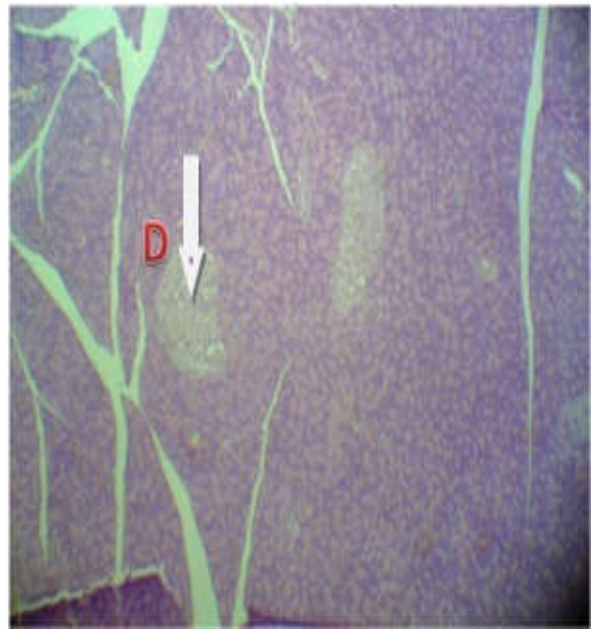
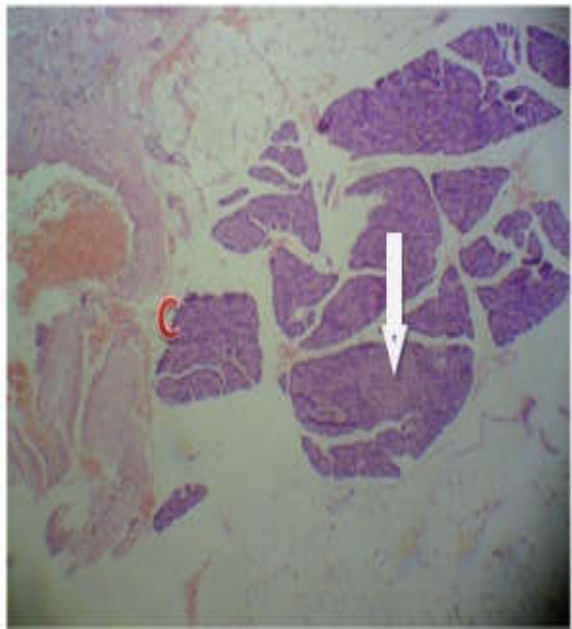
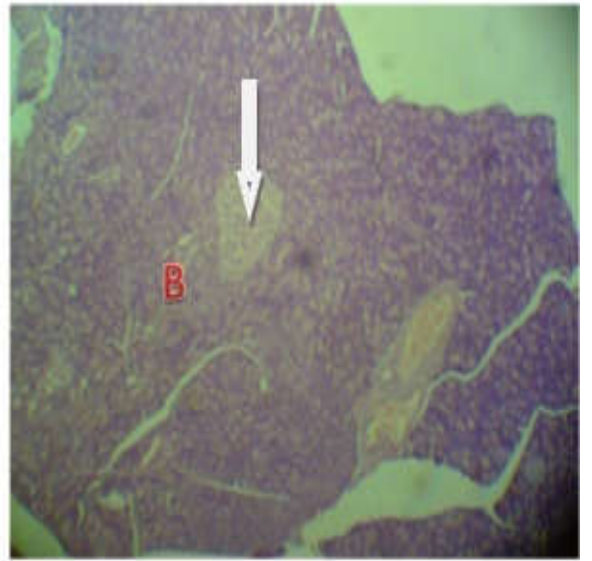
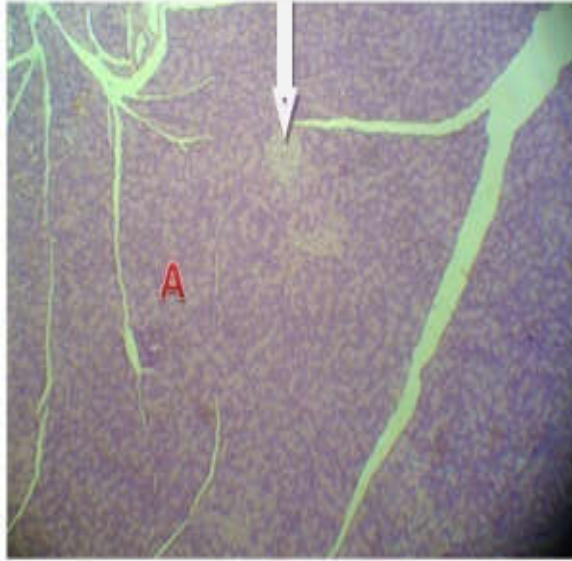
The results are expressed as Mean \pm S.E.M (n=6). ^ap < 0.05 compared with normal control values and ^bp < 0.05 compared with diabetic control.

4.8 Effects of aqueous ethanol extract and its butanol fraction of *Moringa stenopetala* leaves on histopathology of pancreas

The structure of the pancreas of the normal control and diabetic rats are shown after treatment in Table 10 and Figure 14. The pancreas of normal control rats showed normal islets, whereas that of diabetic rats showed hyperplasia of beta-cells and congestion of pancreatic parenchymal cells. Administration of the aqueous ethanol extract and n-butanol fraction of *Moringa stenopetala* leaves as well as metformin treatment increased the number of islets when compared to that of diabetic animals. The size of islets of Langerhans in the groups treated with aqueous ethanol and metformin was slightly reduced as compared to the normal control rats, however the group treated with n-butanol extraction shows islets of a similar size to that of the control group.

Table 10: Effects of *Moringa stenopetala* leaves on histopathology of pancreas.

Treatment Groups	Size of Islets of Langerhan	Inflammation/ neovascularization	Fibrosis	Necrosis
<i>Normal control</i>	Normal	Not observed	Not observed	Not observed
<i>Diabetic Control</i>	Reduced <u>perfield</u>	Marked lymphocyte infiltration	Focal <u>stromal</u>	Not observed
<i>DC + 500mg/Kg ethanol extract</i>	Slightly reduced	Mild lymphocyte infiltration	Not observed	Not observed
<i>DC + 500mg/Kg butanol fraction</i>	Normal	<u>Neovascularization</u> with congested vessels	Not observed	Not observed
<i>DC + 150mg/Kg Metformin</i>	Slightly Reduced	<u>Neovascularization</u>	Not observed	Not observed



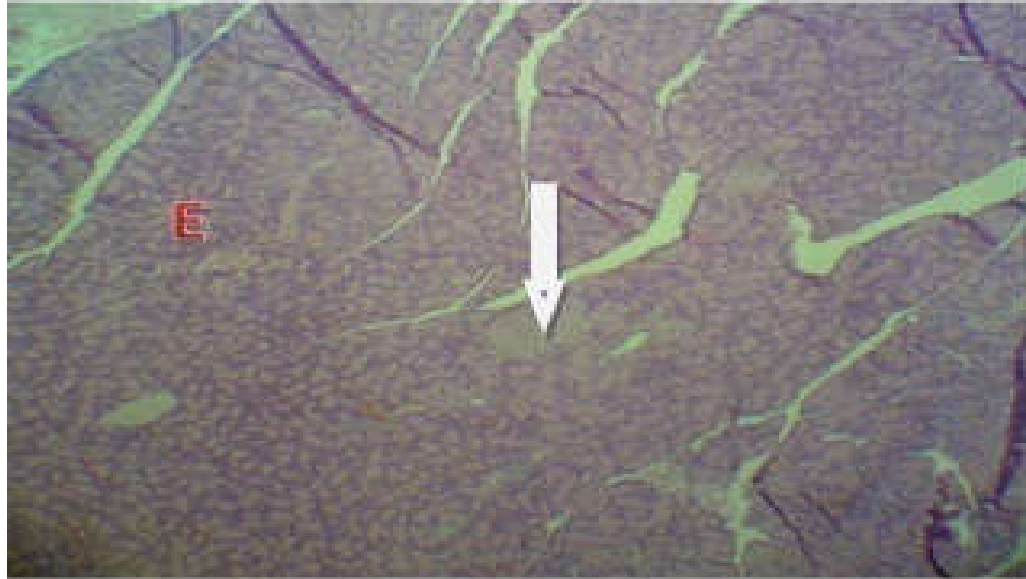


Figure 14: Histopathology of pancreas after administration of *Moringa stenopetala* in diabetic rats.

A, x25: Pancreas of DC animal showing severe congestion of pancreatic parenchymal cells, infiltration of inflammatory cells and hyperplasia of islets cell. B, x25: Pancreas of diabetic animal treated with ethanol extract showing mild hyperplasia of islets cell and congestion of parenchymal cells. C, x25 Pancreas of diabetic animal treated with butanol fraction showing mild hyperplasia of islets cell and congestion of parenchymal cells D, x25: Pancreas of NC animal showing normal histology. E, x25: Pancreas of diabetic animal treated with metformin showing reduced size of islets and devoid of fibrosis and necrosis.

4.9 Antiglycation activity of hydroalcoholic extract of *Moringa stenopetala* leaves.

Figure 15 shows the effect of hydroalcoholic extract of *Moringa stenopetala* leaves at the concentration of 0.5mg/ml, 1mg/ml and 2mg/ml on the total AGEs formation during 14 days of incubation. The fluorescent intensity of BSA incubated with fructose significantly increased about 5.1-fold when compared to BSA, indicating progressive formation of AGEs. When the glycation occurred in the presence of test material, it was observed that *Moringa stenopetala* significantly reduced the formation of AGEs by $54.75 \pm 0.94\%$ at

the concentration of 2mg/ml, as compared to BSA incubated with fructose but less potent than AG ($60.25 \pm 1.13\%$), which was used as control.

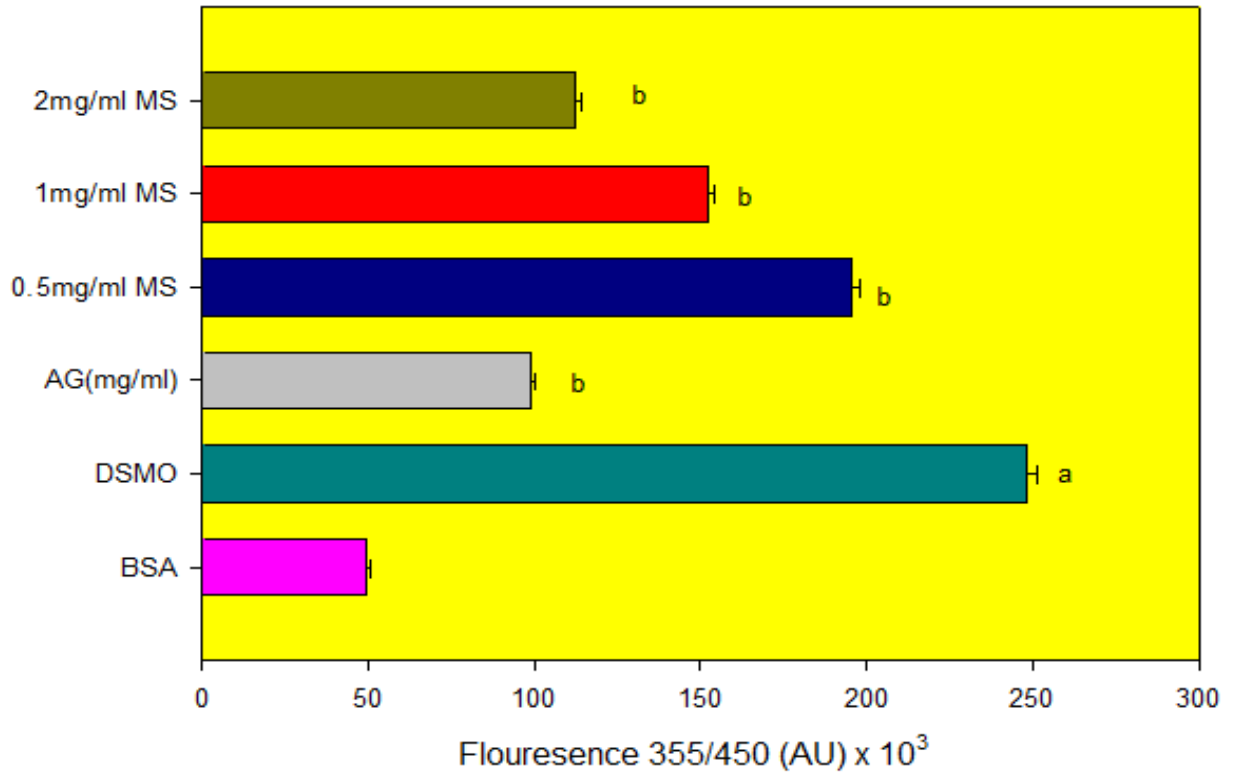


Figure 15: The effects of hydroalcoholic extract of *Moringa stenopetala* (MS) at different concentration and aminoguanidine (AG, 1 mg/ml) on fluorescent AGEs formation in the BSA/fructose system.

Each value represents the mean \pm SEM (n=3). ^aP < 0.05 compared to BSA, ^bP < 0.05 compared to DMSO.

4.10 The effect of *Moringa stenopetala* leaves on protein carbonyl content.

Figure 16 shows the effect of the extract of *Moringa stenopetala* on protein-bound CML formation. The results showed that the formation of CML in BSA incubated with fructose was significantly 3.64-times higher than BSA incubated without fructose. The addition of *Moringa stenopetala* leaves extract at the concentration of 2mg/ml to the solution reduced CML-derived AGE by approximately $19.25 \pm 0.18\%$, whereas AG inhibited the formation of CML by 45.46% when compared to BSA incubated with fructose.

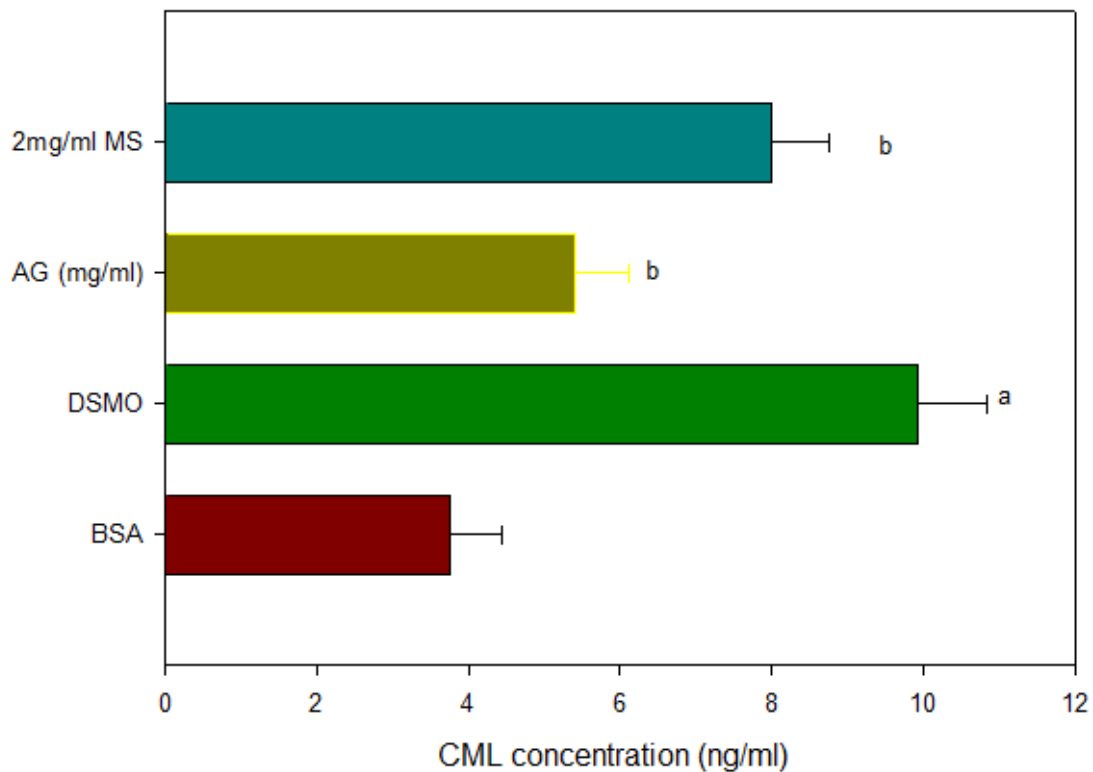


Figure 16: The effects of hydroalcoholic extract of *Moringa stenopetala* (MS) at different concentration and aminoguanidine (AG, 1mg/ml) on the level of N ϵ -(carboxymethyl) lysine (CML) in the BSA/fructose system.

Each value represents the mean \pm SEM (n=3). ^aP < 0.05 compared to BSA, ^bP < 0.05 compared to DMSO

4.11 The effect of *Moringa stenopetala* leaves on the level of fructosamine.

After day 14 of the experiment, the level of fructosamine in BSA incubated with fructose produced a 3.3-fold increase compared to BSA (Figure 17). The results showed that hydroalcoholic extract of *Moringa stenopetala* significantly decreased the level of fructosamine by about $36.1 \pm 0.11\%$.

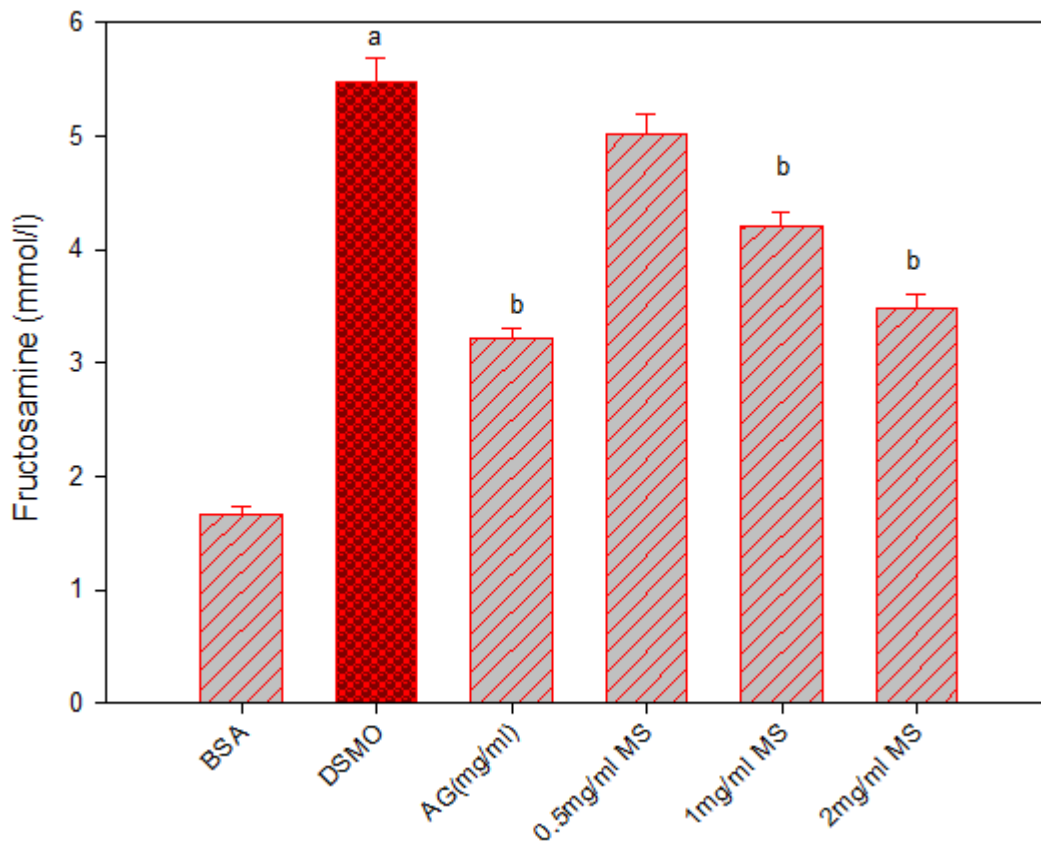


Figure 17: The effects of hydroalcoholic extract of *Moringa stenopetala* (MS) at different concentration and aminoguanidine (AG, 1mg/ml) on the level of fructosamine in the BSA/fructose system.

Each value represents the mean \pm SEM (n=3). ^a P < 0.05 compared to BSA, ^b P < 0.05 compared to DMSO.

4.12 The effect of *Moringa stenopetala* leaves on beta-sheet formation.

To confirm the observation that the plant material inhibits beta-sheet formation of albumin experimentally, Congo red, beta-sheet-specific dye, was used as shown in Figure 18. With this assay, the positive albumin control exhibited the strongest absorbance as expected and all the treatment groups showed significant reduction in absorbance ($P < 0.05$).

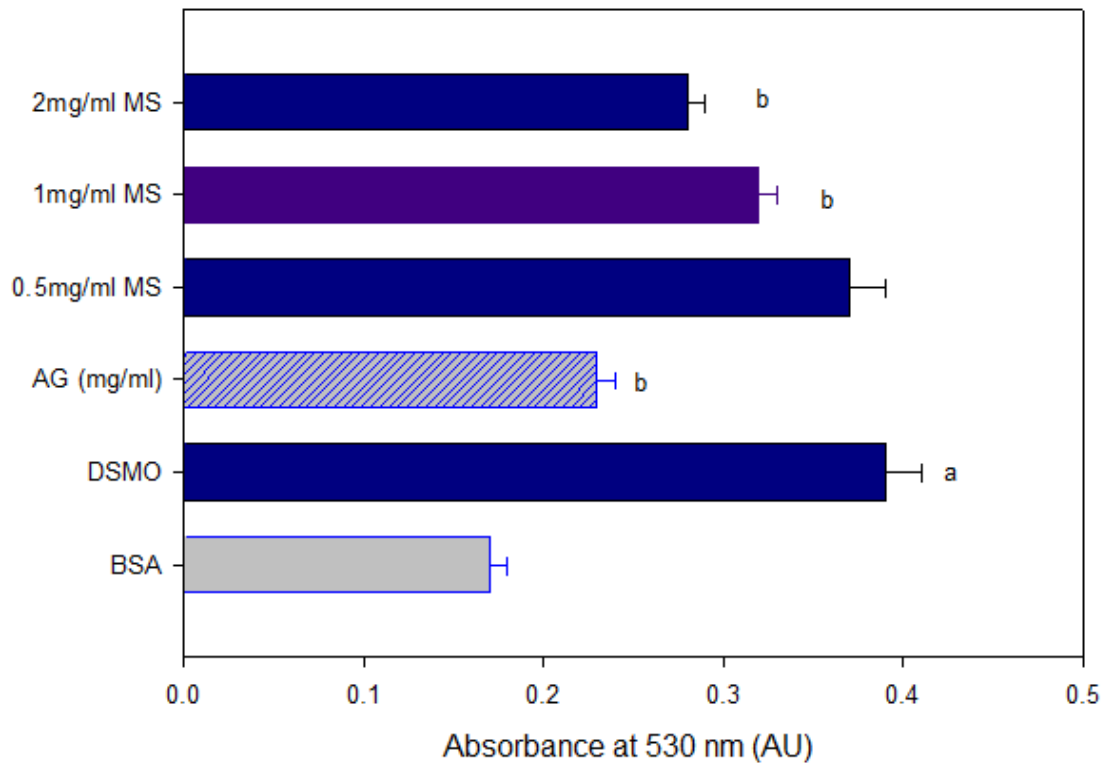


Figure 18: The effects of hydroalcoholic extract of *Moringa stenopetala* (MS) at different concentration and aminoguanidine (AG, 1mg/ml) on absorbance of Congo red in the BSA/fructose system.

Each value represents the mean \pm SEM (n=3). ^a $P < 0.05$ compared to BSA, ^b $P < 0.05$ compared to DSMO.

4.13 The effect of *Moringa stenopetala* leaves on protein thiol group.

Figure 19 shows the results for available free thiol groups of BSA on *Moringa stenopetala* leaves extract. A significant decrease in free thiol groups was observed in BSA incubated with fructose, indicating that protein glycation modified thiol groups to form disulfide in BSA. It was found that the plant material significantly reduced the oxidation of thiol groups by approximately 19.4%, at a concentration of 2mg/ml whereas AG protected the loss of protein thiol groups about 23.6% at a concentration of 1mg/ml, as compared to BSA incubated with fructose.

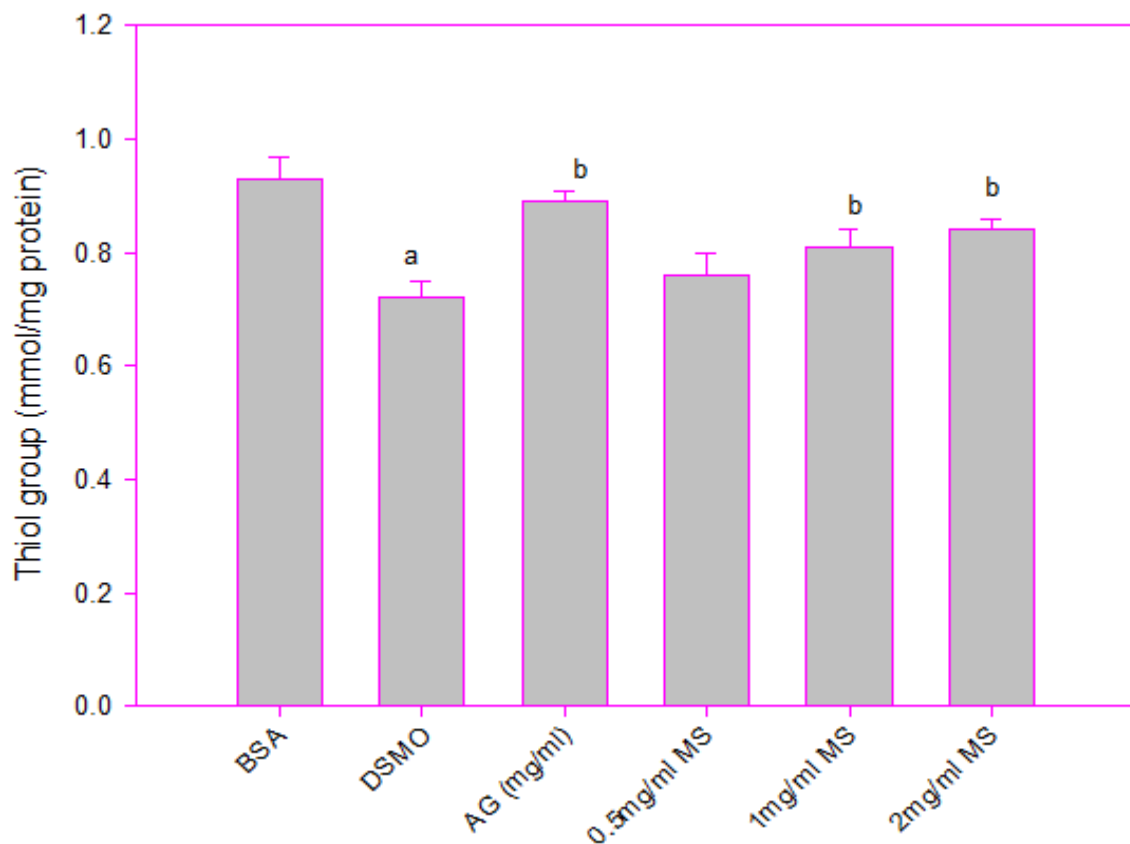


Figure 19: The effects of hydroalcoholic extract of *Moringa stenopetala* (MS) at different concentration and aminoguanidine (AG, 1mg/ml) on thiol content in the BSA/fructose system.

Each value represents the mean \pm SEM (n=3). ^a P < 0.05 compared to BSA, ^b P < 0.05 compared to DMSO.

4.14 The effect of *Moringa stenopetala* leaves on protein carbonyl content.

The addition of fructose to the BSA solution for 14 days significantly increased the extent of protein carbonyl formation, compared to BSA in the absence of fructose. The result indicated that *Moringa stenopetala* leaves suppressed protein carbonyl formation by approximately 18.7% when compared to BSA incubated with fructose. In addition, AG reduced protein carbonyl formation in BSA incubated with fructose by 28.3% (Figure 20).

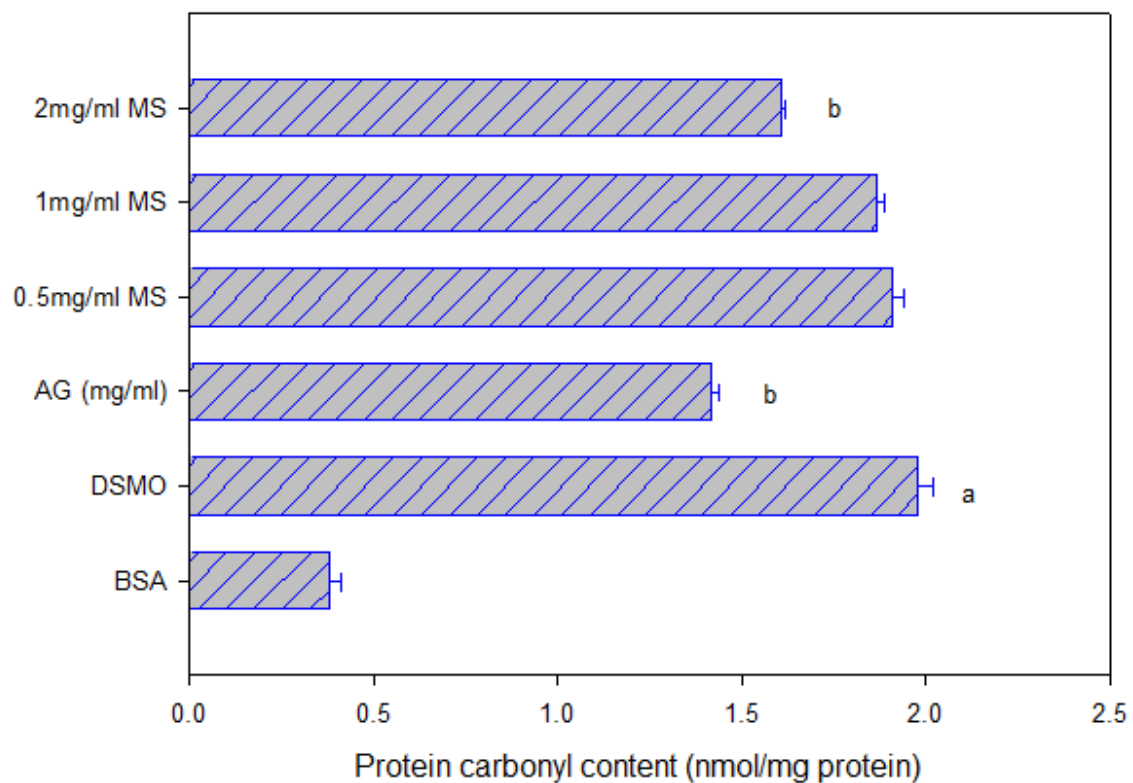


Figure 20: The effects of hydroalcoholic extract of *Moringa stenopetala* (MS) at different concentration and aminoguanidine (AG, 1mg/ml) on Protein carbonyl content in the BSA/fructose system.

Each value represents the mean \pm SEM (n=3). ^a P < 0.05 compared to BSA, ^b P < 0.05 compared to DSMO

4.15 Antioxidant activity of *Moringa stenopetala* leaves.

In order to determine the extent of scavenging effect, ethanolic extract of the leaves of *Moringa stenopetala* was tested for antioxidant activity using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical. The extract showed antioxidant activity as shown in Table 11.

Table 11: DPPH % radical scavenging activity of ethanolic extract of *Moringa stenopetala* leaves

Concentration ($\mu\text{g/ml}$)	%inhibition of DPPH Ascorbic acid	Ethanolic extract
15.625	20.58 \pm 0.14	2.44 \pm 0.71
31.25	87.68 \pm 0.39	5.70 \pm 1.11
62.5	91.71 \pm 1.24	8.42 \pm 1.71
125	93.32 \pm 2.15	15.95 \pm 0.95
250	94.21 \pm 1.01	32.81 \pm 1.33
500	98.34 \pm 1.34	37.51 \pm 1.15

Results are expressed as means \pm S.E.M., n = 3.

5 Discussion

Diabetes mellitus is a metabolic disorder that usually affects carbohydrate, fat, and protein metabolism, followed by multiorgan injury in the later period, and hyperlipidemia is associated with hyperglycemia (Upendra et al, 2010). More powerful new compounds with pan-target activity and proven long-term safety should be highly effective in a clinical setting for patients with coexisting relevant lipid and glucose metabolic disorders. These discoveries pave the way for the development of drugs for treating chronic multigenic metabolic and cardiovascular diseases, for which therapy is presently insufficient or nonexistent (Toma, 2013). This is the first study to investigate the effect of leaf extract of *Moringa stenopetala* on pancreatic and intestinal enzymes related to antihyperglycemic and antihyperlipidemic activities as well as antiglycation activities.

The presence of phytochemicals in plant products gives a great potential for balancing metabolic disturbances. Several phytomolecules including flavonoids, phenolic compounds, alkaloids, glycosides, saponins, glycolipids, dietary fibres, polysaccharides, peptidoglycans, carbohydrates, amino acids and others obtained from various plant sources have been reported as potent hypoglycemic agents. Flavonoids are a heterogeneous group of ubiquitous plant polyphenols, which exhibit a variety of pharmacological activities, including the anti-atherogenic as well as antihyperglycemic effects, lipoprotein oxidation, blood platelet aggregation and vascular reactivity (Mukherjee et al, 2006). A high content of phytochemicals especially total polyphenolic compounds and total flavonoids may contribute to the pleiotropic effects of *Moringa stenopetala* leaves that support the use of the plant for different metabolic disorders in the local community.

It is well known that inhibition of intestinal α -glucosidase and pancreatic α -amylase activity results in delaying carbohydrate digestion of absorbable monosaccharides, causing reduction of postprandial hyperglycemia. The plant extract showed a weaker pancreatic α -amylase activity compared to intestinal α -glucosidase activity. α -glucosidase inhibitors delay intestinal carbohydrate absorption and slow the sharp rise in blood sugar levels that diabetic patients typically experience after snacks. However, none of the

currently available α -glucosidase inhibitors for clinical use are devoid of severe adverse effects (Kurihara et al, 2011; Sama et al, 2012). The search for new group of agents from natural resources especially from plant medicines has, therefore, become an attractive approach for the treatment of postprandial hyperglycemia. Our study showed that hydroalcoholic leaf extract of *Moringa stenopetala* is a potent inhibitor of α -glucosidase activity, and therefore suggests that extracts of *Moringa stenopetala* could be an attractive source of alternative treatment.

Besides hyperglycemia, diabetes mellitus is highly characterized by elevated levels of triglycerides and cholesterol in the blood highly associated with a modern lifestyle and increase consumption of a high fat diet (Jacobson et al, 2007). Reducing absorption of free fatty acids and free cholesterol by inhibiting pancreatic lipase and pancreatic cholesterol esterase reduces hyperlipidemia associated with diabetes mellitus (Biraria and Bhutani, 2007). In our previous findings it was reported that leaf extract of *Moringa stenopetala* has antihyperlipidemic effects (Toma et al, 2012). This effect could be attributed to the inhibition of pancreatic lipase and pancreatic cholesterol esterase which have been observed in the present study.

Diabetes mellitus is a metabolic disorder that usually affects carbohydrate, fat, and protein metabolism, followed by multiorgan dysfunction in the later period, and hyperlipidemia associated with hyperglycemia (Upendra et al, 2010). Effective novel compounds with pan-target antidiabetic activity and proven long-term safety should be targeted in a clinical setting for patients with coexisting relevant lipid and glucose metabolic disorders. These discoveries pave the way for the development of drugs for treating chronic multigenic metabolic and cardiovascular diseases, for which therapy is presently insufficient or nonexistent (Toma, 2013; Mukherjee et al, 2006). This is the first study to investigate the effect of aqueous ethanol and n-butanol fractions of *Moringa stenopetala* leaves on the regenerative behavior of pancreatic islets of Langerhans after streptozotocin induced damage of the pancreas and on biochemical effect after such treatment.

Diabetes can be induced by pharmacologic, surgical or genetic manipulations in several animal species. Most experiments in diabetes are carried out on rodents, although some

studies are still performed in larger animals. The majority of studies published in the field of ethnopharmacology between 1996 and 2006 employed pharmacological models. Streptozotocin (STZ, 69%) and alloxan (31%) are by far the most frequently used drugs and this model has been useful for the study of multiple aspects of the disease. Both drugs exert their diabetogenic action when they are administered parenterally: intravenously, intraperitoneally or subcutaneously. The dose of these agents required for inducing diabetes depends on the animal species, route of administration and nutritional status. According to the administered dose of these agents, syndromes similar to either type 1, type 2 diabetes mellitus or glucose intolerance can be induced (Lenzen et al., 1996; Mythili et al., 2004).

Streptozotocin (STZ) has been proposed to act as a diabetogenic agent due to its ability to destroy pancreatic beta cells by the generation of excessive free radicals. Streptozotocin enters the pancreatic β -cell via a glucose transporter-GLUT2 and causes alkylation of DNA and impairment in glucose oxidation leading to decreased insulin biosynthesis and secretion (Nakatsuka et al., 1990). In adult rats, 60 mg/kg is the most common dose of STZ to induce insulin dependent diabetes, but higher doses are also used. STZ is also efficacious after intraperitoneal administration of a similar or higher dose, but single doses below 40 mg/kg may be ineffective. In general, rats are considered diabetic if tail blood glucose concentrations in fed animals are greater than 126 mg/dl after STZ injection (Patel et al. 2006). Based on these studies we administered 40mg/dl of STZ in overnight fastened rats to induce type 2 diabetes mellitus.

Streptozotocin (STZ) administration generally causes destruction of β -cells after three days in rats (Aseghate and Ponery, 2002). Pancreatic β -cells are particularly sensitive to damage by nitric oxide and free radicals because of the low levels of free radical scavenging enzymes in the tissue. The results of the present study indicate that the extracts of *Moringa stenopetala* produced regeneration/proliferation of the pancreatic β -cells possibly due to prevention of free radical formation induced by STZ. Since pancreas contains stable (quiescent) β -cells which have regenerative capacity, after damage the surviving cells proliferate by replication to supplant the lost cells (Spinas, 1999). Regenerative pancreatic β -cells can be formed by neogenesis or by replication of the

preexisting differentiated cells; since other medicinal plants have shown β -cell regenerative potential, it is possible that the extracts of *Moringa stenopetala* were also responsible for the proliferation of β -cells and the recovery of normal pancreatic morphology as shown in figure 1. Hence this implies that extracts of *Moringa stenopetala* were also responsible for the proliferation of β -cells as there are already reports showing extracts of other medicinal plants which have a β -cell regenerative potential (Yordav et al, 2014). This other medicinal plant material has also shown neovascularization activity in the pancreatic islets of Langerhans and improved stromal fibrosis of pancreatic cells in histopathological investigations.

The presence of phytochemicals in plant products offers a great potential for balancing metabolic disturbances. Several phytomolecules, including flavonoids, total phenolic compounds, alkaloids, glycosides, saponins, glycolipids, dietary fibres, polysaccharides, peptidoglycans, carbohydrates, amino acids and others obtained from various plant sources, have been reported as potent hypoglycemic and antihyperglycemic agents. Flavonoids are a heterogeneous group of ubiquitous plant polyphenols, which exhibit a variety of pharmacological activities, including the anti-atherogenic as well as antihyperglycemic effects, lipoprotein oxidation, blood platelet aggregation and vascular reactivity (Brahmachari, 2011; Kumar et al, 2010). A high content of phytochemicals, especially total polyphenolic compounds and total flavonoids, may contribute to the pleiotropic effects of *Moringa stenopetala* leaves that support the use of the plant for different metabolic disorders in the local community (Toma et al, 2014). A high content of total phenolic compounds and flavonoids may have a significant role in regulating metabolic disturbances which are highly related to diabetes mellitus and its complications due to its protective effect on the pancreas and other essential organs.

Minerals such as chromium and zinc are often reduced in diabetics, and supplementation may improve glycaemic control in cases of mineral deficiency (Bailey C., 1999). Chromium deficiency is common in type 2 diabetes, especially the elderly, and chromium supplementation have improved glycaemic control, without increasing insulin concentrations (Anderson R., et al., 1997). Adequate chromium is necessary for normal insulin sensitivity, but the site of action of chromium is unresolved. Therapeutic

indication for zinc supplements is not clear but high concentration of zinc can exert various insulin-like effects *in vitro* and *in vivo*, but the mechanisms are not established. However, zinc has been reported to protect against β -cell damage and to improve glycaemic control in diabetic patients with liver disease (Ohly P., et al., 2000). As further noted, the leaves have distinctive strong, mustard-like taste, contain calcium, iron, and other trace minerals, and are eaten as a supplement to the major staple foods (Mekonnen, 2005). It is also described by Abuye *et al.* (2003) that raw leaves of *Moringa stenopetala* contain minerals such as potassium, iron, zinc, phosphorus and calcium in significant amount. The presence of these minerals may also contribute to the antihyperglycemic and pancreatoprotective effects of the plant material in this study.

The most common lipid abnormalities in diabetes are hypertriglyceridemia and hypercholesterolemia (Jacobson et al, 2007). Repeated administration of *Moringa stenopetala* leaves extract and fraction for 14 days significantly decreased hypertriglyceridemia and hypercholesterolemia. The observed antihyperlipidemic effect may be due to decreased cholesterologenesis and fatty acid synthesis through inhibition of pancreatic cholesterol esterase and pancreatic lipase inhibition effect, respectively (Birari and Bhutani, 2007; Heidrich et al, 2004). The HDL cholesterol level was significantly improved by *Moringa stenopetala* leaf extract treatment. In our previous reports we have shown that this plant material had inhibitory effects on pancreatic cholesterol esterase and pancreatic lipase (Toma et al, 2014) which may contribute to antihyperlipidemic activities in animal models.

Enzymes indicating liver damage, such as AST, GGT, ALT and ALP level increased in diabetic rats. The elevated serum level of these enzymes was significantly reduced by *Moringa stenopetala* treatment. There is evidence that the diabetic complications such as increased gluconeogenesis and ketogenesis may be due to elevated enzymes (Shanmugasundaram et al, 1983). The restoration of transaminases to their normal levels after treatment indicates revival of insulin secretion and regenerative activities of islets of Langerhans cells of pancreas after administration of the plant material. *Moringa stenopetala* also improved renal functions in diabetic rats by reducing serum urea and

creatinine levels. Our results demonstrate that *Moringa stenopetala* is able to normalize vital organs function in rats.

Streptozotocin has been shown to induce free radical production and cause tissue injury. Previous studies on histopathological investigation of organs such as liver and kidney have shown that *Moringa stenopetala* was non-toxic (Ghebresselasie et al, 2011). The pancreas is especially susceptible to the action of streptozotocin induced free radical damage. *Moringa stenopetala* leaves are rich in total polyphenolic compounds and flavonoid and have an *in vitro* antioxidant effect (Sileshi et al, 2014; Toma et al, 2014). This antioxidant activity of the plant may protect the organ from free radicals. Various studies have shown that diabetes is associated with increasing formation of free radicals and decreasing antioxidant potential (Basha and Subramamian, 2011). The effect of plant material as renoprotective, hepatoprotective and pancreatoprotective parameters may be highly associated with its antioxidant activity.

Accumulation of AGEs has been found in healthy aging persons, and this accumulation is higher when glucose concentrations get higher. Microvascular and macrovascular damage, seen in diabetes, is attributed to the accumulation of AGEs in tissues, but it is also associated with atherosclerosis, Alzheimer's disease, end stage renal disease, rheumatoid arthritis, sarcopenia, cataracts, and other degenerative ophthalmic diseases, Parkinson's disease, vascular dementia and several other chronic diseases (Ardestani and Yazdanparast, 2007).

Protein glycation involves a series of complex reactions that occur between monosaccharides (glucose and fructose) and amino acids or proteins, which produce an unstable Schiff base, and then form an Amadori product such a fructosamine (Adisakwattana et al, 2012). During the propagation reaction, the Amadori products react with the amino acids to form irreversible AGEs. According to the data obtained from this study, the extract showed the strongest anti-glycating inhibition.

In the present study the results on the formation of the AGEs showed that *Moringa stenopetala* extract efficiently inhibited fluorescent and non-fluorescent AGE formation. Furthermore, it also reduced the level of fructosamine and amyloid cross β -structure in

fructose-glycated BSA. A significant increase of protein carbonyl content and oxidation of thiols in BSA were seen when the protein was glycated by fructose. When *Moringa stenopetala* extract was added to the same systems, it significantly suppressed these processes. Our previous study indicates that *Moringa stenopetala* extract has high content of polyphenolic compounds (Toma et al, 2014). Several major mechanisms by which polyphenolic compounds block the carbonyl group in reducing sugars and break the cross linking structure in the formed AGEs have recently been proposed for antiglycation activity (Wu et al, 2011). The reduction of free radical generation by antioxidant activity of polyphenols may highlight other mechanisms for the prevention of AGE formation (Wu et al, 2011; Chiti and Dobson, 2006). Hence, the antiglycation activities observed in this study may be due to the presence of high content of phytochemicals such as polyphenolic compounds in the plant materials.

Glycation is a key mechanism to induce the conformational changes of protein by increasing the level of amyloid cross β -structure, which plays a fundamental role in the protein aggregation. Studies reveal that the deposition of protein aggregation has been associated with the progression of several debilitating degenerative diseases including hemodialysis amyloidosis, diabetes, Parkinson's disease and Alzheimer's disease (Chiti and Dobson, 2006). Notably, accumulation of protein aggregation causes pancreatic islet amyloidosis, which directly induces β -cell damage and impaired insulin secretion (Chiti and Dobson, 2006, Mossina et al, 1999). Our data clearly establish that the plant extract suppress in the level of amyloid cross β -structure of BSA. This beneficial effect of hydroalcoholic extract of *Moringa stenopetala* may help reduce a risk of developing debilitating degenerative diseases in diabetic and other neurodegenerative patients.

Glycation and AGE-induced toxicity are known to be associated with increased free radical production (Mossina et al, 1999). In particular, the process of oxidative degradation of Amadori intermediates can generate free radicals, causing damage by oxidizing proteins (Claudia and Karen, 2010). Major molecular modifications of protein structural changes can be investigated by protein carbonyl formations and the loss of protein thiol groups which are associated with free radical generations (Mossina et al, 1999; Claudia and Karen, 2010). The marked increase in protein carbonyl formation and

the oxidation of thiols in BSA were observed in fructose incubation. Our findings show that the addition of the test material together with fructose to a BSA solution significantly suppresses the protein carbonyl formation and oxidation of thiols. Hydroalcohol extract of *Moringa stenopetala* leaves have, therefore, a potential to ameliorate glycation induced damage to thiols and to decrease carbonyl content invitro.

There is considerable evidence to support the argument that trapping free radicals and reactive carbonyl group formation by antioxidant compounds is a strategy for inhibition of protein glycation (Caengprasath, 2013; Azu et al, 2010). Since the present findings revealed that the plant material has antioxidant activity, the antioxidant(s) may contribute to antiglycation activity of the plant extract. Biochemical mechanisms of anti-glycation reactions have been recently proposed besides antioxidant activity to reduce glycation, such as breaking the cross-linking structures in AGEs that have been formed, blocking the carbonyl or dicarbonyl groups in reducing sugars, Schiff bases or Amadori and inhibiting the formation of late-stage Amadori products (Caengprasath, 2013; Upendra et al, 2010).

In diabetes and Alzheimer's disease, an imbalance in the concentration of zinc and micronutrients such as ascorbic acid is suggested as a causative factor (Caujungco and Faget, 2003). The antioxidant and antiglycation role of zinc and ascorbic acid is well established, though there are many contradictory reports on beneficial or deleterious effects of Zinc and ascorbic acid supplementation in Alzheimer's disease and AGE-related diseases (Caujungco and Faget, 2003; Rashami and Vaishali, 2010). The plant material has been reported for its rich content of zinc and ascorbic acid (Abuye et al, 2003) that may contribute to its antiglycation activities observed in this study.

The phenolic compounds have aromatic rings containing one or more hydroxyl groups, while polyphenols contain multiple phenol rings with in their structures. All classes of phenols possess antioxidant activities, depending on the number of hydroxyl groups present on the benzene ring (packer et al., 2002). Recently, research interest has increased in finding antioxidants that are naturally-occurring for use in food stuffs and medicinal materials, to replace the synthetic ones which are restricted due to their side effects.

Furthermore, natural antioxidants have the capacity to improve food quality and stability; they also can be used as nutraceuticals to terminate free radical chain reactions in biological systems and may thus provide improved health benefits for the consumers. A number of experimental models have been developed for the determination of antioxidant activities (Vayaetal., 2001). High content of the phenolic compounds besides having antioxidant capacity of the plant material may contribute pleiotropic effects observed in our different studies carried out for antihyperglycemic, antihyperlipidemic and antiglycation activities from the extract of the *Moringa stenopetala* leaves.

6 Summary and recommendations

6.1 Summary

The results demonstrate that inhibition of intestinal α -glucosidase by extracts of *Moringa stenopetala* may contribute to antihyperglycemic activity. Extracts of *Moringa stenopetala* also showed antihyperlipidemic activity due to the inhibition of lipase and cholesterol esterase enzymes. Thus *Moringa stenopetala* could be used for prevention/treatment of hyperglycemia and hyperlipidemia.

These findings demonstrated that aqueous extract of *Moringa stenopetala* leaves and its butanol fraction possess antihyperglycemic and antihyperlipidemic properties, and alleviates streptozotocin induced pancreatic damage. Thus plant material of *Moringa stenopetala* leaves use for prevention/treatment of hyperglycemia and hyperlipidemia could be associated with beta cell regenerative potential.

The findings demonstrated that *Moringa stenopetala* leaves effectively protect BSA from fructose-mediated protein glycation *in vitro*. The extract of the leaves also reduced the level of fructosamine, the formation of CML and the amyloid cross β -structure in BSA. In addition, the extract significantly decreased the protein carbonyl content and increased the level of protein thiol, which could be due to free radical scavenging potential of the plant material. Therefore, the *M. stenopetala* leaves have the potential to be used as supplement in the prevention/management of AGE-mediated diseases, particularly for those that are at risk of diabetic complications.

6.2 Recommendations

- ✚ Further studies are required to identify the lead compound(s) present in *Moringa stenopetala* with its (their) molecular mechanism of action on PPAR, insulin sensitization and other insulin targets based on the pathophysiology of diabetes mellitus.
- ✚ Since the present study showed the blood glucose, blood lipid and glycation lowering effects of *Moringa stenopetala* on *invivo* and *invitro* model, prevalence study of DM is recommended in the indigenous people where *Moringa stenopetala* is used as food to evaluate the effect of the plant on the blood glucose of that population.
- ✚ Priority should be given by government, NGOs, researchers and local communities to standardize the products of *Moringa stenopetala* in order to develop safe, effective, and accessible products for prevention/treatment of diabetes mellitus and its complications
- ✚ Since this study and previous studies on this plant material addressed the basic requirements according to WHO recommendation to start clinical trial from the medicinal food products, clinical trial should be initiated.

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8. Annex

8.1 Published articles emanating from the PhD thesis

8.2. Manuscript under Submission emanating from the PhD thesis

Extract of *Moringa stenopetala* leaves alleviates fructose-induced protein glycation *In vitro*

Alemayehu Toma^{1,2*}, Eyasu Makonnen¹, Yelamtsehay Mekonnen³, Asfaw Debella⁴,
Sirichai Adisakwattana⁵

Abstract

Background: *Moringa stenopetala* leaves have shown a variety of pharmacologic properties including effect on diabetes and dyslipidemia. However, little is known about the antiglycation properties of hydroalcoholic extract of *Moringa stenopetala* leaves. The present study was carried out to characterize the protein glycation inhibitory activity of hydroalcoholic extract of *Moringa stenopetala* leaves in a bovine serum albumin (BSA)/fructose system.

Methods: The antioxidant activity was measured using 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) assay. Antiglycation activity was determined with the formation of advanced glycation end products (AGE), fluorescence intensity, N^ε-(carboxymethyl) lysine (CML), the level of fructosamine, and the formation of amyloid cross β -structure. The protein oxidation was examined using the level of protein carbonyl content and thiol group

Results: The results demonstrated that *Moringa stenopetala* leaves significantly inhibited the formation of AGEs by approximately 54.75 \pm 0.94% at a concentration of 2mg/ml. Furthermore, *Moringa stenopetala* leaves reduced the level of fructosamine, the formation of N^ε-(carboxymethyl) lysine (CML), and the level of amyloid cross β -structure. The leaves also prevented oxidative protein damage, including effects on protein carbonyl formation, thiol oxidation of BSA and demonstrated antioxidant activity in DPPH assay.

Conclusions: The findings suggest the importance of *Moringa stenopetala* leaves for preventing AGE-mediated diabetic complications.

Keywords: *Moringa stenopetala*; protein glycation; advanced glycation end products; diabetic complications; fructose

Background

Chronic hyperglycaemia causes the Maillard reaction in which reducing sugars, such as glucose and fructose, react non-enzymatically with amino groups of proteins through a series of reactions, ultimately forming advanced glycation end products (AGE), triggering several non-communicable diseases (Cohen et al, 2007). It has been clearly demonstrated that the accumulation of AGEs in body tissue is the leading cause of several age-related degeneration, atherosclerosis and diabetic complications such as retinopathy, nephropathy and neuropathy (Khazaei et al, 2010; Schmidt and Stern, 2000). Glycated albumin comprises about 6–15 % of the total albumin in normal individuals and rises to 32 –40 % in hyperglycaemia. These modifications affect the properties of albumin in several ways, including altered conformation and consequently altered binding. Diabetes mellitus, liver diseases and nephropathy are just a few disorders in which altered albumin functions have been described (Schmidt and Stern, 2000; Singh et al, 2001)

Moringa stenopetala (Baker f) *Cufodontis* belongs to family Moringaceaeis commonly grown in Southern parts of Ethiopia (Edward et al, 2002). The leaves of *Moringa stenopetala* are cooked and eaten as vegetables and the leaves and roots are used to treat malaria, diabetes, asthma, repelled placenta, hypertension and gastrointestinal problems (Mekonnen and Gessese, 1998, Mengistu et al, 2012). It has been reported that *Moringa stenopetala* leaves and roots showed antitrypanosomal activity (Mekonnen et al, 1999). The antispasmodic effects of the leaves on smooth muscle tissues and antibiotic properties of the seeds (Mekonnen and Gessese, 1998, Mekonnen, 1999) have also been reported. The crude aqueous extract of the leaves demonstrated hypoglycemic activity (Makonnen et al, 1997). The crude aqueous/ethanol extract and fractions of the leaves of *Moringa stenopetala* have been reported to have both hypoglycemic and antihyperglycemic effect (Mussa et al, 2008; Nardos et al, 2011). Moreover, chronic administration of the n-butanol fraction of ethanol extract of *Moringa stenopetala* leaves in alloxan-induced diabetic mice showed antihyperglycemic and antihyperlipidemic

effects with wide margins of safety, indicating its potential for long term management of diabetes (Toma et al, 2012; Sileshi et al, 2014; Toma et al, 2014).

The present study was, therefore, carried out to determine the inhibitory effect of hydroalcoholic extract of the leaves of *Moringa stenopetala* against bovine serum albumin (BSA) in fructose-mediated non-enzymatic glycation. Moreover, we also examined the inhibitory effect of hydroalcoholic extract of the leaves of *Moringa stenopetala* on oxidation-dependent damages to BSA induced by fructose. Finally, the ability of *Moringa stenopetala* to inhibit the formation of the N^ε-(carboxymethyl) lysine (CML), non fluorescent major chemical advanced glycation structure is investigated.

Materials and Methods

Chemicals and Reagents

Congo red, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), nitroblue tetrazolium (NBT), 1-deoxy-1-morpholino-D-fructose (DMF), L-cysteine, and aminoguanidine (AG) were obtained from Sigma (St. Louis, MO, USA). 2, 4-dinitrophenylhydrazine (DNPH) was purchased from Ajax Finechem (Taren Point, Australia). Trichloroacetic acid (TCA) and guanidine hydrochloride were purchased from Merck (Darmstadt, F.R., Germany) and Fluka (Steinheim, Germany), respectively. OxiSelect™ N^ε-(carboxymethyl) lysine (CML) ELISA kit was purchased from Cell Biolabs (San Diego, CA, USA). All other chemicals used were of analytical grade.

Collections and preparation of plant materials

The leaves of *Moringa stenopetala* were collected from Gamo Gofa Zone, South Nation's Nationalities Peoples Region, 520 kilometers south of Addis Ababa. The plant was then identified and authenticated by a taxonomist, and deposited in the Herbarium of Ethiopian Public Health Institute (EPHI) with a voucher number 'AL-001'. It was then dried under shade and crushed to powder for extraction.

Preparation of plant material extract

The powdered leaves (1.2 Kg) were extracted by percolation using 70 % (v/v) ethanol, and the mixture was then filtered using Whatmann filter paper no. 1. The extract was dried by evaporation using rotary vaporizers under reduced pressure at a temperature of 40-45°C. The residue filtrate obtained was then dried by steam bath at 40°C and kept in a refrigerator at 8 °C. The yield of the aqueous ethanol extract was 20.1 % in weight by weight (w/w).

***In Vitro* Glycation of Bovine Serum Albumin (BSA)**

The formation of glycated BSA was done according to a previous method with minor modifications (Sharma et al, 2002). BSA (10 mg/mL) was incubated with fructose (0.5 M) in 0.1 M phosphate buffer (PBS), pH 7.4 containing 0.02% sodium azide (NaN₃) at 37°C for 2 weeks in the absence or presence of *Moringa stenopetala* leaf extract (0.5, 1, and 2mg/ml) and aminoguanidine (1mg/ml). Dimethylsulfoxide (DMSO, 4%) was used as a solvent for this study. Aliquots of the reaction mixtures were then assayed for AGEs formation, fructosamine, protein carbonyl content, thiol group, amyloid cross β structure, and CML.

Determination of AGEs Formation

The formation of AGEs was determined with spectrofluorometer (Wallac 1420 Victor 3 V, PerkinElmer, Walham, MA, USA) at excitation and emission wavelengths of 355 and 460 nm, respectively. The inhibitory effect of extract and aminoguanidine was expressed as percentage inhibition compared with maximum glycation elicited by fructose.

The percentage of fluorescent AGE formation was calculated as follows:

Inhibition of fluorescent AGEs %

$$= [(FC-FCB) - (FS-FSB) / (FC-FCB)] \times 100$$

Where FC and FCB were the fluorescent intensity of control with fructose and blank of control without fructose, FS and FSB were the fluorescent intensity of sample with fructose and blank of sample without fructose.

Fructosamine Measurement

The concentration of the Amadori product fructosamine was measured by NBT assay (Ardestani and Yazdanparast, 2007). The glycated BSA (10 μ L) was incubated with 100 μ L of 0.5 mM NBT in 0.1 M carbonate buffer, pH 10.4 at 37 °C. The absorbance was measured at 530 nm at 10 and 15 min time points. The concentration of fructosamine was calculated compared to DMF, the standard.

Determination of N^ε-(carboxymethyl) Lysine (CML)

After two weeks of incubation, N^ε-(carboxymethyl) lysine (CML), a major antigenic AGE structure, was determined using enzyme linked immunosorbant assay (ELISA) kit. According to the manufacturer's protocol, the glycated samples were diluted to final concentration of 1 μ g/ml (10,000-fold dilution) before used in the assay. Each diluted sample (100 μ l) was incubated in the 96-well protein binding plate at 37°C for at least 2 h. After washing with PBS, an assay diluent was added and further incubated for 2 h at room temperature on an orbital shaker. Three washes with wash buffer were needed before incubating for 1 h each with anti-CML antibody and with secondary antibody HRP conjugate. The substrate solution (100 μ l) was added for 20 min before adding stop solution in an equal volume. The absorbance of samples was measured immediately at 450 nm and compared with the absorbance of CML-BSA standard providing in the assay kit (Adisakwattana et al, 2012).

Determination of Protein Carbonyl Content

The carbonyl group in glycated BSA, a marker for protein oxidative damage, was assayed according to the method of Levine and colleagues with minor modifications (Wu et al, 2011). Four hundred μ L of 10 mM DNPH in 2.5 M HCl was added to 100 μ L of glycated samples. After 1 h incubation in the dark, 500 μ L of 20% (w/v) TCA was used for protein precipitation (5 min on ice) and then centrifuged at 10,000 g for 10 min at 4 °C. The protein pellet was washed with 500 μ L of ethanol/ethyl acetate (1:1) mixture three times and re-suspended in 250 μ L of 6 M guanidinehydrochloride. The absorbance was measured at 370 nm. The carbonyl content of each sample was calculated based on

the extinction coefficient for DNPH ($\epsilon = 22,000 \text{ M}^{-1} \text{ cm}^{-1}$). The results were expressed as nmol carbonyl/mg protein.

Thiol Group Estimation

The free thiols in glycated samples were measured by Ellman's assay with minor modifications (Adisakwattana et al, 2012). Seventy μL of glycated samples were incubated with 5 mM DTNB in 0.1 M PBS, pH 7.4 at 25 °C for 15 min. The absorbance of samples was measured at 410 nm. The concentration of free thiols was calculated from L-cysteine standard and expressed as nmol/mg protein.

Determination of protein aggregation

Amyloid cross β -structure, a common marker for protein aggregation was measured using a congo red assay according to a previous published method with minor modifications (Chiti and Dobson, 2006). Briefly, the glycated BSA (50 μL) was incubated with 50 μL of 100 μM congo red in 10% (v/v) ethanol/PBS for 20 min at 25°C. The absorbance was measured at 530 nm.

DPPH Radical Scavenging Activity Assay

The free radical scavenging activity of the extract was measured in vitro by 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) assay according to the method described earlier. The stock solution was prepared by dissolving 24 mg DPPH with 100 ml methanol and stored at 20°C until required. The working solution was obtained by diluting DPPH solution with methanol to attain an absorbance of about 0.98 ± 0.02 at 517 nm using the spectrophotometer. A 3 ml aliquot of this solution was mixed with 100 μl of the sample at various concentrations (10 - 500 $\mu\text{g}/\text{ml}$). The reaction mixture was shaken well and incubated in the dark for 15 min at room temperature. Then the absorbance was taken at 517 nm. The control was prepared as above without any sample. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as the following equation:

$$\text{Scavenging effect (\%)} = [(A \text{ cont.} - A \text{ test}) / A \text{ cont.}] \times 100$$

Where, A cont is the absorbance of control reaction and A test is the absorbance in the presence of extract (Mishara et al, 2009).

Statistical Analysis

All data are presented as means \pm SEM. Statistical significance was evaluated using one-way ANOVA. A Duncan post-hoc comparison was used to analyze the sources of significant differences. A p-value < 0.05 was considered statistically significant.

Results

Antiglycation activity of hydroalcoholic extract of *Moringa stenopetala* leaves.

Figure 1 shows the effect of hydroalcoholic extract of *Moringa stenopetala* leaves at the concentration of 0.5mg/ml, 1mg/ml and 2mg/ml on the total AGEs formation during 14 days of incubation. The fluorescent intensity of BSA incubated with fructose significantly increased about 5.1-fold when compared to BSA, indicating progressive formation of AGEs. When the glycation occurred in the presence of test material, it was observed that *Moringa stenopetala* significantly reduced the formation of AGEs by $54.75 \pm 0.94\%$ at the concentration of 2mg/ml, as compared to BSA incubated with fructose but less potent than AG ($60.25 \pm 1.13\%$), which was used as control.

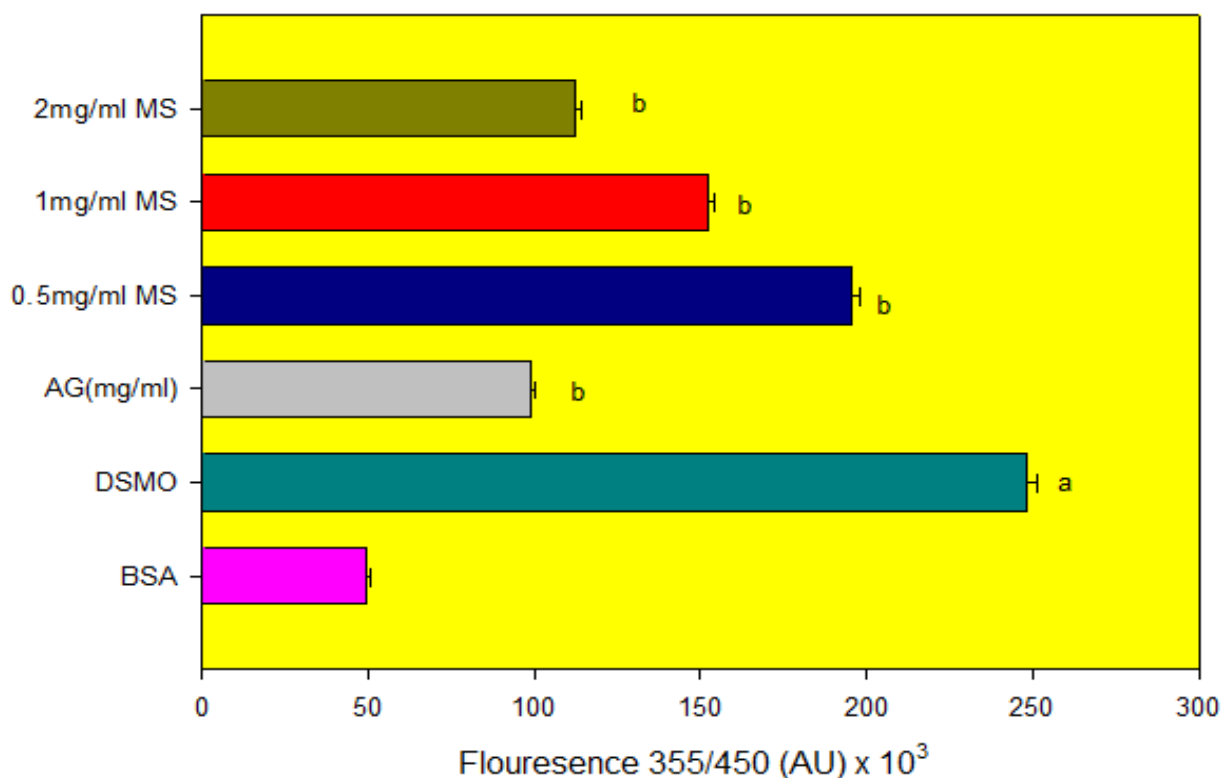


Figure 1: The effects of hydroalcoholic extract of *Moringa stenopetala* (MS) at different concentration and aminoguanidine (AG, 1 mg/ml) on fluorescent AGEs formation in the BSA/fructose system. Each value represents the mean \pm SEM (n=3). ^a P < 0.05 compared to BSA, ^b P < 0.05 compared to DMSO.

The effect of *Moringa stenopetala* leaves on protein carbonyl content.

Figure 2 shows the effect of the extract of *Moringa stenopetala* on protein-bound CML formation. The results showed that the formation of CML in BSA incubated with fructose was significantly 3.64-times higher than BSA incubated without fructose. The addition of *Moringa stenopetala* leaves extract at the concentration of 2mg/ml to the solution reduced CML-derived AGE by approximately $19.25 \pm 0.18\%$, whereas AG inhibited the formation of CML by 45.46% when compared to BSA incubated with fructose.

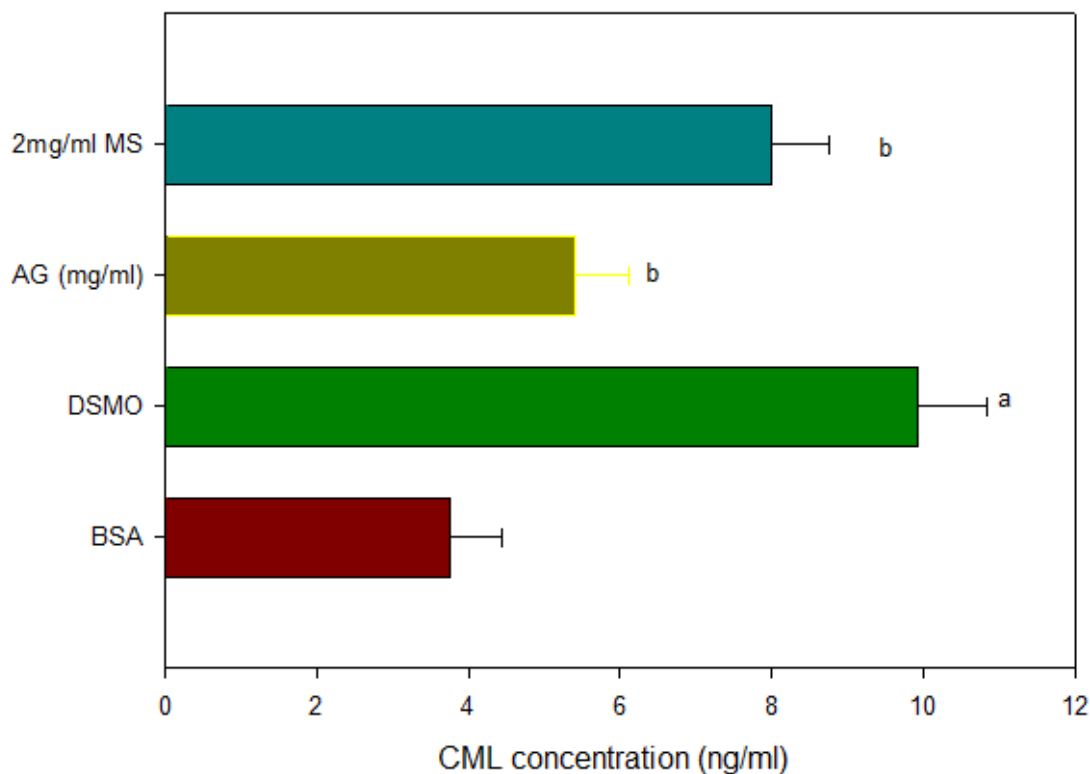


Figure 2: The effects of hydroalcoholic extract of *Moringa stenopetala* (MS) at different concentration and aminoguanidine (AG, 1mg/ml) on the level of N^ε-(carboxymethyl) lysine (CML) in the BSA/fructose system. Each value represents the mean \pm SEM (n=3). ^aP < 0.05 compared to BSA, ^bP < 0.05 compared to DMSO.

The effect of *Moringa stenopetala* leaves on the level of fructosamine.

After day 14 of the experiment, the level of fructosamine in BSA incubated with fructose produced a 3.3-fold increase compared to BSA (Figure 3). The results showed that hydroalcoholic extract of *Moringa stenopetala* significantly decreased the level of fructosamine by about $36.1 \pm 0.11\%$.

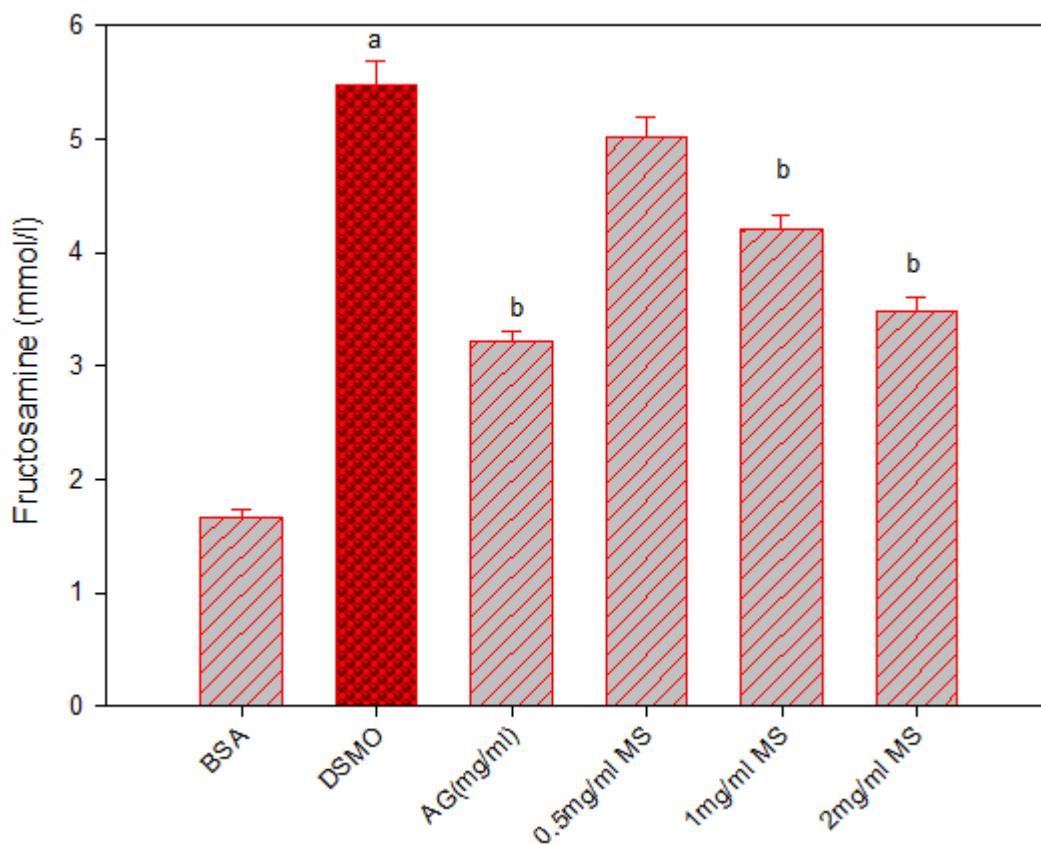


Figure 3: The effects of hydroalcoholic extract of *Moringa stenopetala* (MS) at different concentration and aminoguanidine (AG, 1mg/ml) on the level of fructosamine in the BSA/fructose system. Each value represents the mean \pm SEM (n=3). ^a P < 0.05 compared to BSA, ^b P < 0.05 compared to DSMO.

The effect of *Moringa stenopetala* leaves on beta-sheet formation.

To confirm the observation that the plant material inhibits beta-sheet formation of albumin experimentally, Congo red, beta-sheet-specific dye, was used as shown in Figure 4.5. With this assay, the positive albumin control exhibited the strongest absorbance as expected and all the treatment groups showed significant reduction in absorbance (P < 0.05).

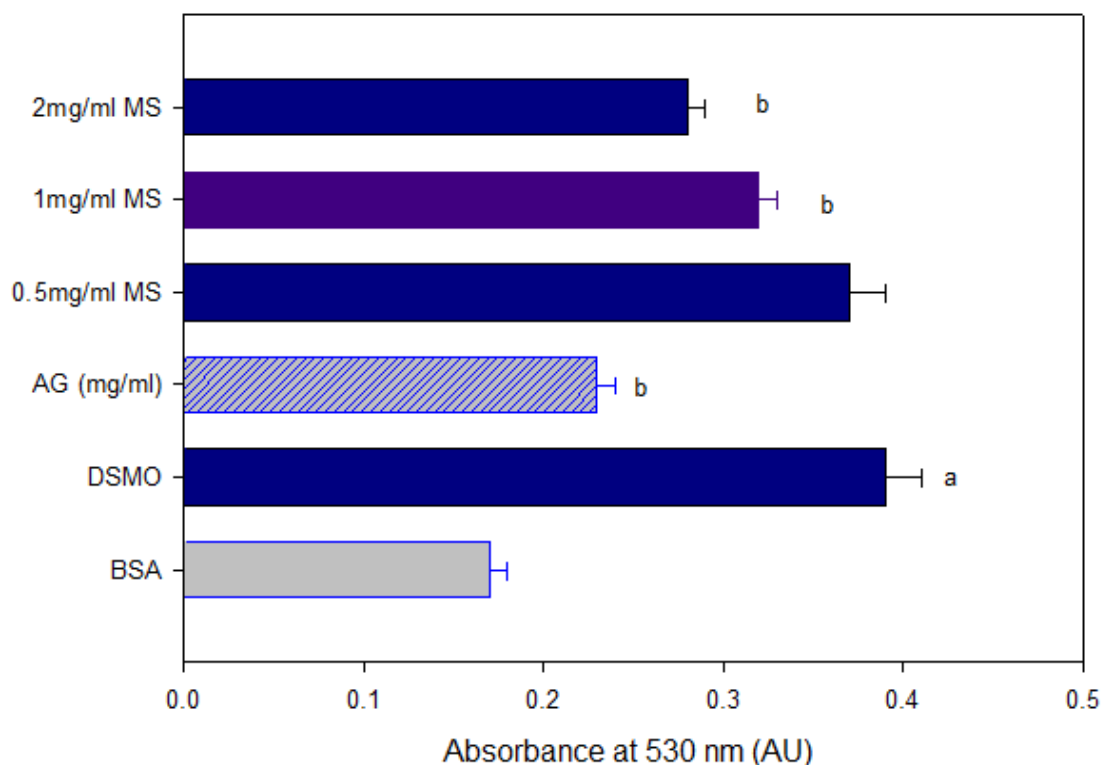


Figure 4: The effects of hydroalcoholic extract of *Moringa stenopetala* (MS) at different concentration and aminoguanidine (AG, 1mg/ml) on absorbance of Congo red in the BSA/fructose system. Each value represents the mean \pm SEM (n=3). ^aP < 0.05 compared to BSA, ^bP < 0.05 compared to DMSO.

The effect of *Moringa stenopetala* leaves on protein thiol group.

Figure 5 shows the results for available free thiol groups of BSA on *Moringa stenopetala* leaves extract. A significant decrease in free thiol groups was observed in BSA incubated with fructose, indicating that protein glycation modified thiol groups to form disulfide in BSA. It was found that the plant material significantly reduced the oxidation of thiol groups by approximately 19.4%, at a concentration of 2mg/ml whereas AG protected the loss of protein thiol groups about 23.6% at a concentration of 1mg/ml, as compared to BSA incubated with fructose.

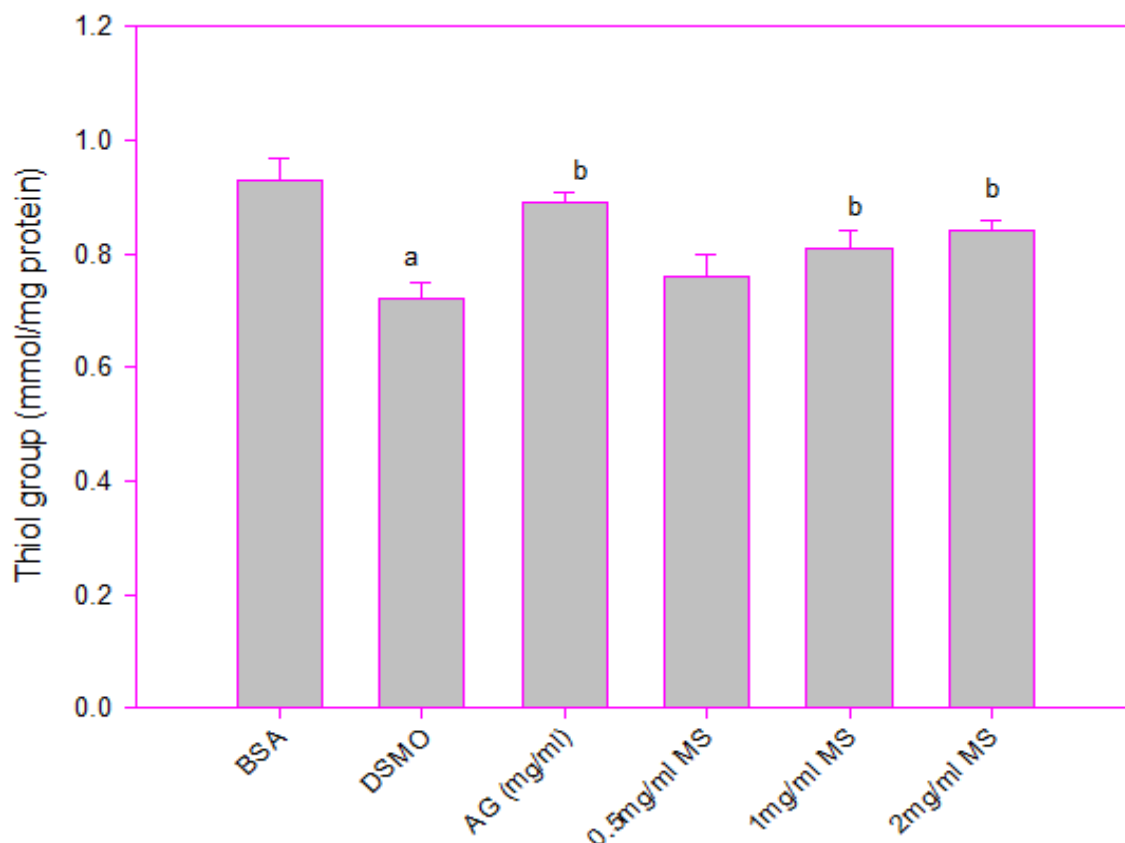


Figure 5: The effects of hydroalcoholic extract of *Moringa stenopetala* (MS) at different concentration and aminoguanidine (AG, 1mg/ml) on thiol content in the BSA/fructose system. Each value represents the mean \pm SEM (n=3). ^aP < 0.05 compared to BSA, ^bP < 0.05 compared to DMSO.

The effect of *Moringa stenopetala* leaves on protein carbonyl content.

The addition of fructose to the BSA solution for 14 days significantly increased the extent of protein carbonyl formation, compared to BSA in the absence of fructose. The result indicated that *Moringa stenopetala* leaves suppressed protein carbonyl formation by approximately 18.7% when compared to BSA incubated with fructose. In addition, AG reduced protein carbonyl formation in BSA incubated with fructose by 28.3% (Figure 6).

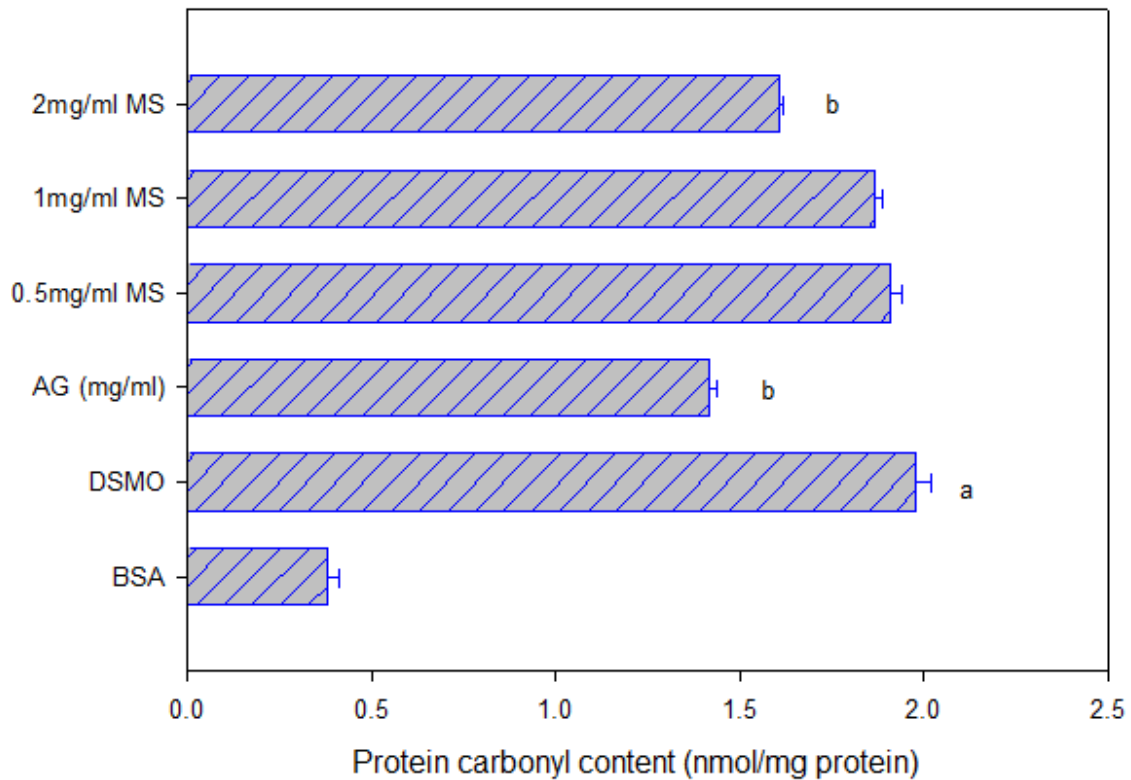


Figure 6: The effects of hydroalcoholic extract of *Moringa stenopetala* (MS) at different concentration and aminoguanidine (AG, 1mg/ml) on Protein carbonyl content in the BSA/fructose system. Each value represents the mean \pm SEM (n=3). ^a P < 0.05 compared to BSA, ^b P < 0.05 compared to DSMO

Antioxidant activity of *Moringa stenopetala* leaves.

In order to determine the extent of scavenging effect, ethanolic extract of the leaves of *Moringa stenopetala* was tested for antioxidant activity using 1, 1-diphenyl-2-picryl hydrazyl (DPPH) free radical. The extract showed antioxidant activity as described in Table 1.

Table 1: DPPH % radical scavenging activity of ethanolic extract of *Moringa stenopetala* leaves

Concentration ($\mu\text{g/ml}$)	%inhibition DPPH Ascorbic acid	of Ethanolic extract
15.625	20.58 \pm 0.14	2.44 \pm 0.71
31.25	87.68 \pm 0.39	5.70 \pm 1.11
62.5	91.71 \pm 1.24	8.42 \pm 1.71
125	93.32 \pm 2.15	15.95 \pm 0.95
250	94.21 \pm 1.01	32.81 \pm 1.33
500	98.34 \pm 1.34	37.51 \pm 1.15

Results are expressed as means \pm S.E.M., n = 3.

Discussion

Accumulation of AGEs has been found in healthy aging persons, and this accumulation is higher when glucose concentrations get higher. Microvascular and macrovascular damage, seen in diabetes, is attributed to the accumulation of AGEs in tissues, but it is also associated with atherosclerosis, Alzheimer's disease, end stage renal disease, rheumatoid arthritis, sarcopenia, cataracts, and other degenerative ophthalmic diseases, Parkinson's disease, vascular dementia and several other chronic diseases (Ardestani and Yazdanparast, 2007).

Protein glycation involves a series of complex reactions that occur between monosaccharides (glucose and fructose) and amino acids or proteins, which produce an unstable Schiff base, and then form an Amadori product such a fructosamine (Adisakwattana et al, 2012). During the propagation reaction, the Amadori products react with the amino acids to form irreversible AGEs. According to the data obtained from this study, the extract showed the strongest anti-glycating inhibition.

In the present study the results on the formation of the AGES showed that *Moringa stenopetala* extract efficiently inhibited fluorescent and non-fluorescent AGE formation. Furthermore, it also reduced the level of fructosamine and amyloid cross β -structure in

fructose-glycated BSA. A significant increase of protein carbonyl content and oxidation of thiols in BSA were seen when the protein was glycated by fructose. When *Moringa stenopetala* extract was added to the same systems, it significantly suppressed these processes. Our previous study indicates that *Moringa stenopetala* extract has high content of polyphenolic compounds (Toma et al, 2014). Several major mechanisms by which polyphenolic compounds block the carbonyl group in reducing sugars and break the cross linking structure in the formed AGEs have recently been proposed for antiglycation activity (Wu et al, 2011). The reduction of free radical generation by antioxidant activity of polyphenols may highlight other mechanisms for the prevention of AGE formation (Wu et al, 2011; Chiti and Dobson, 2006). Hence, the antiglycation activities observed in this study may be due to the presence of high content of phytochemicals such as polyphenolic compounds in the plant materials.

Glycation is a key mechanism to induce the conformational changes of protein by increasing the level of amyloid cross β -structure, which plays a fundamental role in the protein aggregation. Studies reveal that the deposition of protein aggregation has been associated with the progression of several debilitating degenerative diseases including hemodialysis amyloidosis, diabetes, Parkinson's disease and Alzheimer's disease (Chiti and Dobson, 2006). Notably, accumulation of protein aggregation causes pancreatic islet amyloidosis, which directly induces β -cell damage and impaired insulin secretion (Chiti and Dobson, 2006, Mossina et al, 1999). Our data clearly establish that the plant extract suppress in the level of amyloid cross β -structure of BSA. This beneficial effect of hydroalcoholic extract of *Moringa stenopetala* may help reduce a risk of developing debilitating degenerative diseases in diabetic and other neurodegenerative patients.

Glycation and AGE-induced toxicity are known to be associated with increased free radical production (Mossina et al, 1999). In particular, the process of oxidative degradation of Amadori intermediates can generate free radicals, causing damage by oxidizing proteins (Claudia and Karen, 2010). Major molecular modifications of protein structural changes can be investigated by protein carbonyl formations and the loss of protein thiol groups which are associated with free radical generations (Mossina et al, 1999; Claudia and Karen, 2010). The marked increase in protein carbonyl formation and

the oxidation of thiols in BSA were observed in fructose incubation. Our findings show that the addition of the test material together with fructose to a BSA solution significantly suppresses the protein carbonyl formation and oxidation of thiols. Hydroalcohol extract of *Moringa stenopetala* leaves have, therefore, a potential to ameliorate glycation induced damage to thiols and to decrease carbonyl content invitro.

There is considerable evidence to support the argument that trapping free radicals and reactive carbonyl group formation by antioxidant compounds is a strategy for inhibition of protein glycation (Caengprasath, 2013; Azu et al, 2010). Since the present findings revealed that the plant material has antioxidant activity, the antioxidant(s) may contribute to antiglycation activity of the plant extract. Biochemical mechanisms of anti-glycation reactions have been recently proposed besides antioxidant activity to reduce glycation, such as breaking the cross-linking structures in AGEs that have been formed, blocking the carbonyl or dicarbonyl groups in reducing sugars, Schiff bases or Amadori and inhibiting the formation of late-stage Amadori products (Caengprasath, 2013; Upendra et al, 2010).

In diabetes and Alzheimer's disease, an imbalance in the concentration of zinc and micronutrients such as ascorbic acid is suggested as a causative factor (Caujungco and Faget, 2003). The antioxidant and antiglycation role of zinc and ascorbic acid is well established, though there are many contradictory reports on beneficial or deleterious effects of Zinc and ascorbic acid supplementation in Alzheimer's disease and AGE-related diseases (Caujungco and Faget, 2003; Rashami and Vaishali, 2010). The plant material has been reported for its rich content of zinc and ascorbic acid (Abuye et al, 2003) that may contribute to its antiglycation activities observed in this study.

The phenolic compounds have aromatic rings containing one or more hydroxyl groups, while polyphenols contain multiple phenol rings with in their structures. All classes of phenols possess antioxidant activities, depending on the number of hydroxyl groups present on the benzene ring (packer etal., 2002). Recently, research interest has increased in finding antioxidants that are naturally-occurring for use in food stuffs and medicinal materials, to replace the synthetic ones which are restricted due to their side effects. Furthermore, natural antioxidants have the capacity to improve food quality and stability;

they also can be used as nutraceuticals to terminate free radical chain reactions in biological systems and may thus provide improved health benefits for the consumers. A number of experimental models have been developed for the determination of antioxidant activities (Vayaetal., 2001). High content of the phenolic compounds besides having antioxidant capacity of the plant material may contribute pleiotropic effects observed in our different studies carried out for antihyperglycemic, antihyperlipidemic and antiglycation activities from the extract of the *Moringa stenopetala* leaves.

Conclusion

The findings demonstrated that *Moringa stenopetala* leaves effectively protect BSA from fructose-mediated protein glycation *in vitro*. The extract of the leaves also reduced the level of fructosamine, the formation of CML and the amyloid cross β -structure in BSA. In addition, the extract significantly decreased the protein carbonyl content and increased the level of protein thiol. Therefore, the *M. stenopetala* leaves have the potential to be used as supplement in the prevention/management of AGE-mediated diseases, particularly for those that are at risk of diabetic complications.

Competing Interests

The authors declare that they have no competing interests.

Authors Contribution

AT conceived the idea, drafted the proposal and involved in all implementation stages of the project and write up. EM, YM, AD and SA reviewed the study proposal, and involved in all implementation stages of the project and write up. All authors reviewed the proposal and the final manuscript. All authors approved final version of the manuscript.

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