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Effect of Ginger Rhizome Homogenate, Ginger Rhizome Steam Distillate, and Simvastatin on Fasting Blood Glucose and Serum Lipid Profiles of Streptozotocin-induced Diabetic Balb/c Mice

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This is to certify that the thesis prepared by Aweke Mulu Belachew entitled: "Effect of Ginger Rhizome Homogenate, Ginger Rhizome Steam Distillate, and Simvastatin on Fasting Blood Glucose and Serum Lipid Profiles of Streptozotocin-induced Diabetic Balb/c Mice" and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Medical Biochemistry complies with regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Introduction: Diabetes is an increasing problem in Ethiopia, affecting up to 6.5% of Ethiopian adults. There are serious complications associated with diabetes, particularly macrovascular and microvascular complications. Controlling blood glucose significantly reduces the risk of complications of diabetes. Insulin therapy is the only current treatment for type 1 diabetes. Type 2 diabetes can be treated with insulin as well numerous oral and injectable medications. However, current diabetes treatment has disadvantages, such as drug-related hypoglycemia and high cost. Traditional medicine is used to treat diabetes across the world and is readily available and inexpensive. Ginger (*Zingiber officinale*) is widely consumed as a spice, and numerous studies suggest that ginger may have beneficial effects for diabetes and dyslipidemia. However, further studies are needed to evaluate these effects of ginger.

Objectives: To evaluate the effects of homogenized ginger rhizome, and a steam distillate of ginger rhizome containing 25% gingerol, on blood glucose and fasting lipid profiles of streptozotocin (STZ)-induced diabetic mice and to compare these effects with those of simvastatin.

Methods: Balb/c mice were randomly assigned to receive ginger rhizome homogenate, steam distillate of ginger rhizome or simvastatin treatment. Two doses of streptozotocin (33mg'kg) were given intraperitoneally a week apart to induce diabetes. Mice were treated with 2g/kg/day of ginger rhizome homogenate by oral gavage, or about 3 mL/kg/day of steam distillate of ginger rhizome by oral gavage, or 40mg/kg/day simvastatin intraperitoneally. Body weights, blood glucose and lipid profiles were measured.

Results: Oral gavage with ginger rhizome homogenate caused significant blood glucose increases, whereas a steam distillate of ginger rhizome showed significant blood glucose lowering effects, in STZ-induced diabetic mice. Diabetic mice treated with ginger rhizome homogenate for 56 days showed significantly lowered serum LDL and triglyceride, and higher HDL levels compared with non-treated diabetic mice but had no significant effect on total cholesterol. The steam distillate of ginger rhizome, given by oral gavage, lowered LDL but had no significant effect on HDL, total cholesterol or triglyceride levels of STZ-diabetic mice. Simvastatin (40mg/kg) given intraperitoneally decreased blood glucose, decreased LDL and increased HDL, but had no significant effect on triglycerides and total cholesterol in STZ-diabetic mice. None of these treatments, ginger rhizome homogenate, steam distillate, or simvastatin, had a major effect on weights of diabetic mice.

Conclusions: Ginger rhizome homogenate treatment exacerbated hyperglycemia in STZ-diabetic mice, but improved diabetic dyslipidemia, while a steam distillate of ginger rhizome and simvastatin each decreased fasting blood glucose and improved lipid profiles of STZ-induced diabetic mice.

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List of Abbreviations

ATP……Adenosine triphosphate

BMI……Body mass index

DNA……...Deoxyribonucleic acid

HDL……High density lipoprotein

HOMA-IR…..…..……...Homeostatic model assessment of insulin resistance

IFN-γ……..Interferon gamma

IL-1β…….. Interleukin 1-beta

IL6……..Interleukin 6

IRS 2/1……... Insulin receptor substrate 1/2

LDL……Low density lipoprotein

mRNA……Messenger ribonucleic acid

PGE2……..Prostaglandin E2

PI3K……Phosphatidylinositol trisphophatekinase

ROS……Reactive oxygen species

STZ…… Streptozotocin

TG…….. Triglyceride

VLDL…….Very low density lipoprotein

WHO……..World Health Organization

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Chapter 1

INTRODUCTION

1.1 Epidemiology of Diabetes

Diabetes mellitus is the most common metabolic and endocrine disorder worldwide. It is linked to disturbances in carbohydrate, fat and protein metabolism and is especially important because the global prevalence of diabetes is projected to rise in coming years (Chen *et al*, 2011). WHO calculates that 347 million people around the world suffer from diabetes, that this number will double by 2030, and that 80% of diabetics live in developing countries (WHO, 2012). According to WHO studies, the number of diabetic cases in Ethiopia in 2000 was estimated at 800,000 and by 2030 this figure is expected to rise to 1.8 million (WHO, 2012). Over the last 30 years, type 2 diabetes has changed from a relatively mild ailment associated with aging to one of a major cause of premature mortality and morbidity in most countries. The biggest populations of diabetics worldwide are to be found in India (51 million), China (43 million), and the USA (27 million). Nevertheless, there is a worsening global epidemic of diabetes, with almost every country showing a rise in prevalence of diabetes (Centers for Disease Control and Prevention, 2011; Lam and LeRoith, 2012).

1.2. Diabetes in Ethiopia

Ethiopia is one of the top five countries with the highest number of people affected by diabetes mellitus in sub-Saharan Africa. According to International Diabetes Federation (IDF) estimation, 3.5 % of Ethiopians had diabetes in 2011 and by 2030 there will be almost 2 million diabetics in Ethiopia (Abebe *et al*, 2014). The prevalence of diabetes mellitus is considerably higher among

urban compared to the rural populations. Diabetes is largely undiagnosed and untreated in many populations in Ethiopia, especially in rural settings, and over half of individuals with diabetes do not adhere to their medications (Abebe *et al*, 2014). Different studies show a range of prevalence for diabetes in Ethiopia, from 0.5% among high school students in Gondar to 6.5% among bankers and schoolteachers in Addis Ababa (Nhsisso *et al*, 2012; Peters, 1983) One study in a Gondar hospital diabetic clinic found that there was a 12.5% increase in the number of diabetics (type 1 and type 2) visiting the clinic between 2000 and 2010, and the average body mass index of patients increased during that period (Abebe *et al*, 2013).

A study done in 2005 in Addis Ababa showed that only 21% of diabetic patients had access for blood glucose monitoring, and only 5% of diabetic patients were able to do self blood glucose monitoring at home. The emphasis given for diabetic education (24%) was less than expected. Screening of diabetics for diabetic complications was poor, and 75% of diabetics had hospital admissions related to conditions associated with poorly controlled diabetes. A third of diabetics had hypertension and diabetic eye conditions, and one in every five had renal disease (Nigatu, 2012). Not only is more screening of the general population to detect early type2 diabetes needed, but also multiple studies show that diagnosed diabetics who attend diabetic clinics in Ethiopia nevertheless often have poorly controlled diabetes (Nshisso, 2012).

1.3 Pathophysiology of Diabetes

Concepts regarding diabetes are changing. Until recently, diabetes was classified into type 1 and type 2 diabetes, with several other types of diabetes, including gestational diabetes, drug-induced diabetes and others also being defined (Lin and Sun, 2010). Type1 diabetes occurs mainly in children, involves autoimmune destruction of pancreatic islet beta-cells, and renders patients

completely dependent upon insulin. Type 2 diabetes initially involves insulin resistance, leading to hyperinsulinemia, and is associated with obesity and metabolic syndrome, but eventually progresses to involve pancreatic beta cell dysfunction with an insulin deficit and most diabetics (over 90%) are type 2 diabetics (Kahn *et al*, 2014).

However, it is now thought that diabetes is a group of perhaps hundreds of diseases with different etiologies, all of which involve pancreatic beta cell dysfunction. Latent autoimmune diabetes of adults (LADA), for example, resembles type 1 diabetes very closely, but autoimmune destruction of beta cells is a slower process, taking many years, so that LADA presents in later adulthood. LADA is sometimes mistaken for classic type 2 diabetes, but it does not involve insulin resistance. Maturity Onset Diabetes of the Young (MODY) is an autosomal dominant inherited form of diabetes that affects young adults and can be caused by numerous mutations, including mutations of the glucokinase gene and mutations in genes that control beta cell differentiation (Gardner *et al*, 2012). MODY, unlike classic type 1 and type 2 diabetes, does not involve destruction of beta cells in the pancreas or insulin resistance, but consists of malfunctioning beta cells that do not secrete insulin normally in response to increases in blood glucose. "Mitochondrial diabetes" is another group of diabetic diseases, involving mutations in mitochondrial transfer RNA genes and cause defects in mitochondrial function. Mitochondria are involved in signaling in the glucose-stimulated increase secretion of insulin by beta cells, so patients with mitochondrial diabetes have defects in this signaling pathway (Lin and Sun, 2010; Sylvia *et al*, 2014; Steven *et al*, 2014). Mitochondrial diabetes also does not involve insulin resistance or pancreatic beta cell destruction, and so cannot be classified as classic type 1 or type 2 diabetes.

To add to the complexity of diabetes, it is now clear that some diabetics with insulin resistance do not progress to diabetes and that some patients who were thought to be classical type 2 diabetes

do not have insulin resistance. An emerging concept is that there are thin individuals who are prone to type 2 diabetes and there are obese people who are relatively resistant to developing diabetes. This agrees with the idea that diabetes is a heterogeneous group of diseases and that the distinction between type 1 and type 2 diabetes is not as clear as once was thought (Steven *et al*., 2014; Kahn *et al*, 2014).

The common defect in all forms of diabetes is a reduced secretion of insulin by beta cells in response to elevations of blood glucose. This may be due to beta cell destruction as in type 1 diabetes and LADA, poor signaling of glucose-stimulated insulin secretion as in mitochondrial diabetes or MODY, or poor differentiation of beta cells as in some forms of MODY. Insulin resistance in classic type 2 diabetes progresses only to overt diabetes when insulin hypersecretion exceeds the capacity of the pancreas to secrete insulin, due to toxicity of glucose, lipids and other metabolic products causing pancreatic beta cell death. It may be that type 2 diabetes really is a genetic predisposition to beta cell death in the presence of insulin resistance (Kahn *et al*, 2014).

1.4 Pancreatic β-cell Dysfunction in Type 2 Diabetes

Under normal physiological conditions, plasma glucose concentrations are maintained within the range 60 to 100 mg/dL in humans, through a tightly regulated and dynamic interaction between tissue sensitivity to insulin, glucose use by the tissues, and insulin secretion (Kaku, 2010; Ozougwu *et al*, 2013). The pancreatic β-cells constantly synthesize insulin, regardless of blood glucose levels. Insulin is stored within vacuoles and released, once activated, by an elevation of the blood glucose level (Lin and Sun, 2010). In type 2 diabetes, the β cells eventually fail to produce enough insulin to meet the body's demand, because of β-cell insulin secretion dysfunction and/or decreased β-cell mass (Karaca *et al*, 2009).

Many mechanisms contributing to type 2 diabetes may also trigger *β*-cell apoptosis and reduce *β*-cell mass or ability to compensate for insulin resistance. Some of these mechanism include endoplasmic reticulum stress, chronic hyperglycemia, oxidative stress, chronic hyperlipidemia, mitochondrial dysfunction. Circulating cytokines including IL-1β, leptin, resistin, and IFN-γ play important roles in the development of pancreatic *β*-cell dysfunction and type 2 diabetes (Bunner *et al*, 2014; Folli *et al*, 2011; Donath *et al*, 2008). For instance, when pancreatic *β*- cell exposure to chronic IL-1β activates the expression of inducible nitric oxide synthase (iNOS) and results in excessive production of nitric oxide (NO), which interferes with electron transfer, inhibits ATP synthesis in mitochondria, and induces the expression of proinflammatory genes (Wang *et al*, 2010). Also, the molecular mechanisms of pancreatic *β*-cell dysfunction in type 2 diabetes involve increase of IRS 2 serine/threonine phosphorylation, which results in IRS2 ubiquitination and proteosomal degradation, defects in insulin signaling and insulin secretion (Lin and Sun, 2010).

1.5 Role of the Liver in Type 2 Diabetes

The liver is a key target for the anabolic hormone, insulin and its catabolic counterpart, glucagon, and it is the major organ with the ability to form lipids, glycogen, generation of glucose from non-sugar carbon substrates and intracellular energy supply via glycolysis depending on the requirement (D'Alessio, 2011). Impaired insulin sensitivity and dysregulated insulin action in the liver contributes significantly to the pathogenesis of type 2 diabetes (Fritsche *et al*, 2008), because insulin inhibition of the major gluconeogenic enzymes, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase is diminished. Consequently, this leads to increased hepatic glucose production to fasting hyperglycemia.

Insulin receptor substrates play key roles in the orchestration of the complex hepatic metabolic responses to insulin. IRS -1 and IRS-2 are complementary key players in the regulation of hepatic insulin signaling and expression of genes involved in gluconeogenesis, glycogen synthesis, and lipid metabolism (Dong *et al*, 2006, Roncero *et al*, 2013). Dysregulation of this complex system leads to impaired signal transduction resulting in pathological states. Reduced IRS protein levels in the liver and hyperphosphorylation of IRS on serine/threonine residues are hallmarks in the development of insulin resistance and type 2 diabetes mellitus (Fritsche *et al*, 2008). Dysfunction of IRS proteins initially leads to mild hyperinsulinemia, postprandial hyperglycemia, increased hepatic glucose production, and dysregulated lipid synthesis, and is considered as a major pathophysiological mechanism for the development of insulin resistance and type 2 diabetes (Ozougwu *et al*, 2013).

1.6 Insulin Resistance

Type 2 diabetes is characterized by insulin resistance of peripheral tissues, such as skeletal muscle and adipose tissue. Skeletal muscle plays a crucial role in maintaining systemic glucose metabolism, accounting for 85% of whole body insulin stimulated glucose uptake (Peppa *et al*, 2010). In insulin resistant states, decreased insulin-stimulated glucose uptake occurs, due to impaired insulin signaling and multiple post receptor intracellular defects including impaired glucose transport and glucose phosphorylation, and reduced glucose oxidation and glycogen synthesis (Abdul-Ghani and DeFronzo, 2010).

At the molecular level type 2 diabetic subjects have impaired insulin-stimulated tyrosine phosphorylation of IRS-1and impaired PI3K in skeletal muscle (Asano *et al*. 2007). Dysregulation of these specific tyrosine residues in IRS-1 severely impairs the ability of insulin to stimulate

muscle glycogen synthesis, glucose uptake and oxidation, and other acute metabolic- and growth-promoting effects of insulin (Abdul-Ghani and DeFronzo, 2010). Mechanisms for this dysregulation might include tumor necrosis factor alpha (TNFα)-mediated down regulation of mRNA transcription, kinase-mediated serine/threonine phosphorylation, proteasome mediated degradation and phosphatase mediated dephosphorylation (Peppa *et al*, 2010). These signaling abnormalities could result in impaired insulin-stimulated glucose transport in the skeletal muscle of type 2 diabetic subjects (DeFronzo and Tripathy, 2009). Ultimately, these signaling events result in reduction of the translocation of glucose transporter 4 (GLUT4) to the plasma membrane and leading to a decrease in adipocyte and skeletal muscle glucose uptake (Jung and Choi, 2014).

The expression of GLUT4 is down-regulated in muscle tissue as well as adipocytes, following changes in adipose glucose metabolism results in whole-body insulin resistance in patients with type 2 diabetes (Gonzalez *et al*, 2011). Thus, the perturbation of GLUT4 translocation in insulin-resistant adipocytes will not only promote hyperglycemia by reduced glucose flux into adipose tissue but it will also contribute to whole-body insulin resistance via alterations in adipose endocrine functions (McInnes *et al*, 2012).

Studies indicate that adipocyte GLUT4 deficiency may result in generation of circulating retinol-binding protein-4 (RBP4) factors that are responsible for hepatic expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase and impaired insulin signaling in muscle (McInnes *et al*, 2012). In addition to this, Adipocytes secrete excess factors (adipokines), which may alter systemic insulin action and hepatic glucose production including adiponectin, resistin, leptin, cytokines IL6 and TNFα, visfatin as well as FFA (Kaku, 2010). Exposure of cells to TNFα or elevated levels of FFAs stimulates phosphorylation of serine residues of IRS1. This phosphorylation reduces tyrosine phosphorylation of IRS1 in response to insulin (Peppa *et al*,

2010). Both adipose tissue and the macrophages within adipose tissue serve in both endocrine and paracrine fashions to promote inflammation and decrease insulin sensitivity (De Luca and Olefsky, 2008).

1.7 Diagnosis of Diabetes Mellitus

Diabetes is usually diagnosed based on plasma glucose criteria, either the fasting plasma glucose or the 2-h plasma glucose value after a 75-g oral glucose tolerance test. Recently, an International Expert Committee added the A1C (threshold ≥6.5%) as a third option to diagnose diabetes (American Diabetes Association, 2011). In addition, it includes excessive urine production, compensatory thirst, increased fluid intake, blurred vision, unexplained weight loss, lethargy, and changes in energy metabolism (Lin and Sun, 2010), though type 2 diabetes frequently is asymptomatic.

It is recommended that the same test be repeated immediately using a new blood sample for confirmation. If two different tests are both above the diagnostic threshold, this also confirms the diagnosis. On the other hand, if a patient has discordant results from two different tests, then the test result that is above the diagnostic cut point should be repeated. If, for example, a patient meets the diabetes criterion of the A1c (two results ≥6.5%), but not FPG (≥126 mg/dL [7.0 mmol/L]), that person should nevertheless be considered to have diabetes (American Diabetes Association, 2015).

A fasting blood glucose test above 126 mg/dL or above indicates diabetes. In type 1 diabetes, plasma insulin level is very low or undetectable during fasting and even after a meal. In type 2 diabetes, plasma insulin concentration may be several fold higher than the normal level and usually increases to greater extent after a standard glucose load a glucose tolerance test (American

Diabetes Association, 2011). Glycated hemoglobin level provides a reliable measure of chronic glycemic control without the need for a fasting or timed sample, and it correlates well with the risk of long term diabetes complications and mortality. Several population based studies have investigated the utility of the HbA1c level for detecting undiagnosed diabetes and the potential to use the HbA1c level as a good screening tool for type 2 diabetes (American Diabetes Association, 2014). HbA1c of 6.5% is recommended as the cut point for diagnosing diabetes. (World Health Organization, 2011).

1.8 Obesity, Metabolic Syndrome and Diabetes

Accumulating evidence indicates that obesity is closely associated with an increased risk of metabolic diseases such as insulin resistance, type 2 diabetes, dyslipidemia and nonalcoholic fatty liver disease (Jung and Choi, 2014). A recent study of 183 countries showed a worldwide prevalence of overweight/obese people (BMI>25) of 37% for men and 38% for women in 2013. Between 1980 and 2013, there was an increase of 27.5% in men and 47.1% in women, in the prevalence of overweight and obesity (WHO, 2013). Alarmingly, the prevalence of obesity in children is increasing in almost every country, including developing countries. Globally, in developed countries the prevalence of overweight or obesity in children was about 23% in 2013. In developing countries the prevalence of childhood overweight or obesity was about 13%, though the prevalence increased from 8% to 13% in developing countries between 1980 and 2013. Ethiopia has one of the lowest prevalences of overweight/ obesity, with under 5% of adults and under 2.5% of children being obese/overweight. Nevertheless, the global trend is towards an increase in obesity prevalence and this is a concern for all countries (Ng *et al*, 2014).

1.9 Dyslipidemia and Type 2 Diabetes Mellitus

The liver normally synthesizes fat and exports it as VLDL particles. However, when tissues become insulin resistant, VLDL levels are often higher than normal and coupled with lower than normal HDL levels, a condition called dyslipidemia (Rader & Hovingh, 2014). Type 2 diabetes is associated with a cluster of interrelated plasma lipid and lipoprotein abnormalities, including reduced HDL cholesterol, a predominance of small dense LDL particles, and elevated triglycerides (American Diabetes Association, 2003). Insulin resistance is central to the pathogenesis of type 2 diabetes and contributes to dyslipidemia by affecting lipoprotein size and subclass particle concentrations of VLDL, LDL and HDL (Jung and Choi, 2014).

Impaired insulin action at the level of the adipocyte is believed to result in defective suppression of intracellular hydrolysis of TGs with the release of free fatty acids into the circulation (Ragheb and Medhat, 2011). The increased influx of free fatty acids from adipose tissue to the liver promotes TG synthesis and the assembly and secretion of large VLDL; this results in elevated plasma VLDL levels, increase hepatic glucose production, decrease insulin clearance and induce peripheral insulin resistance (Vijayaraghavan, 2010).

In type 2 diabetes, elevated VLDL-asssociated TGs reduce levels of cardio protective HDL as TGs are transferred when these particles interact (Mora *et al*, 2007). The reductions in HDL associated with type 2 diabetes and insulin resistance are multifactorial, but a major factor is VLDL-enhanced TG enrichment of HDL and LDL by exchange of TG for cholesterol and is followed by hepatic lipase-mediated hydrolysis of the TG portion, resulting in small, relatively cholesterol-poor HDL and LDL particles (Ragheb and M. Medhat, 2011). In addition, high TG-rich lipoproteins (after eating), remnant lipoproteins, Apolipoprotein B 100 (Apo B), and small,

dense HDL particles have also been shown to be increased in patients with type 2 diabetes (Chapman *et al*, 2011).

1.10 Management of Diabetic Dyslipidemia

Current recommendations for the management of dyslipidemia in patients with type 2 diabetes include lifestyle interventions such as diet, physical activity, weight loss and smoking cessation are an integral part of any diabetes management plan. Epidemiologic and intervention studies have shown significant improvement in the features of diabetic dyslipidemia with medical nutrition therapy and physical activity (American Diabetic Association, 2015). Although lifestyle interventions can improve diabetic dyslipidemia to some extent, pharmacological therapy will be needed to reach treatment goals in many patients. There are several classes of medications used in the treatment of dyslipidemia associated with insulin resistance and type 2 diabetes. These including statins, fibrates, niacin, and bile acid sequestrants, and phytosterols and ezetimibe (which inhibits intestinal cholesterol absorption) are available to target normalization of the lipid profile (Vijayaraghavan, 2010).

Statins, inhibitors of 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase, thereby slowing cholesterol biosynthesis and reducing low-density lipoprotein cholesterol (Paulweber *et al*, 2010). Statins bind to HMG-CoA reductase at nanomolar concentrations, leading to an effective displacement of the natural substrate HMG-CoA, which binds instead at micromolar concentrations (Gazzerro *et al*, 2011). Statins also deplete nonsterol cholesterol precursors, the isoprenoid metabolites such as geranylgeranyl pyrophosphate and farnesyl pyrophosphate, which are necessary for prenylation of critical membrane proteins that regulate cellular communication, including the inflammatory response (Mills *et al*, 2011). The primary actions of statins on

lipoprotein metabolism are mediated by increased LDL receptor activity and LDL uptake, reduced hepatic lipoprotein secretion, thus lowering serum LDL. Statins also have additional beneficial effects, for example they stabilize plaques, increase of endothelial nitric oxide synthetase, restoration of platelet activity and of the coagulation process to reduce inflammation, lower plasma triglyceride levels and raise HDL cholesterol (Paulweber *et al*, 2010). Nevertheless, most studies have not reported a reversal of the small dense LDL phenotype associated with diabetic dyslipidemia with statins.

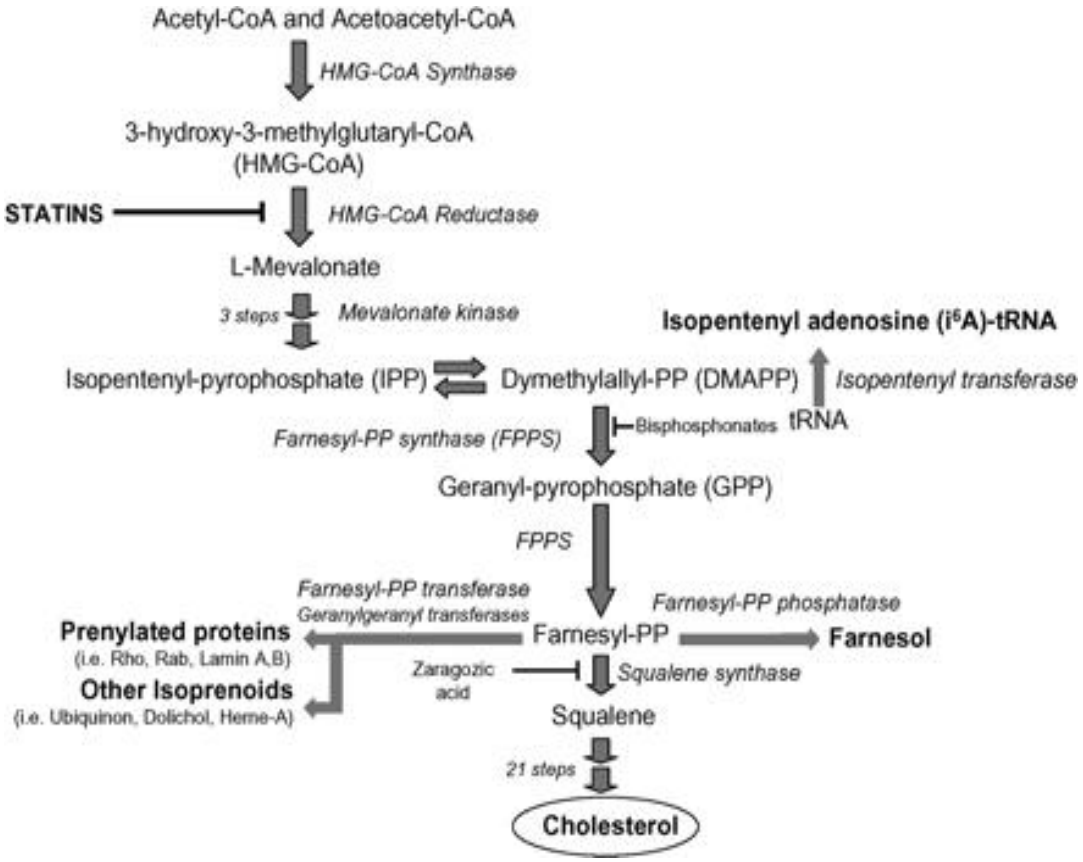


Figure 1. Role of HMG-Co A reductase inhibitors on cholesterol synthesis (Gazzerro *et al*, 2011)

Statins have antiatherosclerotic effects independently of their hypolipidemic action. In type 2 diabetes a great deal of data exists on the action mechanism and efficacy of statins in the prevention

of CVD events (Mills *et al*, 2011). The Collaborative Atorvastatin Diabetes Study assessed the benefits of a statin in type 2 diabetes patients, and showed a 37% reduction in acute CVD events (Colhoun *et al*, 2004). In the Heart Protection Study, simvastatin (40 mg/d) reduced the composite primary endpoints by 33% (Collins *et al*, 2003). Also, atorvastatin 10 mg/d decreased the rate of major CVD events in 23% in the Anglo Scandinavian Cardiac Outcomes Trial subgroup (Sever *et al*, 2005).

However, studies suggest that statins were linked to diabetes risk, but the cardiovascular event rate reduction with statins far outweighed the risk of incident diabetes even for patients at highest risk for diabetes (Ridker *et al*, 2008). Common adverse events associated with statin use include gastrointestinal upset and muscle aches, although dose related hepatoxicity and myotoxicity are the most clinically significant adverse events (Vijayaraghavan, 2010).

1.11 Oxidative Stress in Diabetes

High glucose levels, which can stimulate free radical production and weaken defense systems of the body, result in a condition of imbalance between ROS benefits and their protection; this leads to domination of the condition of oxidative stress (Pandey *et al*, 2010). However, a certain amount of oxidative stress is necessary for the normal metabolic processes since ROS play various regulatory roles in cells (Gomes *et al*, 2012). For instance, they are produced by neutrophils and macrophages during the process of respiratory burst in order to eliminate antigens (Freitas *et al*, 2010). They also serve as stimulating signals of several genes (Gomes *et al*, 2012).

However uncontrolled production of ROS is harmful. Metabolic abnormalities of diabetes cause mitochondrial superoxide overproduction in endothelial cells of both large and small vessels, as well as in the myocardium (Giacco and Brownlee, 2010; Patel *et al*, 2013). Oxidative stress acts

as mediator to inhibit insulin gene expression, suppress insulin gene transcription, and induce alteration of glucose-stimulated insulin secretion by damage to mitochondrial DNA and membrane proteins and by down regulation of glyceraldehyde-3-phosphate dehydrogenase, which results in loss of intracellular and mitochondrial ATP (Chon *et al*, 2014).

In type 2 diabetes, the high nutrient flux and consequent ROS production appear to mediate loss of β-cell function. In insulin-sensitive tissues including muscle, liver, and heart, high fatty-acid flux leads to oxidative damage, whereas noninsulin-sensitive tissues including the eye, kidney, and nervous system are exposed to both high circulating glucose and fatty acid levels and, consequently, ROS-induced diabetic complications (Sivitz and Yorek, 2010).

1.12 Oxidative Stress-induced Alterations in Diabetes

Oxidative stress in diabetes mellitus causes several adverse effects on the cellular physiology. This is particularly dangerous for the pancreatic islet, which is among those tissues that have the lowest levels of intrinsic antioxidant defenses ROS are very reactive chemical species, which can cause oxidation injury to the living cells by attacking lipids, carbohydrates, proteins and nucleic acids (Varashree and Bhat, 2011).

Besides damaging the functions of these molecules, increasing oxidative stress also plays a more important role in advanced glycation end-product (AGE) formation in type 2 diabetes mellitus. Accumulation of AGEs can modify proteins, directly damage the structure and metabolism of extracellular matrix or act via their specific receptors (RAGE, receptor for advanced glycation end-products) (Pandey and Rizvi, 2010). AGE-RAGE interaction activates nuclear factor NF-κB, stimulates the transcription of genes for proinflammatory cytokines, growth factors and adhesive

molecules, induces migration of macrophages and has further toxic effects (Negre-Salvayre *et al*., 2009).

In addition, high reactive dicarbonyl compounds, including glyoxal, methylglyoxal or 3-deoxyglucosone are formed, which subsequently results in the rise of plasma and tissue contents of advanced glycation end products (Rabbani and Thornalley, 2014). Methylglyoxal is formed from breakdown of ketone body metabolism, threonine catabolism, degradation of glycated protein, monosaccharide degradation, food and beverages, DHAP and glyceraldehyde-3-phosphate during glycolysis (Li *et al*, 2013). Glyoxal is formed from degradation of sugars, lipid peroxides and glycated proteins and also, 3-deoxyglucosone is formed from breakdown of fructose-3-phosphate by the polyol pathway (Rabbani and Thornalley, 2015). The fructose produced by the polyol pathway can become phosphorylated to fructose-3-phosphate, which is broken down to 3-deoxyglucosone, which is a powerful glycosylating agents that forms advanced glycation end products (Yan, 2014).

The other alteration induced by ROS is over activation of several PKC isoforms mediating tissue injury induced by diabetes-induced ROS (Giacco and Brownlee, 2010). This results primarily from enhanced *de novo* synthesis of DAG from glucose via triose phosphate, whose availability is increased because increased ROS inhibit activity of the glycolytic enzyme GAPDH, raising intracellular levels of the DAG precursor triose phosphate (Li *et al*, 2013).

Hyperglycemia and insulin resistance–induced excess fatty acid oxidation also appear to contribute to the pathogenesis of diabetic complications by increasing the flux of fructose 6-phophate into the hexosamine pathway (Singh et al, 2014). In this pathway, fructose 6-phosphate is diverted from glycolysis to provide substrate for the rate-limiting enzyme of this pathway, glutamine: fructose 6-phosphate amidotransferase (GFAT) (Pandey *et al*, 2010). GFAT converts fructose 6-phosphate

to glucosamine 6-phosphate, which is then converted to UDP-N Acetylglucosamine. Specific -O-GlcNAc transferases use this for posttranslational modification of specific serine and threonine residues on cytoplasmic and nuclear proteins by O-GlcNAc (Gradinaru *et al*, 2013).

1.13 Streptozotocin and Its Mechanism of Action

Streptozotocin, derived from a fermentation broth of *Streptomyces achromogenes*, has been widely used to study the beta cell destruction both in-vitro and in-vivo models (Singh *et al*, 2012). The drug is a glucose analogue that is rapidly transported into the *β*-cells via the glucose transporter, GLUT2 and is known to be metabolized readily upon entry into the cell (Sheline *et al*, 2012). Intracellular action of STZ results in changes of DNA in pancreatic *β* cells by generating excess ROS and NO leading to DNA breaks by alkylating DNA bases. The N-nitroso-N methylurea portion of the molecule exhibits diabetogenic activity. Glucose may act as carrier for this cytotoxic group (Veerapur *et al*, 2010).

The formation of superoxide anions results from both STZ action on mitochondria and increased activity of xanthine oxidase (Szkudelski, 2001). It was demonstrated that STZ inhibits the Krebs cycle and substantially decreases oxygen consumption by mitochondria (Sheline *et al*, 2012). These effects strongly limit mitochondrial ATP production and cause depletion of this nucleotide in *β* cells (Fukaya *et al*, 2013). Restriction of mitochondrial ATP generation is partially mediated by NO which is generated during its metabolization. This molecule was found to bind to the iron-containing aconitase inhibiting enzyme activity (Szkudelski, 2001).

Augmented ATP dephosphorylation increases the supply of substrate for xanthine oxidase (*β* cells possess high activity of this enzyme) and enhances the production of uric acid – the final product of ATP degradation. Then, xanthine oxidase catalyses reaction in which the superoxide anion is

formed. As a result of superoxide anion generation hydrogen peroxide and hydroxyl radicals are formed (Szkudelski, 2001). Potent alkylating properties of STZ are the main reason of its toxicity. However, the synergistic action of both NO and reactive oxygen species may also contribute to DNA fragmentation and other deleterious changes caused by STZ. NO and reactive oxygen species can act separately or form the highly toxic peroxynitrate (ONOO) (Mabley *et al*, 2001, Alimohammadi *et al*, 2013).

STZ-induced DNA damage activates poly ADP ribosylation which is a nuclear enzyme associated with DNA damage and repair (Mabley *et al*, 2001), the activation of poly ADP ribosylation consumes nicotinamide adenine denucleotide (NAD^+), leading to NAD^+ depletion, further reduction of the ATP content (Fukaya et al, 2013). And subsequent inhibition of insulin synthesis and secretion (Szkudelski, 2001).

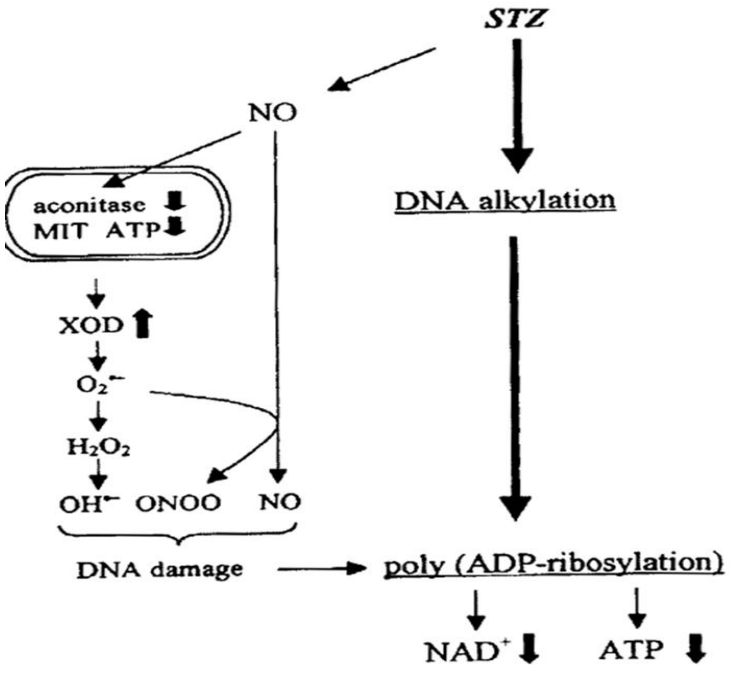


Figure 2. The mechanism of streptozotocin induced toxic events in beta cells of rat pancreas. MIT –mitochondria; XOD – xanthine oxidase (Szkudelski, 2001)

1.14 Control of Hyperglycemia in Type 2 Diabetes

1.14.1 Lifestyle Modifications

Different randomized controlled trials have shown that individuals at high risk for developing type 2 diabetes can significantly decrease the rate of diabetes onset with intensive lifestyle modification programs that have been shown to be very effective (Lin *et al*, 2014; Paulweber *et al*, 2010). For instance in one study the rate of conversion to type 2 diabetes reduced by 58% after 3 years (Li *et al*, 2008). Another study show that lifestyle intervention caused a 43% sustained reduction at 20 years in the Da Qing study (Li *et al*, 2008) and 43% reduction at 7 years in the Finnish Diabetes Prevention Study (DPS) in the rate of conversion to type 2 diabetes (Lindström *et al*, 2006). Also, a 34% risk reduction at 10 years occurred in the U.S. Diabetes Prevention Program Outcomes Study (DPPOS) (Knowler *et al*, 2009). Therefore, according to the result of the above mentioned study lifestyle intervention is one of the treatment and prevention mechanism for type 2 diabetes, and these are cost-effective (Herman *et al*, 2005; Ackermann *et al*, 2008).

1.14.2 Pharmacotherapies for Type 2 Diabetes

Pharmacological agents, such as metformin, sulfonylureas, thiazolidinediones, meglitinides, glucagon like peptide 1 receptor agonists, dipeptidyl peptidase 4 inhibitors, sodium-glucose cotransporter 2 (SGLT2) inhibitors and α-glucosidase inhibitors, natural and synthetic insulins, bile acid sequestrants are widely used and effective in improving hyperglycemia (Thompson, 2013; Ohmura *et al*, 2015). The mechanisms of action implicated in these medications include increased insulin production, decreased hepatic glucose production, sensitization of the insulin receptor pathway, increased excretion of glucose from the kidneys and/or glucagon like peptide 1 secretion (Leahy *et al*, 2010). When pharmacological methods are used to interfere with these

mechanisms, the goal for hemoglobulin A1C (HA1c), an indicator of long-term glycemic control, should be below 6.5 to 7.0% (Esposito *et al*, 2015).

Sulfonylurea-type drugs stimulate pancreatic cells to secrete insulin, leading to lower blood glucose. However, these secretagogues cannot save *β*-cell atrophy (Zaman-Huri *et al*, 2013). Adverse effects of sulfonylureas and meglitinides are frequently linked with weight gain, hypoglycemia and inability to protect cells from death.

Thiazolidinediones and biguanide, metformin, can directly lower insulin resistance and, subsequently, blood glucose (America Diabetes Association, 2014). The adverse effects of metformin include gastrointestinal problems which is the most common and reveal the accumulation of the drug in the small intestine (Lin *et al*, 2014). Also, as a result of lactic acidosis occurs with metformin and it should not be used in unstable or hospitalized patients with congestive heart failure, kidney and liver dysfunction (Ohmura *et al*, 2015). Recognized side effects of thiazolidinediones include weight gain, fluid retention leading to edema and/or heart failure in predisposed individuals, and increased risk of bone fractures (Kahn *et al*, 2006; Inzucchi *et al*, 2012).

Decreasing glucose reabsorption in the kidneys is also a way to lower blood glucose level. Inhibitors of sodium-glucose cotransporter 2 (SGLT2) enhance urinary glucose excretion and thereby lower blood glucose levels in type 1 and type 2 diabetes (Inzucchi *et al*., 2012). Some sodium-glucose cotransporter 2 inhibitors, particularly canagliflozin, may also increase low-density lipoprotein and increase risk of stroke, and so may be detrimental to diabetics. Other adverse effects include urinary tract infections and genital fungal infections (Ohmura *et al*, 2015).

Another new class of diabetes medication is the glucagon like peptide 1 analogues and dipeptidyl peptidase 4 inhibitors. This class controls multiple actions of glucagon like peptide 1 to lower blood glucose, including increasing insulin, reducing glucagon, and gastric emptying, suppressed inappropriately elevated glucagon secretion, reduced body weight, enhanced satiety, and preserved pancreatic *β*-cell function (Parkes *et al*, 2013, Chon *et al*, 2014). Despite the multiple benefits of incretin-based drugs, these drugs are still accompanied by severe gastrointestinal problems such as belching, nausea, vomiting, indigestion, and diarrhea (Chon *et al*, 2014).

Additionally, even in well-managed patients, daily injection of insulin cannot match the natural precise timing and dosing of insulin secretion from the pancreas in response to hyperglycemia, resulting in severe complications (Howlett & Bailey, 1999). Therefore there is a need to develop antidiabetic medicines with improved efficacy and fewer adverse effects. Therefore, many efforts have been made to identify new anti-diabetic agents from different sources, especially medicinal plants because of their fewer adverse effects and relatively low cost.

1.15 Plants as Medicines

Plants have provided humans with medicines for thousands of years. The oldest known document concerning medicinal plants and their uses is the Chinese Pen Ts'ao, which was written 4800 years ago and describes no less than 360 plants, suggesting that herbal medicine was already at an advanced stage in China at this time (Wohlmuth, 2008). In the case of Ethiopian traditional medicine, it is vastly complex and diverse as well as varies greatly among different ethnic groups. Most traditional medical practices in Ethiopia rely on an explanation of disease that draws on both the "mystical" and "natural" causes of an illness and employ a holistic approach to treatment (Bishaw, 1991).

Despite the many advances of biomedicine, the progress afforded residents of first world countries is beyond the reach of the majority of the world's population. For the majority of people, many of whom live in miserable poverty, crude plants preparations are still the main form of medicine. In acknowledgment of this situation, the world health organization is actively promoting the development of traditional medicine (WHO, 2002).

1.16 Origin and Production of Ginger

Ginger originated in South-East Asia, probably in India. Today ginger is cultivated in several parts of the world, the most important producing regions being India, China, Nigeria, Sierra Leone, Indonesia, Bangladesh, Australia, Fiji, Jamaica and Nepal. Among them India and China are the dominant suppliers to the world market (Peter, 2001).

Ginger is the second most widely cultivated spice in Ethiopia, next to chillies (Ayenew *et al*, 2012). Endrias Geta and Asfaw Kifle of Haramaya University who conducted a survey in 2011 on the Production, Processing and Marketing of Ginger in Southern Ethiopia, concluded that ginger had been produced as an important commercial horticultural crop in southern Ethiopia. Farmers produce different varieties and apply traditional management practices (Geta & Kifle, 2011).

1.17 Botany of Ginger

Ginger is a monocotyledon plant, which consists of more than 1200 plant species in 53 genera and belonging to the family *Zingiberaceae* and to the order *Zingiberales*. In the *Zingiberaceae*, it belongs to the subfamily *Zingiberoideae*, which are aromatic with unbranched aerial stems, distichous leaves, open sheaths and hypogeal germination, mainly confined to the old world tropics. Among them, ginger is a slender perennial herb, 30-100 cm tall with branched rhizomes

bearing leafy shoots. The leafy shoot is the pseudo-stem formed by leaf sheath and bears 8-12 distichous leaves (Emmanuel *et al*, 2014).

1.18 Major Ginger-growing Areas in Ethiopia

Ethiopia is a land of diverse climate and soil type that enable prolific growth of several indigenous and exotic spices, herbs, medicinal and other essential oil bearing plants. Ginger has been known in Ethiopia since the beginning of 13th century, when the Arabs brought it from India to East Africa (Sileshi, 2010). The major ginger growing areas in Ethiopia include wetter regions at altitude below 2000 m in Kefa, Illuababor, Gamo Gofa, Sidama and Wellega mostly in gardens and around homesteads. However, large scale production and marketing of ginger are reported from Illubabur, Wolaita, Kembata and Tambaro (Asfaw and Demissew, 2009).

There are also cultivations in Gojam and Gonder regions to cover home consumption. The production of this spice has been expanding in most parts of the country, as it can be grown under varied climatic conditions that do not have frost problem. Ginger thrives well in areas with altitudes from sea level to 1500 m, mean annual temperature of 20-32 ^{o}C and with total rainfall greater than 1200 mm. The ideal soil type for the production of ginger is a well-drained, fertile and friable soil and with enough humus, neutral pH (H/Michael *et al*, 2008).

1.19 Dietary Use and Health Benefits of Ginger

Both fresh and dried ginger rhizomes are used worldwide as a spice, and ginger extracts are used extensively in the food, beverage, and confectionary industries for the production of marmalade, pickles, chutney, ginger beer, ginger wine, liquors, biscuits, and other bakery products (Mesomo *et al*, 2013). In Ethiopia it is among the important spices used in every kitchen to flavor stew, tea, bread and local alcoholic drinks (Asfaw and Abegaz, 2003). Medicinally it is used mainly to

relieve stomach ache, fever, influenza, headache, coughs and tooth ache (Demissew and Asfaw, 2009), though it is used also for anorexia, arthritis, atonic dyspepsia, bleeding, cancer, chest congestion, chicken pox, cholera, chronic bronchitis, cold extremities, colic, colitis, common cold, cough, cystic fibrosis, diarrhoea, difficulty in breathing, dropsy, flatulent, disorders of gall bladder, hyperacidity, hypercholesterolemia, hyperglycemia, morning sickness, prevention of motion sickness, nausea, rheumatism, sore throat, throat ache, stomach ache and vomiting in pregnancy (Haniadka *et al*,2013).

1.20 Chemical Composition of Ginger

The unique flavor properties of ginger arise from the combination of pungency and aromatic essential oil. The main pungent compounds in fresh ginger are a series of homologous phenolic ketones known as gingerols. The gingerols are thermally unstable and are converted under high temperature to shogaol (Mesomo *et al*, 2013).

Similarly, Ethiopian ginger contains aromatic volatile oil in a concentration of 1-3% and the main pungent compound gingerol (Sileshi, 2010). Ethiopian ginger is more pungent and fibrous than Chinese or Indian ginger. In addition to this, the ginger oils were mainly dominated by sesquiterpene hydrocarbons, namely zingiberene (34.8-37.5%), β-bisabolene (9.8- 12.2%) and β-sesquiphelandrene (11.7-12.7%). The physical properties and chemical composition of the oils were found to be comparable with those of the ginger oils in the international market (Abegaz and Asfaw, 2003).

Ginger has been investigated for its chemical constituents and studies have shown it to contains a wide variety of volatile and non-volatile compounds, and also that their concentration varies with

growing conditions, temperature, harvesting and processing. Generally, ginger oil from different places have their own unique compositions (Haniadka *et al*, 2013).

The volatile ginger components consist of n-gingerol (n=6, 8, 12), n-shogaol (n=6, 8, 12), [6]-paradol, [6]-methylgingerdiol, [6]-methylgingerdiacetate,gingerdiones,[6]-gingesulfonicacid,[6]-hydroxyshogaol,and hexahydrocurcumin (Ahui *et al*. 2013, Bhargava *et al*, 2012, Rahmani *et al,* 2014). Ginger also contains other volatile compounds such as α-pinene, camphene, 6-methyl-5-hepten-2-one, myrcene, α- and β-phellandrene, limonene (Mesomo *et al*, 2013), bornyl acetate, 2-undecanone, citronellyl acetate, α-copaene and geranyl acetate (Tang *et al*, 2012).

1.21 Advantage of Ginger Rhizome on Diabetes and Hyperlipidemia

There are relatively few studies of the effects of ginger rhizome on diabetes and hyperlipidemia, but the ones that have been done suggest that ginger might be a useful natural treatment for these disorders. In one study in human diabetics, ginger supplementation significantly lowered the levels of serum LDL-cholesterol and triglyceride levels and improved insulin level and insulin resistance in type 2 diabetics, although there were no significant changes in fasting plasma glucose, serum total cholesterol, HDL cholesterol or hemoglobin A1C (HbA1c) (Mahluji *et al*, 2013). In another study of human type 2 diabetics, ginger supplementation significantly reduced the levels of the inflammatory markers, tumor necrosis factor-alpha (TNF-α) and high-sensitivity C-reactive protein (hs-CRP), though there were no significant changes in interleukin-6 (IL-6). Because diabetes, insulin resistance, metabolic syndrome and cardiovascular diseases are chronic inflammatory states associated with these inflammatory markers, it might be that ginger improves the inflammation in these conditions (Lei *et al*, 2014).

In another study of human diabetics, ginger reduced fasting plasma glucose, HbA1C, HOMA, triglyceride, total cholesterol, CRP, PGE2 and increased insulin level significantly compared with placebo group, but it did not alter HDL, LDL and TNFα levels compared with controls (Arablou *et al*, 2014). Oxidative stress is thought to have a significant role in pancreatic beta cell dysfunction and death in diabetes. Ginger extract treatment of streptozotocin-induced diabetic rats resulted in decreased levels of serum 2, 3-dihydroxybenzoic acid and 2,5dihydroxybenzoic acid metabolites, which are markers of oxidative damage.

The experimental evidence, in humans and laboratory animals, for beneficial effects of *Zingiber officinale* on diabetes, diabetic complications and hyperlipidemia has been reviewed (Li *et al*., 2012). There is evidence from numerous studies that ginger reduces blood glucose in diabetics, although some studies failed to show this. Improvements in parameters of metabolic syndrome and insulin resistance, improved carbohydrate and lipid metabolism, have all been demonstrated. In some cases, there is evidence that specific substances in ginger, including the most active ingredients in ginger, including gingerols and shogaol may mediates one of these benefits. Ginger has also shown protective effects on diabetic microvascular complications (Li *et al*, 2012).

In an *in vitro* pancreatic beta-cell cell culture model of oxidative damage to pancreatic *β* cells, free radical scavenging activities and composition of the isolated n-hexane and ethanolic extracts of ginger root were studied. The ethanolic extract of ginger lowered markers of oxidative stress in the beta cells and improved beta cell viability (Rackova *et al*, 2013).

In a randomized, double-blind, placebo-controlled trial in which 88 human diabetics were randomly given ginger or placebo for 8 weeks, the mean fasting blood glucose showed a decrease of 10.5% in the ginger-treated patients, whereas the mean fasting blood glucose increased by 21%

in the placebo group patients. Hemoglobin A1C also improved in the diabetics. Median fasting insulin level and HOMA-IR also improved in the ginger-fed patients (Ahmadi *et al*, 2013).

Rats treated with ginger extract during a high fat, high carbohydrate diet showed significant improvements of insulin sensitivity using the homeostatic model assessment of insulin resistance (HOMA-IR) after 10 weeks, compared with rats fed the diet alone. The ginger component,[6]-gingerol, the major pungent phenolic principle in ginger, dose-dependently (from 50 to 150 μM) increased adenosine monophosphate (AMP)-activated protein kinase (AMPK) α-subunit phosphorylation in L6 skeletal muscle cells, suggesting that 6-gingerol might be one of the components of ginger that improved the insulin resistance of these rats (Li *et al*, 2014).

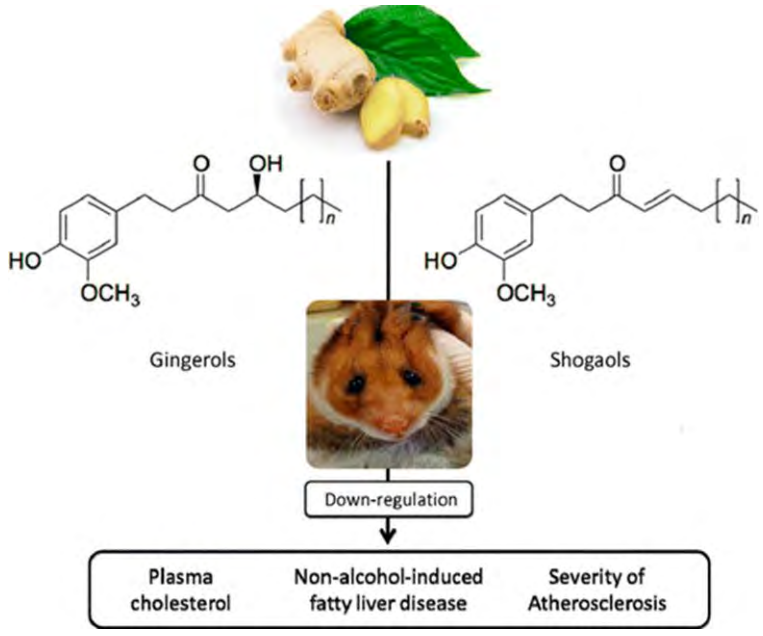


Figure 3. Cholesterol-lowering activity of gingerol and shogaol-enriched ginger extract (GSE) (Lei *et al*, 2014)

To summarise, there is adequate evidence that ginger rhizome may have beneficial effects on diabetes, hyperlipidemia and inflammatory markers of metabolic diseases in experimental animals as well as humans.

1.22 Statement of the Problem and Significance of the Study

Ethiopia has an alarming problem with the prevalence diabetes trending upwards. According to International Diabetes Federation (IDF) estimation, 3.5 % of Ethiopians had diabetes in 2011 and by 2030 there will be almost 2 million diabetics in Ethiopia (Abebe *et al*, 2014). A study of bankers and school teachers in Addis Ababa showed a prevalence of diabetes of 6.5% (Nshisso, 2012). The World Health Organization (WHO) estimated in 2011 that diabetes affects 5% of Ethiopians, and that 34% of the Ethiopian population will die from non-communicable diseases, with a national cardiovascular disease prevalence of 15%, cancer and chronic obstructive pulmonary disease prevalence of 4% each. Another study in Gondar found a prevalence of diabetes of 0.5% in high school students, and of 2.4% in ante-natal clinic attendees (Peters *et al* 1983).

Multiple classes of drugs are used for type 2 diabetes, including various classes of oral antidiabetic agents, insulin and injectable incretin-based drugs (Krentz and Bailey, 2005). These antidiabetic agents often have undesirable side effects in some patients, and can be expensive (Inzucchi *et al,* 2012). Similarly, anti-dyslipidemia drugs often have undesirable side effects (Ridker *et al*, 2008).

Plants are one of the most important sources of medicines. Ginger has been shown in some studies to improve diabetes control, reduce insulin resistance, improve fasting lipids and reduce inflammatory markers of diabetes and other metabolic diseases, yet it is not widely used or recommended for treating diabetes or dyslipidemia in Ethiopia or anywhere else, and data on the effects of ginger on diabetes and dyslipidemia are inconsistent.

Ginger is readily and inexpensively available year-round and widely consumed in Ethiopia. This research project was designed to investigate, in a controlled mouse model, inducing diabetes with streptozotocin, the effect of ginger homogenate and a steam distillate of ginger rhizome on fasting

glucose and lipid profiles of mice, and to compare the results with the effect of simvastatin on cholesterol levels.

This study could add knowledge about the effects of ginger on diabetes and diabetic dyslipidemia that might be useful in encouraging possible treatment of diabetics in Ethiopia with ginger. In Ethiopia, inexpensive, safe and effective natural treatments for diabetes are still not available. It is hoped that ginger and other plants in this family would open up clinical research, trials using humans and biochemical methodologies, and possibly provide new treatment or prevention options for diabetes.

1.23 Hypothesis of the Study

There are multiple hypotheses because numerous parameters were studied. The general hypothesis is that ginger homogenate and/ or steam-distilled extracts of ginger, as well as simvastatin, will improve hyperglycemia and lipid profiles in diabetes induced in mice by STZ.

1.24 Objectives of the Study

1.24.1 General Objectives

The general objective of this study was to evaluate effects of ginger rhizome homogenate and steam-distilled extract of ginger rhizome on diabetes control and fasting lipid profiles of mice, and to compare them with the effects of simvastatin treatment of mice.

1.24.2 Specific Objectives

1. To assess the effect of ginger rhizome homogenate on fasting blood glucose of normal and STZ- induced diabetic mice.
2. To assess the effect of ginger rhizome homogenate on fasting lipid profiles of normal and STZ-induced diabetic mice.
3. To assess the effect of simvastatin on fasting blood glucose and fasting lipid profiles of STZ-induced diabetic mice.
4. To compare the effect of ginger rhizome homogenate on STZ-treated mice with the effect of simvastatin
5. To assess the effects of a steam distillate of ginger rhizome, containing 25% gingerol, on blood glucose and serum lipids of STZ-induced diabetic mice

Chapter 2

MATERIALS AND METHODS

2.1 Chemicals and Instruments

Simvastatin (PRIACIN Medochemic Ltd, UK), pH meter (Calimatic, Germany), sodium acetate (Sigma-Aldrich, USA), Streptozotocin (Sigma-Aldrich, Germany), Sonicator (JANKE & KUNNEL IKA®Labortechnik, USA), liquid nitrogen (Kality Agriculture and Reproduction Center, Ethiopia), weighing balance (Adam Equipment, Switzerland), Auto lab 18 fully automated clinical chemistry analyzer (Italy), Sensocard glucometer.

2.2 Study Setting

The study was conducted at Department of biochemistry, school of medicine, Addis Ababa University.

2.3 Experimental Mice

Due to the absence of estradiol in male mice, they tend to be more susceptible to STZ-induced diabetes (Deeds *et al*, 2011). Therefore, seventy male Balb/c mice aged between 10 and 12 weeks with initial weight of 29 to 33g were used for the study, and were purchased from the Ethiopia Health Institution. To minimize effects of environment-induced physiological changes and endocrine systems changes (Obernier and Baldwin, 2006), they were given two weeks of acclimatization period in the animal house, Department of Biochemistry. School of Medicine, Addis Ababa University. The experiment was performed in the laboratory of Department of Biochemistry, School of Medicine, Addis Ababa University after ethical approval from ethical committee of the Department of Biochemistry, School of Medicine, Addis Ababa University

(protocol number 04/2014). Mice were given free access to tap water and standard mice pellets, made from ground animal food obtained from the Ethiopia Health Institution, Kality. They were housed in polycarbonate cage (6 to 8 mice/cage) at room temperature and with natural night-daytime exposure.

2.4 Plant Rhizome Collection and Authentication

Samples of ginger (*Z. officinale*) rhizomes were purchased from the local markets of Addis Ababa in late February and March, 2015. The seeds were identified and authenticated by the National Herbarium (ETH) of Addis Ababa University, and voucher number 009/zingibil/2015 was given and deposited at the same institute for further reference.

2.5 Preparation of Ginger Rhizome Homogenate

Many methods can be used to extract plant rhizomes (Odey *et al*, 2012). Common extraction methods are ultrasonic extraction and liquid nitrogen freeze-thawing, or a combination of the two. These methods are very effective in terms of time taken for extraction process and can operate at room temperature. In addition to this, they increase the yield of medicinal plant components several fold. Sonication was reported to increase sugar and protein yield by 50% and 46%, respectively. Freeze-thawing with liquid nitrogen breaks plant cells open efficiently, yields more phenolic compounds and produces a less variable extract. Both sonication and liquid nitrogen freeze-thawing have been used separately or in combination, to prepare homogenates and extracts of plant tissues, including rhizomes (Tan *et al*, 2013).

For this study, a kilogram of fresh ginger rhizome was purchased from the local Shola Market, Addis Ababa, Ethiopia in late February and March 2015. Any foreign material such as small stones, leaves and other debris, were first removed. Then the ginger rhizomes were washed, peeled,

cut into small pieces (5 mm cubes) and weighed on a balance. Then about 150mL of liquid nitrogen was poured into the mortar in order to pre-chill the mortar for further treatment. Next the rhizome pieces were transferred into the pre-chilled mortar. Another 150 mL of liquid nitrogen was poured into the mortar containing the rhizome fragments. The frozen rhizome fragments were then ground with a pestle and mortar, and after evaporation of liquid nitrogen, formed a dry powder which was stored at -20^{o}C until use.

For homogenate preparation for administration to mice, 160 g of the ginger powder was dispersed in 300 mL boiled water to form a suspension. The suspension was sonicated with multiple pulses at medium amplitude, each of 10 to 60 seconds. Finally the sample was kept in a refrigerator at 4^{o}C until used for the experiment.

2.6 Administration of Ginger Homogenate and Steam Distillate of Ginger Rhizome to Mice

For ginger rhizome homogenate administration, 160g of ginger rhizome powder, following liquid nitrogen extraction, was suspended in 300 mL water to prepare a concentrated suspension. Large particles were allowed to settle by gravity, then the doses of 15mL/kg per day (about 0.5 mL volume per mouse per day) of the supernatant ginger homogenate, equivalent to 2g/kg per day of dry ginger powder, were administered to each of the treated mouse by oral gavage.

For treatment with steam distillate of ginger rhizome, 0.1 mL of steam distillate was mixed with 0.4 ml of water to form an emulsion and gavaged orally into each mouse. The steam distillate of ginger rhizome was obtained from Original Brand Manufacturing (Guangdong, China) and is a pure 100% water-immiscible ginger distillate containing 25% v/v gingerol. Control mice received water instead of homogenate or distillate.

2.7 Preparation of Simvastatin for Administration

Ten simvastatin tablets (40mg simvastatin per tablet) were weighed then the tablets were grounded to powder with pestle and mortar. It was found that an average 40 mg simvastatin tablet weighed 400 mg, so that 10% of the weight of each tablet was simvastatin and 90% binding material. Aliquots of 0.13g of simvastatin powder (equaivalent to 13 mg of active drug) were transferred into 1.5 mL Eppendorf tubes and kept as powder until use. For administration to mice, simvastatin was prepared as a 0.13g of powder in 4 mL of PBS on the day of use, and 0.4mL of this simvastatin solution was administered intraperitoneally (IP) using a 22-guage syringe needle. Mice received a daily intraperitoneal dose of 40 mg/kg of simvastatin

2.8 Induction of Diabetes with Streptozotocin

To induce diabetes, STZ is typically given through a single intravenous administration or through multiple low-dose intraperitoneal administrations (Deeds *et al*., 2011). For this experiment, diabetes was induced by two intraperitoneal injections of 33 mg/kg streptozotocin, which was dissolved in freshly prepared 0.01M citrate buffer, pH = 4.5. Injections were one week apart. Two weeks after the first STZ injection, mice with random blood glucose levels of 200mg/dL and above were considered as diabetic and included in the study group.

2.9 Blood Glucose Test

Fasting blood glucose was determined by tail vein venipuncture, using a commercial Sensocard glucometer. A drop of blood, obtained by tail vein bleed, using a scalpel blade and cutting 1 mm off the end of the mouse tail, was placed on a Sensocard test strip and blood glucose concentrations read from the glucometer for baseline blood glucose, after 1 week treatment, after 2 weeks treatment and for every two weeks treatment until the experiment ended.

The Sensocard glucometer is based on the principle that glucose oxidase, present in the test strip, converts blood glucose to gluconolactone and hydrogen peroxide. Electrons are generated by using an electron acceptor compound in the glucometer, generating an electric current that is converted to a digital signal that is proportional to the glucose concentration in the blood sample. The upper limit of detection of the Sensocard monitor is a blood glucose concentration of 600 mg/dL.

2.10 Blood Collection for Serum Lipid Profile Determination

Mice were subjected to 3- to- 6 hour fasting prior to biochemical testing. Then, fasting blood samples were collected directly from tail veins for blood glucose determination, by cutting, with a scalpel blade, a small amount (1 mm) of tissue from the end of the tail.

However, larger amounts of blood were required for serum preparation and serum lipid profile analysis, so blood was collected from the facial/temporal vein, which can be done without anesthetic and allows 0.5 cc to 1.5 mL of blood to be obtained terminally (mouse will die from this blood loss), or less blood (0.2 mL) without killing the mouse (Francisco *et al*, 2015). The hairless freckle was located on the side of the jaw. A scalpel blade was used to make an incision about one third the way between the freckle and the inferior part of the ear, and drops of blood were collected by gravity into an Eppendorf tube. In this study, mice were terminally bled, allowing 0.5 to 1.5 mL of blood to be obtained. Mice were then humanely killed by neck dislocation (Francisco *et al*, 2015)

2.11 Estimation of Serum Lipid Profiles

The following parameters were evaluated: Total cholesterol (TC), low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides (TG). These were determined with Auto lab 18

fully automated clinical chemistry analyzer (Italy). Low density lipoprotein cholesterol level was calculated using Friedewald equation (Burtis *et al*, 2008).

Serum was separated after coagulation of blood at room temperature for 30 min and centrifugation at 2000 rpm for 10minutes. Serum was stored at -70^{o}C until biochemical parameters were determined.

2.12 Determination of Total Cholesterol

2.12.1 Principle of the Method

Total cholesterol was measured enzymatically in serum in a series of coupled reactions that hydrolyze cholesteryl esters and oxidize the 3-OH group of cholesterol. Cholesterol esters are hydrolyzed to free cholesterol by cholesterol ester hydrolase (CE). The free cholesterol produced is oxidized by cholesterol oxidase (CO) to cholest-4-en-3-on with the simultaneous production of hydrogen peroxide, which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase to yield Quinoneimine dye with maximum absorbtion between 500-550 nm.

Reactions

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol ester hydrolase}} \text{Cholesterol} + \text{Fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholest-4-ene-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O$$

The test comes in the form of a commercial kit in which serum sample is incubated with enzymes and reagents from the kit and the change in absorption at 500nm is measured spectrophotometrically. This change in absorption is proportional to the concentration of total cholesterol in the serum sample and can be calculated by comparison with absorption changes that occur with standard solutions containing known cholesterol Concentrations.

2.12.2 Procedure

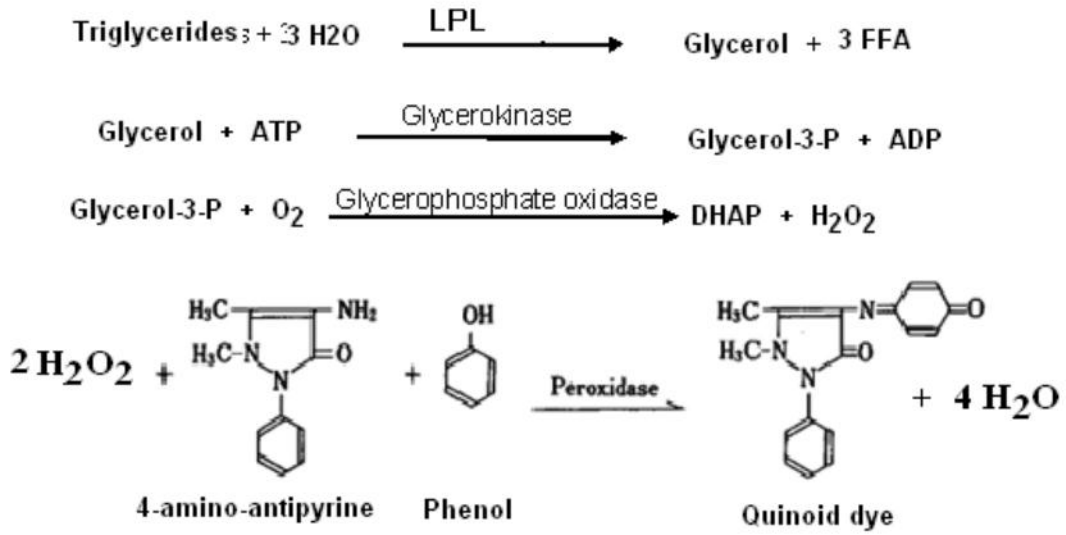
Ten microliter (10µL) serum sample were added into the sample cups and put on the sample disk which rotates to bring the desire sample cup in to position next to the sample probe for specimen sampling. 1000µL reaction reagent (4-Aminophenazone, phenol, peroxidase, cholesterol esterase, cholesterol oxidase) were pipetted into reagent bottles leveled for TC and put on reagent disk and then on the screen menu of the machine TC was entered as a parameter to be tested. The sample probe was pipetted sample from the sample disk and transferred to the reaction disk which contains cuvettes. On the other side of the machine, the reagent probe was pipetted reagents from the reagent disk and transferred it into reaction disk which is a large rotatable disk holding reusable cuvettes with a stirring paddle to stir or mix thoroughly the sample and the reagents. The cuvettes were immersed into reaction water bath and incubated at 37^0C for 5 minutes. Next the reaction disk was rotated the cells to all reaction stations including the photometer light path. Finally, the light was passed through the cuvettes and absorbance of the sample was measured at 500nm.

2.13 Determination of Serum Triglyceride (TG)

2.13.1 Principles of the Method

The method is based on the enzymatic hydrolysis of triglycerides to glycerol and free fatty acids (FFA) by lipoprotein lipase (LPL). Glycerol is converted to glycerol-3-phosphate (G3P) and adenosine-5-phosphate (ADP) by glycerol kinase and ATP. G-3-P is oxidized by glycerol phosphate oxidase to form dihydroxy acetone phosphate (DHAP) and hydrogen peroxide (H_2O_2). In the presence of peroxidase and H_2O_2, 4-aminoantipyrine couples with phenol to form a coloured product (a quininoid dye) that can be measured spectrophotometrically at a wavelength of 500nm.

Reactions Principle:



The triglyceride test comes in the form of a commercial kit containing the reagents, reactants and enzymes needed. Serum samples will be incubated with the kit reagents and enzymes for 5 minutes at 37^0C and absorbance measured at 500 nm against the reagent blank and against known

concentrations of standard triglyceride concentrations. The change in absorbance is proportional to the concentration of triglyceride in the serum sample.

2.13.2 Procedure

Ten micro liter (10µL) serum samples were added into the sample cups and put on the sample disk which rotates to bring the desire sample cup into position next to the sample probe for specimen sampling. 1000µL buffer and 1000µL substrate were pipetted into reagent bottles leveled for TG and put on the reagent disk. Then on the screen menu of the machine TG was entered as a parameter to be tested. The sample probe was pipetted sample from the sample disk and transferred to the reaction disk which contains cuvettes. On the other side of the machine, the reagent probe was pipetted reagents from the reagent disk and transferred it into rotatable reaction disk holding reusable cuvettes with a stirring paddle to stir or mix thoroughly the sample and the reagents. The cuvettes were immersed in to reaction water bath and incubated at 37^0C for 5 minutes. Next the reaction disk was rotated the cells to all reaction stations including the photometer light path. Finally, the light was passed through the cuvettes and absorbance of the sample measured at 500nm.

2.14 Determination of Serum HDL

2.14.1 Principles of the Method

The basic principle of the method is as follows. The apoB containing lipoproteins in the specimen react with antibodies to apoB that renders them nonreactive with the enzymatic cholesterol reagent under conditions of the assay. The enzymes used are also pegylated, and this allows them to react only with HDL and not with antibody-bound LDL, VLDL or chylomicrons. The apoB containing

lipoproteins are thus effectively excluded from the assay and only HDL is detected under the assay conditions.

The HDL-Cholesterol test is a two reagent homogenous system for the selective measurement of serum or plasma HDL-Cholesterol in the presence of other lipoprotein particles. The assay is comprised of two distinct phases. In phase one, it is likely that in the presence of slightly alkaline buffer and magnesium sulfate and dextran sulfate selectively form water-soluble complexes with LDL, VLDL, and chylomicrons, which are resistant to PEG-modified enzymes. In phase two the cholesterol concentration of HDL cholesterol is determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups (approx. 40%).

$$\text{HDL-cholesterol esters} + H_2O \xrightarrow[\text{esterase}]{\text{PEG cholesterol}} \text{cholesterol} + \text{free fatty acids}$$

Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase.

$$\text{cholesterol} + O_2 \xrightarrow[\text{oxidase}]{\text{PEG cholesterol}} \text{cholestenone} + H_2O_2$$

In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase Δ^4 cholestenone and hydrogen peroxide.

$$2\ H_2O_2 + \text{4-amino-antipyrine} + \text{HSDA}^* + H^+ + H_2O \xrightarrow{\text{peroxidase}} \text{Purple-blue pigment} + 5H_2O$$

HSDA= N-(2-hydroxy-3-sulfopropyl)-3, 5-dimethoxyaniline

In the presence of peroxidase, the hydrogen peroxide generated reacts with 4-aminoantipyrine and HSDA to form a purple-blue dye. The color intensity of this dye is proportional to the cholesterol concentration and can be measured spectrophotometrically.

2.14.2 Procedure

Ten micro liter (10μL) serum samples were added into the sample cups and put on the sample disk which rotates to bring the desire sample cup into position next to the sample probe for specimen sampling. 1000μL buffer and 1000μL substrate were pipetted into reagent bottles leveled for HDL-C and put on the reagent disk. Then on the screen menu of the machine HDL-C was entered as a parameter to be tested. The sample probe was pipetted sample from the sample disk and transferred to the reaction disk which contains cuvettes. On the other side of the machine, the reagent probe was pipetted reagents from the reagent disk and transferred it into rotatable reaction disk holding reusable cuvettes with a stirring paddle to stir or mix thoroughly the sample and the reagents. The cuvettes were immersed in to reaction water bath and incubated at 37^0C for 5 minutes. Next the reaction disk was rotated the cells to all reaction stations including the photometer light path. Finally, the light was passed through the cuvettes and absorbance of the sample was measured at 500nm.

2.15 Serum LDL Cholesterol Determination

Most of the circulating cholesterol is found in three major lipoprotein fractions: very low density lipoproteins (VLDL), LDL and HDL. LDL-cholesterol is calculated from measured values of total cholesterol, triglycerides and HDL cholesterol according to the Friedewald equation:

[LDL C] = [Total Cholesterol] - [HDL] - [TG]/5

Where [TG]/5 is an estimate of VLDL-C and all values are expressed in mg/dL. The equation is derived from another equation, [Total Cholesterol] = [VLDL-C] + [LDL-C] + [HDL-C], but TG are easier to estimate than VLDL and [TG/5] is a good estimate of VLDL, although the Friedewald equation is not valid for calculating LDL if the serum TG is above 400 mg/dL.

2.16 Statistical Analysis

The data was subjected to statistical analysis. The whole values of body weight, fasting blood sugar and serum biochemical parameters were expressed as mean ± standard Deviation (SD) and were performed using SPSS statistical software Package Version 21.0. The values were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's post hoc test. The graphs were made by using prism graphpad 6. The p-value less than 0.05 were statistically significant.

Chapter 3

RESULTS

3.1 General Preliminary Observations

Fifteen of 48 mice treated with STZ had fasting blood glucose levels less than 200mg/dL two weeks after initial STZ injection. The remaining 33 STZ-treated mice developed overt diabetes mellitus, with fasting blood glucose over 200 mg/dL, and these STZ-treated mice were chosen for further studies. Fasting blood glucose above 200 mg/dL was chosen to define diabetes in mice, according to previous recommendations of using diabetic cut-off values between 200 mg/dL and 300 mg/dL (Bernstein, 1966). Twenty nine out of these 33 diabetic mice survived the whole experimental period. Four diabetic mice died at week two to three after STZ treatment; these mice showed visibly significant body weight loss, possibly due to severe ketoacidosis. Therefore, only the data of 29 overtly diabetic mice and 22 non-treated control mice were included in this study. All non-treated, non-diabetic mice had fasting blood glucose levels between 111 and 165 mg/dL after 2 to 3 weeks.

Additionally, the mice treated with the steam distillate of ginger rhizome were sluggish and weak, and also had localized sparse hair and irritation around their mouthparts, likely due to irritation from steam distillate organic compounds from the gavage procedure, but this was not true for those treated with ginger rhizome homogenate.

3.2 Blood Glucose Levels of Mice Show Small Increases Immediately Following Intraperitoneal Injections, Regardless of Substance Injected.

It was noticed that within 30 minutes after injection of mice, regardless of whether STZ or buffer vehicle alone were injected, blood sugars increased slightly (Table 1). Therefore a small study was done using three mice per group, in which one group was injected with STZ and the other group was injected with buffer vehicle alone, and blood glucose levels were followed over the subsequent 60 minutes.

Table 1. Mean blood glucose levels of mice injected with STZ (in buffer) or with buffer vehicle alone during the 60 minutes following the injections.

Time	Blood glucose (mg/dL) STZ treated only	Blood glucose (mg/dL) Buffer treatment only (no STZ)
baseline	172.7 ± 6.5	169.3 ± 9.4
16 minutes	213.3 ± 25.2	212.3 ± 27.9
30 minutes	212.3 ± 27.9	182.7 ± 17.4
60 minutes	184 ± 11.0	168.7 ± 9.4

Baseline measurements were taken immediately prior to injections, then at intervals thereafter. Mice received intraperitoneal injections of either 0.54 mL of acetate buffer vehicle alone or 0.54 mL of STZ in acetate buffer at a dose of 33 mg/kg.

According to the result of this study, blood glucose levels of mice appeared to show small increases immediately following intraperitoneal injections, regardless of substance injected, though these

were not significant by statistical analysis. The fasting blood glucose in the group treated with STZ alone increased above 200 mg/dL between 16 minutes and 30 minutes, then fell after 60 minutes to less than 200 mg/dL. Likewise, the control (buffer vehicle only) group had elevated blood glucose at 16 and 30 minutes, followed by a fall to baseline blood glucose after 60 minutes. There were no significant differences between mice treated with STZ alone and mice treated with buffer alone.

These results suggest that there may be a small increase in blood glucose regardless of whether STZ or buffer alone is injected, and this occurs within 30 minutes after injection, but subsequently the blood glucose returns to normal, though more mice need to studied to see if this is significant. It is possible that manipulation of the mice causes anxiety to them and increased blood glucose occur due to catecholamine release, and if mice are allowed to relax the catecholamine response declines. If this were correct, therefore, it is important to ensure that mice are not overly aggressively manipulated prior to or during blood glucose determination, to minimize anxiety-induced hyperglycemia.

3.3 Heterogeneity of Fasting Blood Glucose among Streptozotocin-treated Mice

Over the course of the study, 74.1% of mice developed overt diabetes after STZ treatment (Table 2). After 2 weeks following the second STZ injection, blood glucose was over 550 mg/dL (the upper limit of the Sensocard glucometer is 600 mg/dL) in 25.9% of mice, between 450 mg/dL and 549 mg/dL in 11.1% of mice, between 350 mg/dL and 449 mg/dL in 7.4%, between 250mg/dl and 349mg/dl in 14.8%, between 150mg/dL and 249mg/dL in 37% and less than 150 mg/dL in the remaining 3.7% of mice.

Two apparent peaks were seen for the blood glucose distribution in STZ-treated mice. The first peak occurred in mice with blood glucose less than 250 mg/dL and the second peak occurred in mice with blood glucose above 550 mg/dL (Figure 4), suggesting the possibility that there were two major subgroups of mice within the strain used, with different susceptibilities to STZ-induced diabetes (Figure 4).

Table. 2. Heterogeneity of fasting blood glucose among streptozotocin-treated mice.

Blood glucose (mg/dL)	Number of mice	Percentage
<150	1	3.7
150-249	10	37.0
250-349	4	14.8
350-449	2	7.4
450-549	3	11.1
>550	7	25.9
Total	27	100

Mice were given two STZ injections (33 mg/kg) one week apart, then fasting blood glucose was measured two weeks after the second injection.

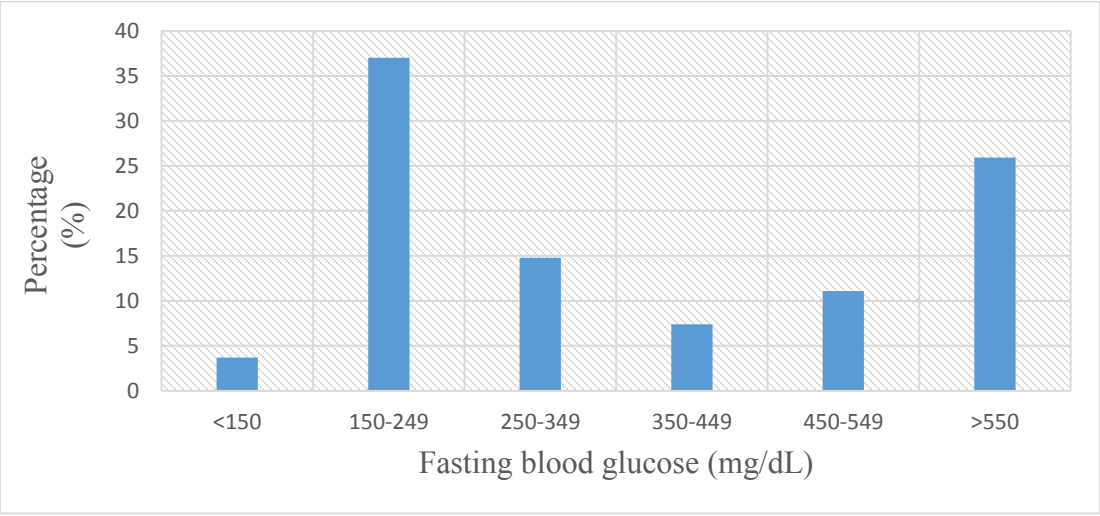


Figure 4. Heterogeneity of fasting blood glucose among mice treated with streptozotocin. Mice were given two intraperitoneal STZ injections (33 mg/kg) a week apart; fasting blood glucose was measured 2 weeks after the second STZ injection

3.4 Effects of Ginger Rhizome Homogenate and Simvastatin on Diabetic Mice

3.4.1 Effect of Ginger Rhizome Homogenate and Simvastatin on Body Weight in Diabetic Mice

As shown in Table 3, mice treated with STZ alone lost a mean of 3.7g of weight after 56 days, whereas control non-diabetic mice gained a mean of 14.4 g in weight. The STZ-diabetic mice treated with ginger rhizome homogenate lost a mean of 2.1g in weight, slightly less than the weight lost (3.7g) by STZ-treated mice that did not receive homogenate. Ginger rhizome homogenate-treated non-diabetic mice gained a mean of 12.3 g, which was similarly to the 14.4 g gained by non-diabetic mice that did not receive homogenate.

Diabetic mice treated with simvastatin lost a little weight (0.5g), and simvastatin compensated only mildly for the weight loss due to diabetes, since non-diabetic mice gained 14.4 g of weight. Hence, ginger homogenate treatment slightly reduced weight loss associated with STZ treatment, as did simvastatin treatment, and the effect was more pronounced in the group of mice were treated with simvastatin than in those treated with ginger rhizome homogenate.

Table 3. Effect of ginger rhizome homogenate and simvastatin on body weight in control and STZ-treated mice.

	Body weight of mice (g)			
Treatment group	Before treatment	After 56 days of treatment	Weight change	p-value
Non-diabetic mice	32 ± 2.9	46.4 ± 3.7	14.4	$p < 0.05$
Non-diabetic mice + ginger rhizome homogenate alone	30.3 ± 1.7	42.6 ± 4.5^{y}	12.3	$p < 0.05$
STZ-diabetic mice + ginger rhizome homogenate	32.1 ± 2.9	30 ± 4.0^{x}	-2.1	
STZ-diabetic mice, non-treated	33.3 ± 5.6	29.6 ± 2.6^{x}	-3.7	
STZ-diabetic mice + simvastatin	35.3 ± 2.5	34.8 ± 7.8^{x,y}	-0.5	

STZ-induced diabetic mice were treated with simvastatin (40 mg/kg/day intraperitoneally) for 56 days, or with ginger rhizome homogenate (2g/kg/day by oral gavage) for 56 days and mice were then weighed. Control non-diabetic mice, with and without ginger homogenate treatment were also weighed after 56 days.

The results show mean ± SD (control non-diabetic mice, non-diabetic mice treated with ginger, n = 8; STZ-diabetic mice, STZ-diabetic mice treated with ginger homogenate, STZ-diabetic mice treated with simvastatin, n = 7). $^{x}p < 0.05$ compared with non-diabetic mice; $^{y}p < 0.05$ compared with STZ-diabetic mice.

3.4.2 Effect of Ginger Rhizome Homogenate and Simvastatin on Fasting Blood Glucose in STZ-induced Diabetic Mice.

The effect of ginger rhizome homogenate on fasting blood glucose levels in mice treated with STZ alone are given in Table 4 and in Figures 6 and 7. In the mice treated with ginger rhizome homogenate, buffer alone (controls) and simvastatin, fasting blood glucose was measured every two weeks after initial treatment. Before induction of diabetes, there was no significant difference in fasting blood glucose among the study groups. Fasting blood glucose in the non-diabetic control mice and non-diabetic mice treated with ginger rhizome homogenate showed no change, and remained normal, throughout the experiment. After 2 months of treatment, mean fasting blood glucose values of STZ-diabetic mice treated with ginger rhizome homogenate (555.4 ± 44.9 mg/dL) were higher than fasting blood glucose of mice treated with STZ alone (461 ± 52.5 mg/dL) which was statistically significant ($P<0.05$). The STZ-diabetic mice treated with simvastatin had a lower mean fasting blood glucose (274.8 ± 223.4 mg/dL) than mice that were treated with STZ alone (461 ± 52.5 mg/dL) ($p < 0.05$, Figure 5), though the range of blood glucose levels in the simvastatin-treated diabetic mice was wide. This result indicates that 56 days of oral gavage administration of ginger rhizome homogenate increased fasting blood glucose levels of diabetic mice, while intraperitoneal injection of simvastatin decreased fasting blood glucose of diabetic mice.

Table 4. Effect of ginger rhizome homogenate and simvastatin on fasting blood glucose in non-diabetic control mice and diabetic mice.

Treatment group:	Blood glucose (mg/dL) day 0	day 14	day 28	day 42	day 56
Non-diabetic mice	141.9 ± 10.3^{z}	126.2 ± 4.9^{y}	127.9 ± 3.9^{y}	136.6 ± 8.3^{y}	129.3 ± 7.8^{y}
Non-diabetic mice + ginger rhizome homogenate	148.1 ± 12.7	$152.7 \pm 8.4^{x,y}$	$99 \pm 10.5^{x,y,z}$	134 ± 26.8^{y}	128.6 ± 7.7^{y}
STZ-induced diabetic mice	145.2 ± 7.4^{z}	398 ± 114.9^{x}	$417 \pm 111.9^{x,z}$	448 ± 102.3^{x}	461 ± 52.5^{x}
STZ-induced diabetic mice + ginger rhizome homogenate	154.2 ± 14.3^{z}	$506 \pm 109.4^{x,y,z}$	$536.3 \pm 56.4^{x,y}$	$589 \pm 20.2^{x,y,z}$	$555.4 \pm 44.9^{x,y}$
STZ-induced diabetic mice + simvastatin	151.5 ± 18.1^{z}	$391 \pm 184.3^{x,y,z}$	$279 \pm 168.8^{x,y}$	$280 \pm 197.6^{x,y}$	$274.8 \pm 223.4^{y,x}$

Non-diabetic control mice or STZ-induced diabetic mice were treated with or without ginger homogenate (2g/kg/day) for 56 days, and STZ-induced diabetic mice were treated for 56 days with or without intraperitoneal 40mg/kg/day simvastatin and blood glucose levels were measured. The results shows mean ± SD (n= 6-8) ^{x} $p < 0.05$, compared with the mice treated with non-STZ diabetic, ^{y} $p < 0.05$, compared with mice treated with STZ-induced diabetic, ^{z} $p < 0.05$ compared mice fasting blood glucose within the group.

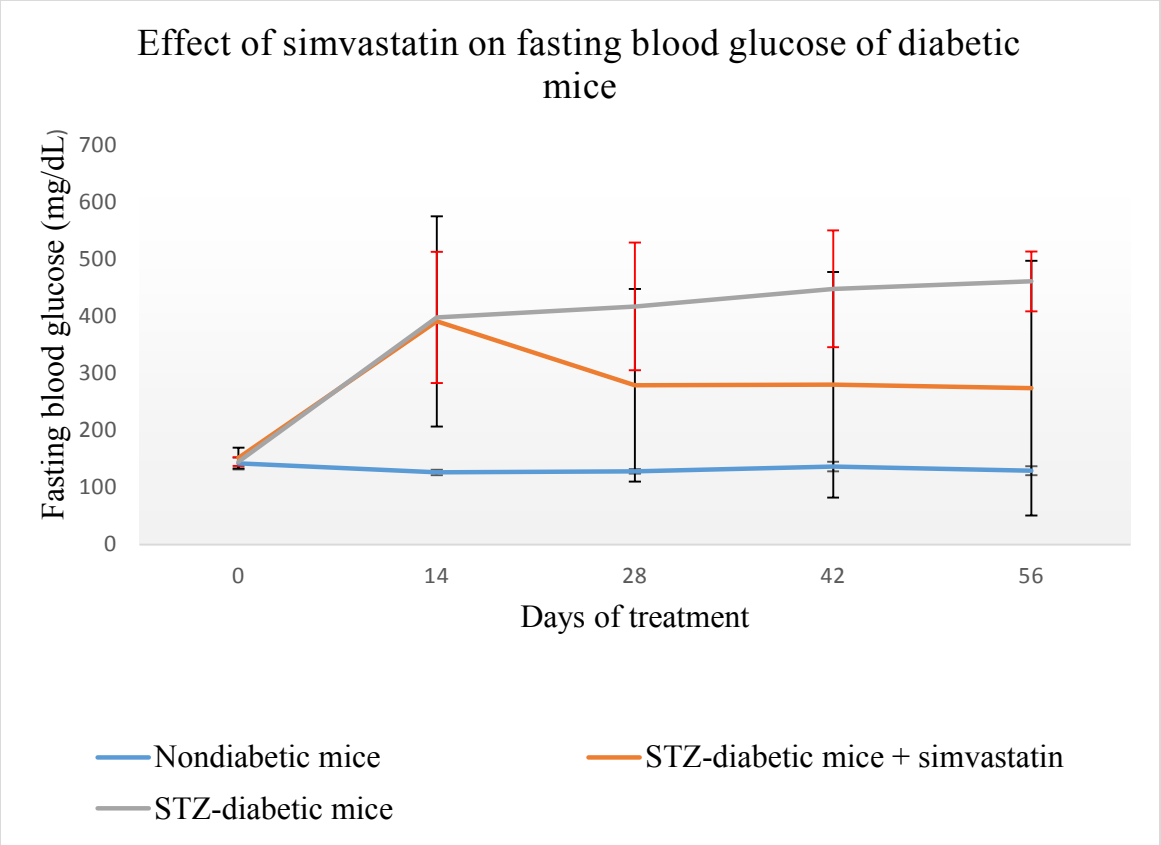


Figure 5. Effect of intraperitoneal simvastatin (40mg/kg/day) on fasting blood glucose of streptozotocin (STZ) induced diabetic mice compared to mice treated with STZ alone and control mice non-diabetic mice. Values are expressed as mean ± standard deviation of mean for 6 to 8 mice

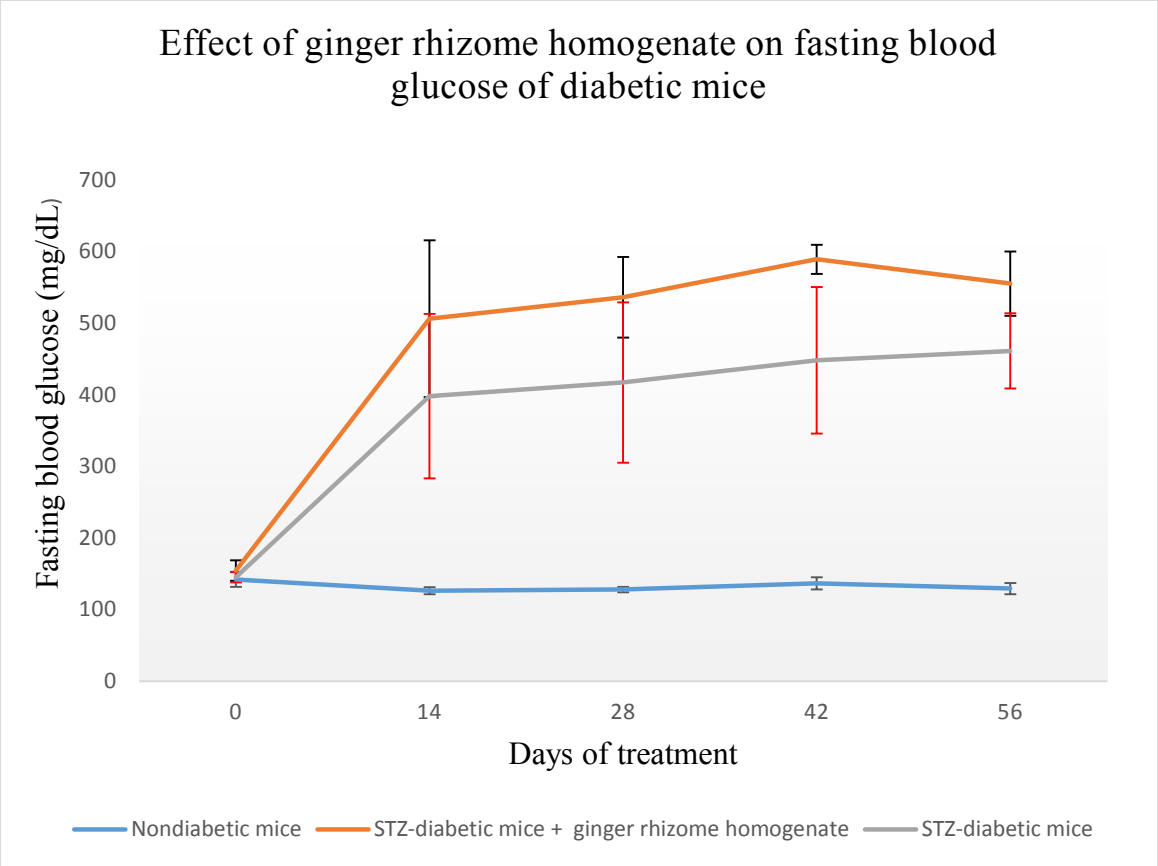


Figure 6. Effect of ginger rhizome homogenate (2g/kg/day) on fasting blood glucose of STZ-induced diabetic mice compared with non-diabetic mice. Values are expressed as mean ± standard deviation

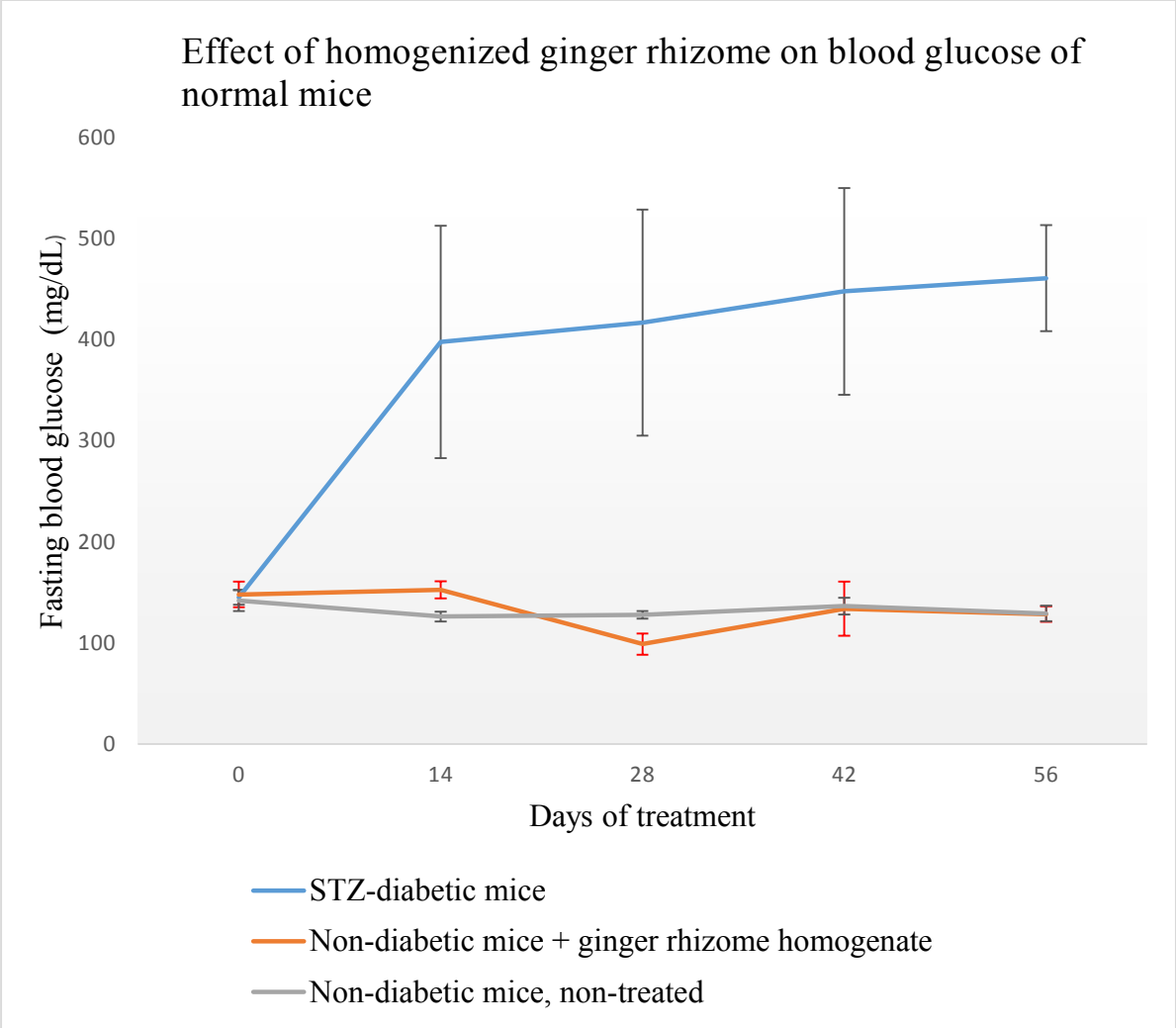


Figure 7. Effect of ginger rhizome homogenate (2g/kg/day) on fasting blood glucose of control non-diabetic mice compared with STZ-induced diabetic mice. Values are expressed as mean ± standard deviation for 6-8 mice.

3.4.3 Effect of Ginger Rhizome Homogenate and Simvastatin on Serum Lipid Profiles in Diabetic Mice

The levels of serum triglyceride, total cholesterol, HDL and LDL cholesterol of experimental mice are shown in Table 5. Serum triglycerides increased (255.9 ± 36.5 mg/dL) significantly in mice

treated with STZ as compared with the control non-diabetic mice (210.6 ± 32.6 mg/dL). Serum triglycerides were significantly reduced (162.9 ± 55.7 mg/dL) in ginger rhizome homogenate-treated STZ-diabetic mice as compared with non-treated STZ-diabetic mice (255.9 ± 36.5 mg/dL). Serum total cholesterol slightly increased (169.4 ± 22.5 mg/dL) in mice treated with STZ as compared with non-diabetic mice (166 ± 24.7mg/dL). Serum TC was not significantly reduced (153.9 ± 36.9 mg/dL) in control non-diabetic mice that were treated with ginger rhizome homogenate as compared to the non-diabetic mice that were not treated with homogenate (Figure 8).

Serum high density lipoproteins (HDL) were decreased (34.2 ± 21.4 mg/dL) significantly in STZ-induced diabetic mice as compared with the (87.8 ± 22.3 mg/dL) non-diabetic control mice. Serum HDL increased (78 ± 20.1 mg/dL) significantly ($p < 0.05$) in STZ diabetic mice treated with ginger rhizome homogenate as compared to non-treated STZ-diabetic mice (Figure 8).

The serum LDL level was significantly ($p < 0.05$) lower in non-diabetic mice (36 ± 13.4 mg/dL) and in STZ-diabetic mice given ginger rhizome homogenate (43 ± 24.8 mg/dL) than it was in untreated STZ-diabetic mice Ginger rhizome homogenate treatment for two months reversed the above-mentioned values to nearly normal (Figure 8).

Serum HDL was increased (80 ± 14.6 mg/dL) significantly in STZ diabetic mice treated with simvastatin as compared to the mice (34.2 ± 21.4 mg/dL) that were treated with STZ alone (Figure 9). The serum LDL level was significantly decreased (22.2 ± 13.9 mg/dL) in mice that were treated with simvastatin. Serum triglyceride were not significantly reduced (215 ± 71.3 mg/dL) in mice treated with simvastatin group as compared to the STZ alone treated mice. Serum total cholesterol

were not significantly reduced (145 ± 16.3 mg/dL) in simvastatin-treated diabetic mice compared to non-diabetic mice (166 ± 24.7 mg/dL). Therefore, simvastatin treatment for 56 days improved HDL and LDL levels to near-normal levels, and had no effect on TG levels, in STZ-induced diabetic mice (Figure 9).

Table 5. Effect of ginger rhizome homogenate on lipid profiles in non-diabetic and STZ-induced diabetic mice.

Serum parameter	Non-diabetic mice	Non-diabetic mice plus ginger rhizome homogenate	STZ-induced diabetic mice	STZ-induced diabetic mice plus ginger rhizome homogenate	STZ-induced diabetic mice plus simvastatin
Total cholesterol (mg/dL)	166 ± 24.7	166.8 ± 16.4	169.4 ± 22.5	153.9 ± 36.9	145 ± 16.3
Triglyceride (mg/dL)	210.6 ± 32.6	224 ± 50.2	255.9± 36.5^{x}	162.9 ± 55.7^{y}	215 ± 71.3
HDL (mg/dL)	87.8 ± 22.3	86.7 ± 14.3^{y}	34.2 ± 21.4^{x}	78 ± 20.1^{y}	80 ± 14.6^{y}
LDL (mg/dL)	36 ± 13.4	32.7 ± 14.6	84 ± 25.5^{x}	43 ± 24.8^{y}	22.2 ± 13.9^{y}

Mice were treated with ginger rhizome homogenate (2g/kg/day oral gavage) or simvastatin (40mg/kg/day i.p.) for 56 days and lipid profiles determined. Each value represents mean ± SD (Buffer alone, Ginger rhizome homogenate alone, n = 8; STZ alone, STZ + ginger rhizome homogenate, STZ + simvastatin, n = 7), ^{x} $p < 0.05$ compared with mice treated with buffer alone and ^{y} $p < 0.05$ compared with mice treated with STZ alone. One-way ANOVA followed by Tukey's post-test.

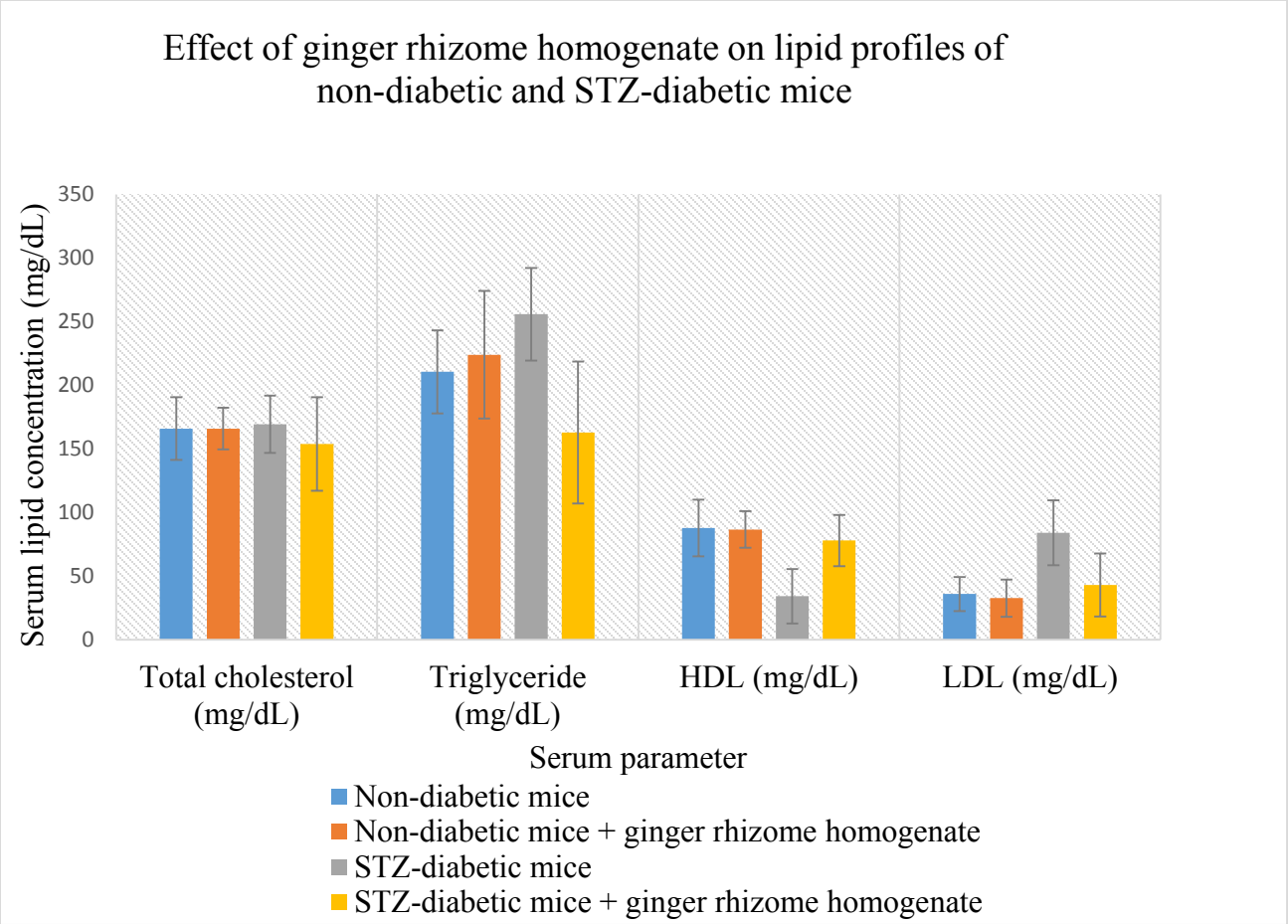


Figure 8. Effect of ginger rhizome homogenate on lipid profiles of diabetic and non-diabetic mice

Non-diabetic mice and STZ-induced diabetic mice were treated with 2g/kg/day ginger rhizome homogenate by oral gavage for 56 days, then serum lipids determined and compared with serum lipids of non-treated non-diabetic mice and non-treated STZ-induced diabetic mice.

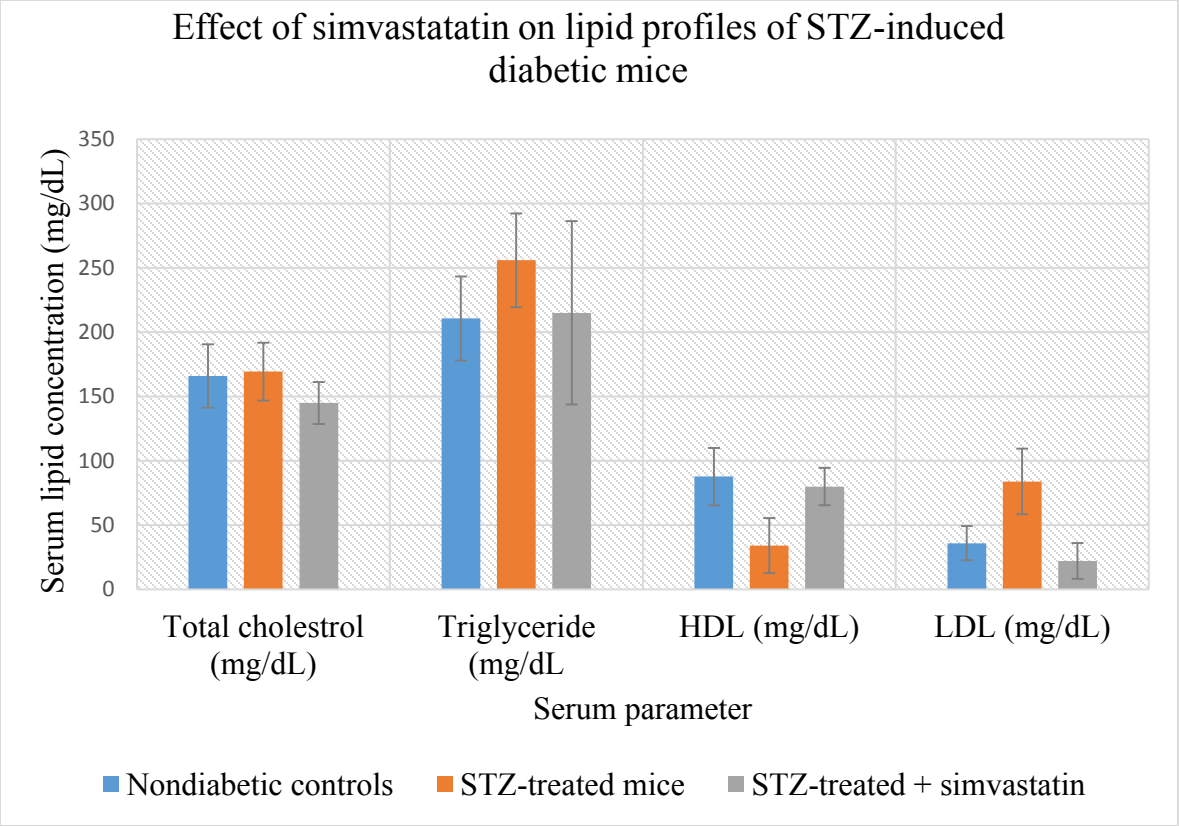


Figure 9. Effect of simvastatin on lipid profiles of STZ induced diabetic mice

STZ-induced diabetic mice were treated for 56 days with 40mg/kg/day of simvastatin intraperitoneally and serum lipid profiles were determined and compared with non-diabetic mice or STZ-induced diabetic mice treated intraperitoneally with buffer vehicle alone.

In Figure 10 the effects of ginger rhizome homogenate on lipid profiles are compared with those of simvastatin in STZ-induced diabetic mice. The ginger homogenate was less effective at lowering LDL in these mice than was simvastatin, but more effective than simvastatin at lowering triglycerides. Ginger homogenate and simvastatin were equally effective at raising HLD levels in STZ-diabetic mice.

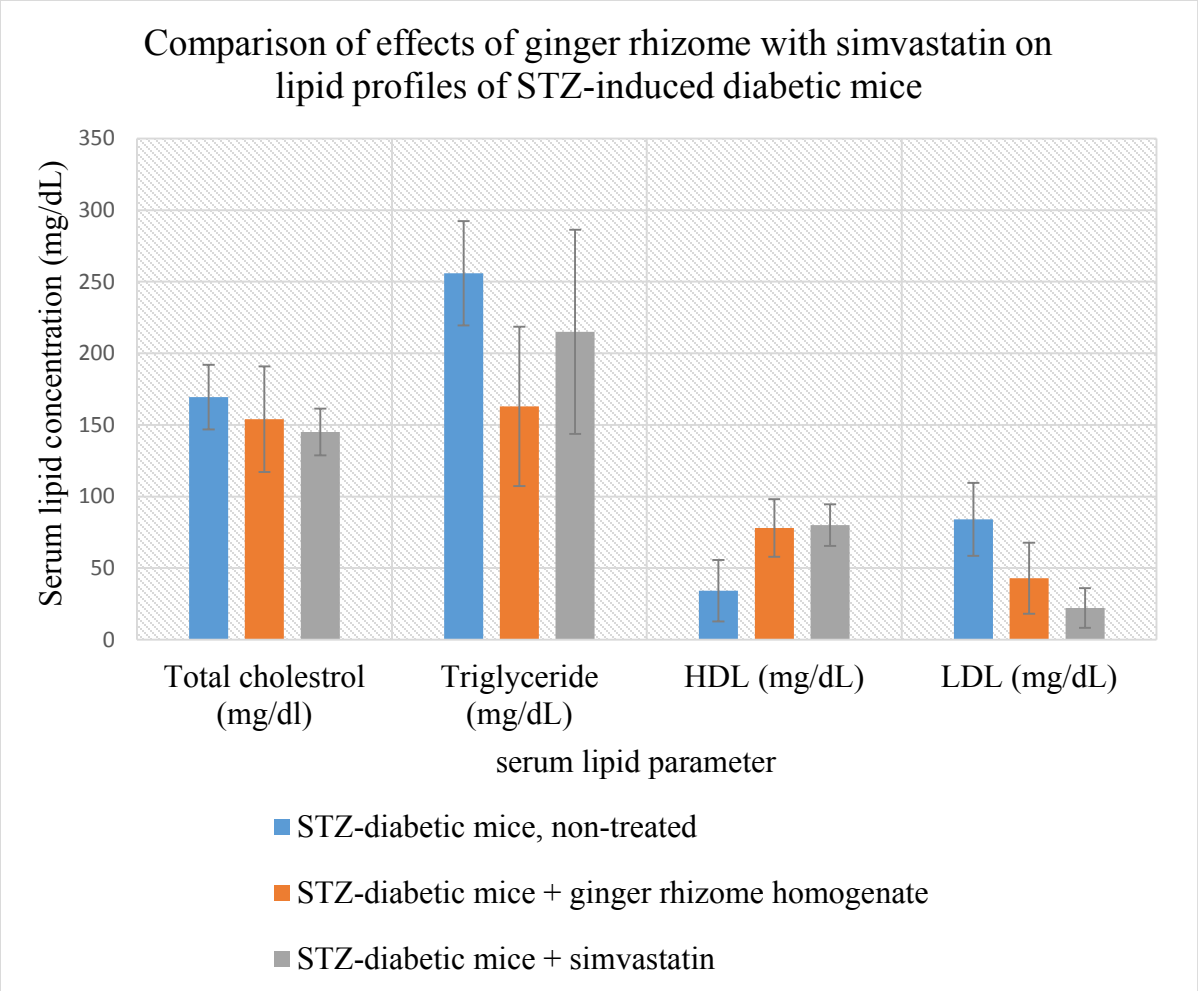


Figure 10. Comparison of the effects of ginger rhizome homogenate with those of simvastatin in STZ-induced diabetic mice

STZ-diabetic mice received no treatment, ginger rhizome homogenate (2g/kg/day oral gavage) for 56 days, or simvastatin (40mg/kg/day intraperitoneally) for 56 days then serum lipid panels were measured.

3.5 Effects of Steam Distillate of Ginger Rhizome on STZ-induced Diabetic Mice

A steam-distillate preparation of ginger rhizome consisting of mainly terpenoids and polyphenols was used to compare ginger homogenate, which contains most components of ginger rhizome, with the distillate, which contains mainly water-immiscible organic components. This distillate contains 25% gingerol, p-cineole, camphene, alpha-terpineol, zingiberene, pinenes, geranial and pentadecaenoic acid, as main components, and at least 65 other organic constituents of ginger.

3.5.1 Effect of Steam Distillate of Ginger Rhizome on Body Weight of STZ Induced Diabetic Mice after One Month of Treatment

The mean body weight of control mice treated with buffer alone increased in a month of treatment from 30.6 to 35.3g. However, the mean body weight of mice treated with STZ alone and mice treated with steam distillate of ginger rhizome extract plus STZ decreased significantly, from a mean of 32.4 g to 29.6g for STZ treatment alone, and from a mean of 32.7g to 302.2 g for STZ-diabetic mice treated with steam distillate ($p < 0.05$) (Table 6).

Table 6**.** Effect of steam distillate of ginger rhizome extract on body weight of STZ induced diabetic mice after one month of treatment.

Body weight (g)				
Treatment groups	Before treatment	One month after treatment	Weight change	p-value
Non-diabetic	30.6 ± 2.3	35.3 ± 4.8	+4.7	*P < 0.05*
STZ-diabetic	32.4 ± 1.5	29.6 ± 1.5 ^{x}	-2.8	
STZ + steam distillate ginger rhizome	32.7 ± 2.9	30.2 ± 2.4^{x}	-2.5	

The results shows mean ± SD (Buffer alone, n = 6; STZ alone, STZ + steam distillate of ginger rhizome extract n = 5), ^{X} $p < 0.05$ compared with mice treated with buffer alone after 1 month experiment and ^{y} $p < 0.05$ compared with mice treated with STZ alone after 1 month experiment.

3.5.2 Effect of Steam Distillate of Ginger Rhizome on Fasting Blood Glucose in Diabetic Mice.

Fasting blood glucose levels were measured after 0, 7, 14 and 28 days of the study (Table 7 and Figure 11). No significant change in fasting blood glucose was observed in control non-diabetic mice treated with buffer alone (no STZ). The fasting blood glucose of mice treated with STZ alone increased to a level of higher than 200mg/dL within two weeks after STZ treatment and these mice maintained their hyperglycemia till the experiment ended (Table 7). In the SZT-diabetic mice treated with steam distillate of ginger rhizome, there was a gradual and statistically significant reduction in mean blood glucose levels from 283.5 mg/dL on day zero to 200.8 mg/dL after one

month of treatment, showing that the steam distillate of ginger rhizome lowered blood glucose in STZ-induced diabetic mice.

Table 7. Effect of steam distillate of ginger rhizome on fasting blood glucose in diabetic mice.

	Blood glucose (mg/dL)			
Treatment	Day 0	Day 14	Day 21	Day 28
Non-diabetic mice	169.2 ± 5.2	174.8 ± 12.4	175 ± 12.5	170 ± 10.3
STZ-induced diabetic mice	178 ± 5.3	282.6 ± 50.7^{x}	302.4 ± 33.1^{x}	308.6 ± 32.9^{x}
STZ-induced diabetic mice treated with ginger rhizome steam distillate	165.6 ± 16.7	283.5 ± 30.5^{x}	249.2 ± 16.2^{x,y}	200.8±29.2^{x,y}

Steam distillate of ginger rhizome (0.1 mL in an emulsion with 0.4 mL of water) was given by oral gavage daily for a month to STZ-induced diabetic mice, and blood glucose measured periodically during the treatment. Non-diabetic control mice and STZ-induced diabetic mice that were not treated with the steam distillate were also assessed.

Each value represents mean ± SD buffer alone treated mice, n = 6; STZ alone treated mice, n = 5, ^{x} $p < 0.05$ compared with the buffer alone treated values and ^{y} $p < 0.05$ compared with STZ alone treated mice. One-way ANOVA followed by Tukey's post-test.

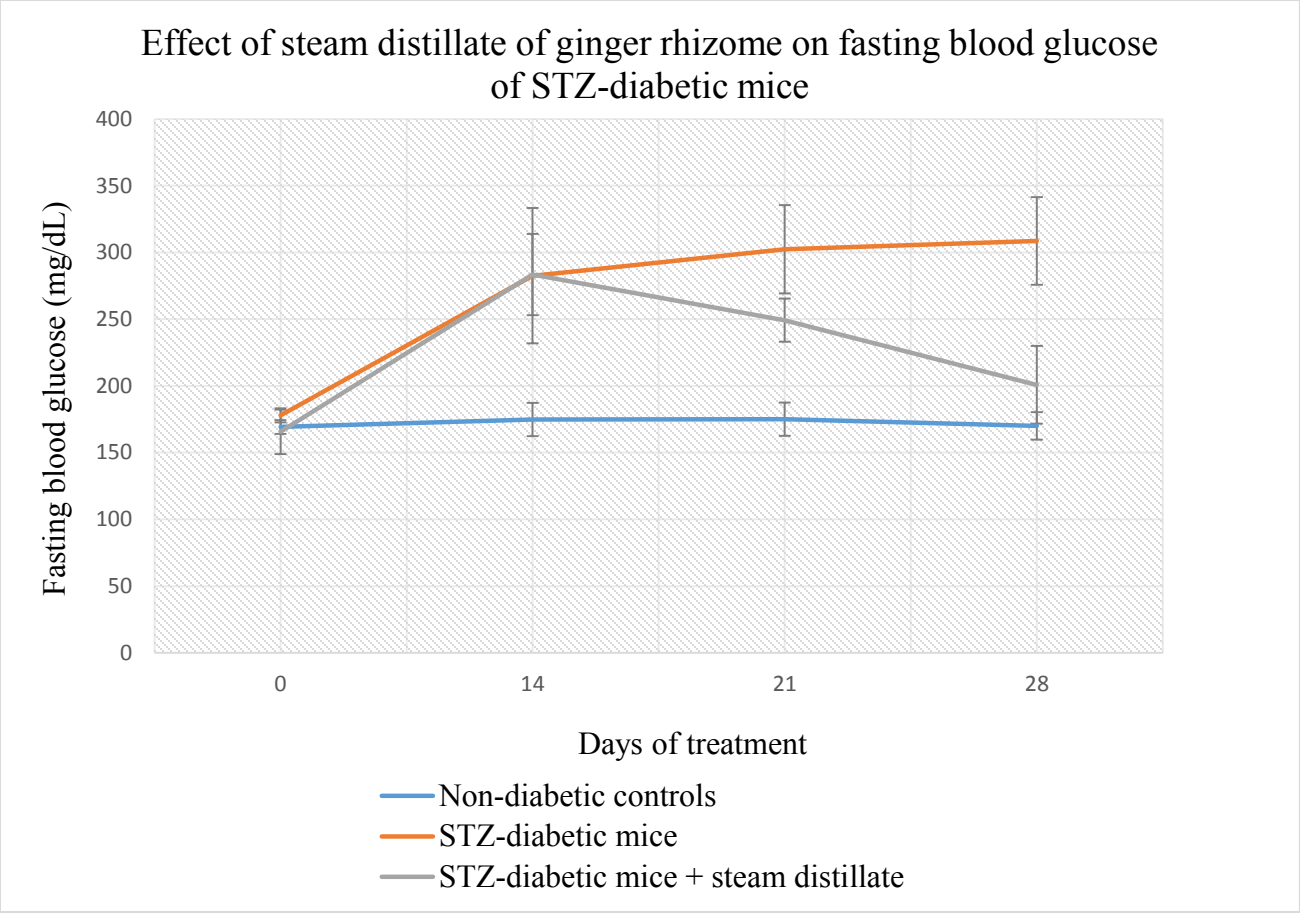


Figure 11. Effect of steam distillate of ginger rhizome on fasting blood glucose in experimental diabetic mice

Steam distillate of ginger rhizome (0.1 mL emulsified with 0.4 mL of water) was given by oral gavage daily to STZ-induced diabetic mice for 28 days, and fasting blood sugars measured periodically and compared with non-diabetic mice and STZ-induced diabetic mice that did not receive the steam distillate.

3.5.3 Effect of Steam Distillate of Ginger Rhizome on Lipid Profiles in Diabetic Mice.

After 28 days of treatment, total blood cholesterol levels were higher (mean, 200mg/dL, $p < 0.05$) in STZ-diabetic mice that were treated with the steam distillate of ginger rhizome compared with control non-diabetic mice (150.3 mg/dL), but not statistically significantly different from STZ-diabetic mice that did not receive distillate (214 mg/dL) (Table 8 and Figure 12). The mean serum HDL levels were 70.9 mg/dL in diabetic mice that were treated with steam distillate of ginger rhizome, compared with 66.2 mg/dL for diabetic mice that did not receive the distillate, and 89.3 mg/dL for non-diabetic mice.

STZ-diabetic mice had a higher mean (397.9 mg/dL, $p < 0.05$) serum triglyceride compared with STZ-diabetic mice (322.5 ± 114.2 mg/dL) that were treated with steam distillate of ginger rhizome extract, showing that the steam distillate lowers TG in diabetic mice. Non-diabetic mice (281.8 ± 2.5 mg/dL) had lower triglyceride levels than diabetic and distillate-treated diabetic mice.

LDL levels were decreased significantly ($p < 0.05$) in STZ-diabetic mice that were treated with steam distillate of ginger rhizome extract (68.4 $\pm$ 38.4 mg/dL in treated diabetic mice compared with 167.9 $\pm$ 15.3 mg/dL in non-treated diabetic mice). Non-diabetic mice had lower LDL levels (102.7 ± 35 mg/dL) than diabetic mice, but higher LDL levels than steam distillate-treated diabetic mice.

Thus, steam distillate improved LDL levels in STZ-induced diabetic mice, but had no significant effect on TC, TG or HDL levels.

Table 8. Effect of treatment for 28 days with steam distillate of ginger rhizome on lipid profiles of diabetic mice.

Serum lipid parameter	Non-diabetic mice	STZ-induced diabetic mice	STZ-induced diabetic mice treated with ginger rhizome steam distillate
Total Cholesterol (mg/dL)	150.3 ± 13.9	214.5 ± 11.6^{x}	200 ± 52.9^{x}
Triglycerides (mg/dL)	281.8 ± 2.5	397.9 ± 66.9^{x}	322.5 ± 114.2^{y}
HDL (mg/dL)	89.3 ± 15.6	66.2 ± 26.6^{x}	70.9 ± 10
LDL (mg/dL)	102.7 ± 35	167.9 ± 15.3^{x}	68.4 ± 38.4^{y,x}

STZ-induced diabetic mice were treated with or without a steam distillate of ginger rhizome (0.1 mL emulsified with 0.4 mL of water) daily for 28 days, then serum cholesterol profiles measured and compared with cholesterol profiles of non-diabetic mice.

Each value represents mean ± SD (Buffer alone, n = 6; STZ alone, Steam distillate of ginger rhizome + STZ; n = 5), ^{x} $p < 0.05$ compared with mice treated with buffer alone ^{y} $p < 0.05$ compared with mice treated with STZ alone group. One-way ANOVA followed by Tukey's post-test.

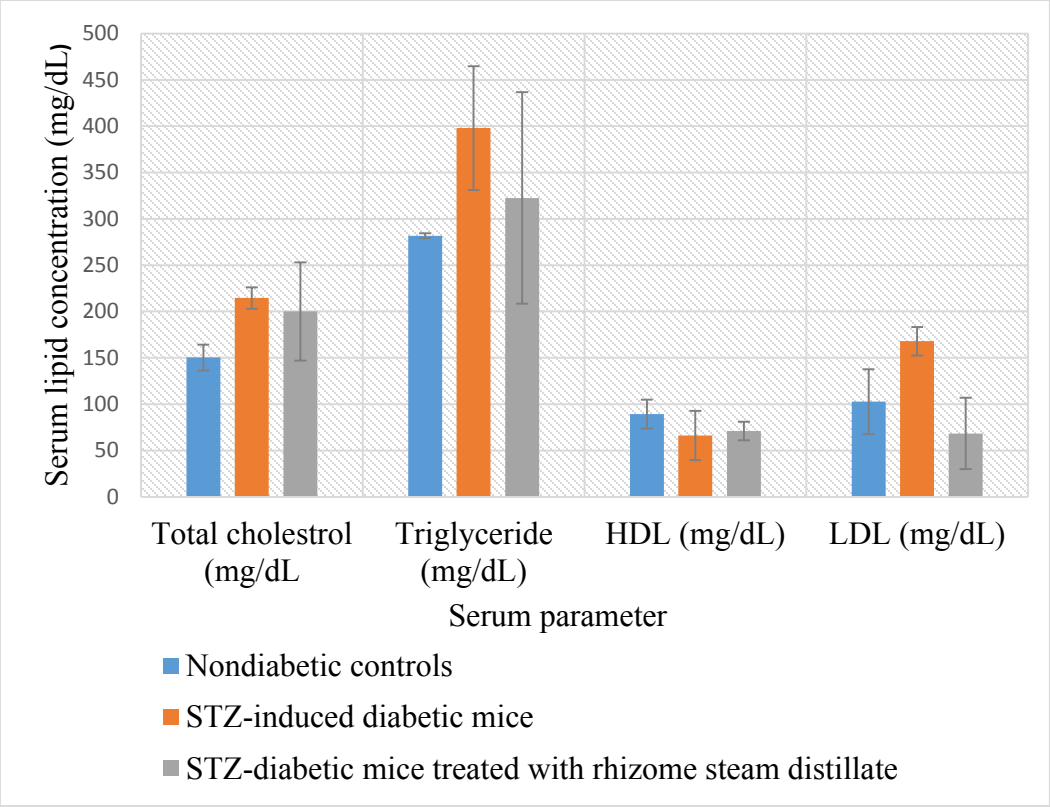


Figure 12. Effect of Steam distillate of ginger rhizome on lipid profiles in diabetic mice

Serum lipid panels were determined in control non-diabetic mice, STZ-induced diabetic mice or STZ-induced diabetic mice treated for 28 days with daily oral gavage of 0.5 mL of an emulsion of 0.1 mL ginger rhizome steam distillate plus 0.4 mL of water.

Chapter 4

Discussion

4.1 Heterogeneity of Fasting Blood Glucose among Streptozotocin-treated Mice

Streptozotocin sensitivity is highly variable in mice, and a dose that causes severe hyperglycemia in most animals may produce mild or no hyperglycemia in other mice. In this study there was a suggestion of heterogeneity according to the baseline fasting blood glucose two weeks after streptozotocin induction, and it therefore cannot be assumed that different mice within one strain of mice will react to streptozotocin in the same manner. Similarly, Arulmozhi *et al* reported that mice are highly heterogeneous in their response to both genetically and chemically induced type 2 diabetes mellitus (Arulmozhi *et al*, 2004). This is consistent with the results of this MSc research project, where there were apparently two populations of mice within those treated with streptozotocin, one with high and the other with low susceptibility to STZ (Figure 4)

Even though genetic susceptibility plays a role in diabetes, there are also other factors, such as the age, sex and weight of animals, which may alter sensitivity to streptozotocin (McNeill, 1999). Since mice of the same age and weight were used for our study the most likely cause of the different susceptibility to STZ genetic variability, though it is possible that some variability occurred due to inequalities in how well the STZ reached the pancreas after intraperitoneal injection. In addition, conditions and stimuli may contribute to variability in streptozotocin induced diabetes outcomes, and steps should be taken to expose animals to uniform conditions to help assure consistent streptozotocin-induced diabetes (Deeds *et al*, 2011).

4.2 Effects of Ginger Rhizome Homogenate and Simvastatin on Body Weight of STZ-induced Diabetic Mice

An increase in body weight of mice implies that anabolic effects have dominated the catabolic ones, while a decrease in body weight would mean that catabolism has dominated (Stanley *et al*, 2000). Diabetics lose weight also because elevated urine glucose causes loss of dietary carbohydrates from the body, with consequent wastage of dietary fuel and less glucose being converted to body fat stores. Diabetic mice showed a reduction in body weight as compared to normal control mice, and the weight further decreased during the experimental period.

Maximum doses of ginger rhizome homogenate (2g/kg) were used in this study, but further experiments using lower doses might be done to assess the dosage of homogenate that is effective. One study showed that treating rats with 5g/kg of ginger rhizome homogenate for 65 consecutive days has a high safety (Shalaby and Hamowieh, 2010). The choice of 2g/kg body weight of dosing of homogenate of *Zingiber officinale* used in the 56-days treatment of diabetic mice in this study was predicated upon the maximal practical and safe dose to give diabetic mice in a single dose of oral gavage administration.

A dose of 2g/kg/day in mice, would be equivalent to a 70 kg human taking 140 g per day of ginger rhizome solids as a homogenate, or eating 140 g of ginger rhizome. This quite feasible, and might be equivalent 4 or 5 cups of strong ginger tea (kesher) daily, for example.

Treatment with ginger rhizome homogenate did not improve the body weight after 2 months in STZ induced diabetic rats (Abdul Sani *et al*, 2014), which was consistent with the findings here in mice, in which STZ induced diabetic mice with ginger rhizome homogenate did not show

improved weight loss. Another study also showed that after treatment with an ethanolic extract of ginger at doses of 100, 200, and 400 mg/kg for 6 weeks, the marked rises in body weight induced by high-fat diet were significantly reduced (Nammi *et al*, 2009).

Similarly, in STZ induced diabetic mice treated with ginger rhizome steam distillate (containing 25% gingerol), weight loss was also observed. Singh *et al* reported that 6-gingerol showed weight reduction in type 2 diabetic db/db mice treated with (100mg/kg) for 12 days (Singh *et al*, 2009). The observed antihyperglycaemic effect of steam distillate in this study is not dependent on body weight reduction. However, another study found that diabetic mice treated with simvastatin had an increased body weight compared with non-treated diabetic mice (Wang *et al*, 2013). This was consistent with this MSc report, which found that simvastatin-treated STZ-diabetic mice showed a body weight reduction compare to both STZ alone and ginger rhizome homogenate-treated STZ-diabetic mice.

4.3. Effect of Ginger Rhizome Homogenate and Simvastatin on Fasting Blood Glucose of STZ-induced Diabetic Mice

Some studies have examined the effectiveness of ginger rhizome extract in hyperglycemia control *in vitro* (L6 myotube cell surface) and (L6 cultured rat skeletal muscle cells) on cells as well as in animal models but different results were also reported in these studies, and ginger extract increases GLUT 4 expression in some studies (Li *et al*, 2012).

It was reported that intraperitoneal injection of aqueous extracts of ginger had no effect on serum glucose of rats when animals were maintained with a standard pellet diet for a 7-week experimental period (Islam and Choi, 2008). Similarly, Abdul Sani *et al* found that aqueous extracts of ginger

alone or in combination with honey had no change on fasting blood glucose after 2 months treatment of STZ induced diabetic rats (Abdul Sani *et al*, 2014). Besides this, Thiraphatthanavong *et al* also found that ginger and purple waxy corn combination had no effect on hyperglycemia on rats treated with streptozotocin (Thiraphatthanavong *et al*, 2014). Bordia *et al* reported that a single oral dose (10 g of powder) of ginger had no fasting or postprandial glucose or lipid lowering effect in type 2 diabetes human subjects (Bordia *et al*, 1997). Ethyl acetate extract of dried rhizomes of *Z. officinale* also had no beneficial effects of blood glucose or insulinotropic effect in rats treated with streptozotocin (Kadnur and Goyal, 2005).

On the other hand, significant reductions of blood glucose using ginger rhizome juice were reported in diabetic mice (Ojewole, 2006) and diabetic rats (Madkor *et al*, 2010). In addition, another study showed that daily oral administration of aqueous extracts of ginger (1 mg/kg of body weight) for 6 weeks significantly reduced fasting blood glucose and increased serum insulin concentrations in streptozotocin-induced type 1 diabetic rats (Elshater *et al*, 2009).

Singhal and Joshi reported an elevation of blood glucose in normal rats treated with ginger powder (Singhal and Joshi, 1983, Oludoyin and Adegoke, 2014). However, in this MSc project, ginger rhizome homogenate significantly increase fasting blood glucose of streptozotocin induced diabetic mice, but not in normal mice. There are no published reports showing that ginger rhizome preprations worsen blood glucose in diabetic animals or humans.

Therefore, from the above reports and as well as this MSc project, it is obvious that the effects of ginger on glycemic control are not the same in all studies or in all experimental conditions, and there is a great deal of variability in the effects of ginger rhizome extracts on blood glucose levels in diabetic and non-diabetic animals and humans. Such inconsistency of results may be due to the

variation in chemical composition of the administered ginger rhizome extracts, depending on the preparation method, product origin, type of rhizome or storage duration (Jolad *et al*, 2005; Jolad *et al*, 2004).

In normal rats fed with fresh squeezed ginger juice for 6 weeks, neither blood insulin nor cholesterol and triglyceride were affected (Akhani *et al*, 2004). Furthermore, a standardized dry ginger ethanol extract (containing 1.9 w/w of gingerol) formulated in corn oil EV.EXT 33 (25, 50 and 100 mg/kg) did not show any effect on blood glucose in normal rats at 3 hours postdose (Weidner and Sigwart, 2000), which is in agreement with the findings of this MSc study.

A dose of 10 mg/kg of simvastatin also reduced fasting blood glucose of STZ induced diabetic rats comparing to mice treated with STZ alone (Mohamadin *et al*, 2011), consistent with this MSc project report, which showed that the mice treated with simvastatin at a dose of 40mg/kg have decreased mean fasting blood glucose compared with mice treating with streptozotocin alone. Another study found that simvastatin clearly potentiated the antihyperglycemic effect of glibenclamide and also administration of simvastatin recovered Langerhans cells from shrinkage in alloxan-induced diabetic rats (Begum *et al*, 2014).

Hydrie *et al* found that simvastatin did not affect all type 2 diabetes human subjects but it improved insulin sensitivity in a selected group of type 2 diabetes subjects who were insulin resistant at the start of the study (Hydrie *et al*, 2007). Similarly, in this study fasting blood glucose was variable among STZ-diabetic mice after eight weeks of simvastatin treatment. In humans, statins increase the risk of type 2 diabetes, though they are still recommended to treat dyslipidemia in diabetics because their positive cardiovascular outcomes outweigh their diabetogenic potential, and blood

glucose levels can be monitored and treated aggressively in diabetics on statins (Chogtu *et al,* 2015).

The accurate mechanisms by which statins may exert any influence on glucose metabolism are unclear but Statins have the potential to alter inflammatory marker. Hu *et al* reported that simvastatin significantly reduce C-reactive protein, TNF alpha and IL-6 levels which are marker of inflammatory processes (Hu *et al*, 2009). Also study show that positive and significant correlation between C-reactive protein and fasting blood glucose among diabetic patients (Baba *et al*, 2010). Another study reported that modest elevations in CRP cause insulin resistance in mice by decreasing skeletal muscle glucose delivery (Tanigaki *et al*, 2013). If this is true, then an argument can be made that statin are useful for treating the pro-inflammatory component of the metabolic syndrome. Therefore, the result of this study also support this concept.

In addition, a high scale study of adiponectin function in animal models of type 2 diabetes provided strong evidence that adiponectin promotes insulin sensitivity in muscle and in the liver (Yamauchi *et al*, 2007). the anti-inflammatory properties along with the capacity of statins to alter circulating levels of several adipokines known to affect glucose homeostasis, including adiponectin, leptin, visfatin and resistin, may beneficially alter glycemic status (Kostapanos *et al*, 2010).

4.4 Effects of Ginger Rhizome Homogenate and Simvastatin on Lipid Profiles of STZ-induced Diabetic Mice

Diabetic dyslipidemia as a metabolic abnormality occurs in diabetes mellitus frequently and is associated with high triglycerides, low HDL cholesterol and high LDL cholesterol level (Mooradian, 2009). He *et al* showed that the most common recognized abnormality was high

triglyceride, very low HDL cholesterol and high LDL cholesterol in STZ induced diabetic rats (He *et al*, 2015). This is consistent with the present study, where triglyceride and LDL cholesterol significantly increased, and HDL-cholesterol significantly decreased, after STZ induction of diabetic mice.

In this study oral gavage with ginger rhizome homogenate caused significant reductions in the levels of serum triglycerides and LDL cholesterol, whereas serum HDL-cholesterol was significantly increased in STZ-induced diabetic mice. Melrokh *et al* reported similarly that treatment with aqueous ginger rhizome extract in streptozotocin-induced diabetes rats caused reduction in the serum levels of triglycerides and LDL-cholesterol, and increased the level of serum HDL-cholesterol (Melrokh *et al*, 2010). Furthermore, Saeid *et al*. also found that oral administration of ginger extract to diabetic chickens for 6 weeks led to decreased serum levels of triglyceride and LDL, and increased serum HDL levels (Saeid *et al*, 2010).

Additionally, in high fat diet-treated rats, ginger extract effectively lowered levels of LDL-cholesterol and increased HDL cholesterol towards control levels, in addition to producing a marked reduction in elevated serum triglycerides (Nammi *et al*, 2009). Similar results were found in mice treated with a high fat diet, where *Zingiber officinale* extract (400 mg/kg for 6 weeks) reduced the enhanced triglyceride levels and showed a small but not significant decrease in hepatic cholesterol levels and increased HDL (Nammi *et al*, 2010).

Ginger rhizome consist of phytosterols (0.3mg/2g) (for example, ergosterol and sitosterol) which are plant sterols that inhibit intestinal absorption of cholesterol and blood cholesterol concentrations (Self Nutrition Data, 2015). It is possible that these may be contributing to the beneficial effects seen in this study.

The hypocholesterolemic effect of ginger may be due to increasing lipoprotein lipase enzyme activity and therefore hydrolysis of circulatory TG and decreasing serum TG (Shirdel *et al*, 2009). Ginger also reduces the Carbohydrate Responsive Element Binding Protein (ChREBP) gene expression in the liver. ChREBP, a transcriptional regulator of lipid and glucose metabolism, converts excess carbohydrates into TGs. Reduced ChREBP expression, decreases expression of acetyl CoA carboxylase (ACC) ACC1, fatty acid synthase, stearoyl CoA desaturase (SCD1), and glucose-6-phosphatase, glucogenic and lipogenic proteins and decreases fat accumulation in the liver, reduces serum TG and improves insulin resistance (Gao *et al*, 2012).

Ginger can increase hepatic cholesterol 7a-hydroxylase enzyme activity and the conversion of cholesterol into bile acids, resulting in reduced serum cholesterol concentration. Also there are observations that the compounds in ginger inhibit the biosynthesis of cholesterol in the liver of rats (Alizadeh *et al*, 2008). In addition to the effect of ginger on increasing bile secretion, increased fecal excretion of cholesterol and phospholipids by ginger can also reduce cholesterol levels (Sharma *et al*, 1991; Arablou *et al*, 2014).

Statins reduce serum cholesterol by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a rate-controlling enzyme for cholesterol biosynthesis. Some statins are obtained after fungal fermentation: lovastatin, pravastatin and simvastatin; others are made by chemical synthesis: fluvastatin, atorvastatin, and cerivastatin (Stancu and Sima, 2001). Plants such as *Pythium ultimum*, *Penicillium citrinum*, oyster mushroom and red yeast rice also contain natural statins (Rea, 2008). Statins remain the first-line therapy option for treating diabetic dyslipidemia. In addition to lowering LDL cholesterol in particular, studies have established that statins also have pleiotropic effects, including anti-inflammatory properties (Sadowitz *et al*, 2010). No publications were found showing that ginger contains a natural statin, though this cannot be ruled

out and further biochemical fractionation and purification methods will be necessary to identify the anti-dyslipidemic components of both the homogenate and distillate.

Simvastatin is the most commonly used statin drug and is widely used for the treatment of hypercholesterolemia (Kalo, 2009). Studies showed that treatment of rats with simvastatin improved lipid profiles, particularly LDL, HDL and triglyceride (Yao *et al*, 2010; Matikainen *et al*, 2010). Similarly, in this MSc study it was observed that intraperitonial injection of simvastatin significantly restored abnormal levels of serum lipid profile markers in diabetic mice, especially HDL and LDL.

In terms of effect of ginger rhizome homogenate on lipid profile it did better job of decreasing triglyceride and increasing HDL, but simvastatin was more effective at lowering LDL than was ginger homogenate. Simvastatin has common adverse side-effects associated with statin use, including gastrointestinal upset and muscle aches, although dose related hepatoxicity and myotoxicity are the most clinically serious adverse events (Vijayaraghavan, 2010). If ginger rhizome homogenate does have such beneficial effects on human cholesterol profiles as this study would suggest, it would preferable to statins because of its having fewer adverse side-effect and is less costly, but caution should be made in diabetics taking ginger.

4.5 Effects of Steam Distillate Ginger Rhizome on Fasting Blood Glucose and Lipid Profiles of STZ-Induced Diabetic Mice

Kazeem *et al* found that treatment of STZ induced diabetic rats with 6 gingerol significantly reduced fasting blood glucose levels (Kazeem *et al*, 2013). In the present study we found that mice treated with steam distillate of ginger rhizome displayed a significant reduction in fasting blood

glucose compare to diabetic mice. However, the blood glucose levels of steam distillate ginger rhizome treated groups still remained in the diabetic range. The major component of the steam distillate of ginger rhizome used in this MSc study is gingerol (25%). This component and 6-shoagol play major role in anti-hyperglycemic activity (Singh *et al*, 2009).

The anti-hyperglycemic effect of ginger may be due to its antagonistic activity against serotonin receptors (Arablou *et al*, 2014). Also ginger may inhibit the activity of intestinal alpha-glucosidase and amylase enzymes activity and thereby reduce absorption of glucose (Li *et al*, 2012). It is possible that they improve glucose uptake by peripheral tissues by increasing the GLUT4 protein, insulin receptors and improving *β*-cells function (Mahluji *et al*, 2013). There is also data suggesting that gingerols improve blood glucose through its effects on key enyzmes involved in glycogen and glucose metabolism. For example, gingerol may lower hepatic glucose-6-phosphatase activity and decrease hepatic gluconeogenesis, and it may inhibit glycogen phosphorylase activity. Khandouzi *et al* (2015) recently reported that human type 2 diabetics fed 2g/day of ginger powder for 12 weeks showed reduced blood glucose levels, improved HbA1c, improved apoB and apoB/apoA1 ratio, and lowered malondialdehyde (a marker for oxidant damage).

It may be due to ginger's rhizome effect on insulin sensitivity is through the activation of PPARɤ or up regulation of adiponectin. Islam *et al*. (2008) stated that the 6-gingerol and 6-shogaol in ginger rhizome, upregulate adiponectin and 6-shogaol has agonistic activity with PPARɤ. Plasma adiponectin levels and its RNA expression are reduced in type 2 diabetes, and increasing adiponectin, improves insulin sensitivity (Nammi *et al*, 2009).

In the present study, the treatment of the streptozotocin induced-diabetic mice with steam distillate of ginger rhizome did not significantly affect HDL or triglyceride levels of the mice, but did reduce LDL levels. Bhandari *et al* (2005) showed that ethanolic extract of ginger produced significant decrease in serum total cholesterol, LDL- cholesterol and triglycerides levels and increased HDL-cholesterol level to diabetic rats, and the extract protected the tissues from lipid peroxidation.

In this study we used a steam distillate of ginger rhizome and ginger rhizome homogenate. The homogenate increased fasting blood glucose significantly, while steam distillate decreased fasting blood glucose, in STZ-induced diabetic mice. The differential effects of homogenate and distillate on LDL, TG and HDL could be due to their different chemical compositions. There may be active substances in the homogenate that are not present in the distillate, and there may be substances in the distillate that are more concentrated and therefore active in the distillate but that are too dilute in the homogenate to be active in this study. The steam distillate has a very high concentration of gingerol which may have anti-hyperglycemic effects (Singh *et al*., 2009). It is also possible that multiple components are responsible for the effects seen, rather than one single component being involved. Further studies involving biochemical fractionation and purification of components of ginger should elucidate which components are involved in the effects seen on blood glucose and cholesterol profiles.

4.6 Conclusions

From this study, it was concluded that ginger rhizome homogenate, when given by oral gavage, appears to be an aggravating factor for fasting blood glucose, but improved serum triglyceride, LDL and HDL in STZ-induced diabetic mice. A steam distillate of ginger rhizome, containing mainly polyphenols (25% gingerol) and terpenoids, improved fasting blood glucose as well as LDL levels. Simvastatin not only improved diabetic dyslipidemia but also beneficially affected hyperglycemia in STZ-treated diabetic mice Simvastatin improved blood glucose, HDL and LDL levels in diabetic mice. These data suggest that ginger homogenate might be considered as a treatment for dyslipidemia in human diabetics, though its potential for exacerbating diabetes needs to be considered and investigated first. Steam distillates of ginger rhizome, however, may be beneficial for lowering LDL specifically, though again studies in humans need to be done.

4.7 Limitations of the Study

- Since there were restricted resources available and costs of the project were limited, more detailed and extensive biochemical analysis of ginger components and their effects on diabetes were not possible to do.
- The ginger rhizome was purchased from the local market, rather than being dug up freshly from the ground, so storage by the sellers may have caused some changes in the final homogenate. Also, the steam distillate took weeks between its transit from China to the USA and then to Ethiopia, and during this time there may have been chemical changes that alter its components.
- Diabetes was induced by streptozotocin in this study, and this may not equivalent biochemically and pathologically to classical type 1 or type 2 diabetes in humans, so the results may be different if they were done in non-STZ models of diabetes.
- The study was in laboratory animals and does not necessarily give a good picture regarding the possible effects of ginger on human diabetes patients. However, studies in animals are more easily controlled and less affected by confounding factors, particularly because all mice used are genetically and phenotypically similar to begin with.
- Additional biochemical tests were impossible to do in single mice because of the limited amount of serum obtained from one mice.
- During the study mice were gavaged with homogenate or with distillate instead of being fed these substances. Also, simvastatin was given intraperitoneally and not orally.

4.8 Recommendations

- Biochemical fractionation, purification and characterization of the active components of ginger rhizome should be carried out to determine and study which components are responsible for the effects of ginger rhizome and distillate on serum lipid profiles.
- Moreover, more experiments should be done with other animal models of diabetes, including high fat fed diabetic animals and genetic models of diabetes (for example, db/db and ob/ob mice), which will allow us to know how useful the ginger rhizome will be in treating metabolic syndrome, diabetes and dyslipidemia, and also elucidate the biochemical mechanisms by which ginger rhizome components produce their effects on blood glucose and dyslipidemia.
- Moreover, further research should be undertaken on the other parts of the ginger plant, including leaf, stem and roots.
- Study of blood and serum biochemical parameters such as insulin levels, organ function tests and glycated hemoglobin, histopathology of vital organs (liver and pancreas, for example) have to be performed to make this study complete in all respects.
- Preparation of ginger rhizome for use as a medicinal, food supplement or food for treatment or prevention of diabetes and dyslipidemia is recommended in the future, including clinical trials on Ethiopian populations who are prone to diabetes.
- Future genetic and biomarker studies may be used to profile patients with type 2 diabetes to identify those responding best to a specific anti-inflammatory drug.

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