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Evaluation of Antihyperglycemic and Hypoglycemic Activities of the Aqueous Leaf Extract of *Rubus Erlangeri* Engl (Rosacea) in Mice

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This is to certify that the thesis prepared by Akebereg Gorems, entitled with: Evaluation of Antihyperglycemic and Hypoglycemic Activities of the Aqueous Leaf Extract of *Rubus Erlangeri* Engl (Rosacea) in Mice and submitted in partial fulfillment of the requirements for the degree of Master of Science in Pharmacology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Evaluation of Antihyperglycemic and Hypoglycemic Activities of the Aqueous Leaf Extract of *Rubus Erlangeri* Engl (Rosacea) in mice.

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Addis Ababa University, 2019

Diabetes mellitus is one of the most common non-communicable disorder in the world. It occurs as a result of either the pancreas not producing enough insulin or the cells of the body not responding properly to the insulin which is produced. Management of diabetes mellitus with currently available modern medicines is costly and chances of side effects are high. These calls for investigations of new and better compounds with least side effects. The experimental plant, *Rubus erlangeri* is among *Rubus* species, which are used for management of diabetes mellitus in many countries but its effect is not yet experimentally validated. Therefore, the aim of the present study was to investigate the antidiabetic effects of this plant both *in vitro* and *in vivo* and find out the relevance of the plant in controlling blood sugar. The *in vitro* study was assessed using a 3, 5-Dinitrosalicylic acid method for α-amylase inhibition activity and DPPH assay method for antioxidant activity. The *in vivo* study on the other hand was carried out in normoglycemic, glucose loaded hyperglycemic and streptozotocin (200 mg/kg) induced diabetic mice. Blood glucose level was then determined using glucometer. In all *in vivo* models, the test groups received various (100, 200 and 400 mg/kg) doses of the *Rubus erlangeri* aqueous leaf extract whereas the positive controls received glibenclamide (5 mg/kg) and the negative control as well as the normal control groups received distilled water (10 mL/Kg). The *in vitro* results demonstrated that this plant has an IC50 of 7.34±0.02 µg/ml for DPPH scavenging activity and 4.90±0.44 µg/ml in α-amylase inhibition activity. In both cases, *Rubus erlangeri* showed comparable effect with the standard drugs (ascorbic acid and acarbose). In the *in vivo* study, the extract significantly reduced blood glucose level with 100 mg/kg at 60 min ($p<0.05$) as well as with 200 mg/kg and 400 mg/kg at the 1^{st} and 2^{nd} h ($p<0.01$) following loading of 2.5 g/kg of glucose. It did not, however, produced significant reduction of glucose level in normal mice compared to the control group. *Rubus erlangeri* also produced significant reduction in blood level in streptozotocin-induced diabetic mice. In the single dose study, the aqueous leaf extract lowered blood glucose level with 200 mg/kg and 400 mg/kg at the 3^{rd} and 4^{th} h ($p<0.05$). Moreover, blood glucose level was reduced with daily

administrations of the extract. The reduction in fasting blood glucose level was significant with lower dose ($p<0.01$) middle and higher dose of the extract at the 2nd week ($p<0.001$) and with all doses of extract at 3rd week ($p<0.01$). In addition, the extract produced less body weight reduction after diabetic induction when it was compared with negative control group. This study revealed that the aqueous leaf extract of *Rubus erlanger* has blood glucose lowering effect with low risk of hypoglycemia.

Key words: Diabetes Mellitus, *Rubus erlangeri*, *in vitro*, *in vivo*, Streptozotocin

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List of Abbreviations and Acronyms

AACE	American Association of Clinical Endocrinologists
ADA	American diabetes association
AMPK	AMP-activated protein kinase
BGL	Blood glucose level
CVB	Coxsackie virus B
DM	Diabetes mellitus
DNSA	3,5-Dinitrosalicylic acid
DPP-4	Dipeptidyl peptidase-4
DW	Distilled water
DPPH	2,2-diphenyl-1-picrylhydrazine
ER	Endoplasmic reticulum
FBG	Fasting blood glucose
GDM	Gestational Diabetes mellitus
GLP-1	Glucagon-like peptide-1
GLUT-2	Glucose transporter-2
GLUT-4	Glucose transporter-4
HbA1c	Hemoglobin A1c
HLA	Human leukocyte antigen
IDF	International Diabetics Federation
IsR	Insulin resistance
NO	Nitric oxide
NODM	Non -obese diabetic mice
OGTT	Oral glucose tolerance test

PPHG	Post-prandial hyperglycemia
RBP-4	Retinol-binding protein-4
RBS	Random blood sugar
SGLT2i	Sodium-glucose co-transporter inhibitors
STZ	Streptozotocin
T1DM	Type 1 Diabetes mellitus
T2DM	Type 2 Diabetes mellitus
TNF-α	Tumor necrosis factor-α
TZD	Thiazolidinediones
WHO	World Health Organization

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1. Introduction

1.1. Overview of diabetes mellitus

The term Diabetes comes from the Greek word "*diabainein*" and it means a "siphon". It was coined by the Greek physician, Aretus. Aretus used the word siphon to describe patients who were passing too much water like a siphon. The word became "diabetes" from the English adoption of the Medieval Latin diabetes. Later on, in 1675, Thomas Willis added mellitus to the term, *Mel in* Latin refers "honey" since the urine and blood of people with this condition contains excess glucose, and glucose is sweet like honey. Moreover, diabetes mellitus (DM) could literally mean, siphoning off sweet water in ancient China people (Suresh, 2016).

According to the American Diabetes Association (ADA), DM is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. It is classified as type 1 (T1DM), type 2 (T2DM) and gestational DM (GDM) based on the pathogenic process leading to hyperglycemia. There are other forms of DM, which is associated with diseases of the exocrine pancreas and drugs or chemicals (Riddle, 2019). T1DM develops because of absolute insulin deficiency due to autoimmune β-cell destruction. On the other hand, insulin resistance that progressively leads to loss of β-cell secretion causes T2DM. Women who develop diabetes during pregnancy are classified as having GDM. GDM is diagnosed in the second or third trimester of pregnancy that was not clearly diabetes prior to gestation. It occurs in about 4% of all pregnancies. Patients with GDM have a 30% - 50% chance of developing DM after delivery (Mane *et al.*, 2012).

The chronic hyperglycemia in diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Failure of these organs is frequently associated with development of macro and microvascular diseases. Neuropathy, nephropathy, and retinopathy are microvascular complications, whereas macrovascular complications consists of cardiovascular disease such as stroke and peripheral artery disease (Papatheodorou *et al.*, 2018). The diagnosis of DM is based on hemoglobin A1c (HbA1c) criteria or plasma glucose criteria. Thus, 8 h fasting blood glucose (FBG) ≥ 126 mg/dL or 2 h post 75 g oral glucose tolerance test (OGTT) ≥ 200 mg/dl or HbA1c ≥ 6.5% or in patients with symptoms of hyperglycemia, a random plasma sugar (RBS) of ≥ 200 mg/dl confirms the presence of DM (Punthakee *et al.*, 2018).

1.2. Epidemiology of diabetic mellitus

DM is becoming a disease of major concern both globally and regionally and is a leading cause of death in most countries (Zimmet *et al.*, 2014). It is one of the four major non-communicable diseases comprising; cardiovascular diseases, cancers and chronic respiratory diseases jointly contributing to 63% of non-communicable deaths worldwide (Mohamed *et al.*, 2018). According to International Diabetics Federations (IDF) by 2017, there were 425 million people with DM. A projection of DM prevalence from this number was expected to rise to 629 million by 2045. In addition, 4 million people died from DM that year. In terms of economic crises, it was estimated to cause a loss of 727 billion dollars in health expenditure which is almost 12% of total money spending on adults for health issue (IDF, 2017). Similarly, based on WHO reports, the number of people with diabetes has risen from 108 million in 1980 to 422 million in 2014. DM is a major cause of blindness, kidney failure, heart attacks, stroke, and lower limb amputation. In 2016, an estimated 1.6 million deaths were directly caused by DM (WHO, 2018).

In terms of regional distributions, more than 60% of people with diabetes live in Asia, with almost one-half in China and India combined. The western Pacific, the world's most populous region, has more than 158.8 million people in 2017 with diabetes, and the number may rise to 183.3 million by 2035 (Nanditha *et al.*, 2016). According to IDF estimates done to determine regional distributions of DM by 2040 using projection from 2015, North America and Caribbean exhibit the highest DM prevalence in comparison to the other regions, whereas Europe would have 71.1 million people with DM, at a prevalence of 10.7% (Sandu *et al.*, 2016). In Africa, the prevalence and magnitude of DM is increasing progressively (Gebre, 2013). The estimated prevalence of diabetes in Africa is 1% in rural areas and ranges from 5% to 7% in urban sub-Saharan Africa (Ogbera *et al.*, 2014). Based on estimations of IDF, 10.8 million people had DM in sub-Saharan Africa in 2006 and this would rise to 18.7 million by 2025 (Levitt, 2008). In Ethiopia, DM is one of the major non-communicable diseases. According to IDF, the number of diabetes cases in the year 20107was 2.6 million people and was the leading among Sub-Saharan Africa (IDF, 2017).

1.3. Normal physiology of insulin secretions

Insulin is a dipeptide hormone secreted by the β-cells of the pancreatic islets of langerhans. It contains A and B chains, where the A chain comprises 21 amino acids and the B chain contains 30 amino acids. The two chains are connected by 2-disulfide bonds, which join the N- and C-terminal helices of the A chain to the central helix of the B chain (Mane *et al.*, 2012). The N-terminal signal peptide drives newly synthesized pre-proinsulin across the endoplasmic reticulum (ER) membrane, where the signal peptide is removed by signal peptidase, forming proinsulin. In the oxidizing environment of the ER lumen, proinsulin rapidly folds, forming three disulfide bonds, including two interchain disulfide bonds (B7–A7 and B19–A20), and one intrachain disulfide bond (A6–A11). Properly folded proinsulin dimerizes and exits the ER for delivery to the Golgi apparatus. As Zn^{2+} concentration begins to rise in the trans golgi network, proinsulin is thought to form hexamers around central coordinating Zn^{2+} ions. Complete conversion of pre-proinsulin to mature insulin takes between 30 and 150 min (Liu *et al.*, 2014).

Insulin plays an essential role in maintaining metabolic homeostasis. It maintains normal blood glucose levels by facilitating cellular glucose uptake, regulating carbohydrate, lipid and protein metabolism, promoting cell division and growth through its mitogenic effects (Wilcox, 2005). As blood glucose increases, such as after eating, insulin will be secreted by β-cells into the bloodstream to promote glycolysis and lower glucose levels (Barrett *et al.*, 2010). In addition to glucose, other stimuli such as arginine and sulphonylureas also promote its secretions (Gebre, 2013). An expulsion of insulin following these stimuli occurs as shown in Figure 1. This is due to high Ca^{2+} sensitive pool which is associated with small portion of granules that are capable of inducing insulin release (Barrett *et al.*, 2010, Fu *et al.*, 2013).

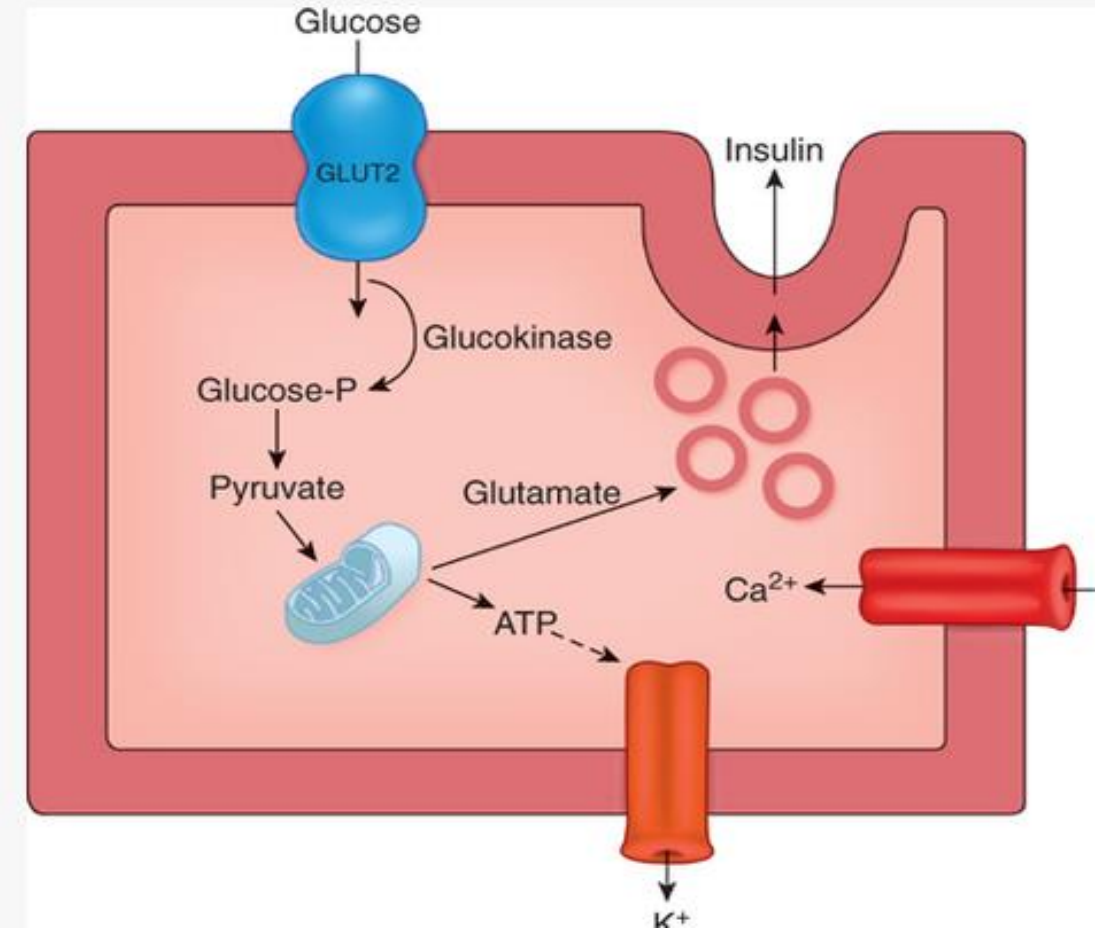

Figure 1: Mechanisms of insulin exocytosis (Barrett *et al.*, 2010): First, glucose is taken up by β-cells through glucose transporter 2 (GLUT-2). But the concentration of glucose should be at least 90 mg/dl to act as glucose sensor and initiate insulin release. After entering to the β-cell, glucose is phosphorylated by glucokinase and will be metabolized to pyruvate in the cytoplasm. Then pyruvate in the mitochondria will be converted to carbon dioxide and water via the citric acid cycle with the formation of ATP by oxidative phosphorylation. The ATP enters the cytoplasm, where it inhibits ATP-sensitive K+ channels to reduce K+ efflux. This depolarizes the β-cell, and Ca^{2+} enters the cell via voltage-gated Ca^{2+} channels. The Ca^{2+} influx causes exocytosis to secrete insulin. Insulin exocytosis can proceed at the rate of 500 granules per second when intracellular [Ca^{2+}] is increased to 17 mmol/L, but it only proceeds at the rate of 3–4 granules per second when [Ca^{2+}] is at 0.17 mmol/L. Exocytosis can also occur with low [Ca^{2+}].

1.4. Pathogenesis of type 1 diabetes mellitus

T1DM is an autoimmune disorder characterized by the destruction of insulin-producing pancreatic β-cells. The autoimmune destruction of β-cells, which is a cause for insulin deficiency, is resulted from genetic, environmental and immunologic factors (Zaccardi *et al.*, 2016).

1.4.1. Roles of genes

Many genes or genetic regions are associated with the inductions of T1DM (Kantarova *et al.*, 2007). From these, human leukocyte antigen (HLA) on chromosome 6p21 was the first known

and major genetic susceptibility determinants (Noble *et al.*, 2010). HLA has two types of cluster of homologous on cell surface proteins; class I and class II (Ji-Won *et al.*, 2005). HLA class I antigens, known as A, B, and C are encoded as a single chain that forms a complex with the essentially nonpolymorphic molecule β-2-microglobulin. On the other hand, the three HLA class II antigens include DR, DQ, and DP. HLA class II molecules are heterodimeric, consisting of α and β chains, encoded by separate genetic loci (Muoio *et al.*, 2008).

Class I found ubiquitously and present intracellular antigen to CD8+ T cells (Bakay *et al.*, 2013). Studies have shown that these HLA class I genes are associated with T1DM. They have a role of binding and presenting peptide antigens. The HLA class I peptide antigen complexes function both in shaping the T-cell in the thymus and in initiating antigen-specific T-cell–mediated cytotoxicity (Noble *et al.*, 2010). Class II molecules, on the other hand, are expressed mainly on professional antigen presenting cells, dendrite cells, macrophages, B-lymphocytes and thymus epithelium (Verdaguer *et al.*, 2004). They are responsible for presenting antigens to CD4+ T cells, which promote inflammation by secreting cytokines upon recognition of their specific targets. Other loci such as insulin gene encoded on chromosome 11p15, the cytotoxic T-lymphocyte-associated protein 4 gene encoded on chromosome 2q33, tyrosine phosphatase encoded on chromosome 1p13, non-receptor type 22, interleukin 2 receptor alpha on chromosome 10p15 and helicase C domain 1gene on chromosome 2q24 were found to be strongly associated with T1DM (Bakay *et al.*, 2013).

1.4.2. Environmental factors

Environmental factors such as viruses, toxins, and nutrients play an important role in the pathogenesis of T1DM (Paschou *et al.*, 2018). Viruses act through direct cytolytic effect, or through triggering an autoimmune process for gradual β-cell destruction. Particularly, coxsackie virus B (CVB) tends to increase the depletion of β-cell and results in an elevated risk of developing autoimmune diabetes in the absence of protective Treg population. However, in individuals who are genetically predisposed to T1DM but not to insulitis, CVB infection may induce a protective Treg population that prevents the development of pathogenic autoimmune islet specific T cells (Paschou *et al.*, 2018). Prenatal and early life exposure to environmental pollutants and toxins such as phthalates, endocrine disruptors (bisphenol A and triclosan), N-nitroso compounds, certain metals (arsenic, organic derivatives of tin), bacterial toxins can have

negative effects on immune system and can increase the risk of developing TIDM (Bodin *et al.*, 2015).

1.4.3. Immunological factors

The role of Immune system in T1DM is studied in non-obese diabetic mice (NODM). The major CD4+T cell response in NODM is the T cells directed to insulin. In particular, the non-conventional 12-20 segment of the insulin B chain is presented by the class II MHC molecule I-Ag7 and elicits pathogenic CD4+T cells. Entrance of CD4+T cells along with a burst of a subset of dendritic cells initiate the autoimmune process (Jarald *et al.*, 2009)**.** Moreover, human studies showed that the inflammatory lesion within islets of those with T1D is typically characterized by a decrease (or absence) of insulin-producing B-cells. The pancreatic islet cell of individuals with this condition is characterized by infiltrations of cells. These cells are composed of T-lymphocytes, B-lymphocytes, macrophages, and lesser numbers of other cells representing the immune response (Bakay *et al.*, 2013).

1.5. Type 2 diabetes mellitus and mechanisms of insulin resistance

Defective insulin secretion and action, Insulin resistance (IsR), leads to multiple metabolic abnormalities in T2DM, including hyperglycemia, and dyslipidemia. IsR is often associated with obesity. It develops when chronic over nutrition and genetic susceptibility synergistically causes impaired insulin signaling as well as a relative insulin deficiency. Moreover, the chronic increase in circulating glucose and lipid levels can further impair insulin secretion and action (Muoio *et al.*, 2008). Other factors including- inter-organ communication networks that are mediated by peptide hormones and inflammatory mediators have a role in developing IsR (Chen *et al.*, 2015)

1.5.1. Adipokines and Insulin Resistance

Insulin has 3 major target tissues—skeletal muscle, liver, and adipose tissue. It has been postulated that the insulin receptor is overexpressed in the cells of these tissues (Samuel *et al.*, 2012). These three sites in the body are capable of glucose deposition and storage. From these, Adipocytes have a regulatory role in the development of IsR because of their capacity to produce adipokines (a group of hormones and cytokines). In obese conditions, the ability of adipokines to store excess lipids becomes saturable as a result there will be abnormal redistribution of lipids to other organs and tissues. Furthermore, adipose tissue also produce other peptide hormones including retinol-binding protein-4 (RBP4) and resistin as well as inflammatory cytokines such

as interleukin-6 and tumor necrosis factor-α (TNF-α) (Zimmet *et al.*, 2014). RBP4 has a negative role in DM. Mice lacking GLUT-4 expressions showed RBP4 which in turn developed IsR. Resistin on the other hand suppresses the uptake of glucose and insulin sensitivity (Muoio *et al.*, 2008).

1.5.2. Inflammatory cytokines

Cytokines are a group of pharmacologically active, low molecular weight polypeptides that possess autocrine, paracrine, and juxtacrine effects (Cieślak *et al.*, 2015). They are clustered into several classes i.e., interleukins, tumor necrosis factors, interferons, colony-stimulating factors, transforming growth factors and chemokines. Cytokines act as pleiotropic polypeptides regulating inflammatory and immune responses through actions on cells. They provide important signals in the pathophysiology of a range of diseases, including DM, chronic low-grade inflammation and activation of the innate immune system (Navarro-Gonzalez *et al.*, 2008). Chronic inflammation due to cytokines plays a pivotal role in endothelial dysfunction, IsR and oxidative stress. Moreover, inflammatory cytokines, mainly IL-1, IL-6, and IL-18 as well as TNF-α are involved in the development and progression of diabetic nephropathy (Navarro-Gonzalez *et al.*, 2008).

1.5.3. Oxidative stress

Oxidative stress can be defined as any disturbance in the balance of antioxidants and pro-oxidants in favor of the later due to different factors such as aging, drug actions and toxicity, inflammation and/or addiction (Agarwal *et al.*, 2012). Several factors induce oxidative stress that may diminish structural and functional integrity of β-cells of pancreatic islets. Oxidative stress may also potentiate the generation of reactive oxygen species along with other proinflammatory cytokines and chemokines around the β-cells that disrupt the blood flow into the β-cells and abolish its function. Moreover, insufficiency of anti-oxidative enzymes such as superoxide dismutase, catalase, and glutathione peroxidase in β-cells provokes destructions of β-cells via oxidative stress. Insufficiency of these anti-oxidative enzymes is also believed to play a role in the development of vascular complications such as neuropathy, nephropathy, retinopathy in diabetes particularly T2DM development of diabetic complications (Akash *et al.*, 2013).

1.5.4. Post-prandial hyperglycemia

Foods may affect body weight control and insulin sensitivity by promoting satiety and stimulating fat oxidation at the expense of carbohydrate oxidation (Ogama *et al.*, 2018). In non-diabetic individuals, pancreatic β-cells increase the release of insulin in response to food consumption and

release a relatively constant level of insulin during the fasting state. After food ingestion, an increase in plasma glucose levels and release of insulin inhibit glucagon secretion. These suppress glucagon release into the circulation and promote glucose uptake in various tissues. In people with postprandial hyperglycemia (PPHG), early insulin release after food ingestion is decreased. In addition, there will be less reduction in glucagon secretion, resulting in increased glucose production in the liver that leads to inefficient glucose uptake, and consequently, increased PPHG. Moreover, there is a decreased tissue glucose clearance and glucose oxidation, with increased non-oxidative glycogen cycling, glycolysis, and glucose uptake in alternative tissues throughout the body. The net result is that more glucose which is both from endogenous and ingested enters the circulation at a faster rate than the body can use it, resulting in prolonged elevations of plasma glucose (Maffettone *et al*., 2018).

1.6. Management of diabetes mellitus

The primary objectives in the management of DM is reducing high blood glucose levels, relieving any symptoms of hyperglycemia and preventing or delaying the onset of diabetes complications (Powers *et al.*, 2018). Different surrogate markers such as FBG, 2-h OGTT, and HbA1c are used to realize these important outcomes (Table 1). To achieve these goals, two major management approaches can be followed: non-pharmacological and pharmacological treatment (Riddle, 2019).

Table 1: Recommended glycemic targets for diabetic patients (Riddle, 2019).

Markers	Target values
FBG	80–130 mg/dl
RBS	<180 mg/dl
2-h 75 g OGTT	<140 mg/dl
HbA1c	
For non-pregnant adults	<7 %
patients with a history of severe hypoglycemia, limited life expectancy, advanced microvascular or macrovascular complications	<8 %

FBG; fasting blood glucose, RBS; random blood sugar, OGTT; oral glucose tolerance test; HbA1c, Hemoglobin A1c

:

1.6.1. Non pharmacological management

Since dietary intake and physical exercise are the two main determinants of the energy balance, they are considered as a base in the treatment of patients with diabetes. Most T2DM patients have some degree of overweight or obesity that results in IsR and thus weight reduction is the mainstay of treatment option. It has been proposed that the caloric intake of the diet prescribed to a diabetic patient with obesity should contain between 500 and 1000 kcal. This weight reduction will improve insulin sensitivity (Marín-Peñalver *et al.*, 2016). Table 2 shows nutritional recommendations for individuals with Diabetes. In addition to diets, regular exercise prevents or delays T2DM. Regular exercise is also recommended for T1DM. It improved cardiovascular fitness, muscle strength, and insulin action. Exercises such as - walking, cycling, jogging, and swimming improve mitochondrial density, insulin sensitivity, skeletal muscle oxidative capacity, glycemic control. According to ADA, lifestyle interventions that include at least 150 min/week of physical activity and dietary changes that results in weight loss of 5%–7% are recommended to prevent or delay the onset of T2DM in populations at high risk and with pre-diabetes (Colberg *et al.*, 2016).

Table 2: Nutritional recommendations for individuals with DM (Gray, 2015).

Types of nutrient	Recommended Amount
Carbohydrates	➢ According to US report, food intake should contain only moderate amount of carbohydrate (~ 45 %) of the total energy intake.
Proteins	➢ According to ADA protein restriction is very essential especially people with other comorbid conditions such as nephropathy ➢ The National Kidney Foundation recommends 0.8 g protein/ kg body weight for people with diabetes and nephropathy.
Fiber	➢ The Dietary Guidelines for Americans advocates consumption of 14 g dietary fiber per 1,000 calories consumed, or an average of 25 g for adult women and 38 g for adult men.
Fat	➢ According to the Institute of Medicine and the Academy of Nutrition and Dietetics for healthy individuals 20% to 35% of total calories should come from fat.
Vitamins	➢ Since diabetes is a state of increased oxidative stress, interest in recommending large of antioxidant vitamins has been high.

1.6.2. Pharmacological management

Although non pharmacological therapy is the cornerstone of treatment for patients with DM, insulin is required for T1DM. Moreover, non pharmacological therapy alone is insufficient for patients with T2DM (Kennedy *et al.*, 2018). Therefore along with diet and exercise oral hypoglycemic drugs are required to achieve the goal of improving glycemic control and preventing both microvascular and macrovascular complications in T2DM (Lorenzati *et al.*, 2010). The American College of Endocrinology and the American Association of Clinical Endocrinologists (AACE) recommend initiation of insulin therapy in patients with T2DM if A1C is > 9 % and, if blood glucose is not controlled by diet, weight loss, exercise and oral medications. Insulin may be used alone or in combination with oral medications (Petznick, 2011).

I. Insulin and its derivatives

Depending on their action profile, insulins can be divided into short, intermediate and long acting. Short acting insulins are used to cover mealtime associated blood glucose level (BGL). They possess a quick onset (30 min) with peaks time of 2-3 hours after injection. Moreover, they have shorter duration of action. Intermediate acting insulins consist of regular insulin modified by adding zinc (lente) or basic protein (NPH). These forms of insulins have duration of action of 12-14 h and usually need to be given twice in a day to provide cover for the entire 24 h. Human long acting insulins on the other hand have a duration of action of approximately 18-20 hours (Bhatia *et al.*, 2007). Nowadays, insulin is prepared in the form of inhalation powder. This form of insulin is usually administered at the beginning of a meal; it is an alternative to injectable bolus/prandial insulin. For patients using this form of insulin pulmonary function must be assessed before initiation, after 6 months, and annually thereafter. Moreover, it is contraindicated in patients with chronic lung diseases and has been associated with acute bronchospasm in patients with asthma and chronic obstructive pulmonary disease (Freeland *et al.*, 2016).

II. Oral Hypoglycemic agents

Different oral anti diabetic drugs with different mechanisms of action are available for treatment of T2DM (Sola *et al.*, 2015). These agents can be categorized into different classes: (i) drugs that bind to the sulfonylurea receptor and stimulate insulin secretion (sulfonylureas and meglitinide analogues); drugs that lower glucose levels by their actions on liver, muscle, and adipose tissue (biguanides, thiazolidinediones (TZD)); (iii) drugs that principally slow the intestinal absorption

of glucose (α-glucosidase inhibitors); (iv) drugs that mimic incretin effect or prolong incretin action (glucagon-like peptide-1 (GLP-1) receptor agonists, dipeptidyl peptidase 4 (DPP-4 inhibitors); and (v) drugs that inhibit the reabsorption of glucose in the kidney (sodium-glucose co-transporter inhibitors SGLT2i) (Kennedy *et al.*, 2018).

a. Sulfonylureas and meglitinide analogues

Sulfonylureas, meglitinides, and D-phenylalanine derivatives work through binding with the sulfonylurea receptor: bind to a specific sulfonylurea receptor on the pancreatic β-cell and blocks ATP-sensitive potassium channels thereby stimulates insulin release. From these, Sulfonylureas were the mainstay of antidiabetic therapy since the early 1950s. Depending on their onset of action, duration of effect, risk of hypoglycemia and incidence of drug interaction, these agents are classified into two categories: first generation (chlorpropamide, tolazamide, tolbutamide) and second generation (glibenclamide, glipizide, gliclazide, and glimepiride). First generation sulfonylureas have longer half lives, greater incidence of hypoglycemia, and more drug interactions (Lorenzati *et al.*, 2010). Following the release of the University Group Diabetes Program study, that the number of deaths due to cardiovascular disease in diabetic patients treated with tolbutamide, the use of first generation drugs was decreased (Sola *et al.*, 2015). On the contrary, second generation agents have quick onsets of action, shorter half lives, and lower incidence of hypoglycemia (Lorenzati *et al.*, 2010).

Meglitinide analogs are secretagogues like sulfonylureas, although not structurally related. Repaglinide, mitiglinide and a newly introduced phenylalanine derivative, nateglinide are prototypes from this class. Both repaglinide and mitiglinide bind to the sulfonylurea receptor on the β-cell, but with lower affinity than sulfonylureas. They stimulate insulin release in the same way. On the other hand, nateglinide is structurally distinct from both sulfonylureas and the meglitinides. It has a quicker onset and a shorter duration of action than repaglinide (Ghosh *et al.*, 2012). These class of drugs are associated with a risk of hypoglycemia particularly if the meal is delayed or skipped (Kennedy *et al.*, 2018).

b. Biguanides and thiazolidinediones

Both biguanides and thiazolidinediones increase the action of insulin on peripheral tissues. Biguanides which include phenformin, buformin and metformin work through activations of AMP activated protein kinase (AMPK). Activations of AMPK inhibits the expression of

transcription factors required for hepatic gluconeogenesis and improves peripheral glucose uptake. The most common adverse effect of biguanides is associated with gastrointestinal disorders. Biguanide also induce lactic acidosis and this is rare but serious side effects of biguanides and was the reason for withdrawing of phenformin and buformin from the market (Behzad *et al.*, 2007, Kennedy *et al.*, 2018).

TZDs or glitazones, on the other hand, exert their antidiabetic effects through activation of gamma isoform of the peroxisome proliferator-activated receptor. Through this, TZDs reduce insulin resistance in adipose tissue, muscle and liver (Elte *et al.*, 2011). Drugs in this class include rosiglitazone and pioglitazone and have been available for use since 1997. Rosiglitazone and pioglitazone have similar clinical efficacy. They reduce FBG and HbA1c from 60- 80 mg/dl and 0.5% - 1.5% respectively. Angina pectoris, heart failure and fluid retention are the main adverse effects of TZDs (Pastromas *et al.*, 2006).

c. Alpha glycosidase inhibitors

Alpha glycosidase inhibitors reduce the rate of glucose absorption through competitive and reversible inhibition of intestinal hydrolase enzymes such as pancreatic α-amylase and α-glucosidases. Drugs in this class include acarbose, miglitol, and voglibose. Their effect is mainly reducing PPHG. In addition, they have modest effect on FBG levels. The main side effects of these drugs are flatulence, abdominal discomfort, bloating and diarrhea, which reduce compliance in patients (Lorenzati *et al.*, 2010).

d. Incretin mimetics

Potentiation or amplifications of glucose induced insulin secretion from pancreas is known as incretin effect. This effect is increased through incretin hormones mainly glucagon-like peptide-1(GLP-1) and glucose-dependent insulinotropic polypeptide (Hansen *et al.*, 2010). In addition of insulin secretions, these hormones take part in reducing glucagon concentrations, delaying of gastric emptying time and induction of satiety. They also play a role in the proliferation of β-cells and decrease in β-cell apoptosis (Pathak *et al.*, 2010). Two pharmacological approaches are available to enhance the incretin effect in T2DM. The first one is administrations of GLP-1 analogs (GLP-1 receptor agonists) and the second approach is to inhibit dipeptidyl peptidase-4 (DPP-4) activities thereby increasing the half-life of incretin mimetic hormones. Antidiabetic drugs such as exenatide, liraglutide, albiglutide, and dulaglutide work through the first approach,

whereas sitagliptin, saxagliptin, linagliptin, and alogliptin lower blood glucose level through inhibition of DPP-4 (Prins, 2008).

e. Sodium glucose co-transporter inhibitors

Sodium-glucose co-transporter-2 inhibitors (SGLT2i) work by inhibiting reabsorption of glucose in the proximal convoluted tubule thereby facilitating its excretion in urine. The mechanism of action is dependent on blood glucose levels and, unlike the actions of insulin secretagogues they have minimal potential for hypoglycemia, and no risk of overstimulation or fatigue of the β-cells. However, they have minimal effect in individuals with renal impairment since their mode of action relies upon normal renal glomerular tubular function (Kalra, 2014). Dapagliflozin, canagliflozin, empagliflozin are SGLT2i approved by European Medicines Agency for treatment of T2DM in 2012, 2013 and 2014, respectively. The AACE recommends that SGLT2i to be considered as a monotherapy option in patients for whom metformin is contraindicated or not tolerated. In addition, SGLT2i may be an option to be added with first line antidiabetic drugs such as metformin as part of dual or triple therapy (Vivian, 2014).

1.6.3. Roles of traditional herbs in diabetic mellitus

Treatment of illness and maintenance of health using herbal medicines is the oldest and most popular form of healthcare practice known to humanity. It has been practiced by all cultures in all ages throughout the history of civilization. According to WHO, 80% of the world's population depends wholly or partly on plant-derived pharmaceuticals. Furthermore, about 25% of modern medicines were descended from plants first used traditionally. For instance, metformin, derived from two linked guanidine units, was initially isolated from a medicinal plant *Galega officinalis* and is currently prescribed in the conventional medicine to treat diabetes (Saraei *et al.*, 2019).

Medicinal plants have been used since ancient times for the treatment and management of DM in traditional medicine systems of many cultures throughout the world. These plants continue to play an important role in the management of DM, especially in developing countries, where many people do not have access to conventional anti-diabetic therapies (Chikezie *et al.*, 2015).

Plants which are endowed with antidiabetic activity have different mechanisms in lowering BGL. Some of them may stimulate insulin kinase or inhibit insulinase activity and others may increase reconstruction of pancreatic β-cells. Moreover, fibers of plants may also interfere in the absorption of carbohydrates and thus have an effect on blood glucose (Bogle *et al.*, 2015).

Antidiabetic activity of these medicinal plants is due to the presence of phenols, flavonoids, terpenoids, coumarins and other constituents which show reduction in BGL (Malviya *et al.*, 2010). Different Medicinal plants have been used for the management of DM in the Ethiopian traditional medicine system (Meresa *et al.*, 2017). Since Ethiopia is rich in varieties of endemic flora, it is imperative to look for medicinal plants with pharmacological activity (Mertens *et al.*, 2016). With this direction, there has been researches conducted on plants that have a traditional claim for treatment of DM using different models and many of them have been demonstrated to exhibit antidiabetic activity. These included, among others, *Moringa stenopetala* (Toma *et al.*, 2014), *Caylusea abyssinica* (Gebreyohannis *et al.*, 2014), *Calpurnia aurea* (Belayneh *et al.*, 2018), *Ajuga remota* Benth (Tafesse *et al*., 2017) and *Pentas schimperiana Subsp* (Dinku *et al.*, 2010).

1.6.4. The experimental Plant, *Rubus erlangeri*

Rubus erlangeri Engl is an endemic plant to Ethiopia and belongs to the family of rosaceae and genus of *Rubus* (Awas, 2016). The family rosacea is mostly found in temperate regions and comprises more than 100 genera and about 3000 species. It includes shrubs or rarely perennial herbaceous plants. In Ethiopia, *Rubus* species are collectively named by different vernacular names njorie (Amharic); goda, gorco, gumere, haltufa (Afaan oromo); kwesheshlla (Tigrigna); njore (Guragegna); hamaroo (Kernbatigna); henjoriya (Welaytigna); argi and melitno (Gamoegna) (Hedberg *et al.*, 1989).

The experimental plant *R.erlangeri* (Figure 2) is 2 m high and its body is covered with sticky hairs. The stem looks purplish brown and is densely covered by stalked glands intermixed with loose hairs and scattered prickles. Its leaves are imparipinnate or trifoliolate, leaflets ovate-acuminate up to 7 x 5 cm, sharply serrate, green above and very densely white-tomentose below, with stalked glands along the veins. Flowers range in the diameter of 2-3 cm where peduncles, pedicels and calyces are densely covered by purplish stalked glands, intermingled with small prickles. Sepals are ovate, long-caudate with 1.5-2 cm long; appressed to pale yellow fruits (Hedberg *et al.*, 1989).

In traditional medicine, many species of the genus *Rubus* were used for the treatment of different disorders. For instance, in traditional Chinese medicine, combination of Chinese raspberry (*R. chingii*) and yang tonic were used to treat infertility, impotence, low backache, poor eyesight, and

frequent urination (Wang, 2011). Different plant parts of this genus are used for the treatment of rheumatism, sore throat, hemorrhoid, diarrhea and similar enteric disorders (Sultana, 2018). In different parts of the world, several different *Rubus* species have been reported to be used for treatment of DM. For instance, *R. imperialis* in Brazil, *R. ulmifolius* in Chilean folk medicine (Rocabado *et al.*, 2008), *R. ellipticus* in India *(Latha et al., 2015),* leaves of *R. fruticosus* in Iran, *R. adenotrichus* in Mexico *(Laguna-Hernández et al., 2017)* and infusion of leaves of *R. apetalus* in Tanzania (Ruffo *et al.*, 2002) are used. In Ethiopia, the leaves of *R. steudneri* (Meresa *et al.*, 2017), *R. apetalus and other Rubus* species are used for DM as decoction. Treatment lasts over 15 days or continued more than these days depending upon weekly urine glucose level (Abate, 1989).

The antidiabetic effects of some of species in this genus were also evaluated experimentally and have shown a promise in reducing BGL (Schädler *et al.*, 2017, Lemus *et al.*, 1999, HL. *et al.*, 2019, Jouad *et al.*, 2002, Carlo *et al.*, 2014). Moreover, the effect was also evaluated for the potential of treating diabetic complication such as neuropathic pain particularly for *R. fruticosus* (Gomar *et al.*, 2015).

A) B)

Figure 2: Photograph of *Rubus erlangeri* Engl.; The shrub (A) and leaves (B).

1.7. Rationale for the study

Despite the promises of the wide range options of prescription medications, these drugs are associated with numerous side effects, which are intolerable for many patients. For instance, Sulfonylureas are known to cause weight gain and hypoglycemia, biguanides carry a risk of lactic acidosis especially among the elderly and in the presence of liver or renal failure, even the most commonly used drug metformin involve numerous gastrointestinal side effect, such as nausea, cramps and diarrhea. α-glycosidase inhibitors, on the other hand, cause flatulence (George *et al.*, 2015). Furthermore, diabetes and the related complications continued to be a major medical problem even if they are still treated by conventional medications. This leads to increasing demand and prescription for natural products having antidiabetic activity with fewer side effects due to their perceived effectiveness, and relatively low costs. In addition, globally, there is a revolution in health care system, resulting in greater acceptance in herbal medicine (Rao *et al.*, 2010). Thus, there is a need to scientifically investigate plants for DM activity. However, there is

no specific information about *R. erlangeri* regarding its use in DM many of the species from this genus have been claimed or sown to have antidiabetic property From chemotaxonomic knowledge and molecular phylogenetic data, species from genera or families known to have an effect such as the abovementioned against certain disease conditions, are believed to be associated with a certain bioactivity or therapeutic potential, thus selection of species from these hot taxa would lead to higher success rates in drug discovery (Atanasov *et al.*, 2015). Therefore, the present study attempted to evaluate antidiabetic activity of the leaves of *R. erlangeri* first *in vitro* then *in vivo* using this notion.

2. Objectives

2.1. General objective

- To evaluate Antihyperglycemic and Hypoglycemic Activities of the Aqueous Leaf Extract of *Rubus Erlangeri* Engl (Rosacea) in Mice

2.2. Specific objectives

- To conduct acute oral toxicity test of the plant extract.
- To evaluate *in vitro* α-amylase inhibition activity of the extract.
- To determine antioxidant activity of the extract.
- To assess hypoglycemic effects of the plant in normal mice.
- To assess glucose lowering effects of extract in normal glucose loaded mice.
- To assess antihyperglycemic effects of single and repeated doses of the extract in streptozotocin (STZ) induced diabetic mice.
- To determine effects of extract on body weight change of STZ induced diabetic mice.
- To determine the phytochemical constituents of the extract.

3. Materials and methods

3.1. Materials

3.1.1. Drugs and chemicals

The following drugs, chemicals and instruments, were used in the experiment during the study period: ammonia, hydrochloric acid and ferric chloride (BDH Laboratory Supplies Ltd, England), acetic anhydride and Mayer‟s reagent (May and Baker Ltd, England), Dragendroff‟s reagent and sulfuric acid (Fisher Scientific, UK), STZ (Sisco Research Laboratories Pvt. Ltd, India), α-amylase (Blulux Laboratories Pvt. Ltd., Faridaban, India), 3,5-Dinitrosalicylic acid (DNSA) (Sisco Research Laboratories Pvt. Ltd. Mumbai, India), Acarbose (Bayer, Germany), starch, sodium chloride, sodium hydroxide, potassium sodium tartrate tetrahydrate, disodium hydrogen phosphate and sodium dihydrogen phosphate (BDH Laboratory Supplies Ltd, England) 2,2-diphenyl-1-picrylhydrazine (DPPH) (Sigm Chemicals Co, St. Louis, MO, USA), glibenclamide (Sanof–Aventis, USA), glucose (Munchen,Germany), and one touch glucometer and glucose standard strip/kits (Smart lab,Gmbh, Germany)

3.1.2. Plant collection

The plant used in this study was collected from Southern Nations, Nationalities, and Peoples' Region, Dawuro zone, Tocha wereda, which is about 500 km southwest of Addis Ababa, Ethiopia. It was collected on March 2019. To avoid change in physical appearance of the leaves during transportations it was packed in plastic holding material. Identification and authentication of the plant specimen was then performed by a taxonomist Ato Melaku Wondafrash and a voucher specimen was deposited (voucher number of AG001) at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University for future reference.

3.1.3. Experimental animals

Healthy swiss albino mice (body weight, 25–35 gm; age 6-8 weeks old) were used for the the experiments. Female mice were used for acute oral toxicity and male mice for STZ induced diabetic model. In addition, randomly selected mice with either sex were used for normoglycemic test and OGTT.

All mice were obtained from the animal house of School of Pharmacy, College of Health Sciences, Addis Ababa University. Mice were fed standard pellet diet and water *ad libitum.* They

were then acclimatized to the environment for one week before commencement of the experiment. All animals were handled according to internationally accepted guidelines (Nrcotn, 2011)

3.2. Methods

3.2.1. Preparations of plant extract

The leaves of *R. erlangeri* were dried under shade and then pulverized using a mortar and pestle to get a coarse powder used for extraction. Decoction method was used for extraction. A portion of *R. erlangeri* powder (150 g) was boiled with 1.2 L of Distilled Water (DW) for 15 min (Remington, 2006, EVANS, 2009) and was allowed to cool. After cooling, it was filtered through muslin cloth followed by Whatman grade No 1 filter paper (Schleicher and Schuell Microscience Gmbh, Germany). The filtrate was then frozen in a deep freezer and dried in a lyophilizer (Operon, Korea). Finally, 14 g brown powder with percentage yield of 9.3 % was obtained. The extract was then packed in a bottle and stored in a desiccator until use.

3.2.2. Acute toxicity study

Acute toxicity for *R. elangeri* aqueous leaf extract was done according to Organization for Economic Co-operation and Development (OECD) guideline 425 (OECD, 2008). For the study, five female mice of 6-8 weeks old were used. All mice were fasted for 4 h before and 2 h after administration of the extract. First, the study was performed in a single mouse with a dose of 2000 mg/kg. Since no death was observed within 24 h, 2000 mg/kg dose of the extract was given for additional four mice. The animals were observed continuously for 4 h with 30 min interval during the first 24 h. Observation was performed for the general signs and symptoms of toxicity, such as unusual skin and fur color, tremors, convulsions, salivation, diarrhea and coma as well as mortality. Observation was continued for a total of 14 days.

3.2.3. *In Vitro* antidiabetic effect study

a. Determinations of α-amylase inhibition activity

A amylase inhibition assay was performed using the DNSA method with a slight modification (Wickramaratne *et al.*, 2016). The leaf extract of *R.erlangeri* was first dissolved in a buffer ((Na2HPO4/NaH2PO4 (0.02 M), NaCl (0.006 M) at pH 6)) and concentrations ranging from 10-500 µg/ml were prepared. Likewise, a positive standard drug, acarbose solution with similar concentrations was prepared. A volume of 200 µl of α amylase solution (2 units/ml) was mixed with 200 µl of each concentration of the extract and acarbose and incubated for 10 min.

Thereafter, 200 µl of the starch solution (1% in water (w/v)) was added to each test tube. The reaction was terminated after 3 min by adding 200 µl of DNSA (DNSA reagent was made by mixing 12 g of sodium potassium tartrate tetrahydrate in 8.0 mL of 2 M NaOH with 96 mM of DNSA dissolved in 20 mL of DW). Test tubes containing all the above added solution were then boiled for 10 min in a water bath at 85°C. The mixture was cooled to ambient temperature and diluted with 5 ml of DW, and the absorbance was measured at 540 nm using a UV-Visible spectrophotometer (Jenway, model 6500). The blank with 100% enzyme activity was prepared by replacing the plant extract/acarbose solution with 200 µl of buffer. In addition, buffer without enzyme solution, and each concentrations of inhibitor (extract or acarbose) without enzyme was prepared as the same procedure mentioned above. The α-amylase inhibitory activity is expressed as percent inhibition and was calculated using the equation given below (Sama *et al.*, 2013). Finally, the % α-amylase inhibition was plotted against the extract and acarbose concentration and the IC50 value which is the concentration of sample required to inhibit 50% α-amylase activity was obtained from the graph for both extract and standard drug. Measurment was carried out three times and the average of IC50 was taken

Inhibition (%) = $\frac{(\mathrm{Ac} - \mathrm{Acb}) - (\mathrm{As} - \mathrm{Asb})}{\mathrm{Ac} - \mathrm{Acb}}$ x100, where Ac refers to the absorbance of control (enzyme and buffer); Acb refers to the absorbance of control blank (buffer without enzyme); As refers to the absorbance of sample (enzyme and inhibitor); and Asb is the absorbance of sample blank (inhibitor without enzyme).

b. **Determinations of antioxidant activity**

Free radical scavenging activity of *R. erlangeri* was examined using DPPH assay (Tadesse *et al.*, 2007). The plant extract was dissolved in methanol and prepared with different concentrations (1000, 500, 250, 125 and 62.5 µg/ml). Ascorbic acid (vitamin C), positive control, was also prepared in the same concentration as the extract. DPPH (5 ml of 0.004% methanol solution) was mixed with 50 µl of each extract and ascorbic acid solution. The mixture was then incubated at a temperature of 37 ^{0}C for 30 min in hot oven. The blank solution was prepared by replacing plant extract or positive standard with 50 µl of methanol and then by adding 5 ml of DPPH. Finally, the absorbance of each sample was measured at 517 nm using UV-spectrophotometer (Jenway, model 6500). For each mixture, absorbance was measured thrice and the average value was taken. Percent inhibition was calculated using the equation below and the IC 50 value of each sample,

which is the concentration of sample required to inhibit 50% of the DPPH free radical, was obtained from dose vs. inhibition curve.

Inhibition (%) = $\frac{(A_o - A_s)}{A_o}$X100. Where, Ao is absorbance of the negative control (0.004% methanol solution of DPPH without test sample) and As is the absorbance of the solution in the presence of sample extract or ascorbic acid.

3.2.4. Measurement of blood glucose level

Blood samples were withdrawn from the tail vein of mouse by cutting the tip of the tail using scissors in all animal models. To prevent infection at the tip of the tail, 70% ethanol using cotton was applied during each measurement. BGL was measured using a glucometer and test strips (Smart lab, Gmbh, Germany). Measurement was carried out three times and the average value was taken. For STZ model, particularly in studying the repeated dose effect BGL from animals without food for 8 h was considered as FBG level (Sun *et al.*, 2016). During fasting time in all *in vivo* models, animals were placed in a bare cage to prevent feeding of sawdust for specified fasting time.

3.2.5. Inductions of experimental diabetes

Diabetes was induced using single high dose of STZ. The dose of this agent required for inducing diabetes depends on the animal species, route of administration and nutritional status (Mythili *et al.*, 2004). Therefore, to determine at what dose STZ would induce DM in this experiment, a pilot study was carried out at three dose levels (150 mg /kg, 180 mg/kg and 200 mg/kg). Accordingly, 200 mg/kg was selected as it produced better results. Prior to STZ injection, mice were fasted for 6 h. STZ was first dissolved in freshly prepared 0.1M citrate buffer whose pH was adjusted to 4.5. It was then given to each mouse immediately. They were then provided with food and 5 % glucose in place of water for the next 24 h to prevent death associated with hypoglycemia. After 72 h, FBG was assayed and animals with FBG $\geq$ 200 mg/dl were considered diabetic (Brosius, 2009, Furman, 2015).

3.2.6. Grouping and dosing of animals

A total of 190 mice were used, 30 each for normoglycemic and OGTT, and 130 for STZ-induced diabetic model. In both normoglycemic and OGTT, animals of either sex were randomly divided into five groups (negative control, positive control and three test groups), each comprising of 6 animals per group. Group I (negative control) and Group II (positive control). Group III, IV and

V were treated with 100 mg/kg (RELE100), 200 mg/kg (RELE200) and 400 mg/kg (RELE400) doses *R. erlangeri* leaf extract, respectively. These doses of extract were selected based on acute toxicity data. For STZ induced diabetic model, diabetic mice were divided into 6 Groups (6 mice per group): one group was normal control without STZ injection and received DW and grouping was made as follows: Group I (Normal control), Group II (negative control), and Group III (positive control). Group IV, V and VI were treated with RELE100, RELE200 and RELE400 doses of the extract, respectively. In all three models (normoglycemic, OGTT and STZ-induced diabetic mice), the negative and normal controls in the STZ-induced diabetic model received DW, whereas the positive control group was treated with 5 mg/kg glibenclamide (GL5). All administration (DW, the aqueous leaf extract and the standard drug) were through oral route with a volume of 10 mL/kg.

3.2.7. Assessment of hypoglycemic activity in normal mice

Hypoglycemic effect of the extract was assessed on normoglycemic mice. Mice were fasted for 6 h with free access to water and randomly divided into 5 groups as described in grouping and dosing section. Blood samples were taken from mice tail veins at 0 h (just before administration of extract, vehicle or standard drug), 1, 2, 3 and 4 h after treatment (Gebreyohannis *et al.*, 2013).

3.2.8. Assessment of antihyperglycemic activity in oral glucose tolerance test

An antihyperglycemic effect of extract was done on overnight fasted mice (14h). After fasting, mice were randomly divided into 5 groups (6 mice per group). Baseline BGL was measured (just immediately before giving each agent based on their grouping). Thereafter DW, extract and standard drug were administered. Thirty minutes post administrations of each agent; animals were loaded with 2.5 g/kg of glucose solution orally. BGL were then measured after 30, 60 and 120 minutes following glucose administration (Tesfaye *et al.*, 2016).

3.2.9. Antihyperglycemic activity of aqueous leaf extract in streptozotocin-induced diabetic mice

a. Single dose study

Effect of single dose of aqueous extract was carried out on STZ-induced diabetic mice. After fasting for 14h, blood sample was collected at 0 h (just before treatment), 1, 2, 3, and 4 h after treatment of DW, standard drug and three doses of extract as per grouping (Gebreyohannis *et al.*, 2013).

b. **Repeated doses study**

Weekly Antihyperglycemic activity of repeated dose of extract was carried out in STZ-induced diabetic mice. Based on their grouping, as mentioned on section 3.2.6, diabetic mice were given DW, standard drug and different doses of extract for 3 weeks whereas The non diabetic group (normal control) was also administered DW. The blood glucose lowering effects of extract was then determined by measuring FBG level every seven days for three weeks. FBG level of diabetic mice were measured just before starting treatment on the 1st day of treatment (3 days after STZ injection) and on the same time the FBG level of normal control mice were measured as baseline. Thereafter it was measured at the 1st , 2nd and 3rd weeks following fasting for 8 h (Sun *et al.*, 2016).

3.2.10. Determination of body weight

Body weight reductions effects of STZ and improvement in body weight change by extract and standard were determined. The body weight of all the treated groups and control group of mice were recorded before treatment (on day 0) and during treatment period i.e. 1st, 2nd and 3rd week. An electronic balance was used for taking body weight of the mice and their weight was expressed as gram (Gao *et al.*, 2018).

3.2.11. Preliminary phytochemical analysis

The aqueous decoction of the plant extract was tested for the presence of secondary metabolites such as saponins, polyphenols, flavonoids, alkaloids, tannins (Debella, 2002), steroids, glycosides and terpenoids following the standard procedures with some modifications (Njoku. et al., 2009).

a. **Test for saponins**

Formation of honeycomb

500 mg of the extract was dissolved in a test tube containing 10 ml of DW and formations of honeycomb froth that persists for half an hour was considered as positive for saponins.

Chemical test

1 ml of 10% solution of sodium nitrate and 3 drops of concentrated sulfuric acid were added into 2 ml of the aqueous solution of the extract, and the formation of a bloody red color was inspected.

b. **Test for Polyphenols**

10 mg of the extract was dissolved in 1ml of water. Half ml of 5% ferric chloride solution was then added to it. And, development of deep blue or black color was taken as an indicator for the presence of phenols.

c. **Test for Flavonoids**

Zinc hydrochloride test

100 mg of the extract was dissolved in five ml of 50% methanol and a zinc powder was added. then five drops of concentrated HCl were added to the test tube. The formation of an orange or red color was taken as positive for the presence of flavonoids.

Lead acetate test

In 2 ml of the extract in the methanol, five drops of 2% lead acetate solution were added and the development of yellow or orange precipitate was inspected.

d. **Test for alkaloids**

500 milligrams of the extract was treated in a test tube with 10 ml of 1% HCl for 30 minutes in a water bath and then filtered through cotton into a test tube. Two drops of the extract was transferred into two test tubes and to one of the test tubes, five drops of Mayer's reagent and to the second five drops of Wagner's reagent were added and the formation of whitish opalescence (Mayer's reagent) or reddish brown precipitate (Wagner's reagent) was considered as presence of alkaloids. .

e. **Test for tannins**

One gm of the extract was heated in a test tube with 10 ml of DW for 5 minutes. After cooling, the solution was filtered through filter paper and 5 ml of 2% NaCl was added to the clear filtrate. Then 5 ml of 1% gelatin was added and the formation of white precipitate indicates the presence of tannins.

f. **Test for steroids**

About 0.25 gm of sample extract was weighed and placed in a test tube. Thereafter, 2 ml of acetic anhydride was added to dissolve it, followed by the addition of 4 drops of chloroform. Two drops of concentrated sulphuric acid was then added to the test tube. The development of a brownish

ring at the interface of the two liquids and the appearance of violet color in the supernatant layer were indicative of the presence of steroids.

g. **Test for glycosides**

0.1 gm of extract was dissolved in 1 ml of DW. Then, 3 drops of 20% sodium hydroxide solution was added and formation of yellow color confirms the presence of glycosides.

h. **Test for terpenoids**

To 0.25 gm of extract 2 ml of chloroform was added. Then, 3ml concentrated sulfuric acid was added to form a layer. Finally, a reddish brown coloration of the interface indicates the presence of terpenoids.

3.3. Data analysis

All statistical analyses were performed using international business machine of statistical package for the social Sciences, (IBM SPSS), version 25 for windows (SPSS inc, Chicago, Illinois, USA). For *in vitro* Studies, independent sample t-test was employed. It was used to determine if there was statistically significant difference in DPPH scavenging and *a*-amylas*e* inhibition activity between extract and standard drugs. For *in vivo* antidiabetic effect, statistical differences between groups and at different time was analyzed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. A two-way ANOVA followed by Tuckey post hoc tests was also used. This test was performed to see the effect of extract overtime for BGL reduction and body weight. In all cases results were expressed as mean ± standard error mean (SEM) and P-values less than 0.05 were considered as statistically significant.

4. Results

4.1. Acute toxicity study

The acute toxicity test result of this study revealed that the aqueous leaf extract of *R. erlangeri* was safe by oral route at a dose of 2000 mg/kg. After 24 h, animals were found to tolerate the administered dose and there were no significant changes in behavior such as motor activity, alertness, restlessness, diarrhea, convulsions, coma and change in skin color. Moreover, there was no mortality within 14 days of observations and lethal dose 50 (LD50) is assumed to be greater than 2000 mg/kg.

4.1.1. *In vitro* antidiabetic effect

a. **Alpha amylase inhibition activity**

The test showed that the extract has an appreciable α-amylase inhibitory effects when compared with acarbose. Figure 3 shows how IC50 can be derived just by taking the values of the measurement of α-amylase inhibition activity. The formula is expressed as a function of Y(AC) for acarbose and Y(EX) for extract. From the graph, the calculated IC50 value was 3.08 and 5.76 for the standard drug and extract respectively whereas the average IC50 value from triplicate measurements was 3.53±0.26 for acarbose and 4.90±0.44 µg/ml for aqueous leaf extract of *R. erlangeri*. Comparing the IC50 values obtained from each triplicate measurement, there was no apparent difference between acarbose and extract.

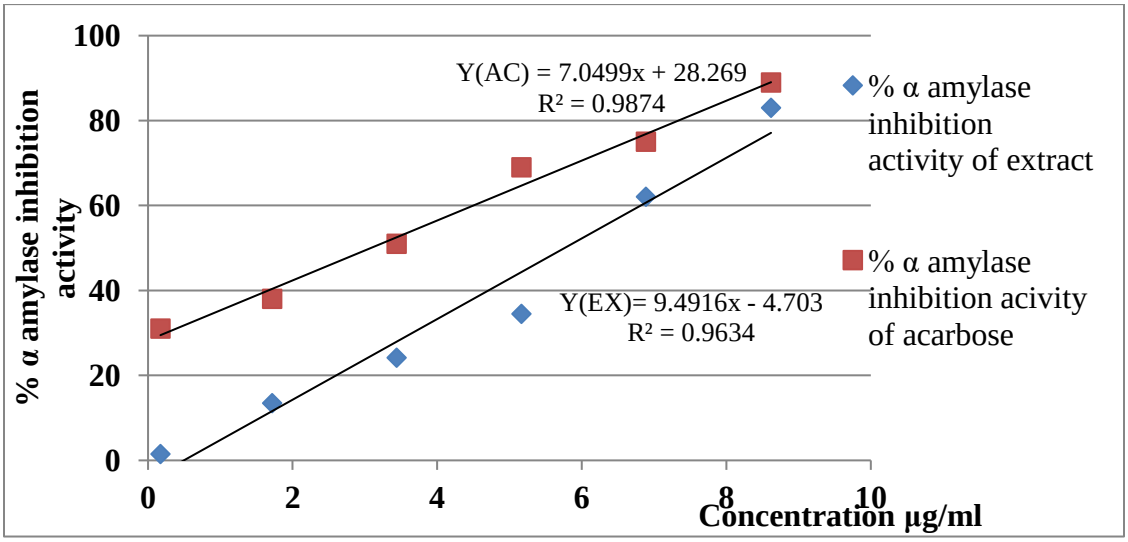

Figure 3: Percentage α-amylase inhibitory effects of the aqueous leaf extract of *Rubus elangeri*.

Data was expressed as IC50 ± S.E.M for each IC50. Analysis was performed by independent sample t-test for (n=3).

b. **Antioxidant activity**

The IC50 values of ascorbic acid and aqueous leaf extract were obtained from the equations represented by a function of Y(AS) and Y(EX) for ascorbic acid and extract respectively (Fig 4). Once again, the figure showed how IC50 values were derived jut by taking the first measurement and was found to be 5.13 and 7.39 for the standard drug and extract respectively. From triplicate measurements, the IC50 value for ascorbic acid was 5.91±0.39 µg/ml whereas for extract was 7.34±0.02 µg/ml. Comparison between the two values did not produce a significant difference.

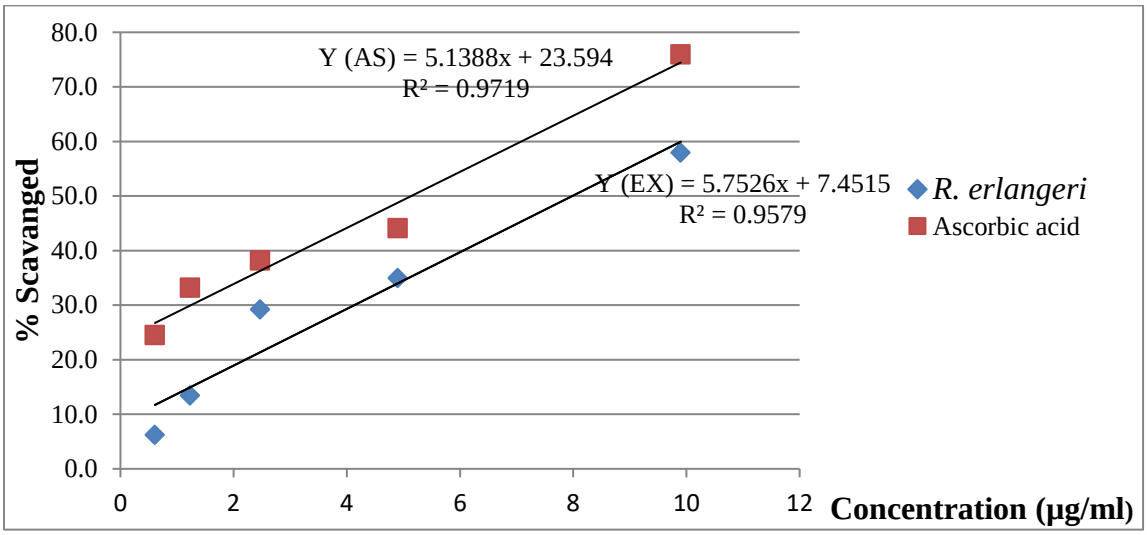

Figure 4: Radical scavenging activity of the aqueous leaf extract of *Rubus erlangeri*; Data was expressed as IC50 ± S.E.M for each; Analysis was performed by independent sample t-test for (n=3).

4.2. Hypoglycemic effects of aqueous leaf extract in normal mice

The FBG level was not significantly different among groups before giving each agent (t=0) (Table 3). GL5 showed significant BGL reduction at the 3rd and 4th h ($p<0.01$) compared to the negative control. But none of the extract doses produced significant decline in BGL at all-time points. In group analysis, there was no significant reduction in BGL difference between the positive control and extract treated groups at all-time points. Moreover, there was also no significant difference among the extract treated groups when compared to one another at all-time points.

Table 3: The effect of aqueous leaf extract of *Rubus erlangeri* on blood glucose level of normoglycemic mice

Groups	Blood glucose Level (mg/dl)				
	0 min	1h	2h	3h	4h
NC	99.4±2.74	89.20±7.91	85.40±5.28	79.20±6.11	76.8±5.24
GL5	101.00±11.58	72.00±8.67	65.00±8.05	55.20±2.20 [a2]	50.60±0.87 [a2]
RELE100	96.20±3.35	83.60±4.06	73.60±2.11	66.00±4.33	63.40±4.87
RELE200	96.60±5.04	83.20±3.27	74.80±4.58	65.60±2.20	60.40±1.20
RELE400	98.40±2.95	78.40±6.37	76.60±5.73	67.80±5.72	63.00±2.34

Each value represents mean ± S.E.M; n=6 for each treatment; Analysis was performed by one way ANOVA; a, compared to negative control; NC, negative control treated with distilled water; GL, glibenclamide; RELE, *Rubus erlangeri* leaf extract; number followed by RELE and GL indicates dose/ in mg/kg; min, minute; h, hour; 2p < 0.01.

Looking at the pattern of BGL decline across time, reduction was observed in each subsequent measurement for all groups with lesser reduction for the control group. The reduction was greater for GL5 treated group. However, comparing the decline in BGL with previous measurements, none of the group showed a significant reduction in BGL at all-time points (Figure 5).

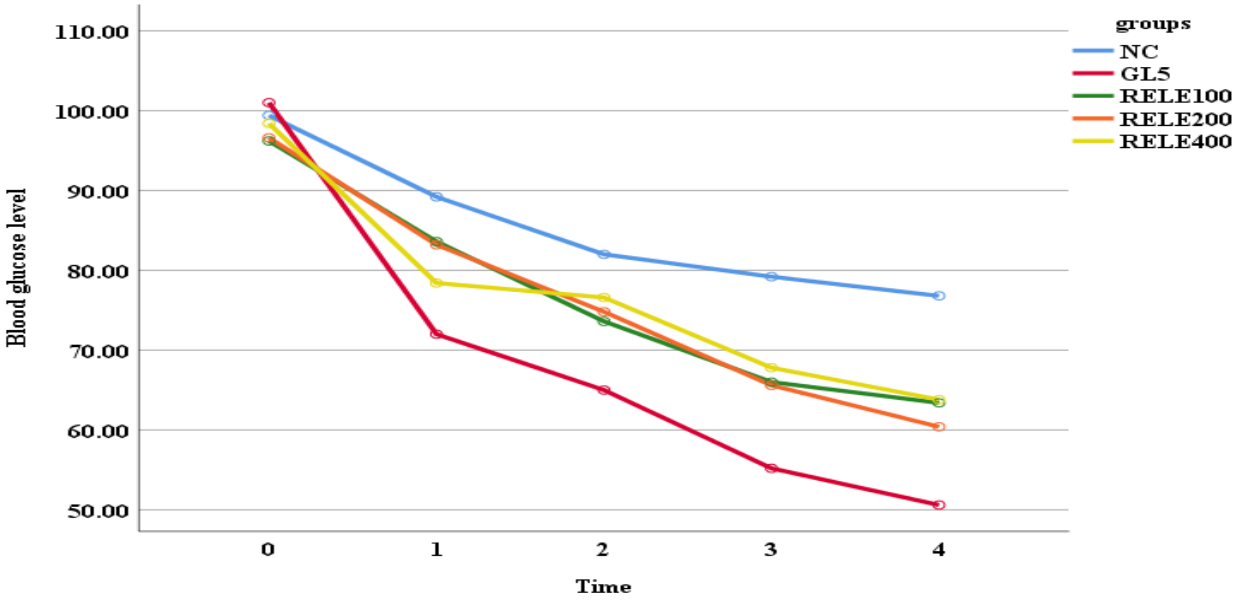

Figure 5: The hypoglycemic effect of different groups across time of blood glucose level measurements*;* Analysis was performed by two way ANOVA; NC, negative control treated with distilled water; GL, glibenclamide; RELE, *Rubus erlangeri* leaf extract; number followed by RELE and GL indicates dose in mg/kg; blood glucose level was compared to the previous time measurement value.

4.3. Antihyperglycemic effect of extract in oral glucose tolerance test

Table 4 shows that there was no significant BGL difference among the negative control, positive control and extract treated groups at t=0.

Glucose loading produced hyperglycemia after 30 min of administration in all groups, with a maximum increase achieved with the negative control (164.6%). Although treatment did manage to reduce BGL at 30 min compared to negative control, only the standard was able to produce a significant reduction ($p<0.05$) (Table 4). It also produced significant reduction at 60 ($p<0.01$) and 120 min ($p<0.001$). RELE00 ($P<0.05$) and the two higher doses of the extract produced significant reduction in BGL at 60 min ($p<0.01$). However, the effect of both RELE200 and RELE400 was maintained up to 120 min the lower dose effect was lost.

Table 4: The Effect of aqueous leaf extract of *Rubus erlangeri* on oral glucose tolerance test

Groups	Blood glucose Level (mg/dl)				% BGL increment
	0 min	30 mins	60 mins	120 mins	at 30 min
NC	94.40±3.93	249.80±59.43	209.40±34.95	123.20±15.72	164.60
GL5	96.2± 6.38	110.60±18.85 [a1]	72.40±9.24 [a2]	56.80±3.02 [a3]	15.00
RELE100	90.40±8.31	166.8±12.35	119.80±8.57[a1]	89.80±5.03	84.50
RELE200	84.80±6.41	137.40±20.21	103.80±3.69 [a2]	70.20±3.07 [a2]	62.02
RELE400	90±2.30	170±12.41	109±10.39 [a2]	81±9.76 [a2]	88.80

Each value represents mean ± S.E.M; n=6 for each treatment; Analysis was performed by one way ANOVA; a, compared to negative control; NC, negative control treated with distilled water; GL, glibenclamide; RELE, *Rubus erlangeri* leaf extract; number followed by RELE and GL indicates dose in mg/kg; min, minute;1p < 0.05; 2p < 0.01; [3]p < 0.001. Time refers after glucose loading.

4.4. Antihyperglycemic effect of aqueous extract in streptozotocin-induced diabetic mice

Of 120 mice were used for diabetic induction each 60 mice for single and repeated dose studies, 76 mice were found to be diabetic with a success rate of 63.3%. Among diabetic, 10 mice were excluded because of their FBG level was above the detection limits of glucometer (BGL>600 mg/dl). In addition, 4 mice died before DM was induced. Moreover, during performing this model, 4 animals died (2 from negative control, 1 from RELE200, and 1 from RELE400) at the end of first week. However, this was compensated by inducing other mice as per protocol.

a. Single dose studies

After DM was induced, there was no significant initial BGL difference among diabetic groups (Table 5). Compared to normal control group, negative group had significantly higher ($p<0.001$) FBG level with mean value >200 mg/dl at all-time points. Group analysis revealed that the extract with 200 and 400 mg/kg doses reduced FBG level significantly ($p<0.05$) at the 3rd and 4th h

compared to the negative control group. Likewise, GL5 lowered FBG level significantly at the 2nd ($p<0.05$), 3rd ($p<0.01$) and 4th h ($p<0.001$) but it showed no significant difference in BGL at all-time points when compared to plant extract treated groups. Moreover, there was no statistically significant difference in FBG level reduction at all-time points when extract treated groups compared with one another. While the negative control group was diabetic at all-time points, GLI5 and RELE400 tended to bring BGL back to the normal control levels at the 4th h. But RELE100 and RELE200 lost to produce this effect.

Table 5: Antihyperglycemic effects of single dose aqueous leaf extract in streptozotocin-induced diabetic mice

Groups	FBG level (mg/dl)				
	Initial	1st h	2nd h	3rd h	4th h
NOC	91.60±1.16	90±0.94	87.80±1.31	84.80±0.66	83.40±0.87
NC	286.00±12.90 a3	284.00±13.36 a3	277.80±13.88 a3	274.60±13.88 a3	269.00±14.20 a3
GL5	280.60±18.10 a3	239.20±12.80 a3	186.60±13.55 a1b1	175.00±11.26 a1b2	133.00±8.56 b3
RELE100	284.00±21.38 a3	266.00±20.96 a3	259.20±18.02 a3	230.00±21.05 a3	202.60±29.80 a2
RELE200	298.80±24.92 a3	258.60±26.70 a3	239.20±36.87 a3	190.80±27.93 a2b1	186.40±26.50 a2b1
RELE400	297.80±13.70 a3	252.00±17.42 a3	200±13.50 a2	173.20±12.99 a1b1	154.20±12.06 b1

Each value represents mean ± S.E.M; n=6 for each treatment; Analysis was performed by one way ANOVA; a, compared to normal control; b, compared to negative control; number followed by RELE and GL indicates dose/ in mg/kg; h, hour; NOC, normal control treated with distilled water; NC, negative control treated with distilled water; GL, glibenclamide; RELE, *Rubus erlangeri* leaf extract; $^1p < 0.05$; $^2p < 0.01$; $^3p < 0.001$.

In the group analysis, the trend over time in the reductions was consistent up to 4th h with all the three doses of extract and positive control group. A decline in FBG level was also recorded with negative and normal control group but with a lesser degree. Compared to the previous time measurement, significant FBG level reduction was produced merely by higher dose of extract and GL5 only at 1st h ($p<0.001$) (Figure 6).

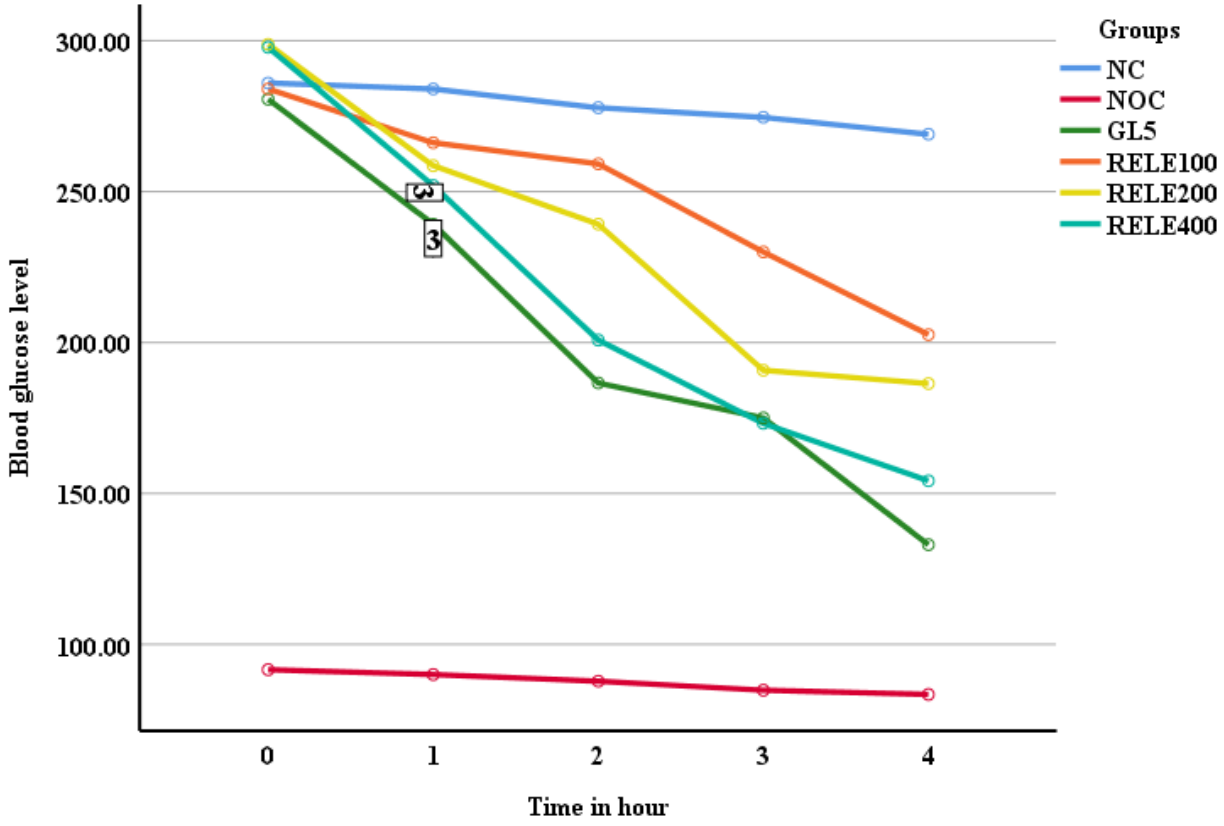

Figure 6: The antihyperglycemic effect of single dose of extract in different groups across time of blood glucose level measurements; Analysis was performed by two way ANOVA; NOC, normal control treated with distilled water; NC, negative control treated with distilled water; GL, glibenclamide; RELE, *Rubus erlangeri* leaf extract; number followed by RELE and GL indicates dose/ in mg/kg; blood glucose level was compared to the previous time measurement value; $^{3}p < 0.001$.

b. **Repeated dose studies**

In repeated dose of the aqueous leaf extract, the effect was determined through measuring weekly FBG level. Group analysis showed that there was no initial FBG difference among diabetic groups (Table 6). Compared to normal control group, negative group had significantly higher ($p<0.001$) FBG level with mean value >200 mg/dl at all-time points. When the effect of extract was compared with negative control group, lower dose ($p<0.01$), middle dose and higher dose ($p<0.001$) started to decrease FBG level significantly at 2nd week. The reduction was continued up to 3rd week with all doses of the extract ($p<0.001$). Likewise, GL5 reduced significantly ($p<0.001$) the FBG level at 2nd and 3rd week when it was compared to diabetic control group. But, it did not produce significant reduction compared to all three doses of extract at all-time points. In addition when the different doses of extract compared to one another, there was no statically significant difference among them at all-time points. While the negative control group was

diabetic at all-time points, GL5, middle and higher doses of extract tends to bring the FBG level to the normal control level at 2nd and 3rd week. But lower dose of extract treated mice had significantly higher FBG level at all-time points compared to normal control ($p<0.05$).

Table 6: Antihyperglycemic activity of aqueous leaf extract of *Rubus erlangeri* on weekly fasting blood glucose level in streptozotocin-induced diabetic mice**.**

Groups	Weekly FBG level (mg/dl)			
	Initial	1st	2nd	3rd
NOC	96.40 ±0.67	97.80±0.97	97.60 ±0.51	96.80±0.86
NC	357.00± 23.01 ^{a3}	374.00±27.72 ^{a3}	393.60±29.61 ^{a3}	394.80±26.19 ^{a3}
GLI 5	323.60 ±45.47 ^{a3}	223.00±42.096 ^{a1}	158.20 ±28.31 ^{b3}	86.40±7.99 ^{b3}
RELE100	320.40± 25.80^{a3}	312.60±54.417 ^{a1}	232.40± 40.26 ^{a1b2}	205.20±31.47 ^{a1b3}
RELE200	307.60± 33.26^{a3}	288.00±35.25 ^{a1}	197.00 ±21.86 ^{b3}	188.00±20.40 ^{b3}
RELE400	315.00± 35.12^{a3}	256.60±29.49 ^{a1}	197.20 ±22.69 ^{b3}	179.60±15.14 ^{b3}

Each value represents mean ± S.E.M; n=6 for each treatment; Analysis was performed by one way ANOVA; a, compared to normal control; b, compared to negative control; number followed by RELE and GL indicates dose in mg/kg; NOC, normal control treated with distilled water; NC, negative control treated with distilled water; GL, glibenclamide; RELE, *Rubus erlangeri* leaf extract; $^{1}p < 0.05$; $^{2}p < 0.01$; $^{3}p < 0.001$.

As time goes weekly FBG level of normal control group was always in a normal range. But the FBG level of negative control group was diabetic throughout the time.

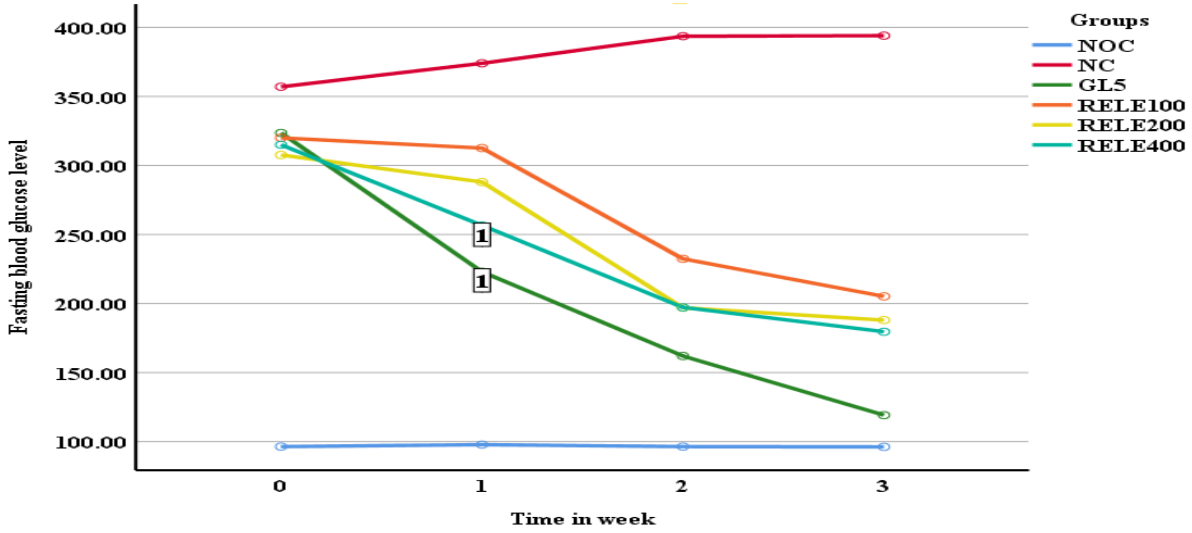

Figure 7**:** The antihyperglycemic effect of repeated doses of extract in different groups across time of blood glucose level measurements**;** Analysis was performed by two way ANOVA; NOC, normal control treated with distilled water; NC, negative control treated with distilled water; GL, glibenclamide; RELE, *Rubus erlangeri* leaf extract; number followed by RELE and GL indicates dose in mg/kg; blood glucose level was compared to the previous time measurement value; $^{1}p < 0.05$.

The FBG level however was decreased in GL5 group and all treatment groups. Compared to previous FBG level only at the 1st week a significant reduction was recorded in GL5 and higher dose of the extract ($p<0.05$) (Figure 7).

4.5. Effect of aqueous leaf extract on body weight change

There was no significant initial body weight difference among groups (Table 7). Group analysis revealed that NC exhibited significant body weight loss after the 2nd and 3rd week following induction of DM compared to normal control group ($p<0.001$). Comparing the effect of extract and GL5 on body weight change with negative control, all doses of extract and GL5 produced significantly less body weight reduction at 3rd week ($p<0.05$). But there was no significant difference in body weight change between the standard drug and extract doses as well as among the three doses of extract. Moreover, unlike that of negative control group, GL5 treated and all doses of the extract did not show significant body weight loss and rather exhibited patterns similar to normal control group.

Table 7: Effect of the aqueous leaf extract of *Rubus erlangeri* on weekly body weight change of diabetic mice

Groups	Body weight in gm			
	Initial	1st Week	2nd Week	3rd Weeek
NOC	30.00 ± 1.41	31.10±1.46	32.00± 1.21	32.87± 1.12
NC	29.20± 1.46	26.10±1.63	22.82±1.08^{a3}	19.60± 1.28 ^{a3}
GLI5	29.70± 1.47	28.60± 1.48	28.10 ± 1.50	28.00± 1.58^{b1}
RELE100	29.18± 1.58	27.40±1.28	26.52±1.29	26.04± 1.42 ^{b1}
RELE200	29.7±0.99	28.32 ±0.98	27.28± 1.31	27.2±1.27^{b1}
RELE400	27.80± 0.86	26.40± 0.83	26.00± 0.83	25.9±0.71^{b1}

Each value represents mean ± S.E.M; n=6 for each treatment; Analysis was performed by one way ANOVA; a, compared to normal control; b, compared to negative control; number followed by RELE and GL indicates dose in mg/kg; NOC, normal control treated with distilled water; NC, negative control treated with distilled water; GL, glibenclamide; RELE, *Rubus erlangeri* leaf extract; $^{1}p < 0.05$; $^{3}p < 0.001$.

Across treatment periods, the body weight of non-diabetic group was increased while those diabetic control group decreased. Compared to the previous body weight, a significant decline was observed at the 1st week in NC group only ($p<0.05$). But no significant body weight change was observed in NC and other groups at the two time points and all-time points, respectively (Figure 8).

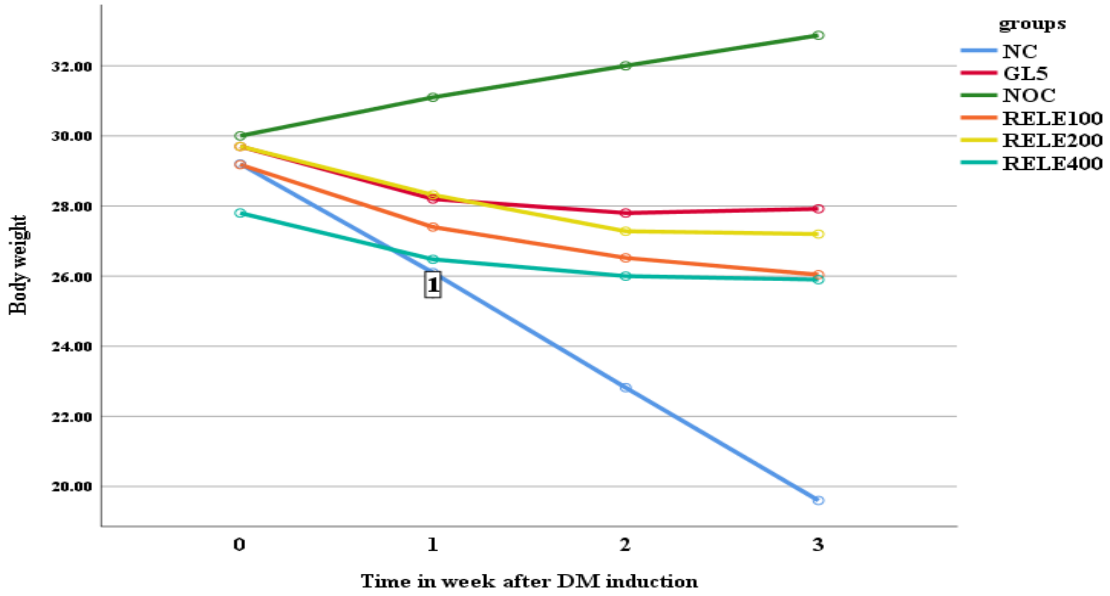

Figure 8: The effect of repeated doses of extract in different groups across time of body weight change; Analysis was performed by one way ANOVA; NOC, normal control treated with distilled water; NC, negative control treated with distilled water; GL, glibenclamide; RELE, *Rubus erlangeri* leaf extract; number followed by RELE and GL indicates dose in mg/kg; blood glucose level was compared to the previous time measurement value; [1]$p < 0.05$.

4.6. Preliminary phytochemical screening

The preliminary phytochemical screening of aqueous leaf extract of R. *erlangeri* revealed the presence of all tested constituents except steroids and terpenoids.

Table 8: Preliminary phytochemical screening of Rubus erlangeri leaf extract

Screened secondary metabolites	Test reagents/methods	Absence/presence
Saponins	Honey comp test	Positive
	10% sodium nitrate and conc. H2SO4	Positive
Polyphenols	5 % ferric chloride	Positive
Flavonoids	Zinc–hydrochloride test	Positive
	Lead acetate test	Positive
Alkaloids	Wagner's reagent	Positive
	Mayer's reagent	Positive
Tannins	Gelatin test	Positive
Steroids	Acetic anhydride and chloroform	Negative
Glycosides	20% sodium hydroxide	Positive
Terpenoids	Chloroform and conc. H2SO4	Negative

5. Discussion

The experimental plant *R. erlangeri* is among *Rubus* species which have a diverse traditional use. The genus *Rubus* consists of many species that are employed in various countries of the world to treat diseases, especially DM (Alonso *et al.,* 1980). Plants in this genus are also used for other disease conditions such as asthma, allergic rhinitis, atopic dermatitis which are related to active oxygen species and free radicals (Sultana, 2018). Plants in this genus are prepared by boiling the leaves with water traditionally (Abate, 1989). Therefore this method of preparation was selected during extraction process.

In the present study, before proceeding to *in vivo* experiment, the antidiabetic effect of the leaves of *R. erlangeri* was first conducted through *in vitro* by determining α-amylase inhibition activity. This enzyme is found in the brush borders of intestine whose function is hydrolyze the complex polysaccharides with the help of α-glycosidase. The later enzyme converts oligosaccharides and disaccharides to monosaccharide and increase postprandial glucose levels. Thus, one therapeutic approach in DM treatment is the prevention of carbohydrate absorption after food intake by inhibition of these enzymes. Moreover, lowering the digestion and breakdown of starch may have beneficial effects on insulin resistance and glycemic index control in people with T2DM (Wickramaratne *et al.,* 2016). The result from α-amylase inhibitory effect suggested that the plant is endowed with antidiabetic effect since there was no significant difference between IC50 values of the standard drug acarbose and extract (3.53±0.26 vs 4.90±0.44 µg/ml). *Rubus* species are potential sources of cyanidin-3-rutinoside, which is an anthocyanin polyphenolic compound (Lim *et al.*, 2015). Combinations of cyanidin-3-rutinoside and acarbose have been shown a synergetic effect in reducing the post prandial blood glucose after maltose and sucrose loading in rats (Adisakwattana *et al.*, 2011). Therefore, the activity of this plant extract against α amylase might be this phenolic compound.

The extract was also shown to have good antioxidant profile, which is comparable with vitamin C. Once again, based on their respective IC50s (5.91±0.39 µg/ml for ascorbic acid, 7.34±0.02 µg/ml for the aqueous extract), there was no significant difference between the two in DPPH scavenging activity. It is believed that oxidative stress plays important role in the impairment of insulin action and exacerbation of DM complications. Antioxidants including ascorbic acid have shown to have a prospect in the treatment of diabetes (Sowndhararajan *et al.*, 2013). Therefore,

the use of antioxidants along with antidiabetic drugs is frequently recommended to avoid such complications (Wickramaratne *et al.*, 2016). Thus, a comparable effect of the extract in DPPH scavenging activity reinforces the notion that the plant could have antidiabetic activity. This plant might also exert antidiabetic activity through enhancement of endogenous free radical scavenging enzymes. There is an evidence that administration of leaf extracts of *R. apetalus* and *R. steudneri*, which are both in the same genus of this experimental plant, increased levels of endogenous free radical scavengers such as superoxide dismutase, glutathione peroxidase, total thiols and catalase in diabetic mice (HL. *et al.*, 2019).

The above findings called for the need to do the antidiabetic activity in *in vivo* model. Therefore, the effect of the extract on BGL in normal mice for hypoglycemic and in oral glucose loaded normal mice as well as STZ induced diabetic mice for antihyperglycemic activity was initiated. The normoglycemic studies revealed that the aqueous extract of *R.erlangeri* had no hypoglycemic activity compared to normal control suggesting a lower risk of hypoglycemia. The slight reduction in BGL for the negative control is attributed to extended fasting time in between measurements.

Drug that is effective in diabetes will have the ability to control the rise in glucose level by different mechanisms the ability of the extracts to prevent hyperglycemia could be determined by glucose loaded hyperglycemic model (Jarald *et al.*, 2009). For the antihyperglycemic activity, in OGTT, mice were first fasted for 14 h before glucose loading. This method is referred to as physiological induction of DM because the blood glucose level of the animal is transiently increased with no damage to the pancreas (Singh *et al.*, 2015). Fasting is required before glucose administration to provide stable baseline glucose measurements and to eliminate fluctuations in BGL by food intake (Bowe *et al.*, 2014), It also stimulates glucose induced insulin sensitivity (Ayala *et al.*, 2010). Although, both oral glucose loading and intraperitoneal (IP) injection is possible (Adisakwattana *et al.*, Hansen *et al.*, 2010), the oral route was selected in this setting since absorption of glucose from the gut leads to the release of gastrointestinal hormones, primarily GLP-1 from the intestinal L-cells. This, in turn, potentiates glucose induced insulin release which would be possible with lower BGL relative to IP (Bowe *et al.*, 2014). Since there is discrepancies among researches regarding sex preference to the sensitivity of glucose induced insulin secretion, either sex were used for OGTT (Wilson *et al.*, 2016, Vital *et al.*, 2006). The effect of extract in lowering BGL might point that it is capable of stimulating insulin release. The secreted insulin would then stimulate peripheral glucose consumption and controls the production

of glucose through different mechanisms. In animal models of OGTT, secreted insulin requires ≥2h to bring back the glucose level to normal (Vital *et al.*, 2006, Chaimum-aom *et al.*, 2017). In this study, whilst >2h was required to bring back BGL to normal level in NC, the extract and GL5 brought down BGL to baseline within or less than 2h. GL5 and all doses of the aqueous extract started to decrease BGL with an onset of around 1h and 90 min respectively. The effect lasted for ≥ 90 min for GL5 and for ≥ 1h for middle and high dose of the extract. But RELE100 lasted for only 30 min in terms of duration of effect. Since, the extract lowered BGL following glucose loading and glucose induces insulin release, the effect behind this antihyperglycemic activity of the extract might involve an insulin-like action, probably, through peripheral glucose consumption or enhancing the sensitivity of β-cells to glucose, resulting in increased insulin release. However, further work might be required such as insulin level determination. From results of *in vitro* α-amylase inhibition activity and *in vivo* OGTT antidiabetic activity of the extract might be attributed to inhibition of glucose absorption.

In the present study, high dose STZ was used to induce diabetes. Mice were fasted before STZ was injected. The reason behind this is that glucose can compete with STZ in fed state, and thus, fasted animals tend to be more susceptible for this chemical (King, 2012). Treatment causes partial destruction of β-cell population, leading to transient increase in blood glucose level. The selective pancreatic β-cell toxicity and diabetic condition results from structural similarity between STZ and glucose. This structural similarity with glucose enables STZ to enter the β-cell via the low affinity GLUT-2 in the plasma membrane (Eleazu *et al.*, 2013a). After entrance to β-cell, the toxic effector mechanism of STZ starts with its decomposed products and the free radicals generated, which damages the pancreatic β-cells by alkylating DNA, impairing mitochondrial activity through inhibiting O-GlcNAcase (Goud *et al.*, 2015). It also methylates DNA and lead to the formation of carbonium ion, which consequently results in NAD+ depletion. Furthermore, through nitric oxide production (NO), it will damage pancreas. NO released in response to exposure leads to self-destruction of these cell. Alternatively, it will also block intra-islet macrophages or endothelial cells from which it diffuses into β-cells (Spinas, 1999).

In this study, the antihyperglycemic activity of single dose of the aqueous leaf extract brought a significant decline in BGL within 3 h after initiations of treatment with middle and high doses of the extract and lasted for around 1h. In addition, the reduction in glucose levels was also

significant after single oral administration of RELE400 and GL5 compared to previous FBG level at 1st h ($p<0.001$). Once again as the case of normoglycemic model effect, the decline in FBG level in negative control group is attributed to extended fasting time.

Studies showed that single IP injection of high dose of STZ can produce sustained hyperglycemia in mice at least for a period of 8 weeks (He-Lin *et al.*, 2010). Similarly, STZ induced persistent hyperglycemia in this study came with no significant change in BGL during the study period of three weeks as observed in the negative control. On the contrary, daily oral administrations of *R. erlangeri* leaf extract reduced FBG level. Particularly, the middle and high doses of extract lowered BGL below 200 mg/dl at the 2nd and 3rd week and there was no significant difference compared to normal control group at these treatment periods. This suggested that administrations of extract beyond this treatment period might reverse the BGL to normal level. Antihyperglycemic activity was also observed in GL5 treated diabetic mice; interestingly, glibenclamide works through binding to the sulfonylurea receptor with final net effect of increasing insulin release especially in a state of hyperglycemia (Lorenzati *et al.*, 2010). This might indicate that single dose IP administration of STZ at a dose of 200 mg/kg did not cause complete destruction of β-cells. Moreover, in a certain study, significant increase in the β-cell density per area of islet in diabetic rats suggested a compensatory response to the STZ damage which shows once again the presence of pancreatic β-cell to some extent in STZ induced diabetic animals (Wang-Fischer *et al.*, 2018). Therefore, antihyperglycemic activity of the *R. erlangeri* aqueous leaf extract in STZ-induced diabetic mice might be due to potentiation of insulin effect probably by increasing the secretion of insulin from β-cells of pancreas or by increasing the peripheral glucose uptake.

In addition to persistent hyperglycemia, STZ induced diabetes is associated with significant weight loss, possibly as a result of the catabolic effects of insulin deficiency and severe hyperglycemia, as well as the volume depletion associated with osmotic diuresis (Deeds *et al.*, 2011, Kitada *et al.*, 2016). Likewise, in this study, the body weight of diabetic mice became significantly lower ($p < 0.001$) than that of normal mice after 2nd week. But, oral administration of *R. erlangeri* leaf extract and GL5 produced significantly less bodyweight ($p<0.05$) when it was compared to negative control group at the third week. The lesser body weight loss in both extract and standard drug treatment group might be attributed to structural protein synthesis or

improvement of glycemic control which suggested the antidiabetic potential of the extract (Eleazu *et al.*, 2013b).

The antidiabetic effects of plants is due to the presence of secondary metabolites such as, phenols including flavonoids, tannins, alkaloids and saponins (Kooti *et al.*, 2016). Polyphenols promote the uptake of glucose in tissues, and therefore improving insulin sensitivity. They also inhibit glucose absorption. Plants in *Rubus* species contain resveratrol, which is a polyphenol. Resveratrol increase pancreatic β-cell function and have a potential of protecting β-cell dysfunction. It also increases glucose induced incretin effect through inhibiting phosphodiesterase (Aryaeian *et al.*, 2017). On the other hand, tannins work through decreasing glycogen content and inducing glucose transport via insulin mediated signaling pathway in adipocytes (Babby *et al.*, 2014). The activities of saponin is linked to in regulating BGL and prevent diabetic complications due to their antioxidant activity (El Barky *et al.*, 2017) and alkaloids exert their effect through inhibiting protein tyrosine phosphatase-1B and their activity is also because of their antioxidant effect (Tiong *et al.*, 2013).

6. Conclusion

The present study confirmed the antidiabetic activity of the aqueous leaf extract of *Rubus erlangeri.* The results obtained from the *in vitro* studies suggested that the extract works through inhibiting free radicals as well as reducing post-prandial hyperglycemia through α-amylase inhibition activity. The *in vivo* study, on the other hand, established that oral administration of the aqueous leaf extract of *Rubus erlangeri* has a beneficial effect in reducing blood glucose level both in oral glucose loaded and streptozotocin-induced diabetic mice, with a minimum risk of hypoglycemia.

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7. Recommendations

The following are recommended for further work.

- Since the crude extract of this plant possessed antioxidant activity, further *in vitro*/*in vivo* free radical scavenging activities should be done to determine the extract effect in augmenting the activities of catalase, superoxide dismutase and glutathione peroxidase.
- Diabetes mellitus is a chronic disease and therefore chronic toxicity study should be performed to evaluate the long-term effect of the plant extract.
- This study demonstrated that the aqueous leaf extract of *Rubus erlangeri* possessed an antidiabetic activity in experimental mice. More researches are needed to understand the mechanism (s) of action of the extract
- Quantifications of secondary metabolites and isolation of compounds responsible for antidiabetic activity.

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