

**ANTIDIABETIC ACTIVITY OF *AJUGA REMOTA* BENTH
(HARMEGUSA) LEAVES
IN STREPTOZOTOCIN INDUCED DIABETIC RATS**



**A Thesis Submitted to the School of Graduate Studies of Addis Ababa
University in Partial Fulfillment of the Requirements for the Degree of
Master of Science in Medical Biochemistry**

By:-

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ABBREVIATIONS

ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
AST	Aspartate aminotransferase
AREt	Ajuga remota leaves ethanolic extract
HDL-C	High Density Lipoprotein cholesterol
LDH	Lactate Dehydrogenase
LDL-C	Low Density Lipoprotein cholesterol
NAD ⁺ / NADH	Nicotinamide adenine dinucleotide oxidized/ reduced
STZ	Streptozotocin
T1DM	Type one diabetes Mellitus
T2DM	Type two Diabetes Mellitus
AS160	160-kDa Akt substrate
CAP	Cbl- associate protein
GLUT4	Glucose transporter-4
Grb2	Growth factor receptor-bound protein 2
IRS	Insulin receptor substrate
MAPK	Mitogen-activated protein kinase
PDK-1	3-phosphoinositide-dependent protein kinase-1
PI3	Phosphatidylinositol 3
PKC zeta/lambda,	Protein kinase C zeta/ lambda
RAS	Retrovirus-associated DNA sequences
Shc	Src homology 2 domain-containing protein
SOS	Son of sevenless protein
PPAR	Peroxisome proliferator activated receptor
DPP-4	Dipeptidyl peptidase-4

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ABSTRACT

Ethnopharmacological relevance: *The leaves of Ajuga remota (A. remota) have been utilized traditionally for the cure of diabetes mellitus and other ailments.*

Aim of the study: *The present study was aimed to assess the antidiabetic activity of A. remota leaves extract in Streptozotocin (STZ) induced diabetic rats.*

Material and methods: *Antidiabetic activity of the ethanol extracts of A. remota leaves (AREt) was studied in STZ induced diabetic rats. The effect of extract on fasting blood glucose, body weight, lipid profile, serum, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, urea, creatinine and total protein were analyzed. Glibenclamide was used as standard reference drug.*

Results: *A. remota leaves ethanol extract (AREt) showed highly significant blood glucose lowering effect. After diabetic rats treated with AREt (200 and 400 mg/kg) for 28 days, there were a significant decrease in fasting blood glucose, total cholesterol, triacylglycerol, LDL-cholesterol, serum enzymes (alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase), and significant increase in body weight, serum total protein, HDL-cholesterol, levels as compared to untreated diabetic rats.*

Conclusion: *The results of the experiments showed that A. remota leaves might be useful for management of diabetes mellitus and other abnormalities associated with this metabolic disorder. The present study might support the traditional use of A. remota for diabetes mellitus treatment.*

Key words: *Ajuga remota, diabetes mellitus and streptozotocin*

1. INTRODUCTION

1.1. Diabetes mellitus

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia or high blood glucose levels with deranged carbohydrates, fats and proteins metabolism (Figure 1.1) resulting from absolute or relative lack of insulin secretion or insulin resistance by peripheral tissues mainly the liver, skeletal muscle and adipose tissues or both. It is also characterized by hyperlipidemia and hyperaminoacidemia (Rao *et al.*, 2010 ; Sudha *et al.*, 2011). If untreated, the long-term effects of diabetes mellitus include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation and features of autonomic dysfunction, including sexual dysfunction. People with diabetes mellitus are at increased risk of cardiovascular, peripheral vascular and cerebrovascular disease (Karimulla and Kumar, 2011).

The global prevalence of diabetes mellitus estimated to increase, from 8.3% (366 million) in 2011 to 9.9% (522 million) by the year 2030. The largest increases will take place in the regions dominated by developing economies such as India and China. United States of America also have great number of patients with diabetes mellitus. There were about four million more men than women with diabetes mellitus (185 million men and 181 million women) in 2011. However, this difference is expected to decrease to two million (277 million men and 275 million women) by 2030 (International Diabetes Atlas, 2012).

In Ethiopia, there is no single national level prevalence study on diabetes mellitus. However, The World Health Organization estimated the prevalence of diabetes to be in the range of 2-3% and the number of people with diabetes mellitus in the year 2000 was approximately 800,000. This number is expected to reach 1.8 million by 2030 (Feleke and Enquesslassie, 2005). Other report also showed that, if there is a constant annual increase in the number of diabetic cases, this figure would reached approximately 1.3 million by 2010 (Wild *et al.*, 2004). In general, International diabetes federation estimated that prevalence of diabetes mellitus to be 2% and 3.5% in 2011 and 2030, respectively (Shaw *et al.*, 2010).

1.2. Classification of diabetes mellitus

Diabetes mellitus is classified on the basis of the pathogenic process that leads to hyperglycemia (Kasper *et al.*, 2011). Different types of diabetes mellitus have been identified and categorized as: type I, type II, gestational and other specific types of diabetes mellitus (American Diabetes Association, 2012).

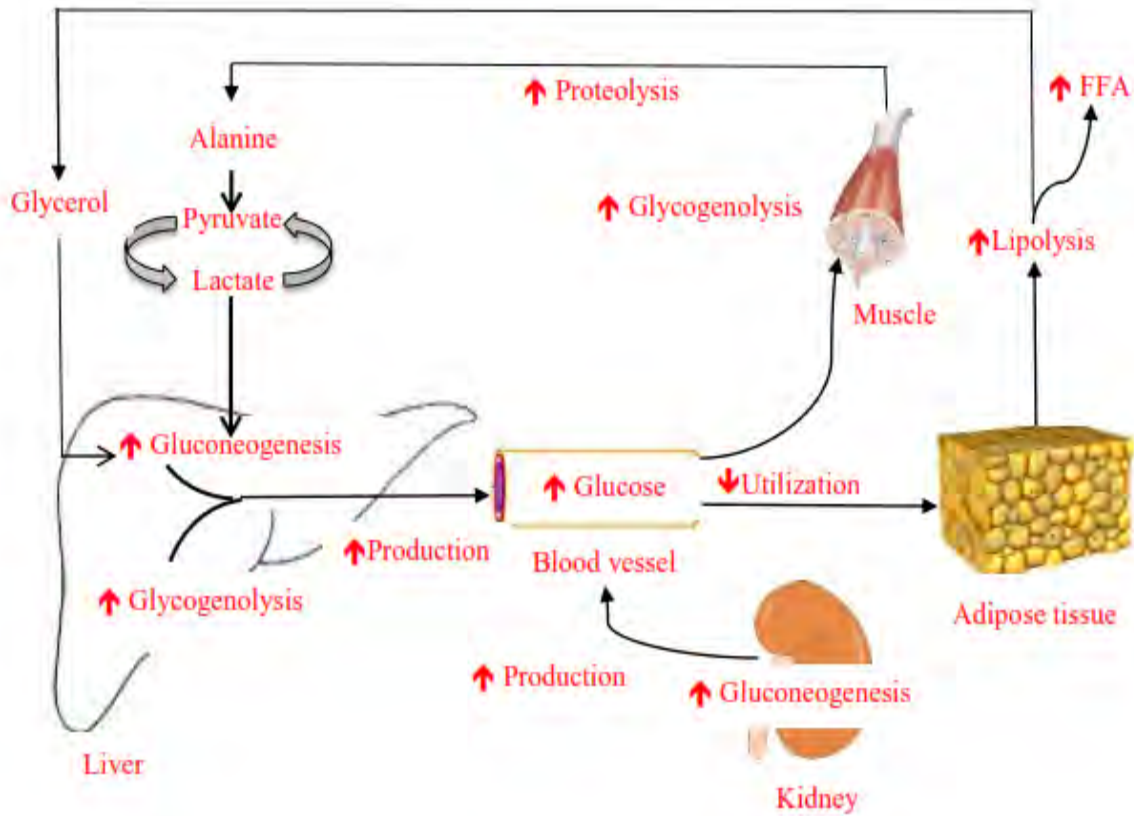


Figure 1. 1: Common metabolic disorders in diabetes mellitus patients. (↑) indicates activation or increase level and (↓) suppression or decrease level.

1.2.1. Diabetes mellitus type I

About 10% of patients with diabetes have type I diabetes mellitus (formerly known as insulin dependent diabetes mellitus, or IDDM). It is usually juvenile onset and results from the autoimmune destruction of the pancreatic β -cells. Decrease plasma insulin concentration results in a very prolonged increase in the plasma glucose concentration. Lack of insulin in continued presence of glucagon results in over production of glucose and ketone bodies by the

liver and reduced ability of the peripheral tissues to utilize glucose. Then, the body enters a catabolic state, with extensive lipolysis and proteolysis. Patients with untreated T1DM often present with dehydration, which is caused by osmotic diuresis when the rate of glucose filtration at the kidney exceeds the maximum rate of renal glucose reabsorption. A complication of type I diabetes is diabetic ketoacidosis due to ketone formation, which is a potentially fatal cause of metabolic acidosis (Kibble and Halsey, 2009).

1.2.2. Diabetes mellitus type II

Type II diabetes mellitus (formerly known as non-insulin dependent diabetes mellitus or NIDDM) is by far the more common form and accounts for 90% of diagnosed cases. However, T2DM is usually a progressive disease that remains undiagnosed in a significant percentage of patients for several years. T2DM is often associated with visceral obesity and lack of exercise indeed; obesity related T2DM is reaching epidemic proportions worldwide. Usually, there are multiple causes for the development of T2DM that are associated with defects in the ability of target organs to respond to insulin (insulin resistance), along with some degree of β -cells deficiency. Insulin sensitivity can be compromised at the level of the insulin receptor (IR) or at the level of post receptor signaling. Figure 1.2 describes insulin-signaling pathways downstream of the insulin receptor in skeletal muscle, likely affected in diabetes mellitus type II patients. T2DM appears to be the consequence of insulin resistance, followed by reactive hyperinsulinemia, but ultimately by relative hypoinsulinemia and β -cell failure (Koeppen and Stanton, 2008).

1.2.3. Gestational diabetes mellitus

Gestational diabetes mellitus is a type of diabetes mellitus that arises during pregnancy (usually the second or third trimester). About 20-50% of affected women develop type II diabetes mellitus later on. It may occur due to insufficient insulin production to meet the extra needs of pregnancy. On the other hand, it may be found during the first trimester of pregnancy, and in these women, the condition most likely existed before the pregnancy. Individuals at high risk for gestational diabetes mellitus include older women, previous history of glucose intolerance and women from certain high risk ethnic groups (Fatima *et al.*, 2012).

1.2.4. Other specific types of diabetes mellitus

Maturity onset diabetes of the young (MODY) is a type of non-insulin dependent diabetes mellitus caused by rare autosomal dominant mutations and impaired insulin secretion, accounting for 1% to 2% of diabetes cases. Patients with MODY usually present before the age of 25 and often have a strong family history of diabetes (Weedon and Frayling, 2007). MODY can result from mutations in at least six different genes, which were numbered according to the sequence of discovery. One of these encodes the glycolytic enzyme, glucokinase (MODY2), which is an important glucose sensor, whereas the others encode transcription factors: hepatocyte nuclear factor (HNF)-4 α (MODY1), HNF-1 α (MODY3), insulin promoter factor-1 (IPF1/Pdx-1; MODY4), HNF-1 β (MODY5) and neurogenic differentiation factor 1 (NeuroD1; MODY6) (Gupta and Kaestner, 2004).

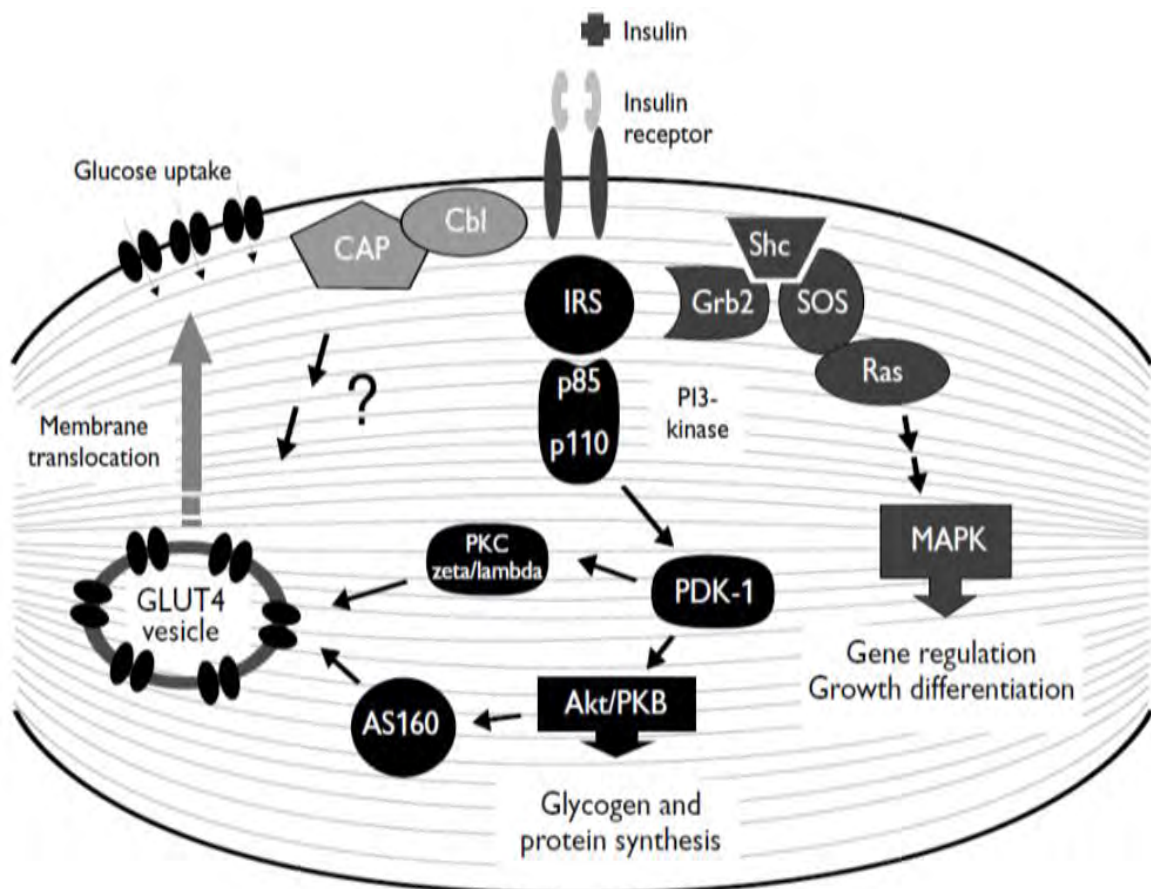


Figure 1. 2: Overview of insulin-signaling pathways in skeletal muscle (Glund and Zierath, 2005).

All forms of diabetes mellitus, both inherited and acquired, are characterized by hyperglycemia, a relative or absolute lack of insulin, and the development of diabetes specific microvascular pathology in the retina, renal glomerulus, and peripheral nerve. Diabetes mellitus also associated with accelerated atherosclerotic macrovascular disease affecting arteries that supply the heart, brain, and lower extremities. Pathologically, this condition resembles macrovascular disease in nondiabetic patients, but it is more extensive and progresses more rapidly in diabetic patients (Kronenberg *et al.*, 2008).

1.3. Pathophysiology of diabetes mellitus

1.3.1. Pathophysiology of type I diabetes mellitus

Type I diabetes is a T-cell mediated autoimmune disease involving destruction of the insulin-secreting β -cells in the pancreatic islets which takes place over many years. Hyperglycemia accompanied by the classical symptoms of diabetes mellitus occurs only when 70-90% of β -cells have been destroyed. Markers of the immune destruction of the β -cells include islet cell autoantibodies, autoantibodies to insulin, autoantibodies to glutamic acid decarboxylase (GAD65), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2 β . One and usually more of these autoantibodies are present in 85-90% of individuals when fasting hyperglycemia is initially detected (American Diabetes Association, 2008).

1.3.2. Pathophysiology of type II diabetes mellitus

Type II diabetes mellitus is a heterogeneous disorder with varying prevalence among different ethnic groups. The pathophysiology of type II diabetes mellitus is characterized by peripheral insulin resistance and declining β -cell function, and both are influenced by genetic and environmental factors (such as obesity, nutrition, and physical activity) (Mahler and Alder, 1999). The former is primarily represented by decreased insulin-stimulated glucose uptake in skeletal muscle and adipose tissues, augmented endogenous glucose production (predominately in the liver), and enhanced lipolytic activity in fat cells. The latter is an apparent progressive process with both functional defects or the substantial reduction in the maximum capacity to secrete insulin in islet cell and, eventually, apparent loss of β -cell mass of Pancreas (Kimmel and Inzucchi, 2005 ; Kim and Lee, 2010).

1.3.3. Pathophysiology of gestational diabetes mellitus

Insulin resistance during pregnancy stems from a variety of factors, including alterations in fetal-placental hormones such as prolactin, growth hormone, progesterone, and cortisol secretion (insulin antagonists), human placental lactogen secretion (which is produced by the placenta and affects fatty acids and glucose metabolism, promotes lipolysis, and decreases glucose uptake), and insulinase secretion (which is produced by the placenta and facilitates metabolism of insulin) (Gilmartin *et al.*, 2008). The pancreas normally compensates the challenge by increasing insulin secretion, but when it fails to do so, or when insulin secretion declines due to β -cell function impairment then gestational diabetes mellitus (GDM) develops. Maternal hyperglycemia, which is typical of GDM, causes a greater transfer of glucose to the fetus, causing fetal hyperinsulinemia and an overgrowth of insulin sensitive (mainly adipose) tissues, with consequent excessive, unbalanced fetal growth, causing more traumas at birth, shoulder dystocia and perinatal deaths. Hyperinsulinemia can also cause numerous neonatal metabolic complications, such as hypoglycemia, hyperbilirubinemia, hypocalcemia, hypomagnesemia, polycythemia, respiratory distress syndrome, and a greater long term risk of diabetes mellitus and obesity in the child (Lapolla *et al.*, 2009).

1.4. Diabetes mellitus and dyslipidemia

Diabetes mellitus is known to cause hyperlipidemia through various metabolic derangements, which is found in about 40% of diabetic patients (Karimulla and Kumar, 2011). The dyslipidemia associated with insulin resistance (also referred to as Atherogenic dyslipidemia) is characterized by moderately increased triglyceride (TG) levels carried in very low density lipoprotein (VLDL) particles, low high density lipoprotein cholesterol (HDL-C) levels carried in small HDL particles, and LDL-C levels that do not differ substantially from those of individuals without type II diabetes (Figure 1.3). In addition, TG-rich lipoproteins (after eating), remnant lipoproteins, apolipoprotein B 100 (ApoB) has also been shown to be increased in patients with type II diabetes. In addition, LDL-C particles are small and dense, carrying less cholesterol per particle; therefore, at any given LDL-C concentration, there are more LDL particles present in an individual with type II diabetes relative to an individual

without the disease, which may make the LDL-C level a misleading measure of risk in patients with type II diabetes (Vijayaraghavan, 2010).

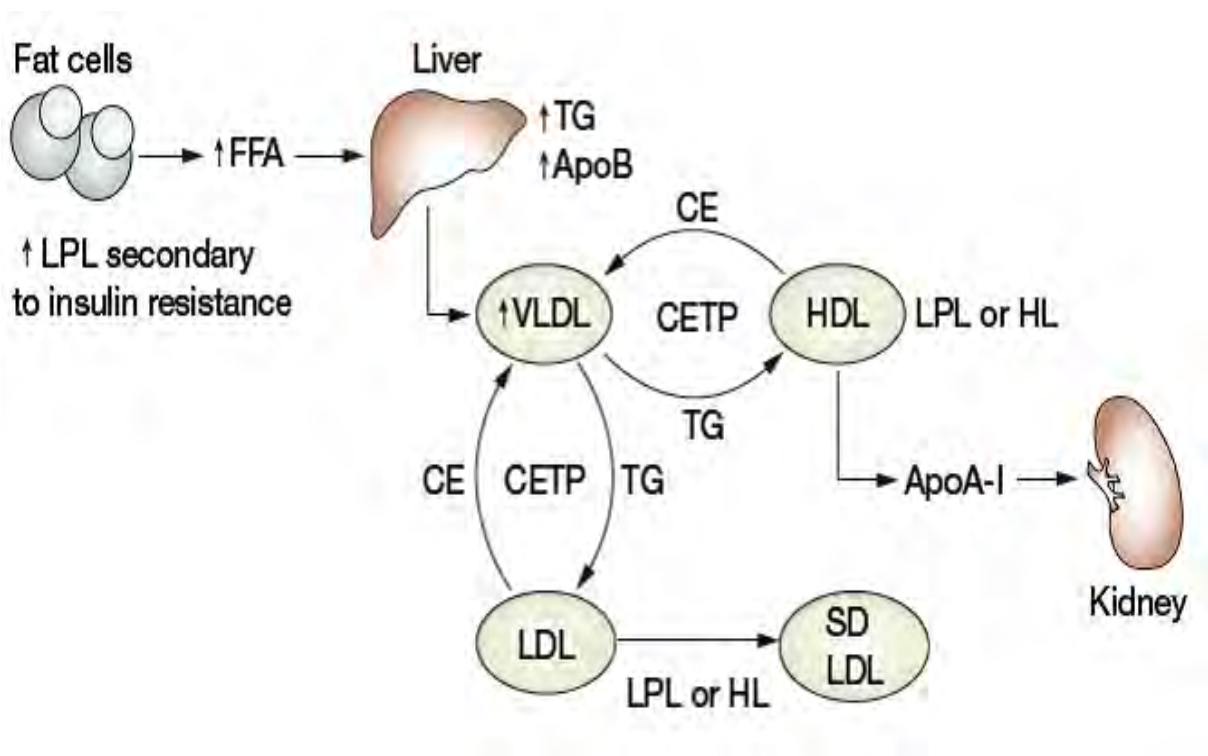


Figure 1. 3: The role of insulin resistance in diabetic dyslipidemia. Insulin resistance initiates the characteristic triad of high triglyceride level, low HDL-C level and high small dense LDL level. If the concentration of VLDL transported triglyceride is high, CETP promotes the transfer of LDL cholesterylester or HDL cholesterylester in exchange for triglyceride. Triglyceride rich HDL-C or LDL-C can undergo hydrolysis by hepatic lipase or lipoprotein lipase. Abbreviations: ↑, increased level; ApoA-1, apolipoprotein A-1; ApoB, apolipoprotein B; CE, cholesteryl ester; CETP, cholesterylester transfer protein; FFA, free fatty acid; HL, hepatic lipase; LPL, lipoprotein lipase; SD LDL, small dense LDL cholesterol; TG, triglyceride (Mooradian, 2009)

1.5. Diagnosis of diabetes mellitus

The blood glucose level of a healthy human is about 100 mg/dL on fasting and up to 160 mg/dL in the postprandial state. Diabetes mellitus is characterized by recurrent or persistent hyperglycemia, and is diagnosed by demonstrating one of the followings (Jarald *et al.*, 2008).

1.5.1. Fasting blood glucose and insulin levels

Fasting blood glucose level at or above 126 mg/dL or 7.0 mmol/L value often indicates diabetes mellitus or at least marked insulin resistance. In type I diabetes mellitus, plasma insulin level is very low or undetectable during fasting and even after a meal. In type II diabetes mellitus, plasma insulin concentration may be several folds higher than the normal level and usually increases to greater extent after a standard glucose load a glucose tolerance test (Guyton and Hall, 2006).

1.5.2. Oral glucose tolerance test

World health organization (WHO) recommended that the oral glucose tolerance test should be used only if the blood glucose concentration is in the uncertain range of 5.5-11.1 mmol/ L (The DECODE study group, 1999). Plasma glucose level at or above 200 mg/dL or 11.1 mmol/L two hours after a 75 g oral anhydrous glucose in water, indicates diabetes mellitus (American Diabetes Associaton, 2011).

1.5.3. Glycated hemoglobin (HbA_{1c})

Glycated hemoglobin level provides a reliable measure of chronic glycemic control without the need for a fasting or timed sample, and it correlates well with the risk of long term diabetes complications and mortality. Several population based studies have investigated the utility of the HbA_{1c} level for detecting undiagnosed diabetes and the potential to use the HbA_{1c} level as a good screening tool for type II diabetes (Choi *et al.*, 2011). HbA_{1c} of 6.5% is recommended as the cut point for diagnosing diabetes. A value of less than 6.5% does not exclude diabetes mellitus, diagnosed using glucose tests (World Health Organization, 2011).

1.6. Treatment of diabetes mellitus

1.6.1. Treatment of diabetes mellitus type I

The primary goal of treatment of diabetes mellitus is to prevent both micro and macro-vascular complications and permit the patient to live out their natural life span by maintaining near normal glycemic control (Tanaka and Itoh, 2011). Insulin is necessary for normal carbohydrates, proteins, and fats metabolism (Figure1. 4). Patients with type I diabetes

mellitus do not produce enough of this hormone to sustain life and therefore they are totally depend on exogenous insulin for survival (American Diabetes Associaton, 2012).

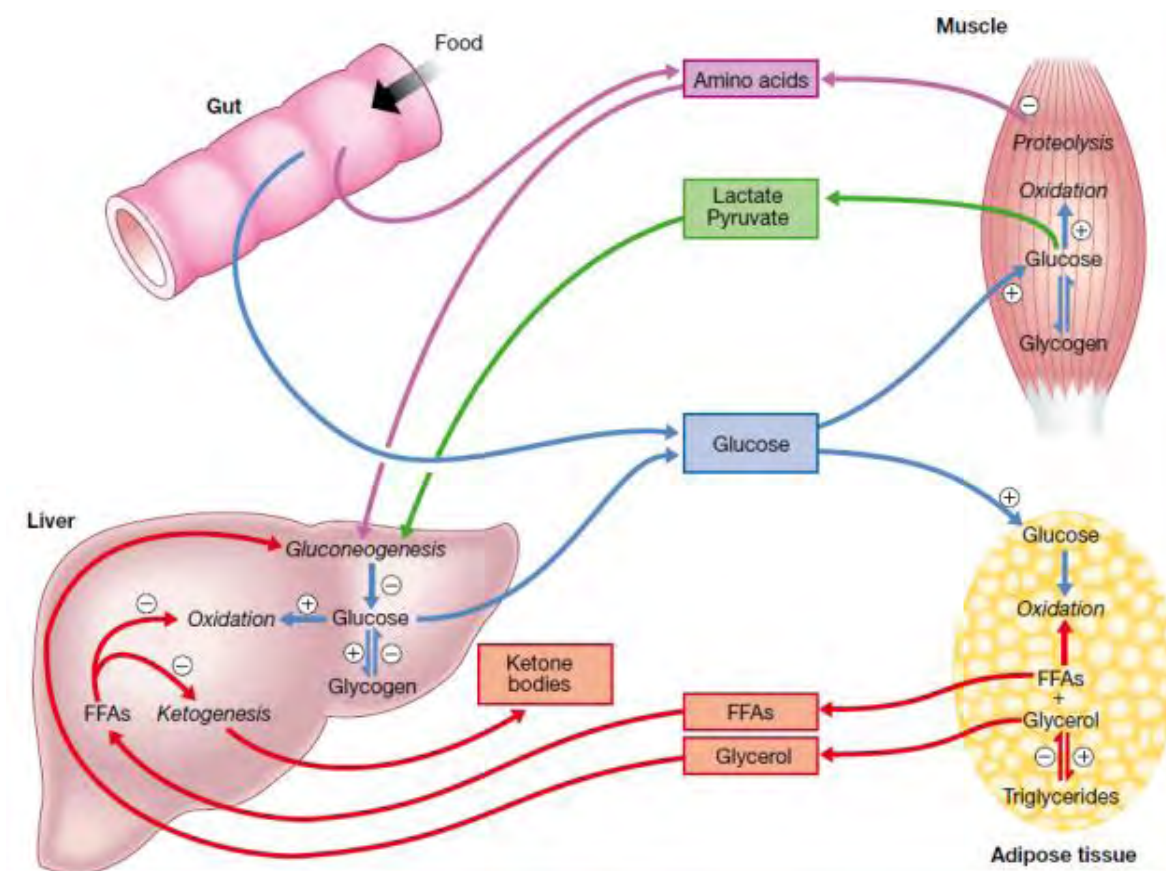


Figure 1. 4 : Major metabolic pathways of fuel metabolism and the actions of insulin. (+) indicates stimulation and (-) suppression by insulin (Colledge et al., 2010).

1.6.2. Treatment of diabetes type II

Diet and exercise are encouraged with oral hypoglycemic agents as the initial pharmacological therapy for type II diabetes mellitus. Current oral treatment options can be subdivided into the hypoglycemic drugs (sulfonylureas and benzoic acid derivatives) and antihyperglycemic drugs (biguanides, α -glucosidase inhibitors, and thiazolidinediones), their action have shown in Figure1.5 (Harrigan et al., 2001). To manage post prandial hyperglycemia at digestive level, glucosidase inhibitors such as acarbose, miglitol and voglibose are used. These inhibit degradation of carbohydrates thereby reducing the glucose absorbtion by cells. To enhance glucose uptake by peripheral cells biguanide such as metformin is used. Sulfonylureas like glibenclamide is insulinotropic and works as

secretagogue for pancreatic cells. New peptide analogs, such as exenatide and sitagliptin, increase glucagon like peptide1 (GLP-1) serum concentration and slow down the gastric emptying. Although, several therapies are used for diabetes mellitus treatment, there are certain limitations due to high cost and side effects such as development of hypoglycemia, weight gain, gastrointestinal disturbances, and liver toxicity (Dey *et al.*, 2002). Table 1.1 summarizes various limitations of current drug therapies of diabetes mellitus. Hence efforts are to find suitable antidiabetic therapy (Prabhakar and Doble, 2011 ; Modak *et al.*, 2007).

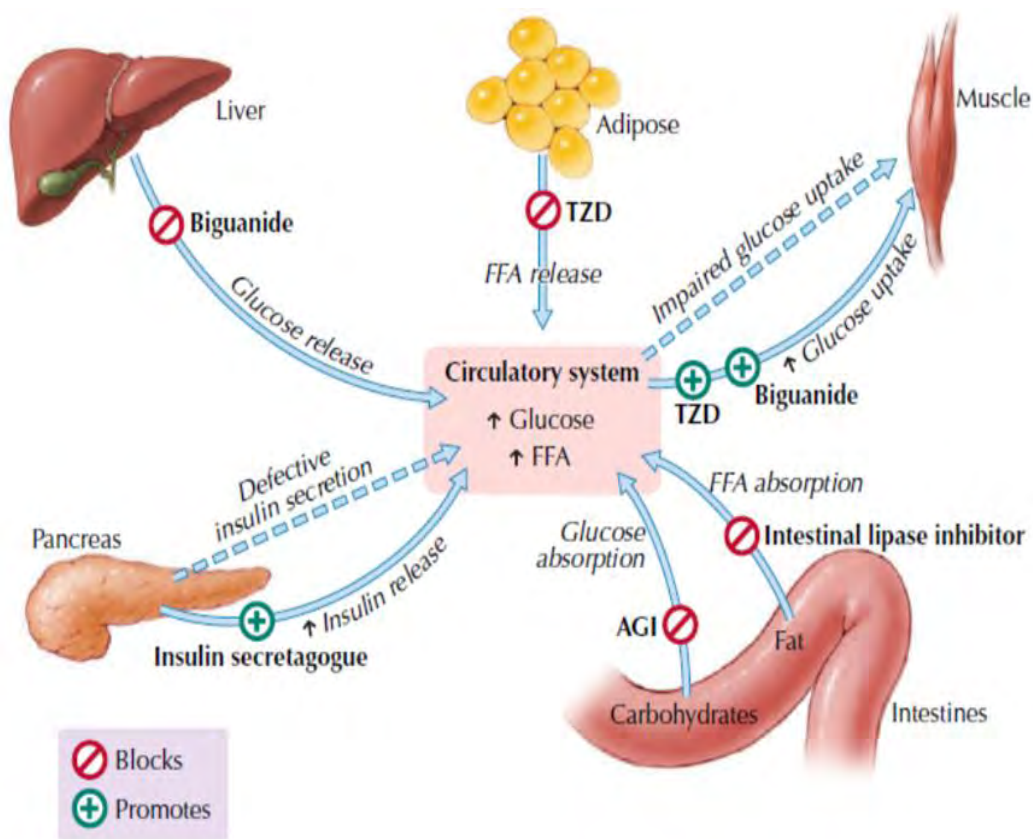


Figure 1.5: Major target organs and actions of oral hypoglycemic and antihyperglycemic agents in T2DM. TZD = thiazolidinedione; FFA = free fatty acid; AGI = α -glucosidase inhibitor (Cheng and Fantus, 2005).

In contrast to type I diabetes mellitus, individuals with type II diabetes are not dependent on exogenous insulin for survival. However, over time, many of these individuals will show decreased insulin production, therefore requiring supplemental insulin for adequate blood glucose control, especially during times of stress or illness or when oral antihyperglycemic and hypoglycemic drugs fail to lower blood glucose (American Diabetes Association, 2002)

Table 1. 1: Synthetic drug for diabetes mellitus, molecular target, site of action and their adverse reactions

Antidiabetic Drugs	Molecular target	Site of action	Limitations/Side Effects
Sulfonylureas	SU receptor, K ⁺ ATP Channel	Pancreatic β -cells	Hypoglycemia, weight gain
Biguanides	Not clear	Liver and muscles	Gastrointestinal disturbances, lactic acidosis
Alpha-glucosidase inhibitors	α -glucosidase	Intestine	Gastrointestinal disturbances
Thiazolidinediones	PPAR- γ	Fat , liver and muscles cells	Liver toxicity, weight gain, high LDL-C, high cost
Exenatide	GLP-1 receptor	Pancreatic α -cells, intestinal mucosal cells	Nausea, hypoglycemia, diarrhea
Sitagliptin	DPP-4	Intestine	Headache, nausea
Insulin	Insulin receptor	Liver, Muscle and fat cells	Hypoglycemia, weight gain

1.7. Medicinal plants and diabetes mellitus

The use of plants as medicines goes back to early man. Certainly the great civilizations of the ancient Chinese, Indians, and North Africans provided written evidence of man's ingenuity in utilizing plants for the treatment of a wide variety of ailments (Seifu *et al.*, 2012). It is estimated that 70-80% of people worldwide rely on traditional herbal medicine to meet primary health care needs (Uprety *et al.*, 2012). In addition, medicinal plants also use for income generation and livelihood improvement (Amri and Kisangau, 2012).

Medicinal plants are being looked up once again for the treatment of diabetes. Many conventional drugs have been derived from prototypic molecules in medicinal plants. Metformin exemplifies an efficacious oral glucose lowering agent. Its development was based on the use of *Galega officinalis* to treat diabetes. *Galega officinalis* is rich in guanidine, the hypoglycemic component. Because guanidine is too toxic for clinical use, the alkyl biguanides synthalin A and synthalin B were introduced as oral antidiabetic agents in Europe in the 1920s but were discontinued after insulin became more widely available. However,

experience with guanidine and biguanides prompted the development of metformin (Dey *et al.*, 2002).

To date, ethnobotanical information indicates that over 1200 plants are used as traditional remedies for the treatment of diabetes mellitus (Fraser *et al.*, 2007) and more than 200 pure compounds have showed lowering blood glucose activities. The World Health Organization (WHO) has encouraged and recommended the use of herbs as an alternative therapy for diabetes mellitus since medicinal plants are often less expensive, easily accessible, less toxic and suitable. Though a wide range of medicinal plants are in use world over, many of them are without valid scientific sanctity. Apparently, a systematic scientific scrutiny of the antidiabetic potentials of these plants has become a matter of utmost importance to justify their application in ethnomedicine (Sellamuthu *et al.*, 2009 ; Ansarullah *et al.*, 2011). A list of some Ethiopian medicinal plants with their antidiabetic efficacy is given in table 1.2.

The main active constituents derived from medicinal plants which have antidiabetic activity include alkaloids, glycosides, galactomannan gum, polysaccharides, peptidoglycan, hypoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic ions. These affect various metabolic cascades, which directly or indirectly affect the level of blood glucose in the human body (Prabhakar and Doble, 2011).

The antidiabetic activity of medicinal plants depends upon variety of mechanisms. Generally, the mechanisms of action could be grouped as: adrenomimeticism, pancreatic beta cell potassium channel blocking, cAMP (secondary messenger) stimulation; providing certain necessary elements like calcium, zinc, magnesium, manganese and copper for the beta-cells; Inhibition of β -galactosidase and α -glucosidase; preventing oxidative stress that is possibly involved in pancreatic β -cell dysfunction found in diabetes (Jarald *et al.*, 2008); Stimulation of glycogenesis, glycolysis and citric acid cycle and hexose monophosphate shunt; inhibition of gluconeogenesis and glycogenolysis (Khanavi *et al.*, 2012 ; Sellamuthu *et al.*, 2009 ; Singh *et al.*, 2001); Improvement in digestion along with reduction in blood sugar and urea; protection of destruction and regeneration of the β -cells, initiate insulin release (Figure1. 6); reduction in insulin resistance and/ or inhibition in renal glucose reabsorption (Narayan *et al.*, 2012).

Table 1. 2: Some Ethiopian medicinal plants with their antidiabetic efficacy

Plant name	Family name	Local Name	Antidiabetic efficacy	References
Allium Cepa	Alliaceae	Qey-shnkurt	Hypoglycemic and antihyperglycemic activity in normal and STZ induced diabetic rats	(Campos <i>et al.</i> , 2003)
Allium sativum	Allium sativum	Nech-shnkurt	Antidiabetic activity in STZ induced Diabetic rats	(Thomson <i>et al.</i> , 2007)
Carica papaya	Caricaceae	Papaya	Hypoglycemic and Antihyperglycemic effect in STZ induced diabetic rats	(Juárez-Rojop <i>et al.</i> , 2012 ; Sasidharana <i>et al.</i> , 2011)
Caylusea-abyssinica	Resedaceae	Rench	Antidiabetic activity in glucose loaded diabetic rats	(Tamiru <i>et al.</i> , 2012)
Curcuma longa	Zingiberaceae	Ird	Antihyperglycemic effect in STZ induced diabetic rats	(Masry, 2012)
Coriandrum-sativum	Apiaceae	Dimbelal	Hypoglycemic effect inn normal rats	(Aissaouia <i>et al.</i> , 2011)
Cuminum-cyminum	Apiaceae	Ensilal	Antidiabetic influence in STZ induced Diabetic rats	(Willatgamuwa <i>et al.</i> , 1998)
Cymbopogon-citratus Stapf	Poaceae	Lomi sar	Hypoglycemic effect in normal rats	(Adeneye and Agbaje, 2007)
Linum-usitatissimum	Linaceae	Telba	Antihyperglycemic effect in alloxan induced diabetic rats	(Ghule <i>et al.</i> , 2012)
Mangifera indica	Anacardiaceae	Mango	Hypoglycemic effect in diabetic human	(Waheed <i>et al.</i> , 2006)
Moringa-stenoptela	Moringaceae	Shiferaw	Hypoglycemic and antihyperglycemic effect in alloxan induced diabetic rats	(Toma <i>et al.</i> , 2012 ; Nardos <i>et al.</i> , 2011)
Nigella sativa	Ranunculaceae	Tikur-Azmud	Antihyperglycemic activity in diabetic rats	(Salama, 2011)
Trigonella-foenum graecum	Fabaceae	Abish	Antidiabetic effect in diabetic rabbits	(Puri <i>et al.</i> , 2012)
Zingiber-officinale	Zingiberaceae	Zinjible	Hypoglycemic effect in alloxan induced diabetic rats	(Jafri <i>et al.</i> , 2011)

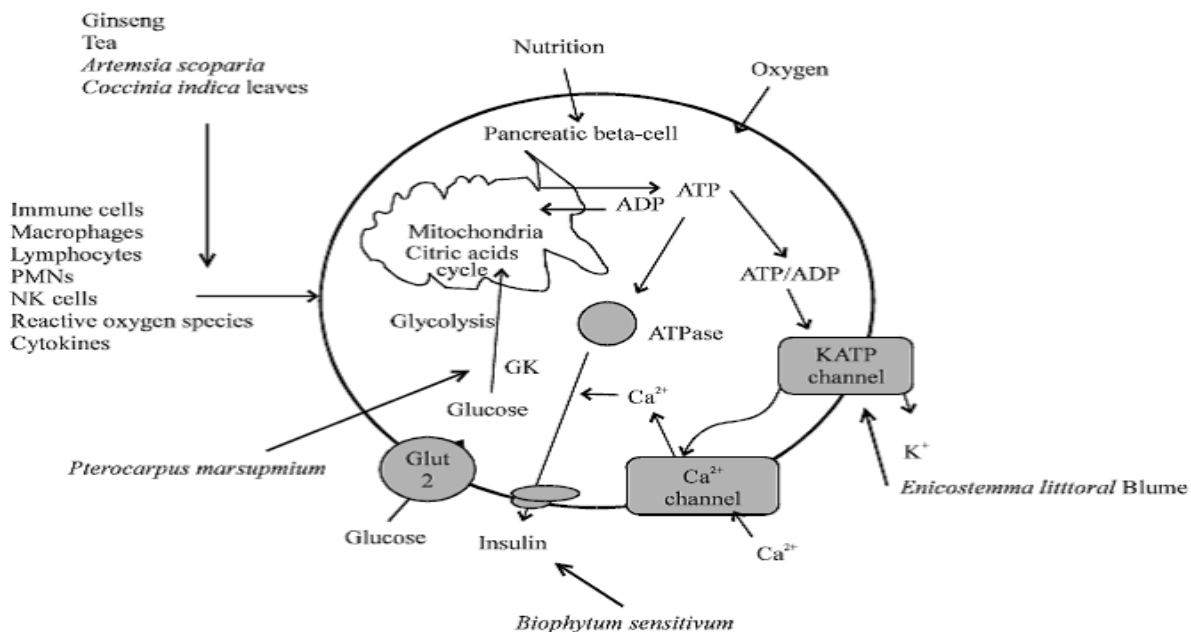


Figure 1. 6: Insulin secretion and the action sites of some hypoglycemia herbs. Glucose is taken up into β -cells via glucose transporters (GLUT-2). It is metabolized in glycolysis and Krebs cycle, resulting in an increased ratio of ATP and ADP in cytoplasm. This closes ATP- sensitive potassium channels (K_{ATP}) leading cell membrane depolarization and subsequently opening voltage-gated Ca^{2+} channels these changes increase free Ca^{2+} concentration in the cytoplasm and eventually triggers insulin secretion (Joseph and Jini, 2011).

1.8. *Ajuga remota* benth

A. remota (Figure 1. 7) is herb belongs to Labiatae family, locally known as ‘harmegusa’ or ‘etse medihanit’. It often lying on the ground and rooting at the nodes, covered with soft hairs, stems growing to 40 cm high. It occurs in different regions of Ethiopia at an altitude of 1600-2200 m. Flowering from late August to October, honeybees collect pollen and nectar from the flowers (Dagne, 2009)

A. remota has bitter taste in almost all its parts. Its leaf is known to relieve stomachache, cold, fever and gonorrhoea (Githinji and Kokwaro, 1993). It has been reported that *A. remota* to have antimalarial (Gitua et al., 2012) and antifungal (Kariba, 2001). In addition, the aerial parts of *A. remota* has shown to have some potent antimycobacterial activity (Cantrell et al., 1999), analgesic and antipyretic activity (Makonnen et al., 2003 ; Debella et al., 2005).

In Ethiopia, the leaf of *A. remota* has been used traditionally for stomachache, diabetes mellitus, and hypertension and hyperlipidemia treatment. The mode of administration usually involves squeezing the fresh leaves and sniffing the juice and drink the infusion or decoction of the leaves.



Figure 1. 7: *Ajuga remota* herb (photo by Freshet Assefa)

A large number of compounds have been identified in phytochemical investigation of *A. remota* herb. Such as neo-Clerodane diterpenoids (Coll and Tandrón, 2005), phytoecdysteroids; β -ecdysone and cyasterone (Kubo *et al.*, 1983), flavonol and iridoid glycosides (Manguro *et al.*, 2006), ergosterol 5,8-endoperoxide, kaempferol 3-O- α -rhamnoside, quercetin 3-O- β -glucoside and quercetin 3-O- rutinoside (Israili and Lyoussi, 2009). In general, phenolics, flavonoids, diterpenoids, glycosides and sugars are major constituents in the aqueous extract leaves of *A. remota*. On other hand, the leaves extract has not detected of alkaloids, cardiac glycosides, steroids triterpenoids, saponins, coumarins, and tanins (Debella *et al.*, 2005).

2. OBJECTIVES

2.1. General objective

To assess antidiabetic activity of ethanol extract of *A. remota* leaves in streptozotocin induced diabetic rats.

2.2. Specific Objectives

- ✓ To determine and compare fasting blood glucose level of controls and under treatment groups;
- ✓ To determine lipid profile of under treatment rats and compare with normal and diabetic control groups;
- ✓ To measure and compare the activity of serum alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase among the controls and under treatment groups;
- ✓ To determine and compare the amount of serum urea, creatinine and total protein of normal control, diabetic control and *A. remota* leaves extract treated rats; and
- ✓ To measure and compare body weight gain and lost on experimental rats during treatment period of *A. remota* leaves extract.

3. MATERIAL AND METHODS

3.1. Drugs, reagents and instruments

In this study, the following drugs, reagents and instruments were used: streptozotocin (Sigma Aldrich, Germany), glibenclamide (Sanofi winthrop industrie, France), citric acid (Schiapparelli Torino, Italy), sodium hydroxide (Labort fine chem pvt. Ltd, India), trisodium citrate (Bio Laboratories Ltd, Israel), ethanol absolute (Research-lab fine chem industries, India), 5% glucose solution (Claris life sciences limited, India), tween 80, diethyl ether, Whatman filter paper No.1, test tube, , gel tube, nect tube, volumetric flask (5 L), beakers (500 mL), funnels, erlenmeyer flasks (500 mL), measuring cylinder (1000 mL), glass rod, spatula, magnetic stirrer, semi-automatic pipettes of 10, 200 and 1000 μ L, gavage (oral feeding syringe), Syringe (1 mL, 3 mL and 5 mL), desiccator , heater, refrigerator, triple balance, digital electronic balance (AB204 model, Switzerland), pH meter (CG843P model, Germany), SensoCard glucometer and strip (77 Electronike Kft, Hungary), water bath (kottermann, Japan), rotavapor (Buchi rotavapor vac R-500, Switzerland), 902 automated chemistry analyzer (Hitachi, Japan) and A 25 BioSystems Chemistry analyzer (BioSystems, Spain).

3.2. Plant materials and authentication

Fresh leaves of *A. remota* were collected from Deneba town, North Shoa about 175 Km North-East of Addis Ababa in late September, 2012. The leaves were identified and authenticated by the National Herbarium (ETH) of Addis Ababa University, and voucher number 001 was given and deposited at the same institute for further reference. The dried leaves were manually grinded and the coarse powder was kept in polyethylene bags at room temperature until used for extraction.

3.3. Preparation of plant crude extract

The coarse powder (600 g) of *A. remota* leaves was macerated in 70% ethanol (1:10 leaves powder to solvent ratio) for 72 hours with mechanical shaking twice a day. This was repeated 3 times until the extract gave faint or no coloration (Kondeti *et al.*, 2010). The extract was then filtered through Whatman filter paper No.1 and solution was evaporated to dryness under

reduced pressure by rotavapor and further concentrated by water bath at 40 °C. Then, gummy residue extract was packed in air tight brown glass bottles with proper label and kept in a refrigerator at 4 °C until used for the experiment.

3.4. Preparation of plant extract solution for administration

The solvent free gummy residue (200 and 400 mg) was dissolved in 10 ml of 1% tween 80 to prepare stock solution of 20 mg/mL and 40 mg/mL daily, respectively. Then, the desired doses of the extract were administered according to the body weight of the rats in respective groups. The dose was selected on the basis of previous reports on the same herb (Debella *et al.*, 2005).

3.5. Experimental animals

Adult male wistar albino rats of weight 150-230 g (initial) with no prior drug treatment were used for the study. Rats were purchased from Ethiopian Health and Nutrition Research Institute (EHNRI). All the rats were acclimatized to the laboratory condition for one week before commencing the experiments and fed with pellete and tap water *ad libitum*. The animals were housed in 12 hours light and dark cycle at room temperature. The experiment was performed in the laboratory of Pharmacology department, school of medicine, Addis Ababa University after ethical approval obtained from ethical committee of the department of Biochemistry, school of medicine, Addis Ababa University (protocol number 0022/2012). All animal handling and care was done as per the guidelines set by the national academies press, Washington, D.C., USA.

3.6. Experimental induction of diabetes mellitus in rats

Diabetes mellitus was induced in overnight fasted rats by a single intraperitoneal injection of freshly prepared solution of streptozotocin (60 mg/ kg body weight) dissolved in 0.1 M citrate buffer, pH 4.5. The negative control rats were injected with the same concentration of citrate buffer only (Mohammadi and Naik, 2008). Streptozotocin injected rats were allowed to drink 5% glucose solution overnight to overcome initial drug induced hypoglycemic mortality (Gandhi and Sasikumar, 2012).

Diabetes mellitus in streptozotocin rats was confirmed by measuring the fasting blood glucose concentration, 72 hours after injection with streptozotocin. The rats with fasting blood glucose above 250 mg/dL were enrolled in the study. The treatment was started on the third day after streptozotocin injection and considered as zero day of treatment (Pillai S.S. *et al.*, 2012).

3.7. Preparation of 0.1 molar citrate buffer

Citric acid (10.5 g) and sodium citrate (14.7 g) were accurately weighted and mixed in 50 ml of distilled water. The volume was made up to 1000 ml with distilled water and the pH was adjusted to 4.5 by sodium hydroxide solution (Rajurkar, 2011).

3.8. Experimental design and treatment protocol

Normal and hyperglycemic rats were randomly assigned into five groups of each comprised six rats receiving the following: Group I: non-diabetic or normal control, received appropriate volume of vehicle that is 1% tween 80, 10 ml/Kg b.w., p.o. (normal control); Group II: Diabetic control, received the vehicle that is 1% tween 80, 10 ml/ Kg b.w., p.o. (diabetic control); Group III: Diabetic treatment, received *A. remota* ethanol extract (200 mg/Kg) b.w., p.o.; Group IV: Diabetic treatment, received *A. remota* ethanol extract (400 mg/Kg) b.w., p.o.; Group V: Diabetic treatment, received glibenclamide (600 µg) b.w., p.o. (Hedayati and Pouraboli, 2012).

The *A. remota* leaves ethanol extract (AREt) and glibenclamide were administered every morning for 28 days by gastric intubation with an oral gavage.

3.9. Measuring fasting blood glucose

Fasting blood glucose was measured with SensoCard glucometer after the collection of blood sample from the tail vein of the overnight (12-15 hr) fasted rats on day 0, 7th, 14th, 21st and 28th.

3.10. Determination of the weight of rats

Body weight gain or lost in each experimental rat was measured and recorded on day 0, 7th, 14th, 21st and 28th with triple balance.

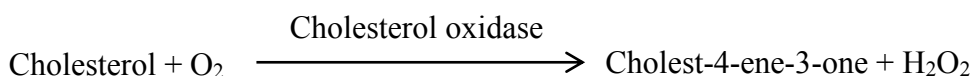
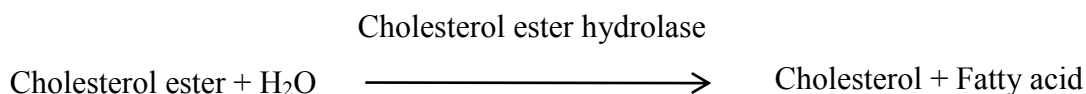
3.11. Estimation of serum biochemical parameters

At the end of the experimental period (on day 29th), all five groups of rats were sacrificed after overnight fast, by anesthetizing with diethyl ether and then blood was collected by direct cardiac puncture. Serum was separated after coagulated at room temperature for 30 min and centrifuged at 3000 rpm for 10 minutes, which was stored at -20 °C until biochemical parameters were determined. Total cholesterol, triacylglycerol (TG), high density lipoprotein cholesterol (HDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), urea and creatinine were determined with 902 automated chemistry analyzer. Low density lipoprotein cholesterol (LDL-C) level was calculated using Friedwald equation (Burtis *et al.*, 2008). Total serum protein was determined with A 25 BioSystems chemistry analyzer.

3.11.1. Measuring serum total cholesterol (Enzymatic method)

Total cholesterol level is determined after enzymatic hydrolysis and oxidation. Cholesterol esters are hydrolyzed to free cholesterol by cholesterol ester hydrolase. The free cholesterol produced is oxidized by cholesterol oxidase to cholest-4-ene-3-one with the simultaneous production of hydrogen peroxide, which oxidatively couples with 4-aminophenazone and phenol in the presence of peroxidase to yield a chromogen. The absorbance is measured at 500 nm.

Reactions



Reagents: enzymatic reagent and standard were ready to use

Specimen: rat serum

Assay

Wavelength: 500 nm

Optical path: 1 cm

Temperature: 37 °C

Measurement: against reagent blank

Procedure

Reagent blank, samples and standard were preincubated at 37 °C for 5 minutes. Reagent blank (1000 µL) and samples (10 µL) or standard (10 µL) were pipetted into cuvette and mixed thoroughly by inversion. The cuvettes were inserted into the cell holder and stopwatch was started to count. The absorbance of sample, standard and the reagent blank were measured at 500 nm within 60 minutes. Finally the absorbance of the sample (ΔA sample) and the standard (ΔA standard) against the reagent blank were calculated.

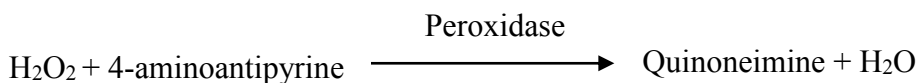
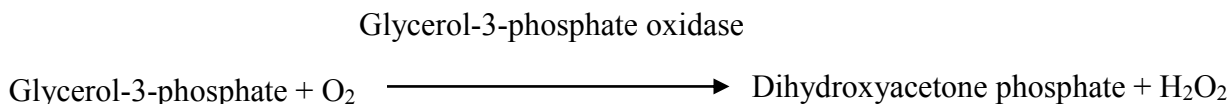
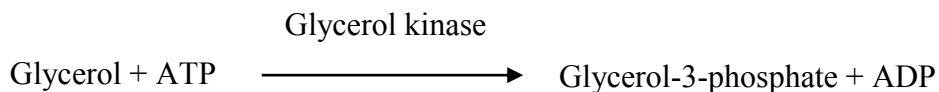
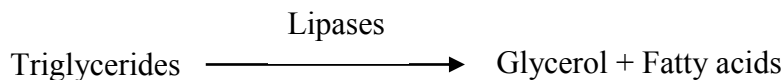
Calculation

$$\text{C of Cholesterol in mg/dL} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{C of Standard in mg/dL}$$

3.11.2. Estimation of serum triacylglycerol (Enzymatic method)

Triacylglycerols is determined after enzymatic hydrolysis with lipases to glycerol and free fatty acids. The glycerol released is subsequently measured by a couple of enzymatic reaction system. The glycerol formed phosphorylated to glycerol-3-phosphate by glycerol kinase. The glycerol-3-phosphate is oxidized by glycerol phosphate oxidase producing dihydroxyacetone phosphate and hydrogen peroxide. Then, Peroxidase catalyzes the redox-coupled reactions of H_2O_2 with 4-aminoantipyrine (4-AAP), producing a brilliant purple color. The absorbance is measured at 540 nm.

Reactions



Reagents: reagent and standard were ready to use.

Specimen: rat serum

Assay

Wavelength: 540 nm

Optical path: 1 cm

Temperature: 37 °C

Measurement: against reagent blank

Procedure

Reagent blank, samples and standard were preincubated at 37 °C for 5 minutes. Reagent blank (1000 µL) and samples (10 µL) or standard (10 µL) were pipetted into cuvette and mixed gently by inversion. The cuvettes were inserted into the cell holder and stopwatch was started to count. The absorbance of sample, standard and the reagent blank were measured at 540 nm within 60 minutes. Finally the absorbance of the sample (ΔA sample) and the standard (ΔA standard) against the reagent blank were calculated.

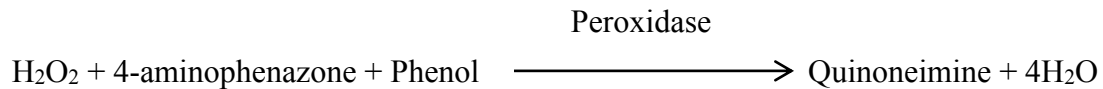
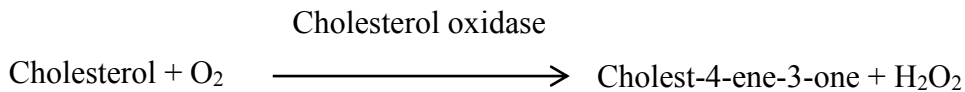
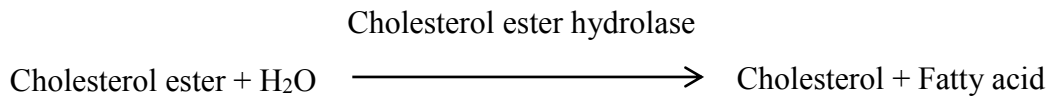
Calculation

$$\text{C of Triglyceride in mg/dL} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{C of standard in mg/dL}$$

3.11.3. Determination of serum HDL cholesterol (Enzymatic method)

The very low density (VLDL) and low density (LDL) lipoproteins from serum are precipitated by phosphotungstate in the presence of magnesium ions. After removed by centrifugation the clear supernatant containing high density lipoproteins (HDL)-fraction and their cholesterol content is determined enzymatically as shown below.

Reactions



Reagents: reagents and enzymes were ready to use

Sample: rat serum

Assay

Wavelength: 593 nm

Optical path: 1 cm

Temperature: 37 °C

Measurement: against reagent blank (RB)

Procedure for precipitation

Reagent (100 µL) and samples (1 mL) were pipetted into centrifuge tube, mixed well, allowed to stand for 5 minutes at 37 °C and centrifuged at 4000 rpm for 20 minutes. The supernatant (sample) was collected for test HDL-C.

Procedure for determination of HDL-C

Reagent blank, samples and calibrator were preincubated at 37 °C for 5 minutes. Reagent blank (10 µL distilled water and 750 µL enzymes) and samples (10 µL samples and 750 µL enzymes) or calibrator (10 µL calibrator and 750 µL enzymes) were pipetted into cuvette and mixed gently by inversion. The cuvettes were inserted into the cell holder and stopwatch was started to count. The absorbance of sample, standard and the reagent blank were measured at 593 nm after 5 minutes. Finally the absorbance of the samples (ΔA sample) and the calibrator (ΔA standard) against the reagent blank were calculated.

Calculation

$$\text{C of HDL-C in mg/dL} = \frac{\Delta A \text{ sample}}{\Delta A \text{ calibrator}} \times \text{Calibrator concentration in mg/dL}$$

Where $\Delta A = A \text{ samples/calibrator} - A \text{ blank reagent}$

3.11.4. Calculation of low density lipoprotein-cholesterol

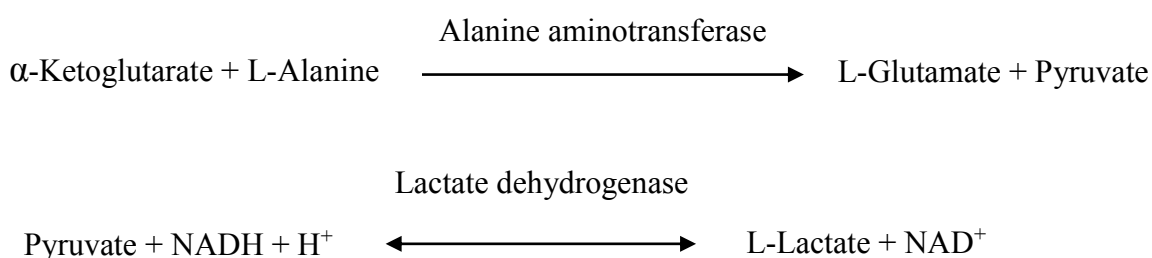
Indirect method, total cholesterol, triglyceride and HDL cholesterol are measured and LDL cholesterol is calculated from the primary measurements by use of the empirical Friedewald Formula equation:

$$\text{C of LDL cholesterol in mg/dL} = [\text{Total cholesterol}] - \frac{[\text{Triacylglycerol}]}{5} - [\text{HDL cholesterol}]$$

3.11.5. Measuring the activity of alanine aminotransferase (Kinetic method)

Alanine aminotransferase present in the sample catalyzes the transfer of the amino group from L-alanine to α -ketoglutarate forming pyruvate and L-glutamate. Pyruvate in the presence of NADH and lactate dehydrogenase (LDH) is reduced to L-lactate. In addition, NADH is oxidized to NAD^+ . The rate of decrease in absorbance of the reaction mixture at 340 nm, due to the oxidation of NADH is directly proportional to the ALT activity.

Reactions



Reagents: reagents were ready to use

Specimen: rat serum

Assay

Wave length: 340 nm

Optical path: 1 cm

Temperature: 37°C

Measurement: against air (decreasing absorbance)

Procedure

Working reagent and samples were preincubated at 37 °C. The spectrophotometer was adjusted to zero absorbance with air. Samples (100 μL) and working reagents (1000 μL) were pipetted into cuvette and mixed gently by inversion. The cuvette was inserted into the cell

holder and stopwatch was started to count. The absorbance was recorded at 340 nm exactly after 1, 2 and 3 minutes. Finally mean absorbance change was calculated.

Calculation

$U/L = 1745 \times \Delta A/\text{min.}$, where ΔA is mean absorbance change per minutes.

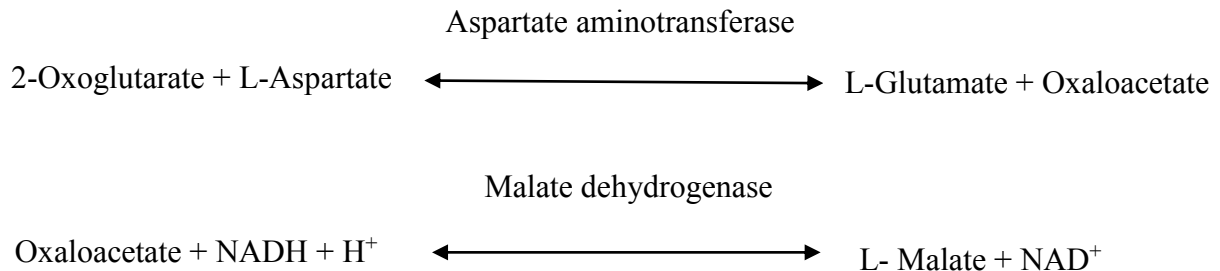
Conversion factor from traditional units (U/L) in SI-units (kat/L)

$$1U/L = 16.67 \times 10^{-3} \mu\text{kat/L}$$

3.11.6. Measuring the activity of aspartate aminotransferase (Kinetic method)

Aspartate aminotransferase catalyzes the transfer of the amino group from L-aspartate to α -ketoglutarate to yield oxaloacetate and L-glutamate. Malate dehydrogenase (MDH) catalyzes the reduction of oxaloacetate with simultaneous oxidation of NADH to NAD^+ . The resulting rate of decrease in absorbance at 340 nm is directly proportional to the AST activity.

Reactions



Reagents: reagents were ready to use.

Specimen: rat serum

Assay

Wavelength: 340 nm

Optical path: 1 cm

Temperature: 37°C

Measurement: against air (decreasing absorbance)

Procedure

Working reagent and samples were preincubated at 37 °C. The spectrophotometer was adjusted to zero absorbance with air. Samples (100 µL) and working reagents (1000 µL) were pipetted into cuvette and mixed gently by inversion. The cuvette was inserted into the cell holder and stopwatch was started to count. The absorbance was recorded at 340 nm exactly after 1, 2 and 3 minutes. Finally mean absorbance was change calculated.

Calculation

$U/l = 1745 \times \Delta A/\text{min}$. where ΔA is mean absorbance change per minutes

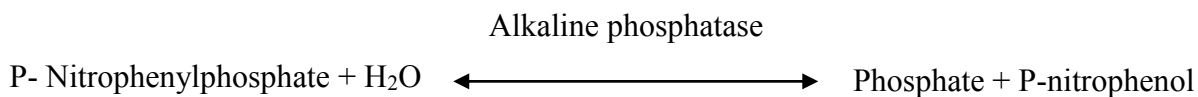
Conversion factor from traditional units (U/L) in SI units (kat/L)

$$1 \text{ U/L} = 16.67 \times 10^{-3} \text{ } \mu\text{kat/L}$$

3.11.7. Measuring the activity of alkaline phosphatase (Kinetic method)

Hydrolysis of p-Nitrophenyl phosphate (p-NPP) gives p-Nitrophenol and Phosphate by Alkaline phosphatase. P-nitrophenol has an intense yellow color in alkaline pH, the intensity of which is measured at 405 nm.

Reaction



Reagents: substrate, buffer and working reagents were ready to use

Specimen: rat serum

Assay

Wavelength: 405 nm

Optical path: 1 cm

Temperature: 37 °C

Measurement: against air (increase absorbance)

Procedure

Working reagent and samples were preincubated at 37 °C. The spectrophotometer was adjusted to zero absorbance with air. Samples (20 µL) and working reagents (1000 µL) were pipetted into cuvette and mixed gently by inversion. The cuvette was inserted into the cell holder and stopwatch was started to count. The absorbance was recorded at 405 nm exactly after 1, 2 and 3 minutes. Finally mean absorbance change was calculated.

Calculation

$U/L = 2757 \times \Delta A/\text{min.}$, Where ΔA is mean absorbance change per minutes.

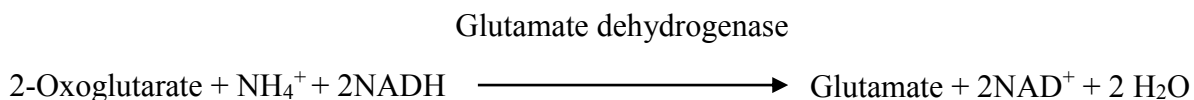
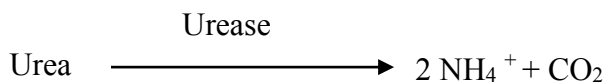
Conversion factor from traditional units (U/L) in SI- units (kat/L)

$$1 \text{ U/L} = 16.67 \times 10^{-3} \mu\text{kat/L}$$

3.11.8. Determination of serum urea

Urea is hydrolyzed by urease to ammonia and carbon dioxide. The ammonia is converted to glutamate by glutamate dehydrogenase (GLDH) in the presence of NADH and oxoglutarate. The reaction is monitored kinetically at 340 nm by the rate of the decrease in the absorbance resulting from the oxidation of NADH to NAD⁺, proportional to the concentration of urea present in the sample.

Reactions



Reagents: standard and working reagent were ready for use

Sample: rat serum

Assay

Wavelength: 340 nm

Optical path: 1 cm

Temperature: 37 °C

Measurement: against air (decreasing absorbance)

Procedure

Working reagent, samples and standards were preincubated at 37 °C. The spectrophotometer was adjusted to zero absorbance with air. Samples (1 µL) or standard (1 µL) and working reagents (10 µL) were pipetted into cuvette and mixed gently by inversion. The cuvettes were inserted into the cell holder and stopwatch was started to count. The absorbance was recorded at 340 nm exactly after 30 seconds (A_1) and exactly 90 seconds later (A_2) of the sample and standard addition. Finally the difference between after 30 and 90 seconds was calculated.

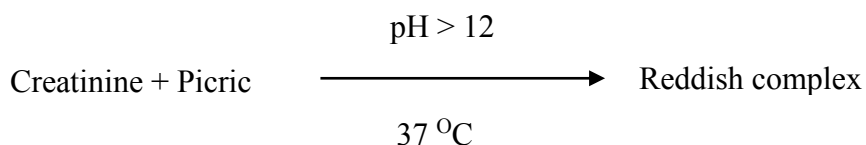
Calculation

$$\text{C of Urea in mg/dL} = \frac{(\text{A}_1 - \text{A}_2) \text{ sample}}{(\text{A}_1 - \text{A}_2) \text{ standard}} \times \text{C standard in mg/dL}$$

3.11.9. Determination of serum creatinine

Creatinine under the alkaline conditions reacts with picrate ion forming a reddish complex. The formation rate of the complex measured through the increase of the absorbance in a prefixed interval of time is proportional to the concentration of creatinine in the sample.

Reaction



Reagents: working reagent was ready to use

Specimen: rat serum

Assay

Wavelength: 500 nm

Optical path: 1 cm

Temperature: 37 °C

Measurement: against air (increase absorbance)

Procedure

Working reagent, sample and standard were preincubated at 37 °C. The spectrophotometer was adjusted to zero absorbance with air. Working reagent (1.0 ml) and sample (100 ml) or standard (100 ml) were pipetted into cuvette and mixed gently. The cuvettes were inserted into the cell holder and stopwatch started to count. The absorbance was recorded at 500 nm after 30 seconds (A₁) and after 90 seconds (A₂) of the sample or standard addition.

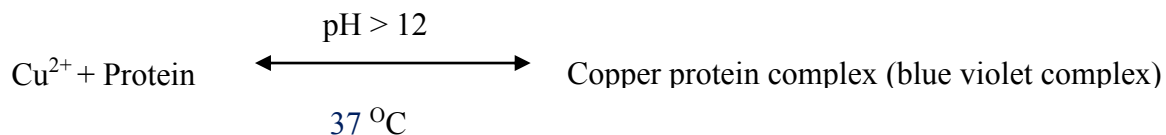
Calculation

$$\text{C of Creatinine in mg/ dL} = \frac{(A_2 - A_1) \text{ Sample}}{(A_2 - A_1) \text{ Standard}} \times \text{C of Standard in mg/dL}$$

3.11.10. Determination of total protein (Biuret method)

Peptide bonds react with Cu²⁺ ions in alkaline solution to form a blue violet colored product complex. A colored chelate is formed between the Cu²⁺ ion, the carbonyl oxygen and amide hydrogen atoms. One Cu²⁺ ion is linked to six peptide bonds. The intensity of the color is proportional to the number of peptide bonds that are reacting and therefore to the amount of protein present in the medium, the absorbance of which is measured at 546 nm.

Reaction



Reagents: single reagent was ready to use

Specimen: rat serum

Assay

Wavelength: 546 nm

Optical path: 1 cm

Temperature: 37 °C

Measurement: against the blank

Procedure

Blank reagent (20 μL distilled water and 1000 μL reagent) and samples (10 μL sample and 1000 μL reagent) or calibrator (10 μL calibrator and 1000 μL reagent) were pipetted into cuvettes and mixed thoroughly at 37 °C by inversion. The cuvettes were inserted into the cell holder and stopwatch was started to count. The absorbance of sample and the reagent blank were measured at 546 nm after 10 minutes. Finally the absorbance of the samples (ΔA sample) and the calibrator (ΔA calibrator) against the blank reagent were calculated.

Calculation

$$\text{C of total protein in mg/dL} = \frac{\Delta A \text{ sample}}{\Delta A \text{ calibrator}} \times \text{C calibration in mg/dL}$$

Conversion factor: g/dL x10 = g/L

3.12. Statistical analysis

All the values of body weight, fasting blood sugar and serum biochemical parameters were expressed as mean \pm standard error of mean (SEM) and were performed using SPSS software package Version 20.0. The values were analyzed by one-way analysis of variance (ANOVA) followed by the Tuckey post hoc test.

4. Results

4.1. Percent yield of *A. remota* leaves

In preparation of crude ethanol extract of *A. remota* from 600 g coarse powder leaves, 11.8% (70.80 g) yield of gummy residue was obtained.

4.2. Effect of *A. remota* leaves extract on fasting blood glucose level in diabetic rats

The effect of different doses of ethanol extract of *A. remota* leaves (AREt) on the fasting blood glucose level in diabetic rats are given in Table 4.1. The fasting blood glucose levels of diabetic control rats were significantly higher than those of normal control rats on day 0, 7th, 14th, 21st and 28th. In diabetic rats, treated with 200 mg/Kg and 400 mg/Kg AREt and 600 µg/Kg glibenclamide, fasting blood glucose level significantly lowered from 21st, 14th and 7th day of treatment as compared with diabetic control rats, respectively. In addition, 200 mg/Kg b.w. dose produced reduction of 18.9% and 28.7% on fasting blood glucose level of diabetic rats on day 21st and 28th, respectively. Whereas, a dose of 400 mg/Kg b.w. resulted in 20.4% and 39.6% reduction of fasting blood glucose on day 21st and 28th, respectively. Treatment with glibenclamide at of 600 µg/kg b.w. dose resulted reduction in 32.3% on day 21st and 47.3% on day 28th which was maximum fall in fasting blood glucose level.

4.3. Effect of *A. remota* leaves extract on body weight in diabetic rats

Table 4.2 demonstrates the body weight of untreated diabetic control rats were significantly reduced as compared with the normal control rats. AREt treated diabetic rats for 28 days significantly improved the body weight gain at 200 mg/Kg and 400 mg/kg as compared to the diabetic control group, comparable to that of standard at 600 µg/Kg. The body weight of normal control rats was significantly increased on days 7th, 14th, 21st and 28th compare to 0 day of treatment, while the diabetic control rats significantly decreased on days 14th, 21st and 28th compare to 0 day. However, the body weight of diabetic rats treated with ethanol extract of *A. remota* leaves was not significantly changed from the 0 day of treatment. Diabetic rats body weight gain treated with 200 mg/Kg AREt, 400 mg/Kg AREt and 600 µg/Kg glibenclamide were -1.63%, 0.67% and 2.95% on day 28th in respective group, respectively.

Table 4. 1: Effect of *A. remota* leaves extract on fasting blood glucose level in STZ induced diabetic rats.

Groups	Fasting blood glucose level (mg/dL)				
	0 day	7 th day	14 th day	21 st day	28 th day
Normal control	97.2±5.2	99.2±3.3	94.50±3.8	98.5±3.9	97.0±5.0
Diabetic control	383.7±15.1 ^a	414.7±13.2 ^a	446.5±9.7 ^{ac}	473.17±11.1 ^{ac}	398.8±21.5 ^a
Diabetic + AREt (200 mg/Kg)	386.5±16.0 ^a	394.7±13.4 ^a	383.3±14.9 ^a	313.3±18.1 ^{abc}	275.7±14.8 ^{abc}
Diabetic + AREt (400 mg/Kg)	381.2±24.1 ^a	387.0±20.9 ^a	359.2±14.2 ^{ab}	303.5±20.1 ^{abc}	230.2±23.1 ^{abc}
Diabetic + Glibenclamide (600 µ/Kg)	393.0±12.8 ^a	336.3±21.6 ^{ab}	328.7±15.7 ^{ab}	266.0±14.2 ^{abc}	207.3±29.7 ^{abc}

The values indicate mean ±S.E.M (n=6).^a p<0.05 compared with normal control values, ^b p<0.05 compared with diabetic control values and ^c p<0.05 compared with the initial level of fasting blood glucose (0 day) of the rats in the respective group.

Table 4. 2: Effect of *A. remota* leaves extract on body weight of STZ induced diabetic rats.

Groups	Body weight (g)				
	0 day	7 th day	14 th day	21 st day	28 th day
Normal control	161.1±5.4	174.1±4.7 ^c	188.7±5.5 ^c	202.5±5.0 ^c	215.5±4.06 ^c
Diabetic control	142.6±3.4	132.6±2.6 ^a	122.2±2.8 ^{ac}	118.6±4.2 ^{ac}	114.0±3.2 ^{ac}
Diabetic + AREt (200 mg/Kg)	147.0±5.3	135.8±4.0 ^a	138.9±2.5 ^{ab}	139.2±2.6 ^{ab}	144.6±3.2 ^{ab}
Diabetic + AREt (400 mg/Kg)	150.2±6.9	137.8±5.8 ^a	144.8±3.9 ^{ab}	147.1±4.4 ^{ab}	149.1±4.6 ^{ab}
Diabetic + Glibenclamide (600µg/Kg)	145.8±2.9	137.1±2.7 ^a	141.4±2.7 ^{ab}	147.4±2.6 ^{ab}	150.1±3.8 ^{ab}

The values indicate mean ±S.E.M (n=6).^a p<0.05 compared with normal control values, ^b p<0.05 compared with the diabetic control group and ^c p<0.05 compared with the initial weight (0 day) of the rats in the respective group.

4.4. Effect of *A. remota* leaves extract on serum lipid profile in diabetic rats

The level of serum lipid profile of experimental rats is described in Table 4.3. Serum total cholesterol, triacylglycerol and LDL-cholesterol (LDL-C) increased significantly in diabetic control rats as compared with the normal control rats. On the other hand, the level of HDL-cholesterol (HDL-C) decreased significantly in diabetic control rats as compared to the normal control rats. Serum TC, TG and LDL-C were significantly reduced whereas HDL-C

was significantly increased with AREt treatment at dose of 200 mg/Kg b.w. and 400 mg/Kg b.w. as compared to the diabetic rats. 400 mg/Kg b.w. dose AREt treatment for 28 days reversed the aforementioned values near normal. The effect was comparable with 600 µg/Kg b.w. glibenclamide with the same period of treatment. A dose dependent reduction in the levels of TC, TG, LDL-C and increase of HDL-C was observed with both doses of AREt treatment.

Table 4. 3: Effect of *A. remota* leaves extract on lipid profile of STZ induced diabetic rats after 28 days treatment.

Groups	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)
Normal control	86.0±1.3	79.5±1.0	28.3±0.7	41.77±1.7
Diabetic control	191.2±6.3 ^a	163.50±3.3 ^a	18.8±0.7 ^a	139.63±6.2 ^a
Diabetic + AREt (200 mg/Kg)	129.5±2.1 ^{ab}	95.67±3.7 ^{ab}	25.2±0.4 ^{ab}	85.20±2.3 ^{ab}
Diabetic + AREt (400 mg/Kg)	98.2±1.5 ^b	80.00±2.1 ^b	29.77±0.5 ^b	52.50±1.6 ^b
Diabetes + Glibenclamide (600 µg/Kg)	88.7±2.1 ^b	69.67±2.9 ^b	35.5±0.9 ^{ab}	39.23±2.5 ^b

The values indicate mean ±S.E.M (n=6). ^a p<0.05 compared with normal control values and ^b p<0.05 compared with diabetic control values.

4.5. Effect of *A. remota* leaves extract on serum enzymes activity in diabetic rats

The activity of serum alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase were significantly elevated in diabetic control rats compared to normal controls. Administration of AREt at 200 mg/Kg b.w. and 400 mg/Kg b.w. doses for 28 days significantly reduced serum enzymes activity (ALT, AST and ALP) in diabetic rats as compared to diabetic control group. Similar effect was also observed with 600 µg/Kg glibenclamide treatment (Table 4.4).

4.6. Effect of *A. remota* leaves extract on serum urea, creatinine and total protein in diabetic rats

Table 4.5 illustrates that serum urea and creatinine levels were significantly elevated in diabetic control rats as compared with the normal control rats. Whereas, a decrease in protein levels was found in diabetic control rats compared with normal control rats. The

administration of AREt at dose 200 mg/Kg, 400 mg/Kg and 600 µg/Kg glibenclamide significantly reduced the serum urea and creatinine level as compared to the diabetic control rats. On the other hand, the level of serum total protein significantly increased as compared with diabetic control rats after 28 days of treatment.

Table 4. 4: Effect of A. remota leaves extract on serum ALT, AST and ALP of STZ induced diabetic rats after 28 days treatment.

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)
Normal control	30.0±1.1	55.8±1.35	51.833±0.8
Diabetic control	72.0±1.3 ^a	94.8±3.5 ^a	87.33±0.84 ^a
Diabetic + AREt (200 mg/Kg)	54.2±1.0 ^{ab}	78.0±4.4 ^{ab}	80.00±2.5 ^{ab}
Diabetic + AREt (400 mg/Kg)	47.7±2.4 ^{ab}	75.0±3.1 ^{ab}	72.83±1.0 ^{ab}
Diabetic + glibenclamide (600 µg/Kg)	42.8±1.6 ^{ab}	71.5±5.5 ^b	65.83±1.2 ^{ab}

The values indicate mean ±S.E.M (n=6). ^a p<0.05 compared with normal control values and ^b p<0.05 compared with diabetic control values.

Table 4. 5: Effect of A. remota leaves extract on serum urea, creatinine and total protein of STZ induced diabetic rats after 28 days treatment.

Groups	Urea (mg/dL)	Creatinine (mg/dL)	Total protein (mg/dL)
Normal control	38.2±0.6	0.8±0.03	5.8±0.1
Diabetic control	80.0±2.5 ^a	1.7±0.2 ^a	4.7±0.2 ^a
Diabetic + AREt (200 mg/Kg)	59.3±1.7 ^{ab}	1.3±0.1 ^{ab}	5.7±0.1 ^b
Diabetic + AREt (400 mg/Kg)	52.5±1.3 ^{ab}	1.1±0.1 ^b	6.0±0.1 ^b
Diabetic + glibenclamide (600 µg/Kg)	49.2±1.8 ^{ab}	1.0±0.0 ^b	5.9±0.1 ^b

The values indicate mean ±S.E.M (n=6). ^a p<0.05 compared with normal control values and ^b p<0.05 compared with diabetic control values.

5. DISCUSSION

The present study was designed to investigate the antidiabetic activity of ethanol extract of *A. remota* leaves in streptozotocin induced diabetic rats.

Streptozotocin (2-Deoxy-2-[[[(methylnitrosoamino)-carbonyl] amino]-D-glucopyranose) has been used to induce diabetes mellitus in experimental rats (Latha and Daisy, 2011). A Single Intraperitoneal (IP) administration of 60 mg/Kg streptozotocin effectively induced diabetes mellitus in rats, which was confirmed by elevated level of fasting blood glucose obtained from the tail of the rats after 72 hours of injection.

The mechanism by which streptozotocin brings about diabetic mellitus includes selective destruction of insulin secreting pancreatic β -cells and lead to poor glucose uptake by peripheral tissues (Szkudelski, 2001). Crude ethanolic extract of *A. remota* leaves reduced high fasting blood glucose level in streptozotocin induced diabetic rats. The biochemical mechanism of actions of *A. remota* extract might be due to an insulin mimetic effect on muscle and adipose tissues by either stimulating glucose uptake and metabolism (Daisy *et al.*, 2010 ; Chadwick *et al.*, 2007), by inhibiting hepatic gluconeogenesis (Im *et al.*, 2009 ; Cetto and Vázquez, 2010) and glycogenolysis (Rawi *et al.*, 2011), by stimulation of regeneration process or increase pancreatic secretion of insulin from existing β -cells (Soleimani *et al.*, 2007 ; Sharma *et al.*, 2008) and/ or inhibition activity against α -glucosidase enzymes in small intestine which convert disaccharides into monosaccharides for sake of absorption (Arai *et al.*, 1999 ; Shinde *et al.*, 2008).

Maximum percent fall of fasting blood glucose was found at the dose of 400 mg/Kg b.w (39.6%) as compared to 200 mg/Kg (28.7%) on day 28. The result was comparable with the standard drug glibenclamide 600 μ g/Kg which reduced fasting blood glucose level by 47.3% on same day. Moreover, significant fasting blood glucose reduction in diabetic rats treated with 400 mg/kg and 200 mg/Kg was observed on day 14th and 21st compared to diabetic control rats, respectively. The difference between the two AREt doses might be attributed to the former contains higher concentration of the active component(s) responsible for more fall of fasting blood glucose than the later.

It was interesting that similar fasting blood glucose lowering effect was observed by glibenclamide known to produce its effect via selective blockage of adenosine triphosphate (ATP) sensitive K^+ (K_{ATP}) channels in the plasma membrane. This leads to membrane depolarization, activate voltage gated Ca^{2+} channels, a rise in cytosolic (Ca^{2+}) and release of endogenous insulin in β -cells of the pancreas (Tomai *et al.*, 1994), this suggest that streptozotocin at 60 mg/Kg i.p. might not sufficient for complete destruction of β -cells and/ or few cells remained to have capability to regenerate and secret insulin.

Fasting blood glucose lowering effect of *A. remota* was similar to *Ajuga iva*, which belongs in the same family, were found to have hypoglycemic effect in streptozocin induced diabetic rats (Hilaly and Lyoussi, 2002). Phytochemical investigations on *A. remota* have reported the presence of bioactive compounds such as iridoid and flavonol glycosides and phytoecdysteroids (Manguro *et al.*, 2006 ; Kubo *et al.*, 1983). It has been suggested that antihyperglycemic effect of AREt extract probably attributed to these constituents through improvement of insulin level (Sundaram *et al.*, 2012a ;Katsube *et al.*, 2010 ; Sundaram *et al.*, 2012b).

The loss of body weight in untreated diabetic rats is due to increased muscle wasting (Flatt *et al.*, 1990) and catabolism of tissue proteins (Ravi *et al.*, 2004). However, *A. remota* leaves ethanol extract treatment improved the body weight in diabetic rats. The ability of AREt to protect massive body weight loss seems to be due to its ability to reduce hyperglycemia which was an indication of proper glucose utilization (Pari and Satheesh, 2004), its protective effect in muscle wasting and controlling protein turn over and/or improvement in diabetes mellitus associated disorders (Oyedemi *et al.*, 2012 ; Saeed *et al.*, 2008). The result revealed that AREt treated groups didn't show significant body weight gain on day 28 from the initial (0) day, this could support that *A. remota* leaves to be a potential strong candidate for treatment of diabetes mellitus over synthetic drugs which are mostly known to cause body weight gain in diabetes mellitus treatment (Prabhakar and Doble, 2011).

Abnormalities in lipid profile are common complications in diabetes mellitus (Gibbons, 1986). Such abnormality represents the risk factors for coronary heart diseases (Shamaony *et al.*, 1994). Activation of hormone sensitive lipase during insulin deficiency causes an increase in free fatty acid mobilization from adipose tissue (Mooradian, 2009). In addition,

hyperglycemia is accompanied by a rise in TC, TG, LDL-C and a fall in HDL-C (Gao *et al.*, 2009) . In the present study, serum total cholesterol, triglycerides and LDL-C levels was decreased and at the same time HDL-C was increased in *A. remota* extracts treated diabetic rats.

The remarkable control of high serum triacylglycerol in ethanolic *A. remota* leaves extract treated diabetic rats could be due to inhibition of endogenous TG synthesis in liver (Xie *et al.*, 2007) or improvement in insulin level or the presence of active component(s) in AREt that suppressed the activity of hormone sensitive lipase in adipose tissue or increased activity of hepatic lipase or lipoprotein lipase accountable for the hydrolysis of excess lipoprotein bound triacylglycerol into fatty acids (Pritchard *et al.*, 1986 ; Mbaka *et al.*, 2012).

Increased level of HDL-C in *A. remota* leaves extract treated groups could be due to the enhancement of lecithin: cholesterol acyltransferase (LCAT) which plays a key role in incorporating the free cholesterol in to HDL which take back to the liver (Ghatak and Panchal, 2012 ; Senoucia *et al.*, 2012). LDL-C reducing effect of AREt presumably attributed to increased expression of low density lipoprotein receptor (LDLR), which enhance LDL particles uptake in liver from the circulation, through the depletion of intracellular cholesterol (Chonga *et al.*, 2011 ; Bursill *et al.*, 2007).

Serum total cholesterol lowering property of *A. remota* leaves extract could be attributed to the presence of hypocholesterolemic compounds in AREt that may act as inhibitor for hepatic hydroxyl methyl glutaryl CoA (HMG CoA) reductase in liver, which take part in cholesterol synthesis (Kumarappan *et al.*, 2007) or increasing the fecal content by inhibiting the absorption of cholesterol from intestine (Raederstorff *et al.*, 2003). Isolated phytoecdysteroids and iridoid glycosides, phytochemical constituent of *A. remota* extract, from other *Ajuga* species have shown antioxidant activity (Mamadaliyeva *et al.*, 2013 ; Fan *et al.*, 2011). Thus, reduction of total cholesterol in AREt extract treated diabetic might also attributed to the aforementioned phytochemical constituents by reducing lipid peroxidation via scavenging free radicals (Sharma *et al.*, 2008).

The decrease in serum total cholesterol, triacylglycerol, and LDL-C and an increase in HDL-C after 28 days treatment showed a dose dependent trend, indicating that efficacy was

proportional to the dose of *A. remota* leaves extract. Similar hypocholesterolemia and hypotriacylglycerol effects were observed by *Ajuga iva* whole plant extract in streptozotocin induced diabetic rats (Hilaly *et al.*, 2007).

In general, 400 mg/Kg b.w. dose was capable to reverse the values of TC, TG, HDL-C and LDL-C near normal over 200 mg/Kg after 28 days of treatment; this could be due to dose dependent effect of the AREt extract.

The activity of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase in serum are generally indicators for liver function. In diabetic rats, the level of these enzymes are elevated due to the necrosis of liver cells by the injection of STZ (Hwang *et al.*, 1996). However, AREt treated diabetic rats showed decreased in the activity of ALT, AST and ALP enzymes that might support its hepatoprotective effect and normalization capability of impaired liver metabolism in diabetic rats (Sharma *et al.*, 2011).

There is increased protein catabolism with the flow of amino acids into the liver, which feeds gluconeogenesis as a result of insulin deficiency during uncontrolled diabetes mellitus (Senthilkumar and Subramanian, 2008). This account for the decrease in serum total protein content in STZ induced diabetic control rats (Narendhirakannan *et al.*, 2006). The results of the present study demonstrated that the treatment of diabetic rats with *A. remota* leaves extract caused a significant increase in serum total protein which might be attributed to an improvement in glycemic control and insulin secretion that increase protein synthesis or decrease protein degradation (Gao *et al.*, 2009).

Negative nitrogen balance is manifested in diabetic rats associated with enhanced proteolysis in muscle and other tissues. Impaired balance of nitrogen coupled with lowered protein synthesis leads to increased concentrations of urea and creatinine in serum (Basha and Subramanian, 2011) indicates progressive renal damage in diabetic rats (Anjaneyulu and Chopra, 2004). Treatment with *A. remota* leaves extract resulted in a considerable reduction to near normal in serum urea and creatinine level indicating the renoprotective role of *A. remota* leaves extract or delay diabetic nephropathy development.

Phytoecdysteroids rich extract from *Ajuga iva* revealed to ameliorate diabetic provoked hepatic and renal toxicity (Hamden *et al.*, 2008). It supports hepatoprotective and

renoprotective effect of *A. remota* leaves extract might be due to these compounds, possibly through their antioxidant activity (Krishnan *et al.*, 2007).

6. CONCLUSION

In the present study, administration of ethanol extract of *A. remota* leaves to STZ induced diabetic rats have prominent reduction in fasting blood glucose level, almost normalization of serum biochemical parameters including lipid profile: total cholesterol, triacylglycerol, high density lipoprotein cholesterol and low density lipoprotein cholesterol; serum enzymes: alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase; serum urea, creatinine and total protein compared to STZ induced diabetic control rats by the mechanism (s) which appear to be similar to that of glibenclamide, which involve insulin sensitization effect.

Therefore, it can be concluded that, especially 400 mg/Kg, the AREt is remarkably effective against streptozocin induced diabetic adult male wistar albino rats thereby validating its ethnomedicinal usage. From the observed antidiabetic activity of *A. remota* leaves extract in STZ induced diabetic rats, the study may supports the use of *A. remota* herb for management of diabetes mellitus by traditional healer.

7. RECOMMENDATION

This study demonstrated that the traditional medicinal plant *A. remota* leaves extract possesses an antidiabetic activity in experimental rats. More researches are needed to establish a solid foundation to this finding and understand the mechanism (s) of action of the extract. Further fractionation should be done to identify the active component(s) responsible for the antidiabetic activity of *A. remota* leaves extract. In addition, researches have to be done on the other parts of the *A. remota*.

Blood and serum biochemical parameters such as glycated hemoglobin, insulin level and histopathology of pancreas, liver and kidney organ have to be done to make the study more complete.

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