# Antidiabetic activity and phytochemical screening of crude extracts of *Stevia rebaudiana* Bertoni and *Ajuga remota* Benth grown in Ethiopia on alloxan-induced diabetic mice

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**List of Abbreviations**

| | |
|---|---|
| AGI | α-glucosidase inhibitor |
| ANOVA | Analysis of Variance |
| EHNRI | Ethiopian Health and Nutrition Research Institute |
| FFA | Free Fatty Acid |
| Glu. | β-D-Glucopiranosil |
| GLP 1-5 | Glucagon-Like Peptide 1-5 |
| HPLC | High Performance Liquid Chromatography |
| IDDM | Insulin Dependent Diabetes Mellitus |
| OGGT | Oral Glucose Tolerance Test |
| $LD_{50}$ | Medium lethal dose |
| NDFS | National Diabetes Fact Sheet |
| NIDDM | Non-Insulin Dependent Diabetes Mellitus |
| Rham | α -L-Rhamnopiranosil |
| SPSS | Statistical Package for the Social Sciences |
| TZD | Thiazolidinedione |
| UV | Ultra violate light |
| WHO | World Health Organization |

**Abstract**

Diabetes mellitus is a major public health problem in the world. The prevalence of diabetes mellitus is increasing with ageing of the population and lifestyle changes associated with rapid urbanization and westernization. According to the World Health Organization estimate 3% of the world's populations (194 million) have diabetes and is expected to double (6.3%) by the year 2025 and that of Ethiopia was about 800,000 cases in 2000, and the number is expected to increase to 1.8 million by 2030.

*Stevia rebaudiana* Bertoni (Asteraceae) and *Ajuga remota* Benth (Lamiaceae) has been used for the treatment of diabetes mellitus and various other ailments in the traditional medical system world wide. This study reports the antidiabetic activity of crude extracts of S*tevia rebaudiana B*ertoni & A*juga remota* Benth grown in Ethiopia on alloxan-induced diabetic mice

A preliminary toxicity study of *S. rebaudiana* and *A. remota* crude extracts was done using Swiss albino mice in oral doses of 1000, 2000 and 5000 mg/kg body weight. The result showed that the medium lethal dose ($LD_{50}$) of the extracts is higher than 5000 mg/kg body weight and hence, in a single dose administration, the plant extracts had no adverse effect. The result obtained is in agreement with that of Geuns (2003) for *S. rebaudiana* and Worku (2005) for *A. remota*.

The preliminary phytochemical screening indicated the presence of flavonoids, saponins, tannins and steroids in both plants and alkaloids in the leaves of *S. rebaudiana*. Extracts of both plants did not contain anthraquinones. The result obtained here is in agreement with that of Anonymous-a (1999) for *S. rebaudiana* and Worku (2005) for *A. remota*.

A concentration of 2.27 % stevioside was obtained in the leaves of *S. rebaudiana*.

The antihyperglycemic activity of the crude aqueous and ethanol extracts of *S. rebaudiana* and *A. remota* were studied in alloxan-induced (200 mg/kg body weight, ip.) diabetic mice, after oral administration at a dose of 300 and 500 mg/kg body weight for two weeks. Administration of crude aqueous extract of *S. rebaudiana* for 14 days lowered elevated blood glucose levels by 30.27% (300 mg/kg) and 42.65% (500 mg/kg) that shows *S. rebaudiana* has retained its antidiabetic activity when

grown out of its natural habitat, i.e. in Ethiopia. In the case of *A. remota*, blood glucose reduction was 27.96% and 38.98% respectively for the dose levels tested. Crude ethanol extract of *S. rebaudiana* and *A. remota* (300 and 500 mg/kg body weight for 14 days) lowered blood glucose level by 28.71% & 33.04% and 28.09% & 28.25% respectively as compared with diabetic untreated mice. Treatment with the known antidiabetic drug, glibenclamide (10 mg/kg body weight) lowered blood glucose level by 51.06%. The effect was more pronounced in the case of the crude aqueous extract as compared with the crude ethanol extract on the respective dose limits for both plant cases.

**Key words**: Diabetes mellitus, *Stevia rebaudiana*, *Ajuga remota*, Stevioside
Antidiabetic activities, Blood glucose level

# 1. INTRODUCTION

## 1.1. Diabetes mellitus

Diabetes mellitus is a metabolic disorder initially characterized by a loss of glucose homeostasis with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both (Barcelo and Rajpathak,2001).

Without enough insulin, the cells of the body cannot absorb sufficient glucose from the blood; hence blood glucose levels increase, which is termed as hyperglycemia. If the glucose level in the blood remains high over a long period of time, this can result in long-term damage to organs, such as the kidneys, liver, eyes, nerves, heart and blood vessels. Complications in some of these organs can lead to death (Pari and Saravanan, 2004).

### 1.1.1. Pathophysiology of diabetes mellitus

The pancreas plays a primary role in the metabolism of glucose by secreting the hormones insulin and glucagon (Figure 1). The islets of Langerhans secrete insulin and glucagon directly into the blood. Insulin is a protein that is essential for proper regulation of glucose and for maintenance of proper blood glucose levels (Worthley, 2003).

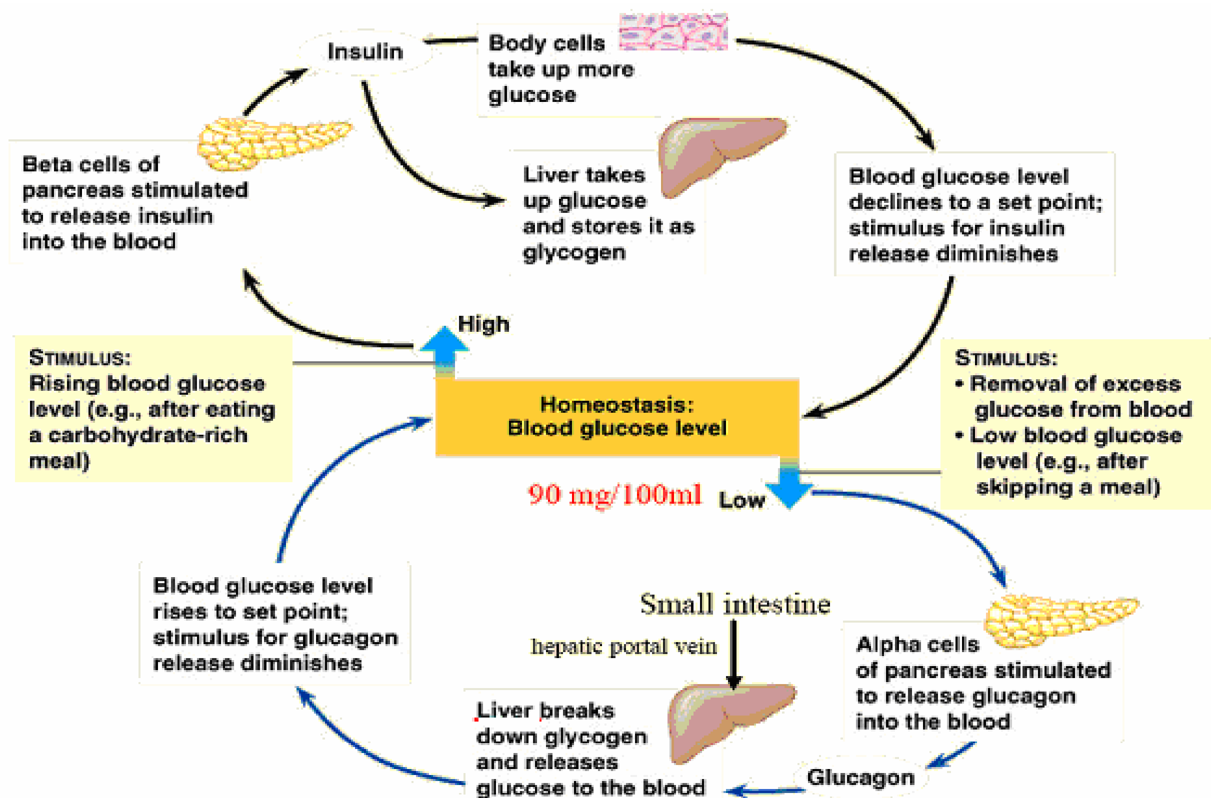



**Figure 1:** The role of pancreas in the body

Glucagon is a hormone that opposes the action of insulin. It is secreted when blood glucose level falls. It increases blood glucose concentration partly by breaking down stored glycogen in the liver by a pathway known as glycogenolysis. Gluconeogenesis is the production of glucose in the liver from non-carbohydrate precursors such as glycogenic amino acids (Sowka *et al.*, 2001).

### 1.1.2. Types of diabetes mellitus

WHO classification of diabetes introduced in 1980 and revised in 1985 was based on clinical characteristics. The two most common types of diabetes were insulin-dependent diabetes mellitus (IDDM) or (type I) and non-insulin-dependent diabetes mellitus (NIDDM) or (type II). WHO classification also recognized malnutrition-related diabetes mellitus and gestational diabetes. Malnutrition-related diabetes was omitted from the new classification because its etiology is uncertain, and it is unclear whether it is a separate type of diabetes (Holt, 2004; Tiwari and Rao, 2002).

#### 1.1.2.1. Type I diabetes mellitus

It is a result of cellular mediated autoimmune destruction of the insulin secreting β-cells of the pancreas, which results in an absolute deficiency of insulin for the body. Patients are more prone to ketoacidosis. It occurs in children and young, usually before 40 years of age, although disease onset can occur at any age. The patient with type I diabetes must rely on insulin medication for survival. It may account for 5 -10 % of all diagnosed cases of diabetes. Autoimmune, genetic and environmental factors are the major risk factors for type I diabetes (NDFS, 2005; Abebe *et al.*, 2003; Cavallerano and Cooppan, 2002).

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

#### 1.1.2.2. Type II diabetes mellitus

Two key features in the pathogenesis of type II diabetes mellitus are a decreased ability of insulin to stimulate glucose uptake in peripheral tissues, insulin resistance, and the inability of the pancreatic β-cell to secrete insulin adequately, β-cell failure. The major sites of insulin resistance in type 2 diabetes are the liver, skeletal muscle and adipose tissue (White *et al.*, 2003; Ostenson, 2001).

Both defects, insulin resistance and β-cell failure, are caused by a combination of genetic and environmental factors. Environmental factors such as lifestyle habits (i.e., physical inactivity and poor dietary intake), obesity and toxins may act as initiating factors or progression factors for type II diabetes. The genetic factors are still poorly understood (Holt, 2004; Lindstrom *et al.*, 2003; Uusitupa, 2002).

Type II diabetes is increasingly being diagnosed at any age nowadays and it accounts for 90-95% of all diagnosed cases of diabetes. It is associated with old age, obesity,

family history of diabetes, impaired glucose metabolism, physical inactivity, and race /ethnicity (NDFS, 2005; Holt, 2004; Li *et al*, 2004).

#### 1.1.2.3. Gestational diabetes mellitus

Gestational diabetes, blood glucose elevation during pregnancy, is a significant disorder of carbohydrate metabolism due to hormonal change during pregnancy, which can lead to elevated blood glucose in genetically predisposed individuals. It is more common among obese women and women with a family history of diabetes. It usually resolves once the baby is born, however, after pregnancy, 5-10% of women with gestational diabetes are found to have type II diabetes and 20-50% of women have a chance of developing diabetes in the next 5-10 years (NDFS, 2005; Worthley, 2003).

### 1.1.3. Epidemiology of diabetes mellitus

The prevalence of diabetes mellitus is increasing with ageing of the population and lifestyle changes associated with rapid urbanization and westernization. The disease is found in all parts of the world and is rapidly increasing in its coverage (Kamalakkanan and Prince, 2003; Sobngwi *et al.*, 2001).

#### 1.1.3.1. Prevalence and incidence of diabetes mellitus

Globally, the prevalence of diabetes (Table 1), without type distinction, was estimated to be 4% in 1995. According to WHO, it is estimated that 3% of the world's population have diabetes and the prevalence is expected to double by the year 2025 to 6.3% (Andrade-Cetto and Heinrich, 2005; Attele *et al.*, 2002). There will be a 42% increase from 51 to 72 million in the developed countries and 170% increase from 84 to 228 million, in the developing countries. Thus, by the year 2025, over 75% of all people with diabetes will be in the developing countries, as compared to 62% in 1995 (Ramachandran, *et al.*, 2002).

The reasons behind this projected increase in prevalence rate are due to urbanization, westernization and their associated lifestyle changes, increase in life expectancy at birth, physical inactivity and obesity and possibly a genetic predisposition (Wild *et al.*, 2004; Sobngwi, *et al.*, 2001). Age, ethnic, regional and racial differences have also been

found to play a role for the diabetic incidence in heterogeneous populations within the same area (Alberti *et al.*, 2007; NDFS, 2005).

**Table 1:** Worldwide prevalence of diabetes (Holt, 2004)

| | Prevalence (n x $10^6$) | | Increase (%) |
|---|---|---|---|
| | **2003** | **2025 (Predicted)** | |
| **Africa** | 13.6 | 26.9 | 98 |
| **Asia** | 81.8 | 156.1 | 91 |
| **Australia** | 1.1 | 1.7 | 59 |
| **Europe** | 38.2 | 44.2 | 16 |
| **Middle East** | 18.2 | 35.9 | 97 |
| **North America** | 25.0 | 39.7 | 59 |
| **South America** | 10.4 | 19.7 | 88 |
| **Total cases worldwide** | 189 | 324 | 72 |

**Diabetes in Ethiopia**

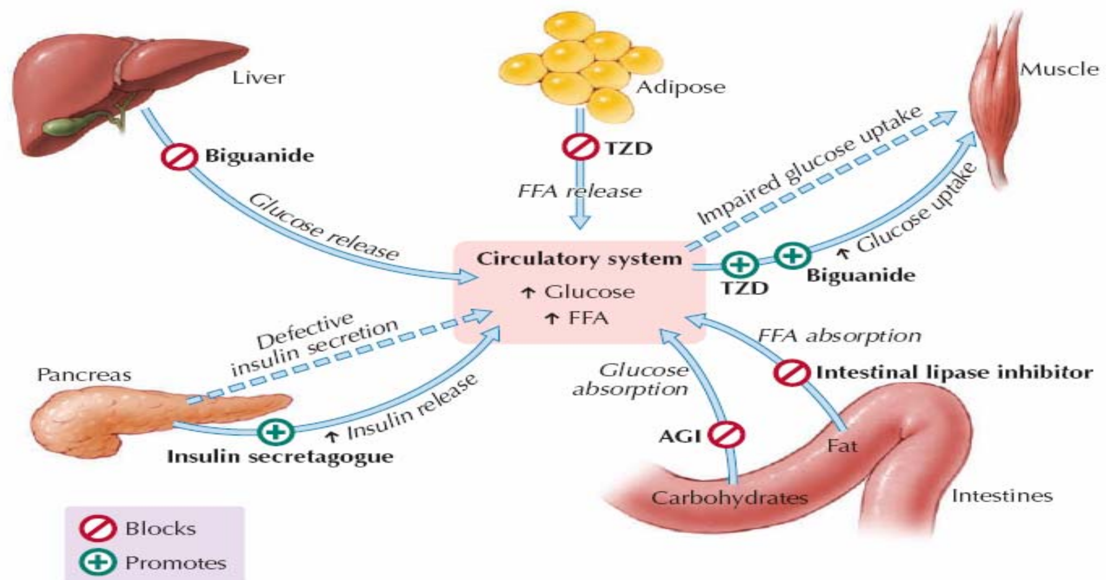
In Ethiopia no population based prevalence study exist but hospital based studies show that the prevalence of diabetes admission has increased from 1.9% in 1970 to 9.5% in 1999 of all medical admissions. It accounts for about 7% of all deaths over the age of 55 years in the medical wards of referral hospitals. According to WHO estimate, the number of diabetic cases in Ethiopia in 2000 were 800,000 and is expected to increase to 1.8 million by 2030 (Feleke and Enquselassie, 2005; Abebe *et al.*, 2003).

### 1.1.3.2. Risk factors

The predisposing factors associated with diabetes mellitus include modifiable and non-modifiable factors. Among the modifiable risk factors, residence seems a major determinant, since urban residents have a 1.5 to 4 fold higher prevalence of diabetes compared to their rural counterparts. This is attributable to lifestyle changes associated with urbanization and westernization, diet, obesity and physical inactivity. Age, ethnicity, history of gestational diabetes and family history of type II diabetes are the main non-modifiable determinants of diabetes prevalence (Libman and Arslanian, 2007; Colagiuri *et al.*, 2006).

### 1.1.4. Management of diabetes mellitus

Diet, exercise, modern drugs including insulin and oral administration of hypoglycaemic drugs such as sulfonylureas and biguanides manage the pathogenesis of diabetes mellitus. Insulin plays a key role in glucose homeostasis along the side of a counter regulatory hormone, glucagon, which raises serum glucose. Carrier proteins (GLUT 1-5) are essential for glucose uptake into cells. In individuals with type II diabetes, a common sequence of therapy starts with diet treatment and exercise followed by oral antihyperglycemic agents. In general, insulin therapy has been considered to be the last therapeutical option when diet, exercise and oral antihyperglycemic agent therapies have failed. Oral agents acting as indicated in Figure 2 are used in type II diabetic patients who fail to meet glycemic goals with medical nutrition therapy and exercise. Traditionally plants are also used for the treatment of diabetes throughout the world (Koski, 2006; Pari and Saravanan, 2004). Management of diabetes without any side effect is still a challenge for the medical system. This leads to an increasing search for improved antidiabetic drugs.




TZD = thiazolidinedione; FFA = free fatty acid; AGI = α-glucosidase inhibitor

**Figure 2:** Major target organs and mechanism of actions of orally administered antihyperglycemic agents in type II diabetes mellitus (Cheng and Fantus, 2005)

### 1.2. Medicinal plants

#### 1.2.1. The importance of medicinal plants & traditional medicines

Medicinal plants, since time immemorial, have been used in virtually all cultures as a source of medicine. It has been estimated that about 80-85% of population both in developed and developing countries rely on traditional medicine for their primarily health care needs and it is assumed that a major part of traditional therapy involves the use of plant extracts or their active principles (Ignacimuthu *et al*., 2006; Elujoba *et al*., 2005; Tomlinson and Akerele, 1998). Due to lack of organized health care systems in developing countries like Ethiopia, people with chronic diseases like diabetes are among the worst suffers in their communities today. Hence, majority of the populations still have limited access or no access, especially those in remote areas, to modern medicines. Instead they use traditional medicines for a range of diabetic complications (Kochhar and Nagi, 2005; Zibula and Ojewole, 2000).

The active principles of many plant species are isolated for direct use as drugs, lead compounds or pharmacological agents. Different species of medicinal plants are used in the treatment of diabetes mellitus. For diabetes treatment, before the discovery of insulin by Banting and Best in 1922, the only options were those based on traditional practices (Ribnicky *et al*., 2006). Till today metformin is the only ethical drug approved for the treatment of non insulin dependent diabetes mellitus patients, which is derived from a medicinal plant *Galega officinalis* (Eshrat, 2002, Oubre *et al*., 1997). Among those plants used traditionally for the treatment of diabetic complications are *Stevia rebaudiana* Bertoni (Gregersen *et al*., 2004; Li *et al*., 2004) and *Ajuga remota* Benth (Abebe *et al*., 2003). Hence, it is essential to study the antidiabetic effect of *Ajuga remota* and whether *S. rebaudiana* retained the antidiabetic activity as it grows in Ethiopia, out of its natural habitat.

#### 1.2.2. The Asteraceae (Compositae) family

Asteraceae is one of the large families containing about one-tenth of the total number of flowering plants, comprising about 950 genera and more than 20,000 recognized

species and characterized by small flowers arranged in a head looking like a single flower (Rendle, 1979).

#### 1.2.2.1. The Genus Stevia

*Stevia*, one of the 950 genera of the Asteraceae family is a genus of more than 200 species. Members of *Stevia* comprise mostly of herbs but also shrubs and trees. Originally it is said to be native to subtropical South America (Paraguay & Brazil) and Central America but now is found over a wide range of areas 500-3500 m altitude, 1,500 – 1,800 mm rain fall and -6 $^{o}$C to +43 $^{o}$C temperatures (De Oliveira *et al*., 2004; Midmore and Rank, 2002; Yao *et al*., 1999).

##### 1.2.2.1.1. *Stevia rebaudiana* Bertoni

*S. rebaudiana* (Figure 3) usually grow in semi-dry mountainous terrains, their habitats range from grasslands, forested mountain slopes, conifer forests, to sub-alpine vegetation. It is an herb of 80 - 180 cm tall with a life span of 3 - 5 years. It grows best in soil that is well drained but with reasonable water holding capacity and preferably with pH 5 - 7; alkaline soil should be avoided (Uddin *et al*., 2006; Midmore and Rank, 2002).

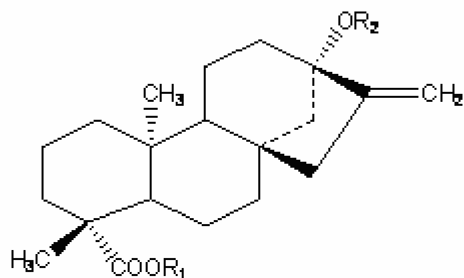
For centuries, the Guarani Indian's in Paraguay and Brazil used *Stevia* species, Primarily *S. rebaudiana*, as a non-calorie sweetener (250-300 times sweeter than sucrose at 0.4% solution) in medicinal green teas for treating heart burn and other ailments (Vanek *et al*., 2001). Although there are more than 200 species of the genus *Stevia*, only *S. rebaudiana* gives the sweetest essence (Savita *et al*., 2004).

**Figure 3**: Picture of *Stevia rebaudiana*

**1.2.2.1.2. Chemical constituents of *Stevia rebaudiana* Bertoni**

In 1931 two French chemists, named Bridel and Lavieille, isolated the glycosides that give *S. rebaudiana* its sweet taste for the first time from the leaves. These glycosides were named stevioside and rebaudioside. Glycosides are organic compounds, which contain a sugar component (glycone) and a non-sugar component (aglycone).

The leaves of *S. rebaudiana* contain diterpene glycosides (Figure 4) namely, stevioside (**1**), steviolbioside (**2**), rebaudioside A-F (**3-8**) and ducloside A (**9**), which are responsible for the typical sweet taste (Mantovaneli *et al*., 2004). Labdane diterpene (E.g. sterebins I-N), triterpenes, sterols and flavonoids are some of the non-sweet secondary metabolites that have also been identified from the leaves of *Stevia rebaudiana* (McGarvey *et al*., 2003; Anonymous-a, 1999).




| Compounds Name | $R_1$ | $R_2$ |
|---|---|---|
| Stevioside | β-Glc | β-Glc-β-Glc(2→1) |
| Steviolbioside | H | β-Glc-β-Glc(2→1) |
| Rebaudioside A | β-Glc | β-Glc-β-Glc(2→1)<br>\|<br>β-Glc(3→1) |
| Rebaudioside B | H | β-Glc-β-Glc(2→1)<br>\|<br>β-Glc(3→1) |
| Rebaudioside C | β-Glc | β-Glc-α-Rha(2→1)<br>\|<br>β-Glc(3→1) |
| Rebaudioside D | β-Glc-β-Glc(2→1) | β-Glc-β-Glc(2→1)<br>\|<br>β-Glc(3→1) |
| Rebaudioside E | β-Glc-β-Glc(2→1) | β-Glc-β-Glc(2→1) |
| Rebaudioside F | β-Glc | β-Glc-β-Xyl(2→1)<br>\|<br>β-Glc(3→1) |
| Ducloside A | β-Glc | β-Glc-α-Rha(2→1) |

**Figure 4**: Structures of diterpene glycosides

#### 1.2.2.1.3. Pharmacological activities of *Stevia rebaudiana* Bertoni

*S. rebaudiana* extracts have been suggested to exert beneficial effects on human health, including antihypertensive, antimicrobial, antiobesity and antioxidant activities and also thought to influence glucose metabolism & renal functions (Savita *et al.*, 2004; Jeppesen *et al.*, 2003). *S. rebaudiana* does not lower blood glucose levels in normal subjects (Ahmed and Smith, 2002).

### 1.2.3. The Lamiaceae family (Mint family)

The Lamiaceae is one of the largest and most distinctive families of flowering plants. It is comprised of about 4000 species distributed over 220 genera world wide. The family has a cosmopolitan distribution but well represented in tropical and temperate regions, especially in those with a seasonal climate (Naghibi *et al.*, 2005).

The leaves emerge oppositely, each pair at right angels to the previous one and stems are usually square. In Ethiopia the Lamiaceae family is represented by 41 genera comprising of annual or perennial herbs or shrubs (Burger, 1985).

#### 1.2.3.1. The Genus Ajuga

*Ajuga* is a genus of about 40-50 species of annual or perennial herbaceous flowering plants in the Mint (Lamiaceae) family, with most species native to Europe, Asia and Africa, but also some species in southeastern Australia. They grow 5-10 cm tall, and are with opposite leaves (Hedberg *et al.*, 2006; De Clavijo, 1997).

##### 1.2.3.1.1. Traditional uses of *Ajuga remota* Benth (syn. *Ajuga integrifolia* Ham-Buch.)

*A. remota* (Figure 5) is a shrub growing widely in East Africa, at an altitude of 1500-3400 m above sea level, Saudi Arabia, Yemen and Afghanistan to East Asia. In Ethiopia it grows in different regions including Bale, Gojam, Gondar, Harerge, Kefa, Shoa, Sidamo, Tigray and Wollo (Hedberg *et al.*, 2006; Coll and Tandrón, 2005).

*A. remota* is locally known as 'Harmagussa' (Ormigna), 'Etse-Libawit' (Ge'ez), 'Akembiye' (Guaragegna), 'Tale' (Welaytigna), and 'Akorarach' (Amharic). It has a very bitter taste, leaves moderately to densely hairy, grayish green, simple, and flowers pale blue, pale violet, light blue or white (Hedberg *et al.*, 2006; Abebe *et al.*, 2003; Abebe and Ayehu, 1993).

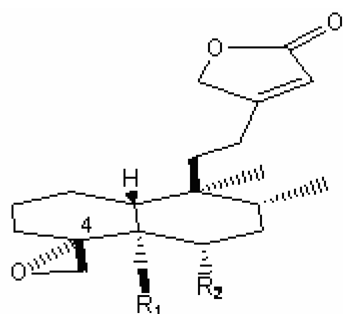
In traditional health system of Ethiopia, the aqueous and sometimes "Arekie" (alcohol) infusion of the fresh or dried leaves of *A. remota* is used for the treatment of various diseases including diabetes, malaria, pain and fevers, toothache, skin disease, hypertension, stomachache, pneumonia, liver problem and swelling of legs (Coll and

Tandrón, 2005; Abebe *et al*., 2003; Kuria *et al*., 2002). Sometimes honey is added in to the preparation to make it palatable, since it has a bitter taste, and to store for longer periods for later use.

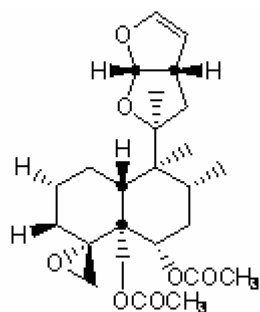
**Figure 5**: Picture of *Ajuga remota*

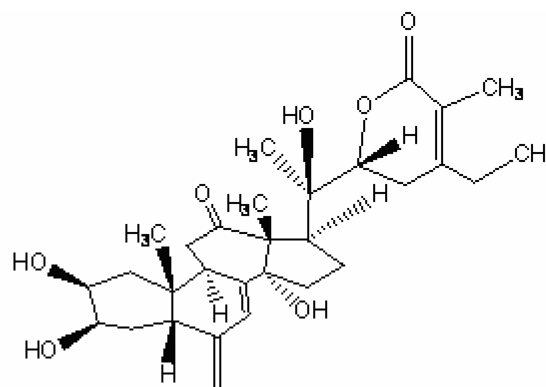
#### 1.2.3.1.2. Chemical constituents of *A. remota* Benth

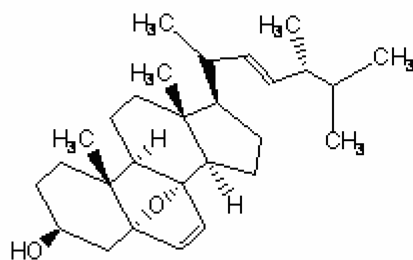
Five different neo-clerodane diterpenes (Figure 6) namely: Ajugarin I**,** II**,** III**,** I**V** and **V** (**10-14**) and clerodin (**15**) were isolated and characterized from the leaves of *A. remota* (Coll and Tandrón, 2005; Coll, 2002). Sterols (ajugalactone (**16**)), triterpenoid (ergosterol-5,8-endoperoxide (**17**) and iridoid glycoside (8-O- acetylharpagide (**18**) were also isolated from the plant. The presence of phenolic acids, flavonoids and saponins was also reported (Kuria *et al*., 2002, 2001; Kariba, 2001).




| **Compounds Name** | **$R_1$** | **$R_2$** | **$C_4$** |
|---|---|---|---|
| Ajugarin I (**10**) | O-$COCH_3$ | O-$COCH_3$ | |
| Ajugarin II (**11**) | O-$COCH_3$ | OH | |
| Ajugarin III **12**) | O-$COCH_3$ | O-$COCH_3$ | α-OH, β-$CH_2OH$ |
| Ajugarin IV **13)** | H | O-$COCH_3$ | α-$COOCH_3$, β-H |
| Ajugarin V (**14**) | H | O-$COCH_3$ | |



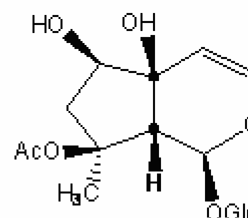




**Figure 6**: Structures of compounds characterized from *A. remota*

#### 1.2.3.1.3. Pharmacological activities of *A. remota* Benth

To verify the traditional uses of *A. remota,* various *in vitro* and some *in vivo* studies have been conducted. The plant was found to have most of the claimed activities including antiviral activity against Human Immunodeficiency Virus type 1, (HIV-1) and Type 2 (HIV-2) (Asres *et al*., 2001), antipyretic (Debela, *et al*., 2005), antifeedant, antihypertensive, insecticidal, antifungal, antimalarial activities (Coll and Tandrón, 2005; Muregi *et al*., 2004). Cyasterone (**19**) with antihyperglycemic activity has also been isolated from *Ajuga reptans* (Abebe *et al*., 2003; Chapman and Hall, 1994).

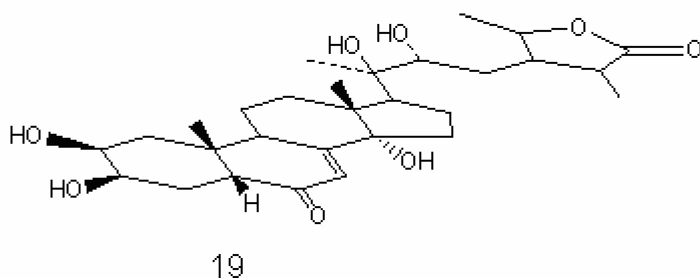



**Figure 7:** Structure of Cyasterone

## 2. Objectives

### 2.1. General objectives

- To show the activity of *S. rebaudiana* and *A. remota* used by traditional practitioners for their diabetic treatment /uses

### 2.2. Specific objectives

- To perform extraction of the study plants using aqueous and 70% ethanol
- To detect the presence/absence of some secondary metabolites in the extracts of the study plants using chemical tests
- To determine the content of Stevioside from *Stevia rebaudiana*
- To investigate the effect of the extracts of the study plants on the blood glucose levels

## 3. Materials and Methods

### 3.1. Materials

#### 3.1.1. Experimental animals

Swiss albino mice weighing 24-35 g of both sexes were purchased from Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa. Before and during the experiment, the mice were allowed free access to standard pellet diet (purchased from Ethiopian Health and Nutrition Research Institute) and water. After randomization in to various groups and before initiation of experiment, the mice were acclimatized to the animal house conditions (Kumar *et al*., 2006; Miura *et al*., 2005; Nagappa *et al*., 2003) at the Department of Biology, Science Faculty, Addis Ababa University, Addis Ababa. Prior to each study, the animals were made to fast for 12-14 hours but had free access to water (Ragavan and Krishnakumari, 2006). All the animal experiments were conducted at biomedical laboratory, Department of Biology, Science Faculty, AAU, Addis Ababa.

#### 3.1.2. Chemicals used

Alloxan (Sigma Chemical Company, USA), Ethanol (BDH Ltd, England), Hydrochloric acid (BDH Ltd, England), Petroleum ether 60-80$^0$C (Labmerk Chemicals LTD India), Chloroform ( ACS, ISO, Merck), Sulphuric acid (Farm Italia Carrloerba, Italy), Acetic anhydride (Techno Pharmchem, India), Ferric chloride (FISHER Scientific Company, New Jersey), n-Hexane (Rathburn Chemicals Ltd, England), Potassium ferrocyanide (BDH Ltd, England), Ferric sulphate (BDH Ltd, England), Lead acetate (BDH Ltd, England), Methanol HPLC grade (Techno Pharmchem, Bahadurgarm, India), Ethyl acetate (ACS, Merck), Acetonitrile (Aldrich, Germany), HPLC grade water (Superchem product, England), Charcoal (BDH Ltd, England), Stevioside 95% (Menge, Germany) and Glibenclamide (Hoechst Pharmaceuticals, Mumbai) were purchased from Germany and Kenema pharmacy no. 2 respectively.

#### 3.1.3. Plant materials

Leave parts of three months old *Stevia rebaudiana* and aerial parts of *Ajuga remota* were collected from Wondo Genet (about 265 km South of Addis Ababa) and Lebu (about 40 km Southwest of Addis Ababa) in December 2006. The plants were identified and authenticated at the National Herbarium, Department of Biology, Science Faculty, Addis Ababa University and voucher specimens (T002 and T001 respectively) were

deposited. The leaves were air-dried under the shed at room temperature at Essential Oils Research Center laboratory, Kaliti, Addis Ababa. The dried plant material was manually powdered and the powder kept in polyethylene bags until used.

## 3.2. Methods

### 3.2.1. Preparation of extracts

#### 3.2.1.1. Ethanol extract

The powdered leaf (100 g) of each plant kept in a thimble was extracted with 70% ethanol in a soxhlet extractor. Extraction process was continued until the color of the final drop of the extract became colorless. The extract was concentrated *in vacuo* at 60 $^{0}$C using a rotary evaporator. To evaporate the remaining solvent, the extract was kept in an oven at a temperature of 40-50 $^{o}$C for 8 hours.

#### 3.2.1.2. Aqueous extract

Powdered leaf (20 g) of each study plant was added to 100 ml hot distilled water (60 $^{o}$c), mixed thoroughly and heated for 20-30 minutes in a water bath with continuous stirring. Hot aqueous extract was filtered under suction. Lypholizer (freeze dryer) (type: Heto power dry LL3000 Wag tech) was used to dry the aqueous extract. The freeze-dried extract was then collected and kept in a refrigerator until used for the experiment.

### 3.2.2. Acute toxicity test

Acute toxicity study was carried out on both plant extracts using female and male Swiss albino mice. The mice were fasted overnight and the weight of each mouse was recorded just before use.

Animals were divided randomly into a control and three treatment groups, each group consisting of four mice (2 male and 2 female). Control group received only the vehicle & each treatment group received orally the 70% ethanol and aqueous extract of the studied plants in a dose of 1000, 2000 and 5000 mg/kg. Animals were kept under close observation for 4 hours after administering the extract (Burger *et al*., 2005), and then they were observed daily for three days for any change in general behaviour and/or other physical activities.

### 3.2.3. Preliminary phytochemical screening

Standard screening test of the extract was carried out for various plant constituents. The crude extract was screened for the presence or absence of secondary metabolites such as alkaloids, steroidal compounds, phenolic compounds, flavonoids, saponins, tannins and anthraquinones using standard procedures (Hymete, 1986).

**i. Test for alkaloids**

a) **Preliminary test**: A 100 mg of an alcoholic extract was dissolved in dilute hydrochloric acid. Solution was clarified by filtration. Filtrate was tested with Dragendroff's and Mayer's reagents. The treated solutions were observed for any precipitation.

b) **Confirmatory test**: Five grams of the alcoholic extract was treated with 40% calcium hydroxide solution until the extract was distinctly alkaline to litmus paper, and then extracted twice with 10 ml portions of chloroform. Chloroform extracts were combined and concentrated *in vacuo* to about 5 ml. Chloroform extract was then spotted on thin layer plates. Solvent system (n-hexane-ethyl acetate, 4:1) was used to develop chromatograms and detected by spraying the chromatograms with freshly prepared Dragendroff's spray reagent. An orange or dark colored spots against a pale yellow background was confirmatory evidence for presence of alkaloids.

**ii. Test for steroidal compounds**

a) **Salkowski's test**: 0.5 g of the alcoholic extract was dissolved in 2 ml chloroform in a test tube. Concentrated sulfuric acid was carefully added on the wall of the test tube to form a lower layer. A reddish brown colour at the interface indicated the presence of a steroid ring (i.e. the aglycone portion of the glycoside).

b) **Lieberman's test**: 0.5 g of the alcoholic extract was dissolved in 2 ml of acetic anhydride and cooled well in an ice-bath. Concentrated sulfuric acid was then carefully added. A colour change from purple to blue to green indicated the presence of a steroid nucleus i.e. aglycone portion of' the cardiac glycosides.

**iii. Test for phenolic compounds**

a) To 2 ml of filtered solution of the aqueous macerate of the plant material, 3 drops of a freshly prepared mixture of 1 ml of 1% ferric chloride and 1 ml of potassium ferrocyanide was added to detect phenolic compounds. Formation of bluish-green color was taken as positive.

b) The dried alcoholic extract (100 mg) was dissolved in water. Few crystals of ferric sulfate were added to the mixture. Formation of dark-violet color indicated the presence of phenolic compounds.

**iv. Flavonoids**

a) **Test for free flavonoids**: Five milliliters of ethyl acetate was added to a solution of 0.5 g of the extract in water. The mixture was shaken, allowed to settle and inspected for the production of yellow colour in the organic layer which is taken as positive for free flavonoids.

b) **Lead acetate test**: To a solution of 0.5 g of the extract in water about 1 ml of 10% lead acetate solution was added. Production of yellow precipitate is considered as positive for flavonoids.

c) **Reaction with sodium hydroxide**: Dilute sodium hydroxide solution was added to a solution of 0.5 g of the extract in water. The mixture was inspected for the production of yellow color which considered as positive test for flavonoids.

**v. Test for saponins**

**Froth test:** 0.5 g of the alcoholic extract was dissolved in 10 ml of distilled water in a test tube. The test tube was stoppered and shaken vigorously for about 30 seconds. The test tube was allowed to stand in a vertical position and observed over a 30 minute period of time. If a “honey comb” froth above the surface of liquid persists after 30 min. the sample is suspected to contain saponins.

**vi. Test for tannins**

a) **Ferric chloride test:** A portion of the alcoholic extract was dissolved in water. The solution was clarified by filtration. 10% ferric chloride solution was added to the clear filtrate. This was observed for a change in colour to bluish black.

b) **Formaldehyde test**: To a solution of about 0.5 g of the extract in 5ml water, 3 drops of formaldehyde and 6 drops of dilute hydrochloric acid were added. The resulting mixture was heated to boiling for 1 minute and cooled. The precipitate formed (if any) was washed with hot water, warm alcohol and warm 5% potassium hydroxide successively. A bulky precipitate, which leaves a colored residue after washing, indicated the presence of phlobatannins.

c) **Test for Phlobatannins**: Deposition of a red precipitate when an aqueous extract of the plant part was boiled with 1% aqueous hydrochloric acid was taken as an evidence for the presence of phlobatannins.

d) **Modified iron complex test:** To a solution of 0.5 g of the plant extract in five milliliter of water a drop of 33% acetic acid and 1 g sodium potassium tartarate was added. The mixture was warmed and filtered to remove any precipitate. A 0.25% solution of ferric ammonium citrate was added to the filtrate until no further intensification of colour is obtained and then boiled. Purple or blackish precipitates which is insoluble in hot water; alcohol or dilute ammonia denotes pyrogallol tannin present.

**Vii. Test for Anthraquinones**

**a) Test for free anthraquinones (Borntrager's test)**

The hydro-alcoholic extract of the plant material (equivalent to 100 mg) was shaken vigorously with 10 ml of benzene, filtered and 5 ml of 10% ammonia solution added to the filtrate. Shake the mixture and the presence of a pink, red or violet color in the ammonia (lower) phase indicated the presence of free anthraquinones.

**b) Test for O-anthraquinone glycosides (Modified Borntrager's test)**

For combined anthraquinones, 5 g of the plant extract was boiled with 10 ml 5% sulphuric acid for 1 hour and filtered while hot. The filtrate was shaken with 5 ml benzene; the benzene layer separated and half its own volume of 10% ammonia solution added. The formation of a pink, red or violet color in the ammonia phase (lower layer) indicated the presence of anthraquinone derivatives in the extract.

### 3.2.4. High performance liquid chromatography (HPLC) analysis

HPLC analysis of *S. rebaudiana* crude extract was carried out to determine the Stevioside content found in the leaves of *S. rebaudiana* in comparison with a reference standard. Acetonitrile-water (80:20 v/v) was used as the mobile phase.

Stevioside was separated by means of a reversed phase Agilent technologies HPLC system comprised of quaternary pump, a column oven, sample freeze and UV detector. A ZORBAX Eclipse XDB-$C_{18}$ column (4.6x150 mm, 5 µm) was employed, at $30^0$C. Separation was made in isocratic mode, using acetonitrile: water (80:20 v/v) at a flow rate of 1ml/min with 20 µl injection volume; detector and column temperature were set at $30^0$C. The detection wave length was 210 nm (Ahmed and Smith, 2002).

**Sample preparation for HPLC analysis**

Silica (25 g) was added to a column. The column was conditioned with hexane (80 ml) and not allowed to dry. The crude extract (250 mg) dissolved in methanol (20 ml), was mixed with silica (5 g) and dried under vacuum. Silica adsorbed concentrated extract was then applied to the column. The analytes were eluted with chloroform: ethyl acetate: water (65:25:4, 100 ml) (Wagner and Bladt, 1996). The eluate was concentrated almost to dryness under vacuum. The residue was re-dissolved in methanol (20 ml). A light yellow solution was obtained and then decolorized using activated charcoal.

Determination of the content of Stevioside in plant material was performed by external standard method. A stock solution of standard Stevioside (Menge Germany) was used and solutions with concentrations of 100, 200, 300, 500 and 800 mg/L were used to

draw calibration curve. Triplicate determinations were carried out and the average taken in drawing the calibration curve.

### 3.2.5. Induction of experimental diabetes

Male Swiss albino mice were fasted overnight (12-14 hours) and their weight and fasting blood glucose level was recorded.

Mice were then made diabetic by a single intraperitoneal injection of alloxan monohydrate (200 mg/kg body weight). Alloxan was first weighed individually for each animal according to their weight & then solublized with 0.5 ml sodium citrate just prior to injection. Food and water were presented to the animals 30 minutes after drug administration (De Carvalho *et al*., 2003; Kamalakkanan and Prince, 2003; Nagappa *et al*., 2003). Two days after alloxan injection, plasma blood glucose level of each animal was determined and animals with a fasting blood glucose range above 200 mg/dl (Kumar *et al*., 2006; Gidado et al., 2005; Pari and Venkateswaran, 2003) were included in the study. The blood samples were collected from the tail of the mice.

### 3.2.6. Experimental design

In the experiment, the mice were divided into 11 groups for the evaluation of fasting blood glucose level and oral glucose tolerance test with five animals in each group.

**Groups 1** & **2** served as negative (took only the vehicle) and positive (diabetes) controls respectively. **Group 3** took standard drug (glibenclamide, 10 mg/kg per day orally), (Nagappa *et al*., 2003). **Groups 4** & **5** were treated with ethanol extract of *S. rebaudiana* (300 & 500 mg/kg respectively). **Groups 6** & **7** were treated with aqueous extract of *S. rebaudiana* (300 & 500 mg/kg). **Groups 8** & **9** received the ethanol extract of *A. remota* (300 & 500 mg/kg), and **groups 10** & **11** received aqueous extract of *A. remota* (300 & 500 mg/kg) in 1 ml distilled water using a gavage daily for 2 weeks (Pari and Venkateswaran, 2003).

Treatment with plant extracts started 48 hours after alloxan injection. Blood samples were drawn at weekly intervals till the end of the study, 2 weeks. Fasting blood glucose

levels and body weight measurement of all the mice were recorded on day 1, 7 and 14 during the experimental period (Nagappa *et al*., 2003).

### 3.2.7. Oral glucose tolerance test (OGTT)

After two weeks of treatment with the plant extracts, the animals were made to fast for 12-14 hours. Their blood glucose level was measured and glucose solution (2 g/kg body weight) was administered orally in a volume of 1 ml. Blood samples were collected 30, 60 and 120 minutes after administration of glucose in order to evaluate their blood glucose level (Kumar *et al*., 2006).

### 3.2.8. Data analysis

Data were statistically evaluated by use of one-way ANOVA, followed by post hoc Scheffe's test using version 13 of SPSS software and Microsoft office excel 2003. The values were considered to be significant if $p<0.05$ was obtained.

## 4. Results and Discussion

### 4.1. Percent yield of plant extracts

In the preparation of crude aqueous extract from the dried leave of S*. rebaudiana* and aerial part of *A. remota* a yield of 38.2% and 21% was obtained respectively.

In the case of crude 70% ethanol extract preparation, a yield of 46.2% for *S. rebaudiana* and 31.7% for *A. remota* was obtained respectively.

### 4.2. Acute toxicity test

A preliminary toxicity study was designed to demonstrate the appropriate safe dose range that could be used for subsequent experiments rather than to provide complete toxicity data on the extract.

Acute toxicity studies conducted revealed that the administration of graded doses of both the crude aqueous and 70% ethanol extracts (up to a dose of 5000 mg/kg) of *S. rebaudiana* and *A. remota* did not produce significant changes in behaviors such as alertness, motor activity, breathing, restlessness, diarrhea, convulsions, coma and appearance of the animals.

No death was observed up to the dose of 5 g/kg body weight. The mice were physically active. These effects were observed during the experimental period (72 hrs). The result showed that in single dose; the plant extracts had no adverse effect, indicating that the medium lethal dose ($LD_{50}$) could be greater than 5 g/kg body weight in mice.

Search for the available literature revealed the non-toxic effect of the leaves of *S. rebaudiana* (Geuns, 2003) and aerial part of *A. remota* (Worku, 2005) in mice.

### 4.3. Preliminary phytochemical screening

Phytochemical screening was done using colour forming and precipitating chemical reagents on the dried leaves of *S. rebaudiana* and aerial part of *A. remota* to generate preliminary data on the constituents of the plant extracts. The results obtained from the

tests were summarized in Table 2 for *S. rebaudiana* and *A. remota*. The chemical tests revealed the presence or absence of major secondary metabolites such as alkaloids, steroidal compounds, phenolic compounds, saponnins, etc in the extracts of the leaves of *S. rebaudiana* and aerial parts of *A. remota.*

The result indicated the presences of flavonoids, tannin, alkaloids and sterols in *S. rebaudiana.* This implied that the plant grown in Ethiopia retained the constituents which were found when it was grown in its natural habitat. *A. remota* was observed to contain flavonoids, tannin and sterols.

Previous study indicated the presence of secondary metabolites like flavonoids, glycosides, and steroids in *S. rebaudiana* (Mantovaneli *et al*., 2004; Anonymous-b, 1999). The presence of flavonoids, saponins and sterols were also reported in the previous study of *A. remota* (Coll and Tandrón, 2005; Kuria *et al*., 2002; Kariba, 2001).

Plants with hypoglycemic and antihyperglycemic activities may contain one or more chemical constituents. Classes of chemical compounds isolated from plants including alkaloids (vindoline), flavonoids (epicatechin), tannin (cathechin), etc. are documented to have the potential to decrease the blood glucose level (Ragavan and Krishnakumari, 2006; Miura *et al*., 2005; Oubre *et al*., 1997). Thus, the significant antidiabetic effect of crude aqueous and ethanol extracts of *S. rebaudiana* and *A. remota* could be due to the possible presence of the aforementioned constituents in the part of the plant used in this particular study, which could act synergistically or independently enhancing the activity of glycolytic and glyconeogenic enzymes.

**Table 2:** Results of phytochemical screening of the extracts of *S. rebaudiana* and *A. remota*

| Test | Reagents | *S. rebaudiana* | *A. remota* |
|---|---|---|---|
| Test for alkaloids | Dragendorff's | ++ | _ |
| | Mayer's | ++ | _ |
| Test for steroidal compounds | Acetic anhydride and conc. sulfuric acid | +++ | +++ |
| | Chloroform and conc. sulfuric acid | +++ | +++ |
| Test for Phenolic compounds | Ferric chloride and potassium ferrocyanide | ++ | ++ |
| Test for flavonoids | 10% Lead acetate | ++ | ++ |
| | Sodium hydroxide | ++ | ++ |
| | Ethyl acetate | ++ | ++ |
| Test for Saponnins | Froth test | ± | ± |
| Test for tannins | Ferric chloride | ± | ++ |
| | Aqueous hydrochloric acid | ± | ++ |
| | Formaldehyde | ± | ++ |
| | Modified iron complex | ± | ++ |
| Test for anthraquinones | Test for free anthraquinones | _ | _ |
| | Test for o-anthraquinone glycosides | _ | _ |

***Key:*** **+++:** Very strong positive

**++:** Strong positive

± **:** Trace

_: Negative

### 4.4. High performance liquid chromatography (HPLC) analysis of *S. rebaudiana*

A reversed phase agilent technologies HPLC/UV system was employed. Separation was made in isocratic mode, using acetonitrile: water (80:20 v/v) as a mobile phase at a flow rate of 1ml/min with 20 µl injection volume.

The sample preparation was done utilizing column chromatography. The crude extract was filtered on a column containing silica gel that was preconditioned with hexane. The residue was re-filled with methanol to a final volume (20 ml). A light yellow solution was obtained and then decolorized using activated charcoal.

The determination of the content of the stevioside in plant material was performed by external standard method. The system was calibrated by quantifying the pure stevioside 95% (Menge, Germany) standard solutions (100 – 800 mg/L). The standard calibration curve (Figure 8) was linear with a correlation coefficient of 0.9946. As indicated in the chromatogram (Figure 9), the stevioside containing peak was identified to be with retention time of 1.276 minutes. The obtained average stevioside content in the leaf preparation of *S. rebaudiana* grown in Ethiopia was 2.27% ± 0.0009 SD. The result obtained was small as compared with the values reported in literature.

The stevioside content from the leaves of *S. rebaudiana* was observed to vary substantially (2-20%) between individual plants from different parts of the world (De Oliveira *et al*., 2004; Yao *et al*., 1999). The variation in the content of stevioside might be because of several reasons, including environmental conditions such as temperature, rainfall and soil. The methods of extraction and analysis, the time of collection and preparation of the samples may also contribute to the variation in percentage content (Midmore and Rank, 2002).

The reverse phase HPLC with UV detection at 210 nm has been used to determine constituents in S*. rebaudiana,* but a problem is that any single UV wave length is a detection compromise as the constituents exhibit a wide range of absorbance maxima (193, 204, 236, 284 nm). Some are only weekly chromophoric (Anonymous-b, 1999).

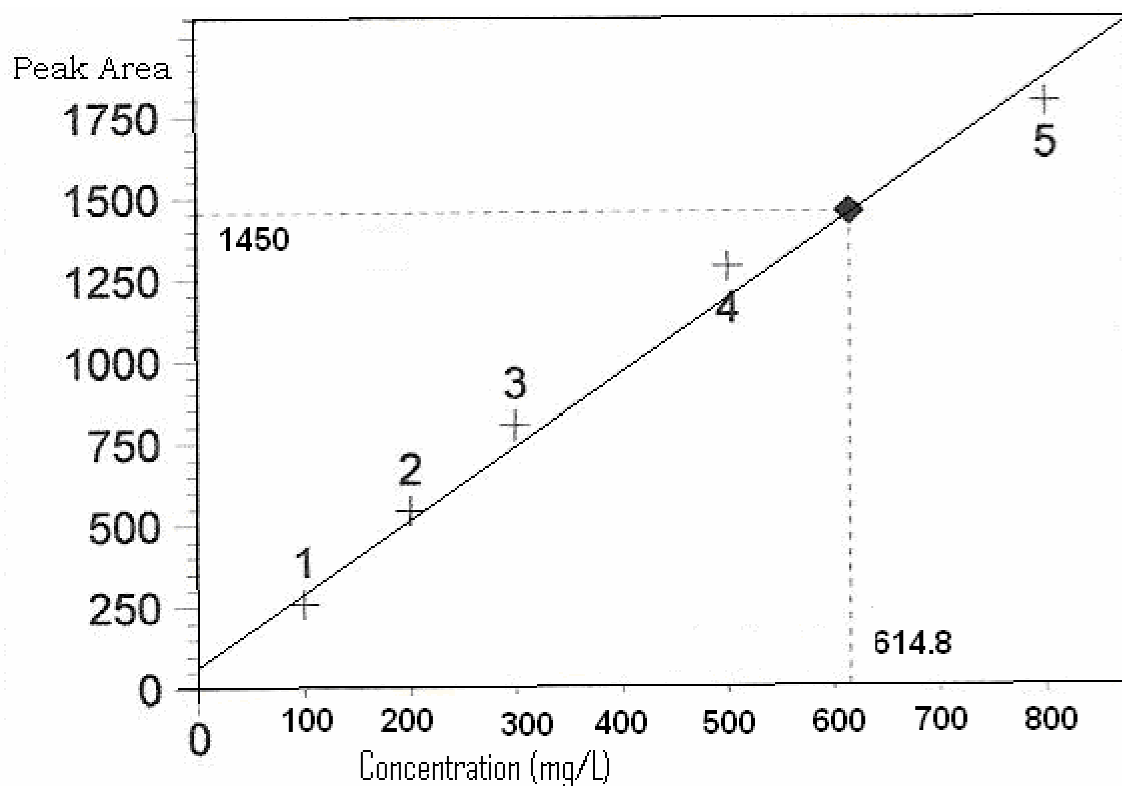



**Figure 8:** Calibration curve for authentic stevioside

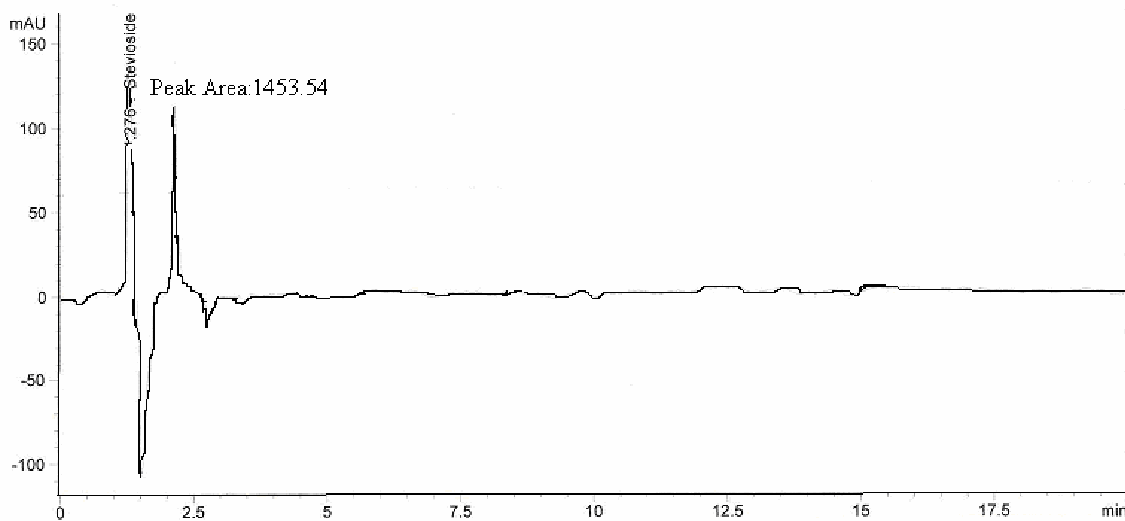



**Figure 9:** HPLC/UV chromatogram of *S. rebaudiana* crude extract

### 4.5. Body weight

The change in the body weight of control and experimental groups of mice treated with *S. rebaudiana* and *A. remota* is shown in Table 3 and 4, respectively. Alloxan-induced (200 mg/kg body weight) mice showed loss in body weight (from 6.29 to 7.89%), which was reversed by oral administration of crude aqueous and ethanol extracts of *S. rebaudiana* and *A. remota* extracts.

The body weight of the normal control mice (negative control), which took the vehicle only, did not show any significant difference, i.e. a 0.55% change on the 14$^{th}$ day. However, the body weight of diabetic control mice (positive control) showed a 4.15% decrease in their body weight after two weeks. In the untreated diabetic control group out of the five animals one mouse died on the 10th day.

A dose-dependent body weight improvement was observed starting from day 1 in diabetic mice treated with both the crude aqueous and ethanol extracts. The effect was more pronounced in case of the crude aqueous extract treated mice for both *S. rebaudiana* (3.26-3.72%) and *A. remota* (2.32-2.60%) as compared with the respective dose of the crude ethanol extract (2.64-3.04% for *S. rebaudiana*) (2.16-2.34% for *A. remota*) effect during the experimental period. During a 14-day treatment, the crude aqueous extract of S. *rebaudiana* and *A. remota* at a dose of 500 mg/kg showed a significant increase in the body weight of the mice from 28.52 ± 1.26 g on day 1 to 29.58 ± 1.23 g (3.72% increment) on day 14 and 31.48 ± 1.10 g on day 1 to 32.3 ± 0.78 g (2.60% increment) on day 14, respectively.

When compared with the aqueous and ethanol extracts, the mice groups that took a dose of 500 mg/kg body weight showed a greater weight gain as compared with the same extract at a dose of 300 mg/kg body weight as shown in Table 3 and 4 for S. *rebaudiana* and *A. remota* respectively.

**Table 3:** Change in body weight of mice treated with *S. rebaudiana* extracts

| Extract | Dose (mg/kg) | Day 0 | Day 1 | Day 14 | %change1 | %change2 |
|---|---|---|---|---|---|---|
| Aqueous | 300 | 31.94 ± 1.00 | 29.42 ± 1.24 | 30.38 ± 1.43 | -7.89 | 3.26 |
| Aqueous | 500 | 30.66 ± 1.20 | 28.52 ± 1.26 | 29.58 ± 1.23 | -6.98 | 3.72 |
| Ethanol | 300 | 30.86 ± 1.21 | 28.76 ± 1.16 | 29.52 ± 1.39 | -6.80 | 2.64 |
| Ethanol | 500 | 26.68 ± 0.58 | 25.00 ± 0.96 | 25.76 ± 0.85 | -6.29 | 3.04 |
| Normal control (Negative) | 1ml (Vehicle) | 25.00 ± 0.96 | 25.38 ± 0.99 | 25.52 ± 0.96 | 1.52 | 0.55 |
| Diabetic Control (Positive) | | 31.92 ± 1.35 | 29.86 ± 1.09 | 28.62 ± 1.33 | -6.45 | -4.15 |
| Glibenclamide | 10 | 30.74 ± 1.18 | 28.96 ± 1.27 | 30.20 ± 0.65 | -5.79 | 4.28 |

Each result is with a mean of 5 mice

%change1 indicates the change between day 0 (before alloxan-induction) and day 1 (after alloxan-induction)

%change2 indicates the change between day 1 and day 14

**Table 4:** Change in body weight of mice treated with *A. remota* extracts

| Extract | Dose (mg/kg) | Day 0 | Day 1 | Day 14 | %change1 | %change2 |
|---|---|---|---|---|---|---|
| Aqueous | 300 | 30.50 ± 1.72 | 28.46 ± 1.63 | 29.12 ± 1.55 | -6.69 | 2.32 |
| Aqueous | 500 | 34.20 ± 0.97 | 31.48 ± 1.10 | 32.30 ± 0.78 | -7.95 | 2.60 |
| Ethanol | 300 | 32.50 ± 0.73 | 30.52 ± 0.64 | 31.18 ± 0.34 | -6.09 | 2.16 |
| Ethanol | 500 | 34.04 ± 0.43 | 31.58 ± 0.54 | 32.32 ± 0.58 | -7.23 | 2.34 |
| Normal control (Negative) | 1 ml (Vehicle) | 25.00 ± 0.96 | 25.38 ± 0.99 | 25.52 ± 0.96 | 1.52 | 0.55 |
| Diabetic Control (Positive) | | 31.92 ± 1.35 | 29.86 ± 1.09 | 28.62 ± 1.33 | -6.45 | -4.15 |
| Glibenclamide | 10 | 30.74 ± 1.18 | 28.96 ± 1.27 | 30.20 ± 0.65 | -5.79 | 4.28 |

Each result is with a mean of 5 mice

%change1 indicates the change between day 0 (before alloxan-induction) and day 1 (after alloxan-induction)

%change2 indicates the change between day 1 and day 14

### 4.6. Blood glucose level

The fasting mean blood glucose level values before and after treatment for two weeks in normal (negative control), diabetic untreated (positive control) and diabetic mice treated with crude aqueous and ethanol extracts of *S. rebaudiana* and *A. remota* is presented in Table 5 (Figure 10) and 6 (Figure 11), respectively. Diabetic control mice were compared with normal control mice and diabetic mice that were treated with the crude extracts were compared with diabetic untreated mice.

The fasting mean blood glucose levels did not show a significant difference in normal control (negative control) mice. In the case of diabetic untreated (positive control) mice the fasting mean blood glucose level showed a statistically significant ($p<0.05$) difference as compared to the normal control mice after two weeks during the experimental period.

On treatment with crude aqueous extract of *S. rebaudiana* (300 and 500 mg/kg), the fasting mean blood glucose levels on day 1 (after being diabetic), i.e. 335.6 ± 14.01 mg/dl reduced to 234.00 ± 16.20 mg/dl and 370.00 ± 19.46 mg/dl reduced to 221.2 ± 18.94 mg/dl respectively. This reduction accounts for 30.27% and 42.65%, respectively. In the case of crude ethanol extract treatment (300 and 500 mg/kg), the fasting mean blood glucose level was reduced by 28.71% and 33.04% respectively. The fasting mean blood glucose level of diabetic mice treated with glibenclamide showed a reduction of 51.06% as compared with diabetic control (positive control) mice.

The improvement in blood glucose homeostasis was in a dose-dependent manner after 14 days of treatment. The effect of the crude aqueous extract of *S. rebaudiana* at a dose of 500 mg/kg body weight showed significantly better reduction as compared with the respective extract at a dose of 300 mg/kg and also with that of ethanol extract.

The effect of the aqueous extract of *S. rebaudiana* on blood glucose in this study had close similarity with the results of a previous study on Goko-Kakizaki rats. The leaves of *S. rebaudiana* contain stevioside, one of the main components of the leaves, which is a

diterpene glycoside. Oral stevioside treatment was done on Goko-Kakizaki rats by Jeppesen, *et al*. (2003). Their finding showed that stevioside has antihyperglycemic activity and it stimulates the first phase of insulin secretion and suppressed the glucagon levels. It also induced the β-cell genes involved in the glycolysis and nutrient-sensing mechanisms (Jeppesen, *et al*., 2003). Even though the content of stevioside obtained from the plant grown in Ethiopia was relatively small; the plant had retained its antihyperglycemic activity.

**Table 5:** Effect of crude extracts of *S. rebaudiana* on fasting blood glucose level (mg/dl) in normal control and alloxan-induced diabetes mice

| **Groups** | **Day of Treatment** | | | |
|---|---|---|---|---|
| | **0** | **1** | **7** | **14** |
| Normal Control (Negative Control) | 110.00 ± 11.47 | 105.40 ± 10.99 | 111.40 ± 10.94 | 109.80 ± 7.49 |
| Diabetes Control (Positive Control) | 123.40 ± 9.29 | 371.20 ± 37.20* | 391.80 ± 31.26* | 405.00 ± 40.97* |
| Glibenclamide | 116.40 ± 3.97 | 349.40 ± 27.57 | 285.00 ± 22.49* | 171.00 ± 18.29* |
| Aqueous Extract 300 mg/kg | 119.20 ± 4.66 | 335.60 ± 14.01 | 290.40 ± 26.56* | 234.00 ± 16.20* |
| Aqueous Extract 500 mg/kg | 127.80 ± 5.31 | 370.00 ± 19.46 | 313.40 ± 14.39* | 212.20 ± 18.94* |
| Ethanol Extract 300 mg/kg | 115.00 ± 4.42 | 262.60 ± 22.23* | 241.20 ± 10.16* | 187.20 ± 17.92* |
| Ethanol Extract 500 mg/kg | 125.60 ± 4.56 | 249.40 ± 21.56* | 213.20 ± 20.36* | 167.00 ± 13.19* |

Values are given as mean ± standard deviation for groups of five animals. Values are statistically significant at * $p < 0.05$.

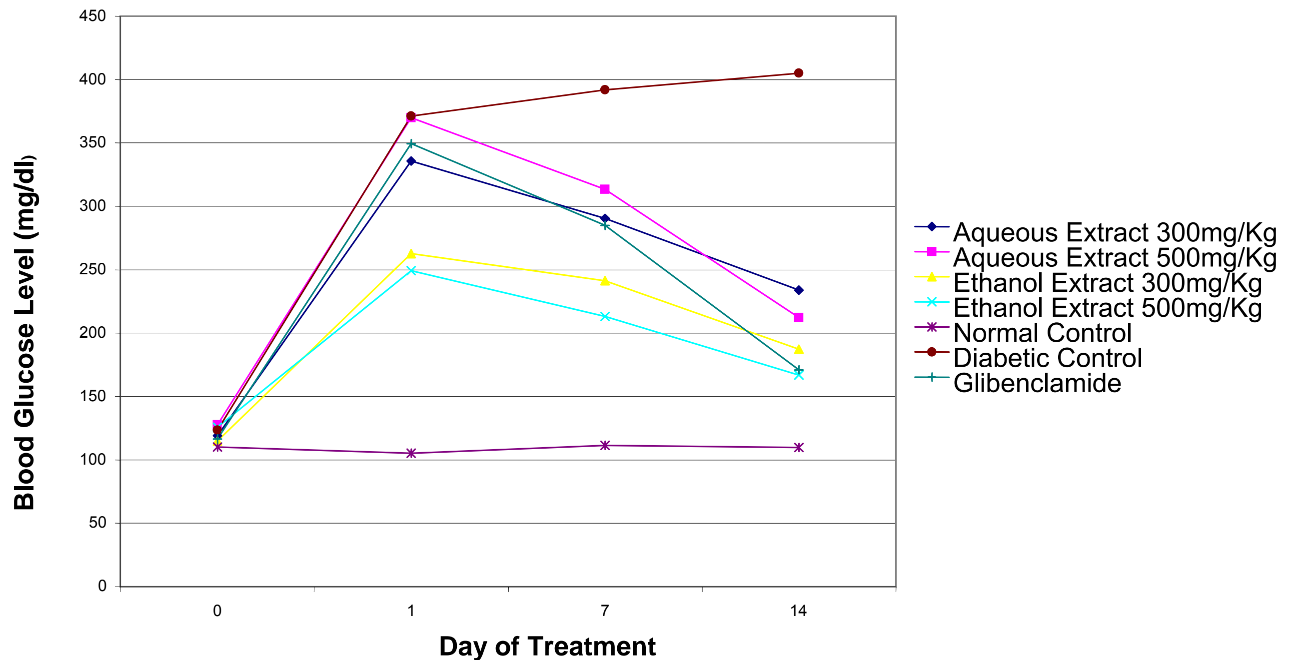



**Figure 10:** Effect of the crude extracts of *S. rebaudiana* on blood glucose level in alloxan-induced diabetic mice

The fasting mean blood glucose level of diabetic mice treated with crude aqueous extract (300 mg/kg and 500 mg/kg body weight), and crude ethanol extract (300 mg/kg and 500 mg/kg body weight) of *A. remota* was reduced by 27.96%, 38.98%, 28.09%, and 28.25% respectively as compared to diabetic control (positive control) mice. The effect was more pronounced in the case of crude aqueous extract as compared with the same dose limit of crude ethanol extract. The result of the ethanol extract did not show dose-dependence where as the aqueous extract showed a dose-dependent reduction of blood glucose levels. The result supported the traditional claim in Ethiopian, particularly in rural parts, for the treatment of diabetes.

**Table 6:** Effect of crude extracts of *A. remota* on fasting blood glucose level (mg/dl) in normal and alloxan-induced diabetes mice

| Groups | Day of Treatment | | | |
|---|---|---|---|---|
| | 0 | 1 | 7 | 14 |
| Normal Control (Positive Control) | 110.00 ± 11.48 | 105.40 ± 10.99 | 111.40 ± 10.95 | 109.80 ± 7.49 |
| Diabetes Control (Negative Control) | 123.40 ± 9.29 | 371.20 ± 37.20* | 391.80 ± 31.26* | 405.00 ± 40.97* |
| Glibenclamide | 116.00 ± 3.97 | 349.40 ± 27.57 | 285.00 ± 22.49* | 171.00 ± 18.29* |
| Aqueous Extract 300 mg/kg | 112.00 ± 6.93 | 349.80 ± 29.32 | 289.60 ± 23.76* | 252.00 ± 17.28* |
| Aqueous Extract 500 mg/kg | 107.40 ± 9.21 | 343.80 ± 20.22 | 240.4 ± 17.98* | 209.8 ± 12.85* |
| Ethanol Extract 300 mg/kg | 112.80 ±9.23 | 242.80 ± 22.62* | 200.00 ± 21.04* | 174.60 ± 12.66* |
| Ethanol Extract 500 mg/kg | 116.00 ± 10.19 | 335.60 ± 31.89* | 261.20 ± 25.64* | 240.80 ± 23.89* |

Values are given as mean ± standard deviation for groups of five animals. Values are statistically significant at * $p < 0.05$.

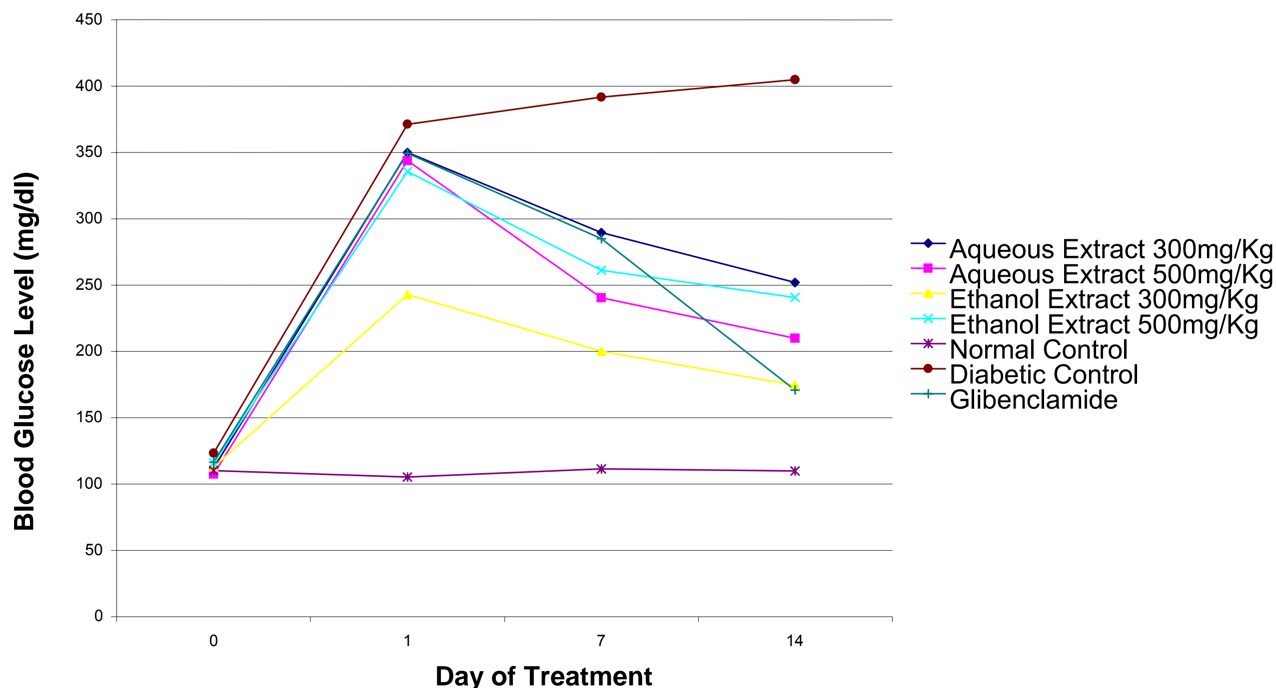



**Figure 11:** Effect of the crude extracts of *A. remota* on blood glucose level in alloxan-induced diabetic mice

In general, the aqueous extract showed a better reduction towards blood glucose level as compared with the ethanol extract in both the study plant cases. This study indicated that the reduction of blood glucose level in aqueous and ethanol extracts of both *S. rebaudiana* in alloxan-induced diabetic mice were a dose dependent. In the case of *A. remota* only the aqueous extract reduced the blood glucose level in a dose-dependent manner.

### 4.7. Oral glucose tolerance test (OGTT)

Results in Table 7 and 8 show the mean blood glucose levels of normal (negative control), diabetic mice untreated (positive control) and diabetic mice treated with *S. rebaudiana* and *A. remota* extracts that were subjected to glucose tolerance test after two weeks respectively. The animals in each group were fasted for 12-14 hrs and then

the mean blood glucose level was evaluated after oral administration of glucose (2 g/kg body weight).

As shown in Figure 12 and 13 on fasting, the blood glucose level of the mice demonstrated basal hyperglycemia and this hyperglycemia was exacerbated by the oral glucose-load (2 g/kg body weight).

The mean blood glucose value in the normal control (negative control) mice rose to a peak value 60 min after glucose load and decreased to near normal level at 120 min. In diabetic control (positive control) mice, however, the peak increase in mean blood glucose concentration was observed after 60 min and remained high over the next 60 min.

The animals that were subjected to oral glucose tolerance test showed a reduction in the mean blood glucose levels after 60 min load of glucose. At 60 min the blood glucose level reached the maximum in both the crude aqueous and ethanol extracts treated animals and then significant reduction was observed in the blood glucose level of diabetic treated with glucose-loaded mice as compared with diabetic control (positive control) mice, loaded only glucose.

The mean blood glucose level at 120 min. after glucose administration was near to the baseline (fasting) in the crude aqueous extract treated mice at a dose of 500 mg/kg body weight for both *S. rebaudiana* and *A. remota* as compared with diabetic mice untreated (positive control). In the case of glucose tolerance test, a great reduction effect was also observed in the aqueous extracts of both plants as compared with that of the ethanol extracts.

**Table 7:** Glucose tolerance test of *S. rebaudiana* extracts on alloxan-induced diabetic mice

| | Aqueous Extract | | Ethanol Extract | | Normal Control | Diabetic Control | Glibenclamide |
|---|---|---|---|---|---|---|---|
| | 300mg/Kg | 500mg/Kg | 300mg/Kg | 500mg/Kg | | | |
| Base-line | 234 ± 16.2 | 212.2 ± 18.94 | 187.2 ± 17.92 | 167 ± 13.19 | 109.8 ± 7.49 | 405 ± 40.97 | 171 ± 18.29 |
| 30' | 284.2± 24.95 | 251.6 ± 20.98 | 247.4 ± 18.31 | 252.2 ± 9.15 | 151.6 ± 15.95 | 422 ± 27.78 | 201.4 ± 19.94 |
| 60' | 312.6 ± 27.13 | 266.2 ± 13.89 | 258.4 ± 16.96 | 257 ± 7.11 | 156.8 ± 7.79 | 433.8 ± 30.06 | 214.8 ± 20.15 |
| 120' | 256.8 ± 18.46 | 224.8 ± 5.02 | 231.6 ± 17.78 | 213.2 ± 16.75 | 131.4 ± 11.23 | 432.6 ±28.52 | 183.8 ± 17.51 |

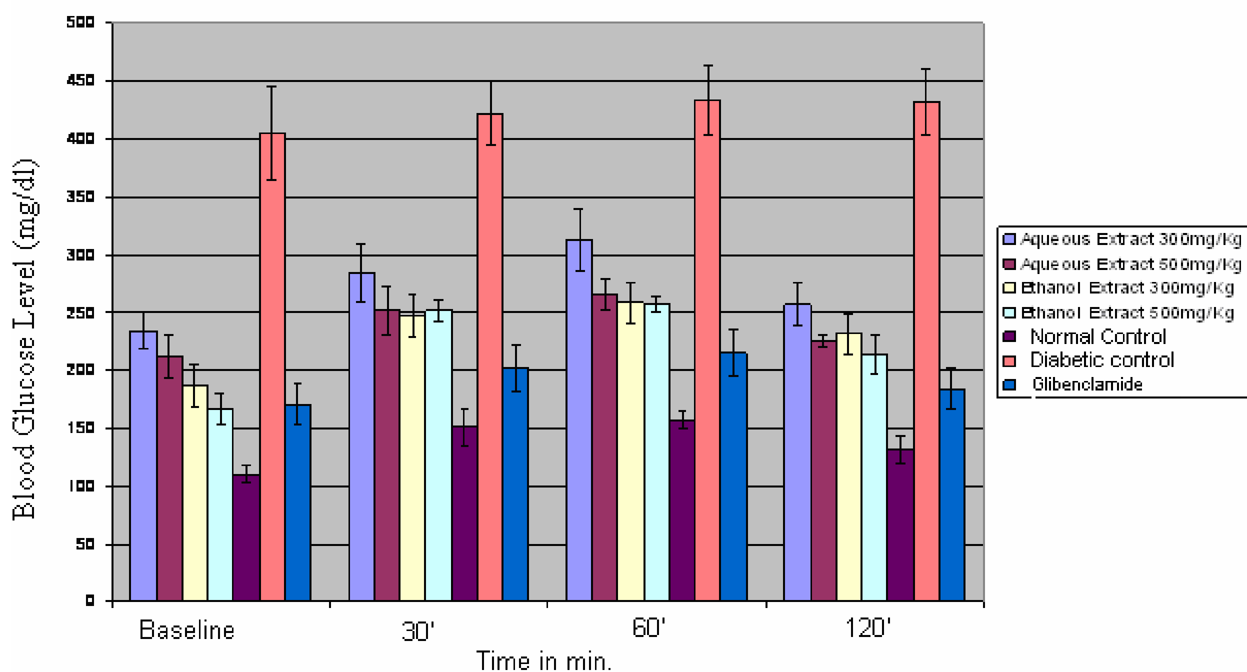



**Figure 12:** Glucose tolerance test of *S. rebaudiana* extracts on alloxan-induced diabetic mice

**Table 8:** Glucose tolerance test of *A. remota* extracts on alloxan-induced diabetic mice

| | Aqueous Extract | | Ethanol Extract | | Normal Control | Diabetic Control | Glibenclamide |
|---|---|---|---|---|---|---|---|
| | 300mg/Kg | 500mg/Kg | 300mg/Kg | 500mg/Kg | | | |
| Base-line | 252 ± 17.28 | 209.8 ± 12.85 | 174.6± 12.66 | 240.8 ± 23.89 | 109.8 ± 7.49 | 405 ± 40.97 | 171 ± 18.29 |
| 30' | 305.4 ± 15.71 | 256.2 ± 15.91 | 209.4 ± 17.21 | 310.6 ± 29.37 | 151.6 ± 15.95 | 422 ± 27.78 | 201.4 ± 19.94 |
| 60' | 331.2 ± 17.68 | 275.4 ± 14.81 | 229 ± 21.34 | 333.6 ± 33.19 | 156.8 ± 7.79 | 433.8± 30.06 | 214.8 ± 20.15 |
| 120' | 312 ± 23.86 | 228 ± 8.60 | 207.4 ± 18.23 | 293 ± 28.68 | 131.4 ± 11.23 | 432.6 ± 28.52 | 183.8 ± 17.51 |

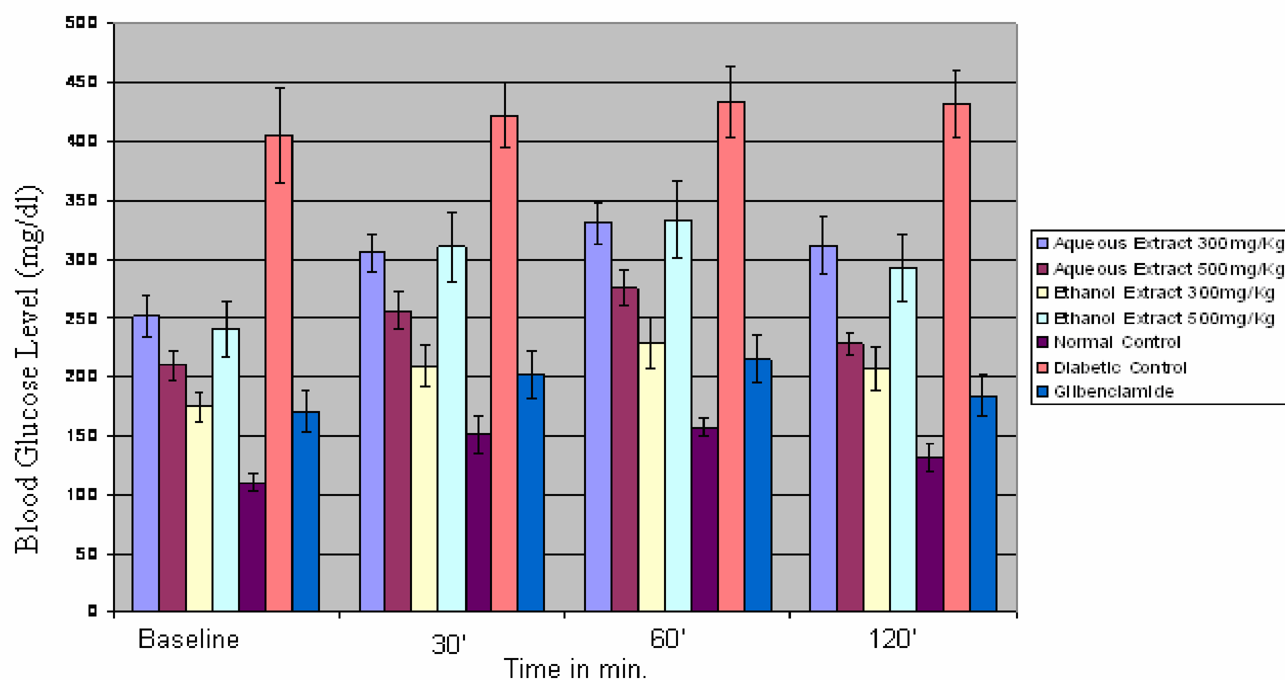



**Figure 13:** Glucose tolerance test of *A. remota* extracts on alloxan-induced diabetic mice

## 5. Conclusions

Oral administration of the extracts in doses from 1000 to 5000 mg/kg/day did not produce significant changes in behaviors. In a single dose administration no adverse effects was observed for the crude extracts of both *S. rebaudiana and A. remota*, indicating that the extracts are not toxic under the observable condition.

Phytochemical investigation of *S. rebaudiana* and *A. remota* revealed the presences of flavonoids, tannins and sterols in both plants and alkaloids in *S. rebaudiana* also contained alkaloids. Compounds belonging to these chemical groups are known to be bioactive for the management of diabetes.

The content of stevioside that was obtained from the extract of *S. rebaudiana* grown in Ethiopia using HPLC analysis was found to be low (2.272%) when compared with what was obtained in other countries, which ranged between 2 and 20%.

A significant antidiabetic activity for the leaf extracts of *S. rebaudiana* was observed in a dose-dependent manner. This indicated that the plant retained its antidiabetic activity when grown out of its natural habitat, at Wondo Genet, Ethiopia. The aerial part extracts of *A. remota* showed a significant antidiabetic activity as well, which supported the traditional use for the control of diabetes. In both plant, the aqueous extract showed a pronounced activity as compared to the ethanol extract. Hence, it might help in preventing diabetic complications and may serve as a good alternative in the present armamentarium of antidiabetic drugs.

## 6. Recommendations

Further studies are required to fractionate, purify and identify the active principle(s) present in the aerial part of A*. remota*.

The stevioside content determined was relatively small. Hence, further agricultural studies should be done in order to improve its content.

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