ANTIHYPERGLYCEMIC AND ANTIHYPERLIPIDAEMIC EFFECTS OF EXTRACTS AND FRACTIONS OF *CLEOME RUTIDOSPERMA* DC AND *SENECIO BIAFRAE* (OLIV. & HIERN) IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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DECLARATION

The research work in this dissertation entitled**: "Antihyperglycemic and Antihyperlipidaemic Effects of Extracts and Fractions of *Cleome rutidosperma* DC and *Senecio biafrae (OLIV. & HIERN)* in Streptozotocin-induced Diabetic Rats"** has been conducted by me in the Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria under the supervision of Prof. I. A. Umar, Prof. S. E. Atawodi and Dr. K. M. Anigo. The information derived from the literature has been duly acknowledged in the text and the list of references provided. No part of this dissertation was previously presented for another degree or diploma at any University.

Israel Oghenevwodokohwo OKORO	----------	----------
(Name of Student)	Signature	Date

CERTIFICATION

This dissertation entitled**: "Antihyperglycemic and Antihyperlipidaemic Effects of Extracts and Fractions of *Cleome rutidosperma* DC and *Senecio biafrae (OLIV. & HIERN)* in Streptozotocin-induced Diabetic Rats"** by Israel Oghenevwodokohwo Okoro meets the regulations governing the award of Doctor of Philosophy of Ahmadu Bello University, Zaria, Nigeria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

To God Almighty and to the memory of my late father; Ven. S. B. F. Okoro, who though saw the commencement of this programme, did not live to see the end of it. May his soul continue to rest in peace at the bosom of our Lord Jesus Christ. Amen.

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ABSTRACT

The antihyperglycemic and antihyperlipidaemic effects of leaves of *Cleome rutidosperma* and roots of *Senecio biafrae* were investigated in streptozotocin (STZ)-induced diabetic mice and rats. Both short term and long term effects of four different solvent extracts (petroleum ether, acetone, ethanol and water extracts) of both plants were evaluated. Single administration of the extracts at a dose of 500 mg/kg body weight was done for the first phase of the study (acute study) while graded daily doses (125 mg/Kg, 250 mg/Kg and 500 mg/Kg) were administered orally to diabetic rats in the second phase of the study (long term, 28 days). To further evaluate the antidiabetic activity of the most active extract (aqueous) of both plants, a bioassay guided fractionation of this crude extract was carried out. Fractions of both plants were tested in different prandial states; fasting, postprandial and post glucose loaded. All the extracts as well as the standard drug, glibenclamide (at 600 μg/kg body weight) significantly ($p< 0.05$) lowered blood glucose level of the diabetic mice and rats for both phases. The effect was highest with the aqueous extracts of both plants. The oral median lethal dose (LD_{50}) of the extracts in rats was > 5000 mg/kg body weight. The aqueous extract of both plants at different doses administered for 28 days, produced significant ($p < 0.05$) changes in the diabetes indices evaluated; there was significant ($p<0.05$) decrease in the level of fasting blood glucose, feed and water intake as well as serum cholesterol, triacylglycerols, low density lipoprotein (LDL), creatinine, urea, bilirubin and activities of liver marker enzymes (AST, ALT and ALP). Significant ($p < 0.05$) increases occured in body weight, High density lipoprotein (HDL), and activities of Superoxide dismutase (SOD), Catalase (CAT), Glutathione-S-Transferase (GST) and Glutathione Reductase (GR). The aqueous extracts of both plants stimulated hepatic glucokinase activity and inhibited the gluconeogenic enzyme, glucose-6-phosphatase.

Fractions of both plants produced significant reduction of blood glucose level at different prandial states. Fractions of both plants at doses of 125 and 62.5 mg/kg b.w., significantly ($p<0.05$) reduced postprandial hyperglycemia after 6 hour of administration. Likewise, glucose tolerance activity was significantly ($p<0.05$) improved in the presence of fractions of the plants to varying extent. Therefore, the results suggests that the plant possess antidiabetic and antilipidaemic properties mediated through: (a) Reduction of postprandial hyperglycemia and improvement of glucose tolerance activity and (b) Inhibition of glucose 6-phosphatase resulting in reduction of hepatic gluconeogenesis and glycogenolysis. *C. rutidosperma* and *S. biafrae* hold promise for development as new and safe oral antidiabetic agents for the management of diabetes mellitus.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FBG	Fasting blood glucose
G6PDH	Glucose-6-phosphate dehydrogenase
GPx	Glutathione peroxidase
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
HDL-C	High density lipoprotein cholesterol
IDDM	Insulin dependent diabetes mellitus
LC-IR	Liquid chromatography coupled with infrared spectroscopy
LC-MS	Liquid chromatography coupled with mass spectrometry
LC-NMR	Liquid chromatography coupled with nuclear magnetic resonance spectroscopy
LC-UV	Liquid chromatography coupled with ultra-violet spectroscopy
LDL-C	Low density lipoprotein cholesterol
LDL-C	Low density lipoprotein cholesterol
MODY	Maturity Onset Diabetes of the Young
NAD^+	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
NIDDM	Non-insulin dependent diabetes mellitus
$O_2 \bullet^-$	Superoxide

OD	Optical density
OGTT	Oral glucose tolerance test
OH•	Hydroxyl radicals
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
STZ	Streptozotocin
TBARS	Thiobarbituric acid reactive substances

CHAPTER ONE

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases characterized by chronic disorder of carbohydrate, fat and protein metabolism that results from defects in both insulin secretion and/or insulin action and leads to hyperglycemia, lipoprotein abnormalities, raised basal metabolic rate, defect in enzymes and high oxidative stress induced damage to pancreatic beta cells (Sharma *et al*., 2010). The disease is associated with reduced quality of life and increased risk factors for morbidity and mortality (Ali *et al.,* 2013). The long term hyperglycemia is an important factor in the development and progression of micro- and macrovascular complication, which include cerebrovascular diseases (Altan, 2003; Strojek, 2003) neuropathy, nephropathy and cardiovascular diseases (Shim *et al*., 2011). Several distinct types of diabetes mellitus exist and are caused by a complex interaction of genetics and environmental factors (Chitra *et al*., 2010).

From ancient period, people have been using medicinal plants for the treatment of diabetes and WHO estimates that 80% of the World's Population presently uses herbal medicine for primary health care (Atmakuri and Dathi, 2010). Antidiabetic plants have the ability to restore the function of damaged pancreatic tissue by increasing the production of insulin or inhibiting the intestinal absorption of glucose (Malviya *et. al.,* 2010). Administration of appropriate antioxidants from plant source could prevent or retard diabetic complications to some extent (Muthulingam, 2010). The level of lipid peroxidation in cells is controlled by various cellular defense mechanisms consisting of enzymatic and non-enzymatic scavenger systems (Halliwell and Gutteridge, 1984); the levels of which are altered in diabetes (Wohaieb and Codin, 1987). Recent experimental and clinical studies

have uncovered new insights into the role of oxidative stress in diabetic complications, and these reports have demonstrated different and innovative approaches to employ natural, antioxidant therapies, e.g., flavonoids and saponin. Flavonoids possess several antioxidant mechanisms including scavenging or quenching free radicals, chelating metal ions, and inhibiting enzymatic systems responsible for free radical generation (Sudha Rani, 2013). The potent antioxidant activity of flavonoids may provide the best protection against elevated oxidative stress (Dias and Takahashi 1994; Blaha *et al*., 2004). Phytochemicals isolated from plant sources are used for the prevention and treatment of cancer, heart disease, diabetes mellitus and high blood pressure (Waltner-Law *et al*., 2002).

1.1 Statement of the Research Problem

According to the WHO (2004), there were 150 million diabetic patients worldwide by the year 2000, with a projection of 221 million people in 2010 and 300 million in 2025. The number of people with diabetes in urban areas is 172 million while 119 million live in rural areas (WHO, 2004). By 2030, the difference is expected to widen with 314 million people living in urban areas and 143 million in rural areas (Shrestha *et al*., 2013). The prevalence in Nigeria varies from 0.65% in rural Mangu village to 11.0% in urban Lagos (International Diabetes Federation, 2009). It is well established that good glycemic control and control of other cardiovascular risks in diabetic patients lead to reduced morbidity and mortality (Mannucci *et al.*, 2013). With the incidence of diabetes in the African population on the rise, the incidence of late diabetic complications is also expected to increase correspondingly (Chinenye *et al*., 2012). This will eventually pose major health care and economic problems. The WHO (2004) suggests that Nigeria has the greatest numberof

people living with diabetes in Africa, with an estimated burden of about 1.7 million which will increase to 4.8 million by 2030 (Chinenye *et al*., 2012).

Various drugs such as biguanides, sulfonylurea and thiazolidenediones are presently available to reduce hyperglycemia in diabetes mellitus (Jung *et al*., 2006; Matsui *et al*., 2006). Management of diabetes without side effect is still a challenge to the medical system. Currently available synthetic antidiabetic agents produce serious side effects such as hypoglycemic coma and hepatorenal disturbances (Gupta *et al*., 2008). Moreover, their usage during pregnancy is not recommended (Ho et al., 2007). Hence, the search for safer and more effective hypoglycemic agents has continued. Ethnobotanical information indicates that plant species are used in the traditional management of diabetes (Oubre *et al*., 1997; Li *et al*., 2004; Abo *et al*., 2008; Van Huyssteen *et al*., 2011). Medicinal plants may provide the useful source of new oral hypoglycemic compounds for the development of pharmaceutical entities or as dietary adjuncts to existing therapies (Kavishankar *et al*., 2011).

Following the WHO's recommendation for research on the beneficial uses of medicinal plants in the treatment of diabetes mellitus (WHO, 2002) and the increased demand by patients to use the natural product with antidiabetic activities, investigation on hypoglycemic agents have gained momentum. Several investigations have been conducted and many plants have shown hypoglycemic activity (Nagappa *et al*., 2003; Otimenyin *et al*., 2008; Momoh *et al*., 2011). Though active principles have been isolated from some plants (Ribnicky *et al*., 2006; Latha *et al*., 2009), some still remain to be identified. These reasons have necessitated the search for a new medication with proven and effective antidiabetic property.

1.2. Justification of the Study

Cleome rutidosperma, commonly known as Spider plant or fringed spider flower has been well studied by different researchers in terms of its antimalarial properties(Bidla *et al*., 2004), nutritional and anti-nutritional compositions (Ojiako and Igwe, 2007), analgesic, anti-inflammatory and antipyretic activities (Bose *et al*., 2007), diuretic and laxative activities (Bose et al., 2006), wound healing activity (Mondal and Suresh, 2012), antimicrobial activity(Bose *et al*., 2007), anticonvulsant activity(Jena *et al*., 2009) and anthelmintic activity(Bose *et al*., 2007), arthritic activity (Chakraborty and Roy, 2010) and antifungal activity(Khuntia and Mohanty, 2011), however, there is paucity of literature on the antidiabetic property of this plant.

Senecio biafrae also known as English spinach has also been extensively studied. Aspects such as chemical composition (Olaposi and Adunni, 2010), physicochemical properties (Famurewa, 2011), effect on sexual maturation (Lienou *et al*., 2012), nutritional quality (Dairo and Adanlawo, 2007) and antimicrobial activity (Gbadamosi and Okolosi, 2013) have been subjects of intense scrutiny. Its use in the treatment of women infertility in Baham subdivision, Western Region of Cameroon (Telefoa *et al*., 2011) and its antihypertensive property (Gbolade, 2012) have also been studied, but the antidiabetic property of this important plant has not been studied or reported.

1.3 Aim and Objectives

1.3.1 Aim of the Study

The aim of the study was to investigate the antihyperglycemic and antihyperlipidaemic effects and possible mechanism (s) of action of the extracts and fractions of *Cleome rutidosperma* and *Senecio biafrae*.in streptozotocin-induced diabetic rats and mice.

1.3.2 Specific Objectives

i. To determine the LD_{50} of the various solvent extracts of *Cleome rutidosperma* and *Senecio biafrae*.

ii. To determine the antidiabetic effects of different solvent extracts of *Cleome rutidosperma* and *Senecio biafrae* in Streptozotocin-induced diabetic rats and mice.

iii. To partially purify the antidiabetic principle in the most active extract of the two plants.

iv. To investigate the possible mechanism of action of the constituent(s) of the active fraction(s).

CHAPTER TWO

LITERATURE REVIEW

2.1 Brief History of Diabetes

The earliest description of diabetes was documented in the writings of Hindu scholars as long as in 1500 BC (Das and Shah, 2011). They had already described "a mysterious disease causing thirst, enormous urine output, and wasting away of the body with flies and ants attracted to the urine of people." The term diabetes was probably coined by Apollonius of Memphis around 250 BC, which literally meant "to go through" or siphon as the disease drained more fluid than a person could consume. Later on, the Latin word "mellitus" was added because it made the urine sweet (MacCracken, 1997).

Sushruta, Arataeus and Thomas Willis were the early pioneers of the treatment of diabetes (MacCracken, 1997). Greek physicians prescribed exercise, preferably on horseback, to "employ moderate friction" and alleviate excess urination. Wine, overfeeding to compensate for loss of fluid weight, starvation diet, potato therapy, and oat cure were some of the other curious forms of remedy suggested for the therapy of diabetes in olden days. Sir William Osler, in the year 1915, was said to have even recommended opium! Early research linked diabetes to glycogen metabolism, and the islet cells of pancreas were discovered by Paul Langerhans, a young German medical student (MacCracken, 1997). In 1916, Sharpey-Shafer of Edinburgh suggested that a single chemical was missing from the pancreas and proposed its name as "insulin." The term insulin originates from the word Insel, which is German for an islet or island (Dods, 2013).

2.2 The Incidence and Prevalence of Diabetes Mellitus (DM)

It has been stated that in 2014 there were globally 386.7 million (8.2 %) people who suffered from this disease. This number is estimated to increase to 591.9 (10.1%) million by 2035 in the absence of better control or cure. Two main reasons for the increase of this disease are an increasing ageing population and obesity (International Diabetes Federation, 2014).

Similarly, the International Diabetes Federation (IDF) estimates that about 21.5 million people (5.1%) suffered from the disease in Africa in 2014 and the number is expected to increase to 41.5 million (5.3%) by 2035(International Diabetes Federation, 2014).

The incidence and prevalence of diabetes mellitus (DM) has continued to increase in Nigeria, despite a great deal of research and resources (Chinenye and Young, 2011). DM is an increasing problem in sub-Saharan Africa, especially Nigeria, with type 2 DM being the most common. It is becoming more prevalent owing to the increasing rates of obesity, physical inactivity, and urbanization. The Framingham study revealed that the burden of DM, which also increases the risk for CVD, has remained fairly constant over the last 50 years (Maiyaki and Garbati, 2014).

2.3. 1 Complications of Diabetes

Metabolic complications of diabetes consist of diabetic ketoacidosis (DKA), hyperosmolar non-ketotic coma (HNC), lactic acidosis (LA), and hypoglycemia. DKA and HNC are related to insulin deficiency. Hypoglycemia results from the treatment of diabetes,

either with oral agents or insulin. Although hypoglycemia may occur in conjunction with oral hypoglycemic therapy, it is more common in patients treated with insulin. LA is usually associated with other factors that may be related to diabetes, such as cardiovascular disease (acute myocardial infarction) associated with hypoxia and excess lactic acid production (Siperstein, 1992). Long term abnormalities in diabetes can result such as eye complications, heart disease, kidney and foot problems if blood sugar levels are poorly controlled (Brophy *et al*., 2007). These complications are of two types-microvascular complications that include retinopathy, nephropathy,neuropathy and peripheral vascular disorders and macrovascular complications that include cardiovascular and cerebrovascular disorders (see Table 2.3). The complications of diabetes can involve multiple systems throughout the body that are susceptible to the detrimental effects of oxidative stress and apoptotic cell injury (Maiese *et al*., 2010). Diabetes also leads to long-term complications throughout the body involving cardiovascular, renal and nervous disorders (Daneman, 2006). The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of various organs especially the eyes, kidneys, nerves, heart and blood vessels (Chandramohan *et al.*, 2009).

The importance of protecting the body from hyperglycemia cannot be overemphasized; the direct and indirect effects on the human vascular system are the major source of morbidity and mortality in both type 1 and type 2 diabetes. Generally, the injurious effects of hyperglycemia are separated into macrovascular complications (coronary artery disease, peripheral arterial disease and stroke) and microvascular complications (diabetic nephropathy, neuropathy, and retinopathy) (Fowler, 2008).

Table 2.1: Complications of Diabetes Mellitus (Shrestha *et al*., 2013).

S/N	Body Location/Disease	Description
1	Eyes	Retinopathy, cataract formation, glaucoma and periodic visual disturbances; leading cause of new blindness.
2	Mouth	Gingivitis, increased incidence of dental cavities and periodontal disease.
3	Pregnancy	Birth of large babies, miscarriages, neonatal deaths and congenital defects.
4	Nervous system	Motor, sensory and autonomic neuropathy leading to impotence, neurogenic bladder, parathesias, gangrene.
5	Skin	Numerous infections and specific lesions due to small vessel disease, increased lipids in blood and pruritus.
6	Kidneys	Diabetic glomerulosclerosis causing nephropathy.
7	Infections	Diabetics have a higher incidence of cystitis, tuberculosis and skin infections; moniliasis is common in diabetic women.

2.3.2 Causes of Diabetic Complications

The complications of diabetes have been tied to oxidant stress (Szabo, 2009). Studies with diabetic animals have shown that oxidative stress leads to DNA damage in renal cortical cells (Simone *et al.*, 2008). Although early effects of elevated glucose may increase the presence of potentially protective pathways, more prolonged exposure of elevated glucose with the rise in insulin levels can lead to reactive oxygen species (ROS) and can be detrimental even if glucose levels are controlled (Barbosa *et al*., 2008).

2.4. Classification of Diabetes

Diabetes mellitus is classified on the basis of aetiology and clinical presentation of the disorder into four types: type 1 diabetes, type 2 diabetes, gestational diabetes, and other specific types.

2.4.1 Type I Diabetes (Insulin Dependent Diabetes Mellitus-IDDM)

Type 1 diabetes or juvenile is an autoimmune disease in which the immune system attacks pancreatic β-islet cells (Dods, 2013). T1D is found for 5–10% of those with diabetes. Destruction of β-islets results in insulinopenia. Often, 70–80% of the β-islet cells are destroyed before detection of the illness. This condition is best defined by the presence in the blood of specific β-islet cell autoimmune antibodies (Dods, 2013). Risk factors are less well defined for type 1 diabetes than for type 2 diabetes (Maiyaki and Garbati, 2014), but autoimmune (Maiyaki and Garbati, 2014), genetic (International Diabetes Federation,

2014), and environmental factors are involved in the development of this type of diabetes (Talaviya *et al*., 2014).

Type 1 diabetes usually is first diagnosed in children and young adults, although it can occur at any age. There is no known way to prevent it, and effective treatment requires the use of insulin. Inheritance of type I diabetes is polygenic (Thevenod, 2008), if the father has type I diabetes the child has 6% risk of developing the syndrome, a 3% risk if mother has and 30% risk if both the parents have the disease(Thevenod, 2008). In studies using identical twins if one twin has type I diabetes there is only 30-40% chance that the other twin will develop type I diabetes (Warjeet, 2011).

The incidence of childhood T1DM varies worldwide (Silink, 2002). Data on incidence of childhood onset T1DM are very limited. Data from large epidemiological studies worldwide indicate that on an annual basis, the overall increase in the incidence of T1DM is around 3% and about 78 000 children under age 15 years develop T1DM worldwide (International Diabetes Federation, 2013).The incidence of childhood type 1 diabetes (T1DM) varies based upon geography, age, gender, family history, and ethnicity (El-Ziny *et al.*, 2014).

2.4.2 Type II Diabetes (Non-Insulin Dependent Diabetes Mellitus-NIDDM)

Type II Diabetes Mellitus (Non-Insulin dependent diabetes mellitus) or adult-onset diabetes is a form of diabetes mellitus that is characterized by hyperglycemia resulting from impaired insulin utilization coupled with the body's inability to compensate with increased insulin production (Srilakshmi, 2007). It is is mainly due to insulin resistance or abnormality in insulin secretion develops slowly and is usually milder and more stable. In type II diabetes, insulin may be produced by the pancreas but action is

impaired, hence the patient is not completely dependent on exogenous insulin (Bamji *et al*., 1999; Srilakshmi, 2007). Type 2 diabetes may account for about 76 - 85 % of all diagnosed cases of diabetes (Talaviya *et al*., 2014). Risk factors for Type 2 diabetes include old age, obesity, and family history of diabetes, prior history of gestational diabetes, impaired glucose tolerance, physical inactivity, and race/ethnicity (Thevenod 2008; Warjeet 2011). The risk of a child developing Type II diabetes is about 15% if one parent has the disease, 70-75% if both are affected (Braxton *et al*., 1993) and 60-100% chance for identical twins if the mother is affected coupled with poor intrauterine nutrition(Chiuand *et al*., 1997). In the case of genetic predictors of NIDDM, the concordance rate in monozygotic twins has been found to be around 70-80% compared with10-20% in di-zygotic twins. Early onset may occur in children born to two diabetic parents who receive a double dose of NIDDM gene (Chiuand *et al*., 1997).

The slow onset of type 2 diabetes, and its usual presentation without the acute metabolic disturbance seen in type 1 diabetes, means that the true time of onset is difficult to determine. There is also a long pre-detection period, and up to one-half of cases in the population may be undiagnosed (Forouh and Wareham, 2014). Because the ratio of detected to undetected cases may vary over time and between places, epidemiological research aimed at defining the true prevalence of type 2 diabetes has relied on special studies in which the presence and absence of disease are defined by the oral glucose tolerance test (Forouh and Wareham, 2014).

Variation in prevalence by geographical location, ethnicity, age, and sex shows the age-standardized prevalence of type 2 diabetes and impaired glucose tolerance (IGT). As in type 1 diabetes, there is marked geographical variation, but the pattern is different

(Forouh and Wareham, 2014). The prevalence is lowest in rural areas of developing countries, generally intermediate in developed countries, and highest in certain ethnic groups, particularly those that have adopted Western lifestyle patterns. Populations with the highest prevalence have a high prevalence of obesity. It is hypothesized that genetic susceptibility to obesity would be disadvantageous in times of food abundance, but advantageous when food is scarce, driving its persistence by natural selection (Dabelea *et al*., 2014). This 'thrifty genotype' hypothesis is supported by evidence of gene–environment interaction; individuals who migrate from low-prevalence areas to the West are at increased risk of type 2 diabetes. Diabetes is up to fourfold to sixfold more prevalent in South Asians and African-Caribbeans in the UK compared with European white populations (Forouh and Wareham, 2014). There is only a small gender difference in the global numbers of people with diabetes, with about 14 million more men than women estimated to have diabetes in 2013(International Diabetes Federation, 2014). The prevalence increases sharply with age in both sexes (International Diabetes Federation, 2014).

2.4.3 Gestational Diabetes

Gestational diabetes mellitus (GDM) is defined as impaired glucose tolerance with onset or first recognition during pregnancy (American Diabetes Association, 2013). It can complicate pregnancy leading to prenatal morbidity and mortality, so clinical detection is important (Talaviya *et al*., 2014). Gestational diabetes is fully treatable but requires careful medical supervision throughout the pregnancy. About 20% - 50% of affected women develop type 2 diabetes later in life (Talaviya *et al*., 2014).

Gestational diabetes occurs more often in African American, Hispanic/Latino, and American Indian women, as well as in women who are obese or have a family history of type 2 diabetes (Craig *et al*., 2009). It requires treatment to bring maternal blood glucose to normal levels and avoid complications in the infant (Craig *et al*., 2009).

The true prevalence of gestational diabetes mellitus (GDM) is unknown (DeSisto *et al*., 2014). GDM prevalence has been steadily increasing with the rise of obesity and type 2 diabetes (Albrecht *et al*., 2010). Both birth certificates and the Pregnancy Risk Assessment Monitoring System (PRAMS), which includes a questionnaire completed by mothers, can provide population-based prevalence estimates of GDM. However, birth certificate data are limited and studies have shown that whereas specificity for GDM is high on the birth certificate, sensitivity is as low as 48% (Devlin *et al*., 2009).

2.4.4 Other Specific Types of Diabetes

Other specific types of diabetes include: Malnutrition related diabetes mellitus, maturity-onset diabetes of youth (MODY) and Neonatal diabetes.

2.4.4. 1 Malnutrition Related Diabetes Mellitus (MRDM)

In tropical developing countries young diabetes with a history of nutritional deficiency and a constellation of symptoms, signs and metabolic characteristics failed to come under the two clinical sub classes of diabetes IDDM and NIDDM. Some young diabetic require high dose of insulin to control blood glucose but don't develop ketoacidosis on withholding insulin which indicates that they are relatively resistant to ketoacidosis (Bamji *et al*., 1999). Hence WHO (1985) included a great number of such cases in the new class of diabetes; Malnutrition Related Diabetes included tropical diabetes, endocrine pancreatic syndrome and ketosis.

2.4.4. 2 Maturity Onset Diabetes of the Young (MODY)

Maturity-onset of diabetes of the young, or MODY, is a form of monogenic diabetes that is caused by mutations occurring in a number of different genes. Mutations in different genes tend to cause a slightly different variant of diabetes (Siddiqui *et al*., 2015). MODY is typically diagnosed during late childhood, adolescence, or early adulthood and is usually observed to develop in adults during their late 50's. One of the main drawbacks in its diagnosis is that many people with MODY are misdiagnosed as having type 1 or type 2 diabetes. (Siddiqui *et al*., 2015). Hyperglycemia is mild and begins in early childhood. Usually dietary restriction is required except during pregnancy as microvascular complications are rare during the period. It is thought to account for 22.5% of all cases of type 2 Diabetes mellitus (Nobre *et al*., 2002).

2.4.4.3 Neonatal Diabetes Mellitus (NDM)

Diabetes in neonates and infants has been first described more than two centuries ago (Greeley *et al*., 2010) but before the discovery of insulin by Nicolae Paulescu in 1921 these infants did not survive to childhood (Greeley *et al*., 2011). Diabetes clinically diagnosed within the first 6 months of life is defined as neonatal diabetes mellitus (Kataria et al., 2014). Depending on whether diabetes resolves later in life or is permanent throughout life, two phenotypes were described: Permanent neonatal diabetes (PNDM) or transient neonatal diabetes (TNDM) (Kataria et al., 2014).

Transient neonatal diabetes accounts for approximately 50% cases of NDM (Greeley *et al*., 2011). Its main clinical feature is that it may either spontaneously remit or be so mild as not to require treatment. However, usually diabetes will often relapse, most often during adolescence (Greeley *et al*., 2011). TNDM is characterized by severe

intrauterine growth retardation (due to deficient insulin secretion in utero) and diagnosis of diabetes within days from birth. Insulin treatment is usually required initially but remission of diabetes typically occurs between 3 and 6 months after onset, only to reappear (usually) later in life (Kataria et al., 2014).

Permanent neonatal diabetes accounts for the other 50% of NDM cases (Hattersley *et al*., 2009). Although the majority of cases of PNDM involve isolated diabetes, some of the known monogenic causes are characterized by a variety of syndromic features. It may be either isolated or form part of a syndrome associating diabetes and other extra-pancreatic manifestations (Proks, 2013).

2.5 Classification of Antidiabetic Agents

Antidiabetic agents can be classified into two categories:

1. Insulin injections and
2. Oral antidiabetic drugs

2.5.1 Insulin injections

These are mostly used on serious cases of diabetes. The hormone insulin is endogenously release from the β-cells of pancreas. Effects of various substitutions on insulin molecule and other modification has led to multiple types of insulin. These are characterized and administered based on their pharmacodynamic and pharmacokinetic characteristics such as onset, peak and duration of action. Most significantly they are classified as rapid-acting, short- acting, intermediate-acting or long-acting types of insulin. The mechanism of action of Insulin is by lowering the blood glucose by peripheral glucose

uptake, especially in skeletal muscle fat and by inhibiting hepatic glucose production (Mane *et al*., 2012).

Stimulus-secretion coupling is an essential biologic event in pancreatic β cells. Ca^{2+}, ATP, cAMP, and phospholipid-derived signals such as diacylglycerol and inositol 1,4,5-triphosphate are the major intracellular signals in insulin secretion (Prentki and Matschinsky, 1987). Glucose-stimulated insulin secretion (GSIS) is the principal mechanism of insulin secretion (Ashcroft, 2005). Glucose is transported into the β cell by glucose transporters, and then metabolized, leading to increases in the ATP concentration (or ATP/ADP ratio), closure of ATP-sensitive K^+ (KATP) channels, depolarization of the β cell membrane, and opening of the voltage-dependent Ca^{2+} channels (VDCCs), thereby allowing Ca^{2+} influx (Ashcroft, 2005). The resultant rise in intracellular Ca^{2+} concentration $[Ca^{2+}]$ in the β cell triggers insulin secretion as illustrated in Figure 2.1. In addition to this KATP channel–dependent pathway, which triggers GSIS, there is also a KATP channel–independent pathway that amplifies the effects of Ca^{2+} on exocytosis (Henquin, 2000) and does not require an additional increase in $[Ca^{2+}]$.

There are different types of insulin and they fall into three main categories: rapid -acting insulin, intermediate- acting insulin and long-acting insulin. The categories and their examples with generic names are as follows:

1. Rapid-Acting Insulin– works very rapidly to lower the blood sugar (e.g.Insulin lispro, Insulin aspart and Insulin glulisine).
2. Intermediate – Acting Insulin - works more slowly and last longer (e.g. Novolin and Humulin).

3. Long-Acting Insulin – works up to 24 hours as a background or “basal” insulin (e.g. Insulin Glargine and Insulin Detemir).

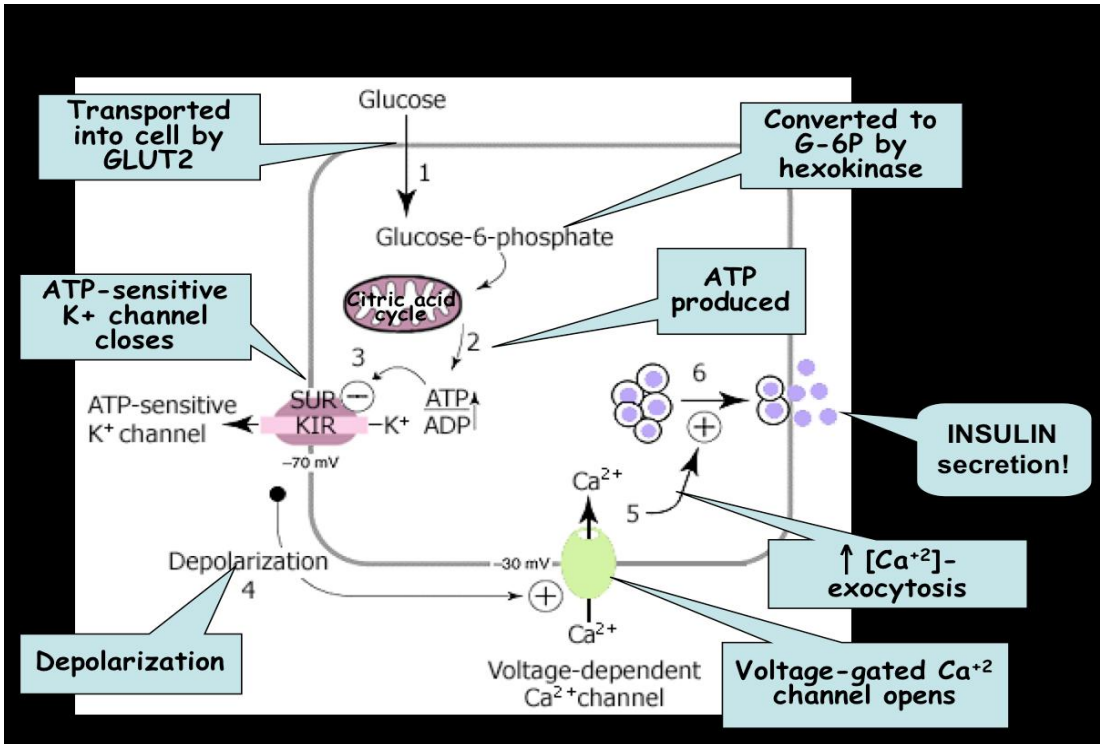


Figure 2.1: Mechanisms of Insulin secretion, Adapted from Ashcroft (2005)

2.5.2 Oral Antidiabetic drugs

Oral antidiabetic drugs are classified into two:

1. Hypoglycemic

2. Antihyperglycemic

1. Hypoglycemic drugs

Primarily increase insulin secretion (insulin secretagogues):

The main side effect is hypoglycemia. Examples include: sulfonylureas and meglitinides

2. Antihyperglycemic drugs

They prevent or reduce hyperglycemia and do not induce hypoglycemia.

Examples are: α-glucosidase inhibitors, thiazolidinediones, and biguanides

2.5.2.1 Insulin Secretagogues: Sulfonylureas

All sulfonylureas (Table 2.1) contain a central S-substituted sulfonylurea structure with various groups terminating the urea N' end group (Ceras *et al.,* 2012). These drugs

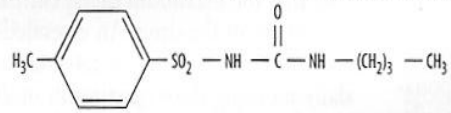
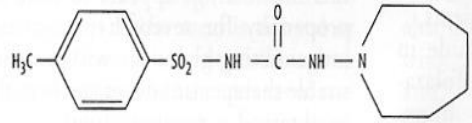
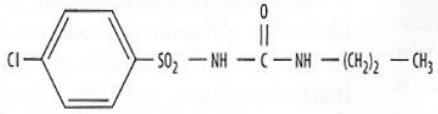
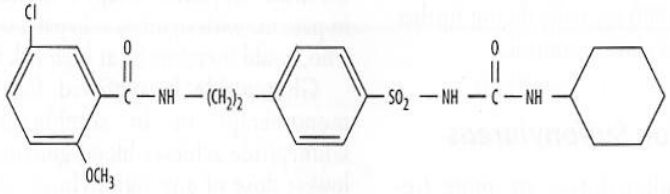
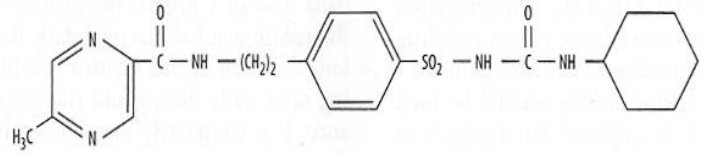
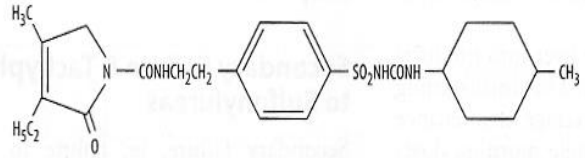
have long been established in the treatment of diabetes and still a large number of people who are taking sulfonylureas, either as a first-line diabetes treatment or in combination with another diabetes medication, accounting for around 25% of newly initiated oral diabetes medications (Desai *et al.*, 2012).

Glibenclamide, an example of the sulfonylureas, has prolonged biological effect due to the formation of active metabolites. It is metabolized primarily in the liver by oxidation of the cyclohexyl ring (omega and omega-1 type oxidations), of the four possible isomeric metabolites, the cis-3-OH and trans-4-OH compounds are the major ones formed (Kharbanda and Alam, 2013).

The mechanism of action of this class of compounds has been found to be related with Adenosine triphosphate (ATP) sensitive potassium (K+) channels (Figure 2.2). Their major action is to increase insulin release from the pancreas. Sulfonylureas binds to a 140kDa high-affinity sulfonylurea receptor that is associated with a beta cell inward rectifier-type ATP-sensitive potassium channel. The binding of a sulfonylurea inhibits the efflux of potassium ions through the channel and results in depolarization. Depolarization, in turn, opens a voltage-gated calcium channel that results in a calcium influx and the release of insulin. Insulin synthesis is not stimulated and may even be reduced by sulfonylureas (Kharbanda and Alam, 2013). Some evidence indicates that after prolonged sulfonylurea therapy, serum insulin levels no longer increase but may even decrease. Also, it has been established that chronic administration of sulfonylureas to type 2 diabetic patients reduces serum glucagon levels and increases the binding of insulin to the tissue receptors (Nolte and Karam, 2001). Sulfonylurea drugs are divided into first and second generation agents, which differ primarily in their potency (Nolte and Karam, 2001). The

first-generation includes: tolbutamide, tolazamide, acetohexamide and chlorpropamide, and the second generation includes: glyburide, glipizide and glimepiride (Kharbanda and Alam, 2013).

Table 2.2. Structure of Sulfonylureas (Karam, 1998).

Sulfonylurea	Chemical Structure	Daily Dose	Duration of Action (hours)
Tolbutamide (Orinase)		0.5–2 g in divided doses	6–12
Tolazamide (Tolinase)		0.1–1 g as single dose or in divided doses	10–14
Chlorpropamide (Diabinese)		0.1–0.5 g as single dose	Up to 60
Glyburide (glibenclamide[1]) (Diaβeta, Micronase, Glynase PresTab)		0.00125–0.02 g	10–24
Glipizide (glydiazinamide[1]) (Glucotrol, Glucotrol XL)		0.005–0.04g (0.02 g in Glucotrol XL)	10–24[2]
Glimepiride (Amaryl)		0.001–0.004 g	12–24

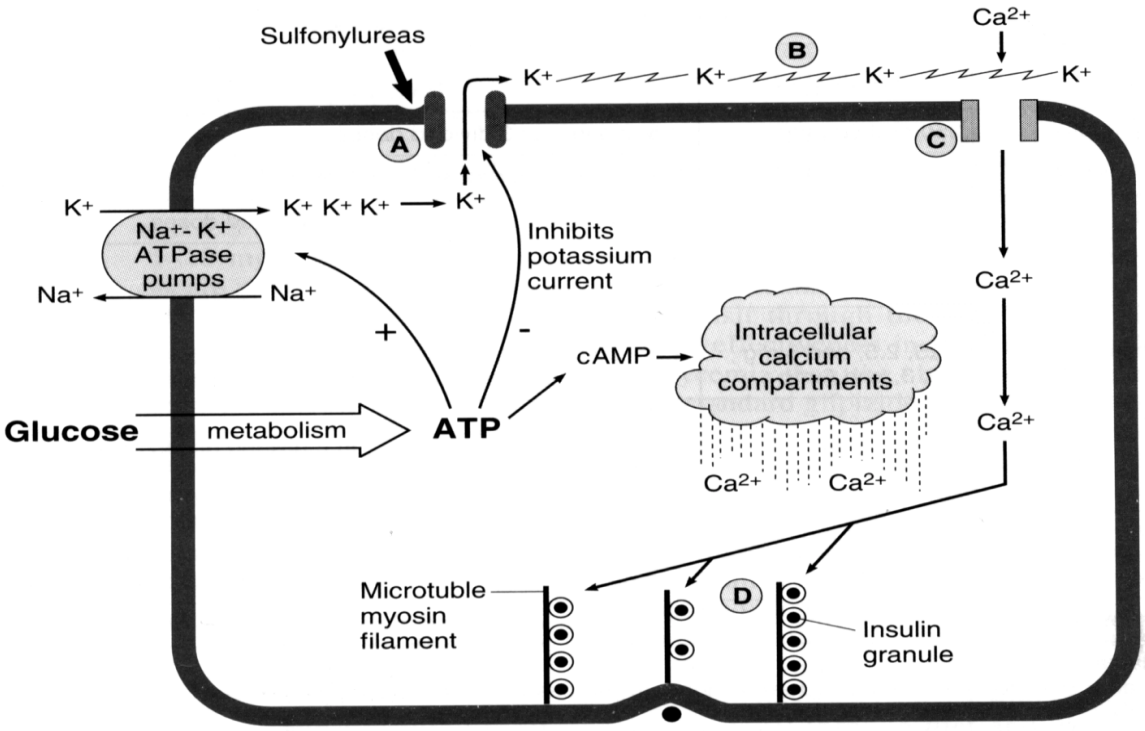


Figure 2.2: Action of sulfonylureas. Adapted from Karam (1998).

2.5.2.2 Insulin Secretagogues: Meglitinides

Meglitinides are a new class of insulin secretagogues. Repaglinide, the first member of the group, was approved for clinical use by the FDA in 1998. These drugs modulate Beta cell insulin release by regulating potassium efflux through the potassium channels. Meglitinides and sulfonylureas overlap in their molecular binding sites since meglitinides have two binding sites in common with sulfonylureas and one unique binding site. They have however, no direct effect on insulin exocytosis as does sulfonylureas (Nolte and Karam, 2001).

2.5.2.3 Biguanides

Three types of biguanides are being used in the treatment of diabetes namely phenformin, buformin and metformin. The use of the first two mentioned has been discontinued due to their association with lactic acidosis (Nolte and Karam, 2001). Metformin originates from the *French lilac*, *Galega officinalis* L., a perennial herb known for centuries to reduce the symptoms of diabetes. The active compound is galegine, a guanidine derivative. While many biguanides have been synthesized, only a few exert a glucose-lowering effect. As can be seen in Figure 2.3, the biguanides have a shared basis, originating from two linked guanidines. The pharmacological differences between the biguanides are determined by differences in their non-polar hydrocycarbon side chains. As a result of these non-polar side chains the biguanides bind to membrane phospholipids and other biological structures (Bailey and Day, 2004).

The full extent of the mechanism of the action of biguanides is unknown, but its blood glucose-lowering action does not depend on the presence of functioning pancreatic beta cells. Proposed mechanisms of action includes direct stimulation of glycolysis in the tissue, and the increase of glucose removal from the blood; reduced hepatic

gluconeogenesis; slowing of glucose absorption from the gastrointestinal tract; with increase glucose to lactate conversion by enterocytes and the reduction of plasmaglucagon levels (Nolte and Karam, 2001). They do not increase insulin levels or cause weight gain. Alone, they rarely cause hypoglycemia (Bridges *et al.,* 2014).

Biguanides are absorbed from the intestines and are not bound to plasma proteins. They are not metabolized and are rapidly eliminated by the kidneys. Drug levels increase markedly in renal insufficiency. Lactic acidosis is a rare, but serious, complication that may occur with drug accumulation (Witters, 2001).

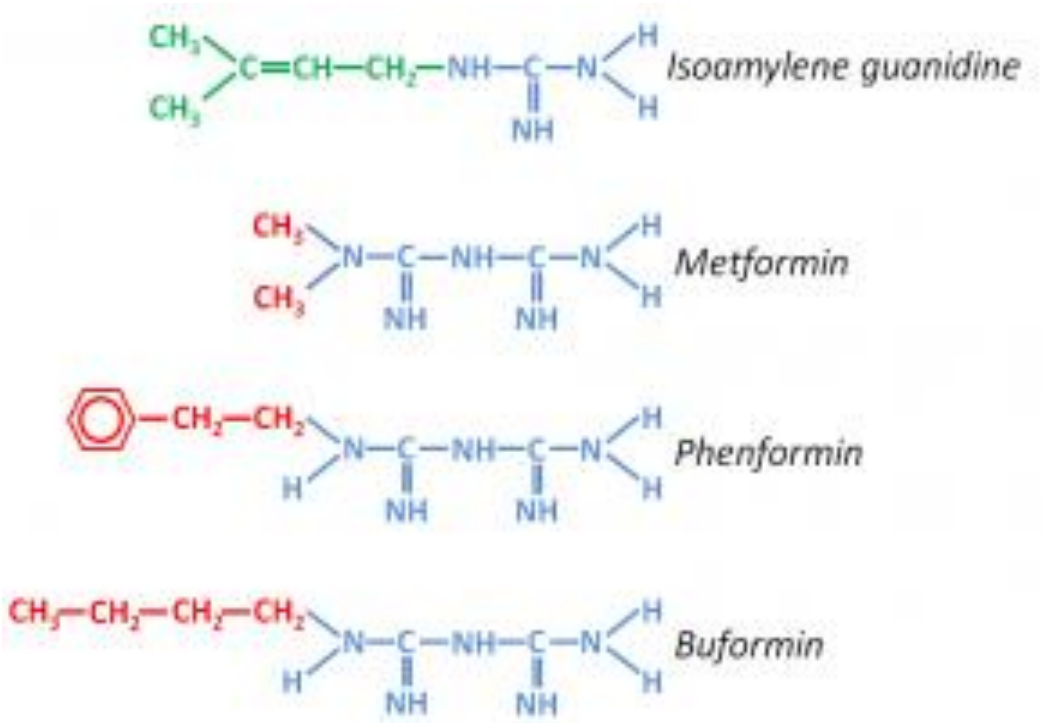


Figure 2.3. The guanidine isoamylene guanidine, and the biguanides metformin, phenformin and buformin. Adapted from Witters (2001).

2.5.2.4 Thiazolidinediones

Thiazolidinediones are oral medications that help lower blood sugar in type 2 diabetes. In addition to a healthy diet and exercise, they are another way to control blood sugar levels. Thiazolidinediones currently available include Actos (pioglitazone), Avandia (rosiglitazone) and a combination drug, Avandamet (rosiglitazone and metformin) (Drazen *et al.,* 2007).

Thiazolidinediones are a newer class of drugs that reduce insulin resistance in the periphery (ie, they sensitize muscle and fat to the actions of insulin) and perhaps to a small degree in the liver (ie, insulin sensitizers, antihyperglycemics) (Verma *et al.,* 2014).

All thiazolidinediones (Figure 2.4) share a common thiazolidine-2-4-dione structure, with chemical modifications selected to enhance their bioactivities, although it is not known whether the differences in potency among the drugs result from changes in bioavailability, metabolism, or mechanistic efficacy (Saltiel and Olefsky, 1996).

The molecular mechanism of thiazolidinedione is activation of peroxisome prolifertoractivated receptor-γ (PPAR-γ). The PPAR-γ nuclear receptors are a class of transcription factor proteins that play an important role in the regulation of lipid and glucose metabolism in vertebrates. They are linked to severe human diseases such as cardiovascular disease and INDDM (Willson *et al.,* 2001; Rangwala and Lazar, 2004).

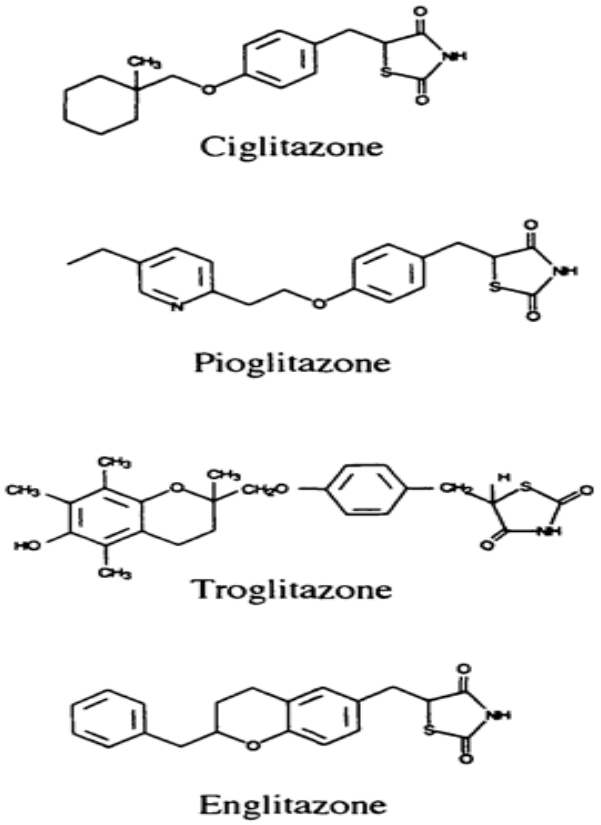
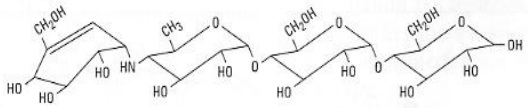
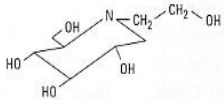


Figure. 2.4. Structures of some thiazolidinedione insulin-sensitizing agents. Adapted from Saltiel and Olefsky (1996).

2.5.2.5 Alpha-glucosidase Inhibitors

Alpha glucosidase inhibitor (GI) prevents the digestion of carbohydrates including starch and table sugar, attenuates postprandial hyperglycemia and delays the development of type 2 diabetes in patients with IGT (Martin and Montgomery, 1996). Their induction of flatulence greatly limits their use. (Chiasson, *et al.,* 2002). Acarbose and miglitol are the two agents available in this class (Table 2.2). Miglitol, an alpha GI, improves endothelial dysfunction assessed by the response of forearm blood flow to reactive hyperemia and flow-mediated dilatation (FMD) in patients with type 2 diabetes and coronary artery disease. It is not absorbed, so liver function abnormalities do not occur. Its modest effect on glycemia and high degree of GI adverse effects (flatulence) limit its use (Hiki *et al.,* 2010; Emoto *et al.,* 2012). Acarbose, an alpha GI, improves postprandial endothelial dysfunction in patients with type 2 diabetes and reduces the risk of cardiovascular events in patients with type 2 diabetes and IGT (Shimabukuro*et al.,* 2006; Kato *et al.,* 2010).

Table 2.3. Structure of Alpha-glucosidase inhibitors (Karam, 1998)

Alpha-glucosidase Inhibitor	Chemical Structure	Oral Dose
Acarbose (Precose)		25–100 mg before meals
Miglitol (Glyset)		25–100 mg before meals

2.6 Limitations of Currently-Available Oral Antidiabetic Medications

The modern medications for diabetes management have their own drawbacks, ranging from the developement of resistance and side effects to lack of receptiveness in large segment of patients population (Zia *et al.,* 2001). The United Kingdom Prospective Diabetes Study (UKPDS) (UKPDS, 1998) has clearly showed that most people with type 2 diabetes will experience progressive pancreatic β cell dysfunction, despite excellent control. The secondary failure rate in this study was 44% after six years of diabetes (UKPDS, 1998). Also, these treatments are associated with side effects or toxic effects (e.g., thiazolidinediones may cause liver toxicity). Sulphonylureas may cause heart disease and increase in the body weight. Other side effects of sulfonylureas include nausea and vomiting, cholestatic jaundice, agranulocytosis, aplastic and hemolytic anemias, generalized hypersensitivity reactions, and dermatological reactions (Seshiah and Balaji, 2000). Acute side effects of Biguanides include diarrhoea, abdominal discomfort, nausea, metallic taste, and anorexia (Derek, 2001). Contraindications include hepatic disease, a past history of lactic acidosis, cardiac failure requiring pharmacological therapy, or chronic hypoxic lung disease. Flatulence, bloating and abdominal discomfort and pain are the major complaints with glucosidase inhibitors. (Michael *et al*., 2005).

The limitations of currently-available oral antidiabetic medication either in terms of efficacy/safety coupled with the emergence of the disease into a worldwide epidemic have encouraged a concerted effort to discover newer drugs that can be used to manage type 2 diabetes more efficiently (Dey *et al*., 2002). Also, with increasing incidence of diabetes mellitus throughout the world and due to adverse effects of allopathic medicinal agents, there is an obvious need for development of indigenous, economical anti-diabetic

crude or purified drugs from botanical or natural sources (DeFronzo, 1999; Venkatesh *et al*., 2003).

2.7 Different Pharmacological Action of Herbal Antidiabetic Remedies

Mechanism of action of an herbal antidiabetic is dependent on the presence of active chemical component in plant material (Chauhan *et al.*, 2010). Different mechanism of action of herbal medicine have been repeated. Excerpts:

- ❖ Adrenomimeticism, pancreatic beta cell potassium channel blocking, cAMP (2nd messenger) stimulation (Marles and Farnsworth, 1996)
- ❖ Stimulation of insulin secretion from beta cells of islets or/and inhibition of insulin degradative processes (Pulok *et al*., 2006)
- ❖ Prevention of pathological conversion of starch to glucose (Sepha and Bose, 1956)
- ❖ Stimulation of insulin secretion (Esmaeili and Yazdanparast , 2004)
- ❖ Preventing oxidative stress that is possibly involved in pancreatic ß-cell dysfunction found in diabetes (Hideaki *et al*., 2005)
- ❖ Reduction in insulin resistance (Pulok *et al*., 2006)
- ❖ Inhibition in renal glucose reabsorption (Eddouks *et al*., 2002)

- ❖ Regenerating and/or repairing pancreatic beta cells (Mohamed *et al*., 2006)
- ❖ Increasing the size and number of cells in the islets of Langerhans (Mohamed *et al*., 2006)
- ❖ Protective effect on the destruction of the betacells (Kim *et al*., 2003a)
- ❖ Inhibition of β -galactocidase and α-glucocidase (Sharma and Mujumdar, 1990)
- ❖ Stimulation of glycogenesis and hepatic glycolysis (Miura *et al*., 2001)
- ❖ Inhibition of alpha-amylase (Heidari *et al*., 2005)

2.8 Reactive Oxygen Species (ROS) and Oxidative Streess in Diabetes Mellitus

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals (Halliwell and Gutteridge, 1999). This unpaired electron(s) usually gives a considerable degree of reactivity to the free radical. Radicals derived from oxygen represent the most important class of radical species generated in living systems (Miller *et al*., 1990). Molecular oxygen (dioxygen) has a unique electronic configuration and is itself a radical.

The addition of one electron to dioxygen forms the superoxide anion radical (O2 •−) (Miller *et al*., 1990) (see Fig. 2.5). Various pathways of ROS formation are outlined in

Figure 2.5. The production of superoxide occurs mostly within the mitochondria of a cell (Valko *et al.*, 2004).

The mitochondrial electron transport chain is the main source of ATP in the mammalian cell and thus is essential for life (Kovacic *et al.*, 2005). During energy transduction, a small number of electrons "leak" to oxygen prematurely, forming the oxygen free radical superoxide, which has been implicated in the pathophysiology of a variety of diseases (Cadenas and Sies, 1998).

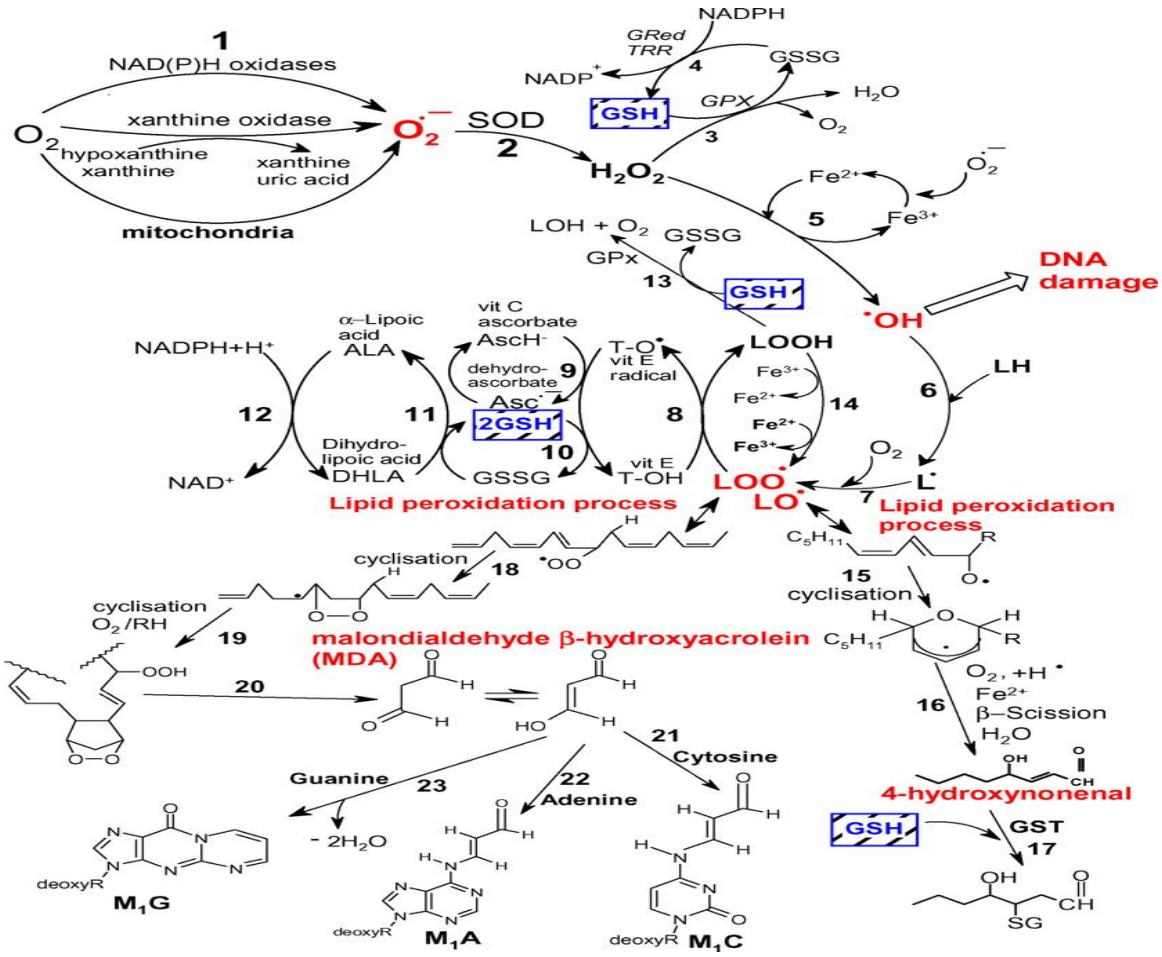


Figure 2.5: Pathways of ROS formation, the lipid peroxidation process and the role of glutathione (GSH) and other antioxidants (Vitamin E, Vitamin C, lipoic acid) in the management of oxidative stress (Valko *et al*., 2007). Reaction 1: The superoxide anion radical is formed by the process of reduction of molecular oxygen mediated by NAD (P)H oxidases and xanthine oxidase or non-enzymatically by redox-reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain. Reaction 2: Superoxide radical is dismutated by the superoxide dismutase (SOD) to hydrogen peroxide. Reaction 3: Hydrogen peroxide is most efficiently scavenged by the enzyme glutathione peroxidase (GPx) which requires GSH as the electron donor. Reaction 4: The oxidised glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase (Gred) which uses NADPH as the electron donor. Reaction 5: Some transition metals (e.g. Fe2+, Cu+ and others) can breakdown hydrogen peroxide to the reactive hydroxyl radical (Fenton reaction). Reaction 6: The hydroxyl radical can abstract an electron from polyunsaturated fatty acid (LH) to give rise to a carbon-centred lipid radical (L•). Reaction 7: The lipid radical (L•) can further interact with molecular oxygen to give a lipid peroxyl radical (LOO•). If the resulting lipid peroxyl radical LOO• is not reduced by antioxidants, the lipid peroxidation process occurs (reactions 18–23 and 15–17). Reaction 8: The lipid peroxyl radical (LOO•) is reduced within the membrane by the reduced form of Vitamin E (T-OH) resulting in the formation of a lipid hydroperoxide and a radical of Vitamin E (T-O•). Reaction 9: The regeneration of Vitamin E by Vitamin C: the Vitamin E radical (T-O•) is reduced back to Vitamin E (T-OH) by ascorbic acid (the physiological form of ascorbate is ascorbate monoanion, AscH-) leaving behind the ascorbyl radical (Asc•-). Reaction 10: The regeneration of Vitamin E by GSH: the oxidised Vitamin E radical (T-O•) is reduced by GSH. Reaction 11: The oxidised glutathione (GSSG) and the ascorbyl radical (Asc•-) are reduced back to GSH and ascorbate monoanion, AscH-, respectively, by the dihydrolipoic acid (DHLA) which is itself converted to α-lipoic acid (ALA). Reaction 12: The regeneration of DHLA from ALA using NADPH. Reaction 13: Lipid hydroperoxides are reduced to alcohols and dioxygen by GPx using GSH as the electron donor. Lipid peroxidation process: Reaction 14: Lipid hydroperoxides can react fast with Fe2+ to form lipid alkoxyl radicals (LO•), or much slower with Fe3+ to form lipid peroxyl radicals (LOO•). Reaction 15: Lipid alkoxyl radical (LO•) derived for example from arachidonic acid undergoes cyclisation reaction to form a six-membered ring hydroperoxide. Reaction 16: Six-membered ring hydroperoxide udergoes further reactions (involving scission) to from 4-hydroxy-nonenal. Reaction 17: 4-hydroxynonenal is

rendered into an innocuous glutathiyl adduct (GST, glutathione S-transferase). Reaction 18: A peroxyl radical located in the internal position of the fatty acid can react by cyclisation to produce a cyclic peroxide adjacent to a carbon-centred radical. Reaction 19: This radical can then either be reduced to form a hydroperoxide (reaction not shown) or it can undergo a second cyclisation to form a bicyclic peroxide which after coupling to dioxygen and reduction yields a molecule structurally analogous to the endoperoxide. Reaction 20: Formed compound is an intermediate product for the production of malondialdehyde. Reactions 21, 22, 23: Malondialdehyde can react with DNA bases Cytosine, Adenine, and Guanine to form adducts M1C, M1A and M1G, respectively.

Oxidative stress results from an imbalance between radical-generating and radical-scavenging systems, i.e. increased free radical production or reduced activity of antioxidant defenses or both. Free radicals and reactive oxygen species (ROS) are formed under normal physiological conditions but become deleterious when not being quenched by the antioxidant systems (Fang *et al*., 2002). There are convincing experimental and clinical evidences that the generation of reactive oxygen species is increased in the diabetic state and that the onset of diabetes is closely associated with oxidative stress (Rosen *et al*., 2001; Johansen *et al*., 2005). Free radicals are formed disproportionately in diabetes by glucose autoxidation, polyol pathway and non-enzymatic glycation of proteins (Obrosova *et al*., 2002). Abnormally high levels of free radicals and simultaneous decline of antioxidant defense systems can lead to the damage of cellular organelles and enzymes, increased lipid peroxidation and development of complications of diabetes mellitus (Maritim *et al*., 2003).

In diabetes mellitus, alterations in the endogenous free radical scavenging defense mechanisms may lead to ineffective scavenging of reactive oxygen species, resulting in oxidative damage and tissue injury. Implication of oxidative stress in the pathogenesis of diabetes is suggested, not only by oxygen free-radical generation, but also due to nonenzymatic protein glycosylation and auto-oxidation of glucose (Mullarley *et al*., 1990), impaired glutathione metabolism (Mclennan *et al*., 1991), alteration in antioxidant enzymes(Strain, 1991), lipid peroxides formation and decreased ascorbic acid levels (Young *et al*., 1991). In addition to GSH, there are other defense mechanisms against free radicals like the enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) whose activities contribute to eliminate superoxide, hydrogen peroxide and Hydroxyl radical (Soto *et al*., 2003). The level of these antioxidant enzymes critically influences the susceptibility of various tissues to oxidative stress and is associated with the development of complications in diabetes. Also this is particularly relevant and dangerous for the beta islet being among tissues that have the lowest levels of intrinsic antioxidant defenses (Lenzen *et al*., 1996; West, 2000; Robertson, 2004).

2.9 Brief History of Medicinal Plants

The association of humans and animals with plants obviously originated with the beginning of life on earth, when plants supplied much of the shelter, oxygen, food and medicine needed by higher life forms (Akerele, 1992). According to World Health Organization (1985), about 70 percent of the world's population relies on plants for their primary health care and some 35,000 to 70,000 species has been used as medicaments, a figure corresponding to 14-28% of the 250,000 plants species estimated to occur around the

world (Akerele, 1992; Padulosi *et al*., 2002), and equivalent to 35-70% of all species used world-wide (Padulosi *et al*., 2002).

From about 250,000 species of higher plants around the world, only 17% have been scholarly investigated for medical potential (Fransworth 1992). The chemical and biological diversity of plants represent a potentially limitless renewable source for the use in the development of new pharmaceuticals. Currently, major biologically active compound in fighting malaria is artemisinin, a sesquiterpene lactone, first isolated in 1972 from wormwood (*Artemisia annua*) by Chinese chemists studying traditional Chinese herbal medicine (Mamedov and Craker, 2008). Research on traditional medicinal plants in the U.S. has resulted in the discovery of alkaloids from Madagascar periwinkle (Catharanthus roseus), used in the chemotherapy of childhood leukemia and for treatment of Hodgkin's disease (Moerman, 2009).

Understanding the relationship among medicinal plants used in traditional medicine systems can help identify plant materials with potential constituents applicable to modern medicine (Zhu, 1998).

2.10 Importance of Herbal Products in Diabetes Mellitus

In modern medicine no satisfactory effective therapy is still available to cure DM. Although insulin therapy, oral hypoglycemic agents, restricted diet, exercises either singly or in combination constitute a major regimen of therapy available for the present day diabetic patients, in a large number of cases, treatment with traditional medicine in the form of plant extracts has been reported to give remarkably good results (Sumana and Suryawanshi, 2001). The presence of hypoglycemic property in leaves and flowers of Vinca Rosea has been reported (Banakar *et al*, 2007). Shroti *et al* (1963) reported a mild

hypoglycemic effect of crude aqueous extract of Vinca Rosea whole plant except roots on alloxan induced diabetic rabbits. Extracts of various parts of the plant viz. methanolic extract of root bark (Hikino *et al*, 1985), ethanol insoluble extract of the leaves (Chen *et al*, 1995) and the leaves (Kim *et al*, 1999) have been reported to possess antidiabetic effect.

Extracts of ripe leaves, tender leaves, fruits and flowers of *Azadiracta indica* have been reported to possess anitdiabetic and antiviral activity (Rao *et al*, 1969). Plant products investigated for antidiabetic effect have been exhaustively reviewed (Ivorra *et al*, 1989) such as *Phyllanthous amarus* (Srividya and Periwal, 1995), *Pterocarpus marsupium* (Manickam *et al*, 1997), *Salacia oblonga* (Augusti *et al*, 1995) etc. Leaves of *Coccinia indica* (Basu, 1992), *Catharanthus roseus* (Rastogi and Mehrotra, 1990), *Momordica charantia* (Day *et al*, 1990), *Gymnema sylvestre* (Rastogi and Mehrotra, 1990) and also the various plant extracts of *Aegle marmelose* (Sumana and Suryawanshi, 2001), *Eugenia jambolana* (Upadhyay *et al*, 1996), *Cassia auriculata* (Pari *et al*, 2001), *Withania somnifera* (Andallu *et al*, 2000) and *Curcuma longa* (Nischino *et al*, 2000), *Alium cepa*, *Alium sativum*, *Ficus bengalensis* and *Trignella foenum graecum* (Twaij and Al-Badr, 1988) have been reported to have potent hypoglycemic and antidiabetic activity.

2.11 Medicinal Plants used in Current Study:

The medicinal plants used in the present study are: *Cleome rutidosperma* and *Senecio biafrae*

2.11.1 *Cleome rutidosperma*

2.11.1.1 Scientific classification of *Cleome rutidosperma*

Kingdom: Plantae

Order: Capparidales

Family: Capparaceae

Genus: Cleome

Species: *Cleome rutidosperma*

(Source: Ruiz-Zapata *et al*., 1996)

2.11.1.2. Vernacular names of *Cleome rutidosperma*

HAUSA: garseya (Yates); IGBO: àkidìmmọ́ó = beans of the dead; YORUBA: ẹtarẹ (auctt.).

Cleome rutidosperma (Capparidaceae) commonly known as Fringed Spider Flower or Purple Cleome is a plant with spreading and prostrate branches and leaves divided in 3 leaflets (see Plate 2.1). It is a low-growing herb, up to 70 cm tall, found in waste herb, grounds and grassy places with trifoliate leaves and small, violet-blue, 5 mm long, borne on long stalks from the leaf axil of the flowering stems. Fruit is cylindrical and curved, about 5 cm long, with numerous round, reddish-brown to black seeds, 1.5 mm diameter with barred surfaces which native to Tropical Africa , although it has become naturalized in various parts of tropical America as well as Southeast Asia (Waterhouse and Mitchell, 1998; Chakraborty *et al*., 2010). Flowering and fruiting plants can be found throughout the year, although most abundantly in the rainy season (Jansen, 2004).



Plate 2.1. Picture of leaves of *Cleome rutidosperma*

2.11.1.3 Traditional Uses of *Cleome rutidosperma*

According to traditional use, the different parts of the plants of Cleome genus are used as stimulant, antiscorbutic, antihelmintic, vesicant, rubefacient and carminative (Kirtikar and Basu, 1991). *C. rutidosperma* is traditionally used in the treatment of paralysis, epilepsy, convulsions, spasm, earache, pain and skin disease. The popular use of the roots, however, refers mainly to its analgesic, anti-inflammatory and antihelmintic activity (Chakraborty *et al*., 2010).

The leaves of *C.rutidosperma* are collected from the wild and eaten as a cooked vegetable or added to soup. They have a bitter taste like mustard and in Uganda clarified butter (‘ghee’) is sometimes added to give it more flavor (Chakraborty *et al*., 2010). It has similar medicinal uses as *C. gynandra* L. Leaf sap is applied in Ghana, Gabon and DR Congo to cure earache and deafness (Chakraborty *et al*., 2010). In Ghana a leaf extract is used to treat irritated skin and in Nigeria it is used to treat convulsions (Kirtikar and Basu, 1991). In Malaysia, planting of *C.rutidosperma* around field edges may be considered as part of an insect control programme (Kirtikar and Basu, 1991), diverting oviposition of diamondback moth (*Plutella xylostella*) away from cultivated plants (Jansen, 2004). In

some areas (e.g. the Philippines, Australia) *C. rutidosperma* is a troublesome weed (Jansen, 2004) with several reported biological activities such as Anti-plasmodial properties, Analgesic, anti-inflammatory and antipyretic activities, diuretic and laxative activities, wound healing and antimicrobial activities amongst others (Table 2.4). Also, the proximate composition of *C. rutidosperma* had been reported (Leung, *et al*., 1968).

Table 2.4: Biological Activities Reported for *Cleome rutidosperma*

S/N	Activity	Reference
1	Anti-plasmodial properties	Bidla *et al*., 2004
2	Analgesic, anti-inflammatory and antipyretic activities	Bose *et al*., 2007
3	Diuretic and laxative activity	Bose at al., 2006
4	Wound healing activity	Mondal and Suresh,2012
5	Antimicrobial activity	Bose *et al*., 2007
6	Diuretic and antibacterial activity	Bose *et al*., 2007
7	Anticonvulsant activity	Jena *et al*., 2009
8	Anthelmentic activity	Bose *et al*., 2007
9	Anti-Arthritic activity	Chakraborty and Roy, 2010
10	Antifungal Activity	Khuntia and Mohanty, 2011

2.11.1.4 Proximate Composition of *Cleome rutidosperma*

Fresh leaves of *Cleome rutidosperma* contain per 100 g edible portion: water 81.0 g, energy 239 kJ (57 kcal), protein 5.5 g, fat 0.9 g, carbohydrate 10.1 g, fibre 1.7 g, Ca 454 mg, Mg 38 mg, P 59 mg, Fe 2.7 mg (Leung, *et al*., 1968).

2.11.2 *Senecio biafrae*

2.11.2.1 Scientific Classification of *Senecio biafrae*

Kingdom: Plantae

Order: Asterales

Family: Asteraceae

Genus: Senecio

Species: *S. biafrae*

(Source: Adebooye, 2004)

2.11.2.2 Vernacular Names of *Senecio biafrae*

IGBO: ota eke; YORUBA: akọ amùnimúyè or worowo or rọrọwọ.

Senecio biafrae (Oliv. & Hiern) also known as english spinach (see Plate 2.2), occurs naturally in the forest zone from Guinea to Uganda (Adebayo, 2009). It is cultivated on a small scale only, mainly in Nigeria and Cameroon (Adebayo, 2009). *Senecio biafrae* is a perennial climbing herb, with stem up to 3 m long, strongly branched; branches succulent, glabrous. Leaves alternate, simple or deeply pinnately lobed, more or less succulent; stipules absent; petiole 1–8(–10) cm long; blade triangular to hastate or with up to 3 lobes on each side, 5–15 cm × 3–14 cm, margin sparsely toothed, surface glossy. Inflorescence a narrowly campanulate head 9–12 mm × 3–5 mm, arranged in a terminal, compound, dense corymb; head many-flowered, homogamous; peduncle 5–11 cm long; involucral bracts 4–6. Flowers bisexual, tubular, 5-merous; corolla c. 6 mm long, pale yellow; stamens with fused anthers; ovary inferior, 1-celled, style bifid. Fruit a cylindrical, glabrous achene c. 3 mm long, black when ripe, with pappus consisting of long, white, silky hairs (Adebooye, 2004).



Plate 2.2: Picture of leaves of *Senecio biafrae*

2.11.2.3 Traditional Uses of *Senecio biafrae*

Senecio biafrae is known for its therapeutic virtues, notably in Nigeria where it is used in the treatment of diabetes or pulmonary defects (Burkill, 1985; Iwu, 1993; Adebayo, 2009). In Benin, Côte d'Ivoire, Congo, or Cameroon it is used in traditional medicine to treat many diseases such as bleeding from cuts, sore eyes, cough, heart troubles, rheumatic pain, or localized oedemas (Adebooye, 2004). In the Western and North-western Regions of Cameroon, ethnobotanical studies revealed its utilization in the treatment of cases of women infertility (Focho *et al*., 2009; Lienou *et al*., 2010). Among the Yoruba speaking people of south-western Nigeria, a leaf extract of worowo is used to stop bleeding from cuts or injury and in Sierra Leone and Cameroon a leaf extract is used to treat sore eyes (Focho *et al*., 2009). In Congo worowo is used to treat cough and heart troubles, as a tonic and to relieve rheumatic pain, prurient allergies and localized oedemas. In Congo it also has cultural uses in initiation and funeral rituals and in Yoruba culture it is associated with rituals to ward off smallpox (Jansen, 2004).

Fresh succulent leaves of worowo are used as a leafy vegetable in Sierra Leone, Ghana, Benin, Nigeria, Cameroon and Gabon (Adebayo, 2009). They are especially popular in south-western Nigeria (Adebayo, 2009). They are usually cooked with pepper, tomato and onions. In such dishes there is no need for meat or fish because of the excellent properties of the vegetable, reflected in the Yoruba proverb 'vegetable soup prepared with worowo does not need meat'. However, fish or meat may be added to the soup. In Sierra Leone, where it is called 'bologi', the leaves are eaten as a steamed vegetable in combination with okra and fish (Adebooye, 2004). They are first steamed in boiling water and later squeezed to remove the mucilage from the leaves (Adebooye, 2004). The

squeezing is followed by 2–3 rinses with cold water to remove the mucilage as completely as possible. An infusion of the leaves is taken as a drink (Jansen, 2004).Its antimicrobial, antihypertensive and anti-infertilityactivities had also been reported (Table 2.5).

2.11.2.4 Proximate Composition of *Senecio biafrae*

Leaves of *Senecio biafrae* contain per 100 g dry matter: crude protein 12.3 g, crude fibre 11.8 g, Ca 342 mg, P 39 mg, Fe 52 mg. Leaves of purple-stemmed types contain per 100 g dry matter: crude protein 11.6 g, crude fibre 10.5 g, Ca 320 mg, P 46 mg, Fe 53 mg (Adebooye, 2000).

Table 2.5: Biological Activities Reported for ***Senecio biafrae***

S/N	Activity	Reference
1	Antimicrobial activity	Gbadamosi and Okolosi, 2013
2	Antihypertensive	Gbolade, 2012
3	Anti-infertility	Telefoa *et al*., 2011

2.11.3 Phytochemical Composition of *Cleome rutidosperma* and *Senecio biafrae*.

Mondal *et al.,* (2009a) had reported from preliminary phytochemical screening, the presence of terpenoids, flavonoids, tannins and saponins in ethanolic extract of the root of *Cleome rutidosperma.* Also, Edeoga *et al.,* (2005) reported the quantitative phytoconstituents (alkaloids, phenols, tannin, flavonoid and saponin) for aqueous leaves extract of *Cleome rutidosperma*.

Similarly, Gbadamosi *et al.,* (2012) found from quantitative phytochemical assay of the ethanolic root extracts: alkaloids, tannins, βCarotene, saponins, flavonoids, steroids, cardenolides, anthraquinones and glycosides for *Senecio biafrae*. Also, Ajiboye *et al.,* (2013) reported alkaloids, saponin, tannin, phenol, flavonoids and glycosides from quantitative estimation of aqueous leaves extracts of *Senecio biafrae*.

2.12 Phytochemicals in Health

Phytochemicals are chemical compounds formed during the plant normal metabolic processes (Harborne, 1973). These chemicals are often referred to as "secondary metabolites". Secondary metabolites are generally produced in response to climatic stress, infection, defense against predators, for propagation etc. Most of the pharmacologically active natural products such as phenolic compounds, alkaloids, terpenoids etc are products of secondary metabolism(Okwu, 2010). They are present in a variety of plants and utilized as important components of food (fruits, seeds, herbs and vegetables) in both human and animals (Okwu, 2010). Phytochemicals of pharmacological significance are briefly discussed below.

2.12.1 Terpenoids

The terpenoids form a large and structurally diverse family of natural products derived from C5 isoprene units, joined in a head to tail fashion therefore these are also referred to as 'isoprenoids'(Dzubak *et al.*,2006). Typical structures contain carbon skeletons represented by (C5)n, and are classified as hemiterpenes (C5), monoterpenes (C10, eg; geraniol), sesquiterpenes (C15, eg; farnesol), diterpenes (C20, eg; geranylgeraniol), sesterterpenes (C25), triterpenes (C30, eg; Squalene) and tetraterpenes (C40, eg; phytene).

2.12.2 Steroids

The steroids are modified triterpenoids containing the tetracyclic ring system of lanosterol (Nah, *et al*., 2000). Cholesterol exemplifies the fundamental structure of steroids (Moreira *et al.,* 2008). Modifications on the side-chain result in a wide range of biologically important natural products. Steroids include a variety of bioactive compounds such as sterols, steroidal saponins, cardio active glycosides, bile acids, corticosteroids and mammalian sex hormones (Moreira *et al.*, 2008). Many natural steroids together with a considerable number of synthetic and semi-synthetic steroidal compounds are routinely employed in medicine (Kris-Etherton, *et al*.,2002)

2.12.3 Carotenoids

Carotenoids are the most common tetraterpenoids with wide distribution in plant kingdom (DellaPenna and Pogson, 2006). Carotenoids are generally derived from lycopene through the cyclization of end groups. Carotenoids not only impart bright colors to plants, but are also involved in photosynthesis and antioxidant (AO) defense mechanism (Fraser and Bramley, 2004).

2.12.4 Phenolic Compounds

They are compounds that have one or more hydroxyl groups attached directly to an aromatic ring (Abu-Amsha *et al.,* 1996). Because of the aromatic ring, the hydrogen of the phenolic hydroxyl is labile, which makes phenols weakly acidic (Figure 2.6). Polyphenols are compounds that have more than one phenolic hydroxyl group attached to one or more benzene rings. Phenolic compounds can be classified in a number of ways. Harborne and Simmonds classified these compounds into groups based on the number of carbons in the molecule (Harborne and Simmonds 1964).

(i) **Simple phenolics**: Simple phenolics are substituted phenols. Catechol (1,2-dihydroxybenzene), resorcinol (1,3-dihydroxybenzene) and phloroglucinol (1,3,5-trihydroxybenzene) etc are typical examples for simple phenolics.

(ii) **Phenolic acids**: Phenolic acids are important phenolic compounds of non flavonoid family, which constitute a large group of phenolic compounds in plants. Phenolic acids includes two main groups namely, hydroxybenzoic acid and hydroxycinnamic acid derivatives. Phenolic acids are reported to have a wide spectrum of pharmacological activities including AO, antimutagenic, antitumor and anticarcinogenic properties (Gerhauser, 2005; Stevenson and Hurst, 2007).

(iii**) Coumarins**: Coumarins have a C6-C3 skeleton, with an oxyge heterocycle as part of the C3-unit. There are numerous coumarins, many of which play a role in disease and pest resistance, as well as UV-tolerance. Umbelliferone is a popular coumarin used in enzyme assays (Prithiviraj, *et al*., 1997).

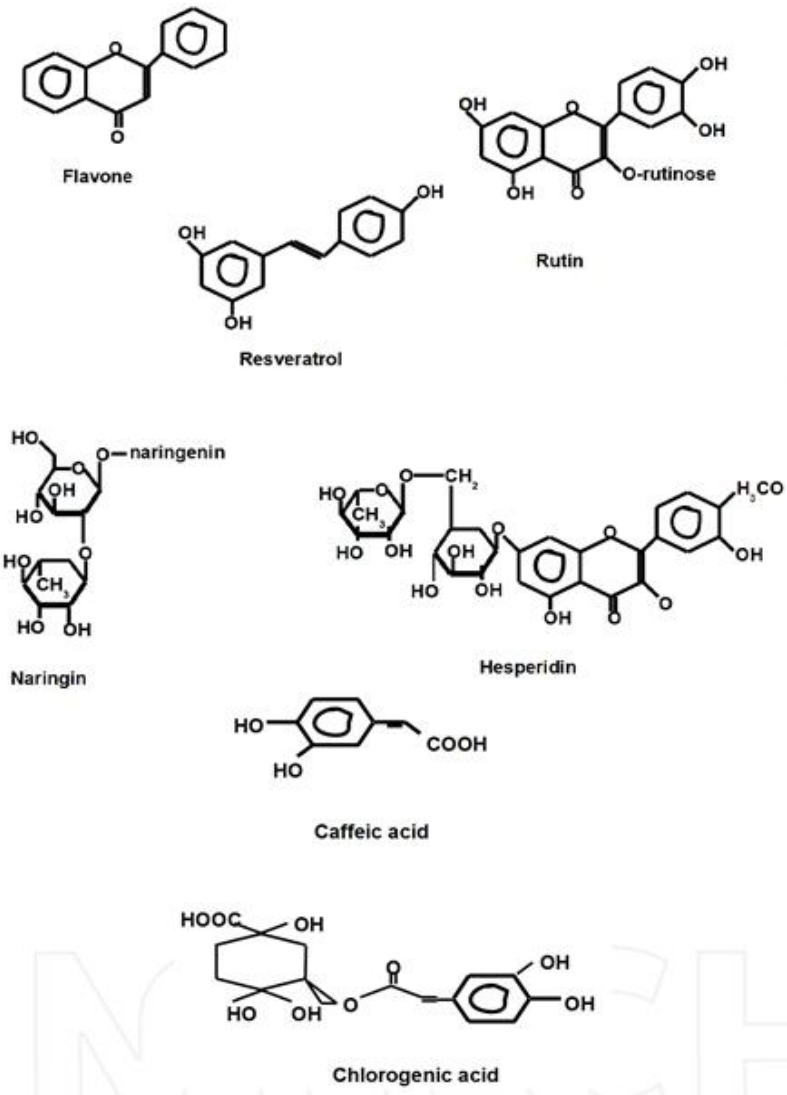


Figure 2.6: Basic structures of some pharmacologically important plant derived phenolics. Adapted from Gerhauser (2005).

(iv) **Flavonoids**: Flavonoids are C15 compounds all of which have the structure C6-C3-C6. Flavonoids may be grouped into different classes based on their general structure (Rice-Evans, 2001).

Typical flavonoids, such as flavanone, have a six-membered heterocycle. The A-ring originates from the condensation of three malonyl-CoA molecules, and the B-ring originates from p-coumaroyl-CoA (Figure. 2.7).

The heterocycle of flavanones also contains a ketone group with saturated C2-C3 carbon-carbon bond (eg; naringenin). Dihydroflavonols are known as flavanonols and often occur in association with tannins in heartwood (eg: Taxifolin or dihydroquercetin).

The heterocycle of flavones contains a ketone group, and has an unsaturated carbon-carbon bond. Flavones are common in angiosperms. The most widely distributed flavones in nature are kaempherol (5,7,4Ihydroxyflavone2), quercetin (5,7,3I,4I hydroxyflavone), and myricetin (5,7,3I,4I,5Ihydroxyflavone).

Flavonoids with pyrilium cation are refered to as anthocyanidins. Widely distributed, colored anthocyanins such as pelargonidin, cyanidin, delphinidin, petunidin, and malvidin are found as their aglycones. The most common anthocyanidin is cyanidin. These compounds are present in the vacuoles of colored plant tissues such as leaves or flower petals (Cornwell, *et al*., 2004).

(v) **Biflavonyls**: Biflavonyls have a C30 skeleton. They are dimers of flavones such as apigenin or methylated derivatives and are found in gymnosperms. The most familiar is ginkgetin from Ginkgo biloba.

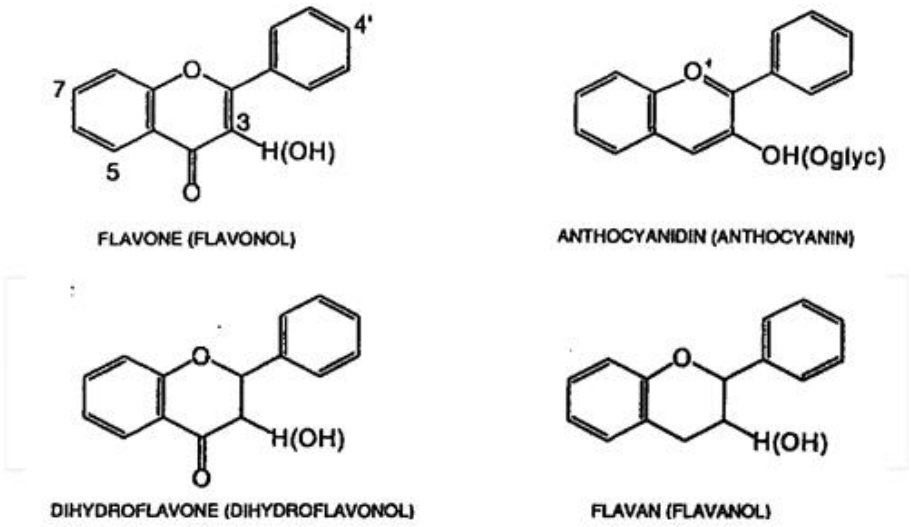


Figure 2.7: Basic structures of some pharmacologically important plant derived flavonoids. Adapted from Cornwell *et al*. (2004).

(vi) **Benzophenones and xanthones**: Benzophenones and xanthones have a C6-C1-C6 structure. Xanthones are yellow pigments in flowers.

(vii) **Stilbenes:** Stilbenes have a C6-C2-C6 structure and are localized in woody stems and seeds. Resveratrol, a well known stilbene found in grape is reported to have cardioprotecive effects.

(viii) **Benzoquinones, anthraquinones and naphthaquinones**

Ubiquinones, the benzoquinone with isoprenoid sidechains, are also known as Coenzyme Q and have a role in electron transport in the mitochondria (Szkopisńka, 2000). Naphthaquinones are rare (Budzianowski, 2000). Among the naphthaquinones juglone is relatively common and it is found in walnuts. Anthraquinone is the most widely distributed of the quinones in higher plants and fungi (Gill, 2001).

(ix) **Betacyanins**: They are unique compounds to the Centrospermae with absorption spectra that resembles anthocyanins, but they contain nitrogen eg; betanidin. Betacyanins are normally found as their glycosides.

(x**) Lignans**: Lignans are dimers or oligomers that result from the coupling of monolignols – p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, with coniferyl alcohol being the most common monolignol used in lignin biosynthesis. Lignans are present in ferns, gymnosperms and angiosperms. Most lignans are optically active, and typically only one enantiomer is found in a given species. Examples of lignans include (+)-pinoresinol, (+)-sesamin, and (–)-plicatic acid.

(xi) **Lignin**: Lignin, the second most abundant bio-polymer on earth (after cellulose) is a phenolic polymer. It provides structural support to plants, facilitates water transport through

the vascular tissue and acts as a physical barrier against insects and fungi (Kim, *et al*., 2003b).

(xii) **Tannins:** Tannins comprise a group of compounds with a wide diversity in structure and commonality in their ability to bind and precipitate proteins (Okuda, 2005) (Figure 2.8). The process of tanning animal skin to make leather utilize tannin and hence the name tanning (Khanbabaee and van Ree, 2001). Tannins can be classified under three groups: condensed tannins, hydrolysable tannins, and complex tannins. Hydrolysable tannins are further divided in to gallotannins (GT) and elligitannins (ET) (Khanbabaee and van Ree, 2001).

(a) **Gallotannins** (GTs): GTs are hydrolysable tannins with a polyol core (a compound with multiple hydroxyl groups) substituted with 2- 12 gallic acid residues. The most commonly found polyol is D-glucose, although some GT contain catechin and triterpenoid units as the core polyol.

(b) **Condensed tannins**: Condensed tannins are also referred to as proanthocyanidins (PAs). PA are mixtures of oligomers and polymers of flavan-3-ols, mainly formed through C4 C8 and/or C4 C6 (B type) or linked through an ether bond between C2 C7 (A type).

(c) **Ellagitannins** (ETs): ETs are also hydrolysable tannins derived from pentagalloylglucose, but unlike GT, they contain additional C-C bonds between adjacent galloyl moieties in the pentagalloylglucose molecule.

(d) **Complex tannins**. Complex tannins are defined as tannins in which a catechin unit is bound glycosidically to either a GT or an ET unit. As the name implies, the structure of these compounds can be very complex (eg; acutissimin A). Acutissimin A has been shown

to be a powerful inhibitor of DNA topoisomerase II, an enzyme required for the division of cancer cells, and a target for chemotherapeutic drugs (Quideau, *et al*., 2003).

(e) **Phlobaphenes**: Phlobaphenes are phenolic polymers present in floral organs of maize (Zea mays L.), Accumulation of phlobaphenes results in red pigmentation.

2.12.5 Amines and Alkaloids

2.12.5.1 **Amines**: Compounds containing nitrogen as a part of their side chain are generally referred to as amines. These molecules impart fishy odor and believed to act as insect attractants. Aliphatic polyamines such as putriscine (Tabur, 1984). Most of the plant aromatic amines are physiologically active. Mescalin (from *Lophophora willimasii*) is a potent hallucinogen. Noradrenaline, histamine and serotonin occurring in common plants are critical to brain metabolism (Brenner, 2002).

2.12.5.2 Alkaloids: Alkaloids are classically defined as the plant derived basic compounds with pharmacological effects and contain one or more nitrogen atoms (Figure 2.9). In practice most of the nitrogen containing secondary metabolites are considered as alkaloids unless they may be readily classified otherwise (Van der Heijden *et al.,* 2004). Alkaloids impart a wide spectrum of physiological effects in plants and animals. Alkaloids in plants serve as chemo protective, anti-herbivory agents or as growth regulator such as indole-3-acetic acid. Also, they have pharmacological applications as anesthetics and CNS stimulants (Madziga *et al*., 2010).

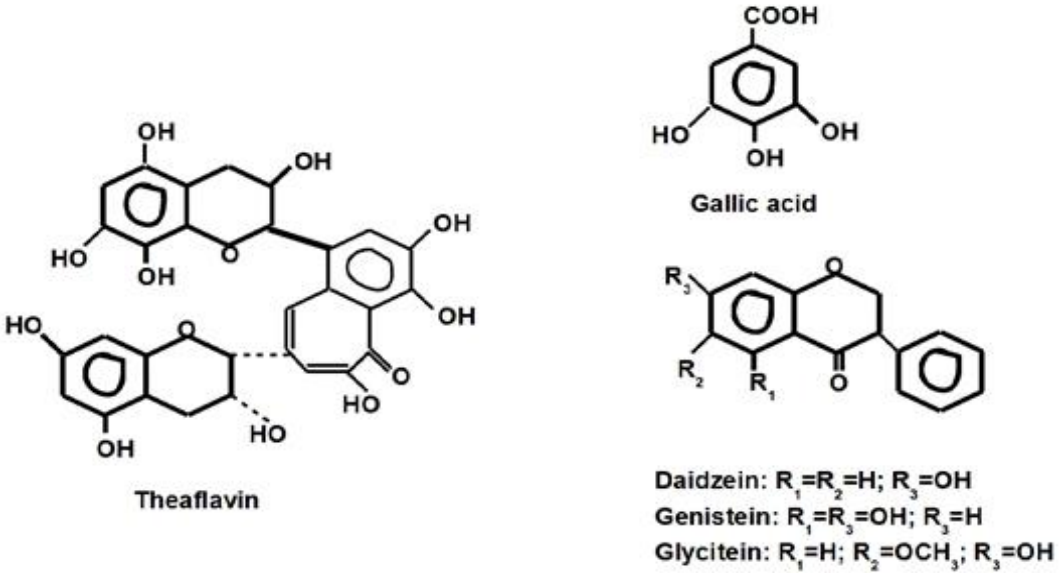


Figure 2.8: Basic structures of some pharmacologically important plant derived tannins. Adapted from Quideau *et al*., (2003).

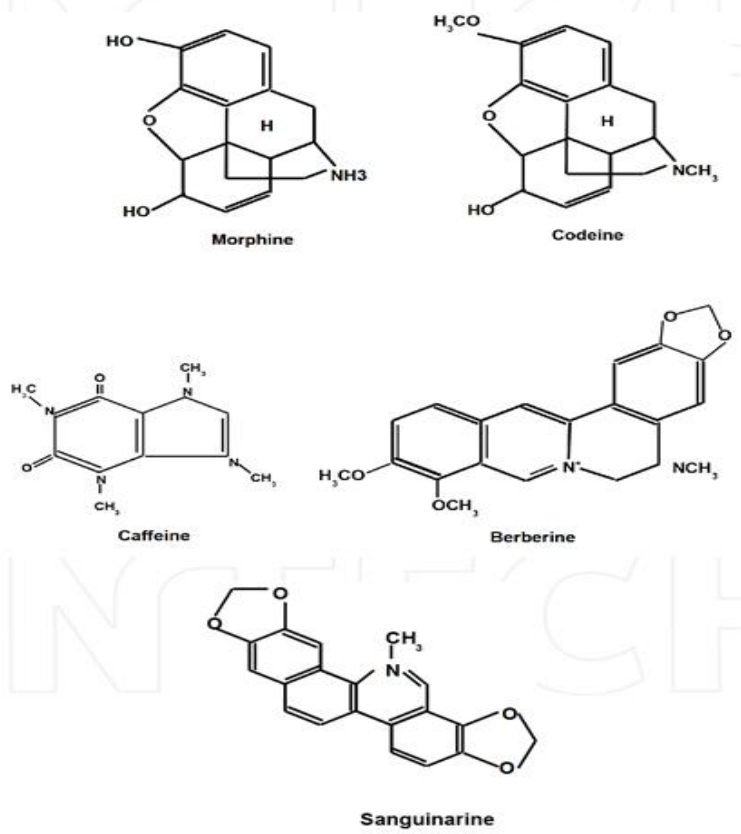


Figure 2.9: Basic structures of some pharmacologically important plant derived alkaloids. Adapted from Madziga *et al*. (2010).

2.13 Mechanism of Action of Phytochemicals

Different mechanisms of action of phytochemicals have been suggested. They may inhibit microorganisms (Kris-Etherton *et al*., 2002), interfere with some metabolic processes (Manson, 2003) or may modulate gene expression and signal transduction pathways (Surh, 2003). Plant extracts and essential oils may exhibit different modes of action against bacterial strains, such as interference with the phospholipids bilayer of the cell membrane which has as a consequence a permeability increase and loss of cellular constituents, damage of the enzymes involved in the production of cellular energy and synthesis of structural components, and destruction or inactivation of genetic material. In general, the mechanism of action is considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport, and coagulation of cell contents (Kotzekidou *et al*., 2008).

2.14 Phytochemicals as Antioxidants

Natural products and secondary metabolites formed by living systems, notably from plant origin, have shown great potential in treating human diseases such as cancer (Vickers, 2000), coronary heart diseases (Ralph and Provan, 2000), diabetes and infectious diseases (Lai *et al.*, 2010). Phytochemical screening is an important step in the chemical and pharmacological study of a medicinal plant (Vijayalakshmi and Ravindhran, 2012).

There are several standard methods used for the phytochemical screening of medicinal plants as described for alkaloids, steroids, phenols, flavonoids, saponins, glycosides and tannins (Mohammad *et al.*, 2011).

Antioxidants are secondary metabolites found to occur naturally in plants such as fruits and vegetables (Hollman, 2001). An antioxidant can be defined in simple terms as a

compound that inhibits or prevents oxidation of a susceptible substrate (Hollman, 2001). Plants produce a very impressive array of antioxidant compounds that includes carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols and tocotrienols to prevent oxidation of the susceptible substrate (Alam, 2009). Common antioxidant includes vitamin A, vitamin C, vitamin E, carotenoids, lutein and -carotene (Dahanukar *et al.,* 2000). These plant based dietary antioxidants are believed to have an important role in the maintenance of human health because our endogenous antioxidants provide insufficient protection against the constant and unavoidable challenge of reactive oxygen species (Fridovich, 1998).

Metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress (Erhola *et al.,* 1997). Oxidative stress plays a major role in diabetes (Maritim *et al.,* 2003). Supplementation of dietary antioxidants reduces the risk of oxidative damage and thus arising a necessity to extract these antioxidants from the plant matrices (Urisni *et al.,* 1994).

DPPH assay (1, 1 diphenyl 2, picryl hydrazyl), nitric oxide scavenging activity, superoxide scavenging activity, lipid peroxidation assay, reducing power ability, FRAP method (Ferric Reducing Ability of Plasma), cytochrome C test and xanthin oxidase method are the various *in vitro* models which are used to evaluate the potency of the plant extract to scavenge free radicals (Wang and Curtis, 2006). Hence, in this study phytochemical screening was carried out to confirm the presence and quantify the phytoconstituents in the leaves of *Cleome rutidosperma* and roots of *Senecio biafrae* using various solvents based on polarity.

CHAPTER THREE

Materials and Methods

3.1 Materials

3.1.1 Chemicals and Reagents

Adenosine 5′-triphosphate (ATP), L-Glutathione (reduced), L-Glutathione (oxidized), (−)-Epinephrine, Bovine serum albumin, Acetic acid, 2-Thiobarbituric acid, Ammonium molybdate, β-Nicotinamide adenine dinucleotide phosphate, reduced tetra(cyclohexylammonium) salt, 1-Chloro-2,4-dinitrobenzene, 5,5′-Dithiobis(2-nitrobenzoic acid), D-Glucose 6-phosphate sodium salt, Streptozocin, Alpha amylase, 3,5-dinitrosalicylic acid, Alpha glucosidase, Glucose 6-phosphatase, 4-Nitrophenyl α-D-glucopyranoside, Glucose 6-phosphatase, Acarbose, Sodium citrate; Citric acid; Petroleum ether; Acetone; Methanol; nButanol; Ethanol; Hexane; Chloroform; HCl; H_2SO_4; Dodecyl sulphate sodium (SDS); Potassium dihydrogen phosphate (KH_2PO_4); Phosphoric acid; Potassium chloride; Sucrose; Trichloroacetic acid (TCA) were all products of Sigma-Aldrich (St Louis, MO, USA). All other chemicals and reagents used in the present study were of analytical grade.

Randox kits obtained from Randox Laboratories Limited, Ardmore, Co Antrim, UK, were used for the analyses of; serum transaminases (ALT and AST), alkaline phosphatase, albumin, total protein and bilirubin (total and direct), Serum total triacylglycerols concentrations, total cholesterol level, HDL-cholesterol and glucose concentrations.

3.1.2 Equipment

Buchi rotary evaporator R-144 (Buchi Labortechnik AG, Switzerland), Buchi water bath (Grant JB Series), Heraeus Labofuge 300 Centrifuge, JENWAY 6405 UV/Vis Spectrophotometer, Orbital shaker (Balart Products, USA), Glucometer (Accu-chek® Active, Roche diagnostic, Mannheim, Germany) and pH Meter- Elico Ltd.

3.1.3 Plant Materials

The plants (leaves of Spider plant and roots of English spinach) were collected from Abraka in Delta State, Nigeria and Akoko in Ondo State, Nigeria, respectively. They were identified at the Herbarium Unit, Department of Plant Science, University of Benin, Benin City, Edo State-Nigeria, where voucher specimens were deposited with numbers: UBHc0148 and UBHs0149.

3.1.4 Animals

Male Wistar Albino rats weighing 120-200g and mice weighing 25-35g were obtained from National Veterinary Research Institute (NVRI) Vom, Jos, Plateau State, Nigeria. The animals were allowed 3 weeks of acclimatization before commencement of the experiment. They were fed on standard laboratory diet (Vital Feed Nig. Ltd, Jos, Nigeria) and water *ad libitum* throughout the duration of the experiments.

3.2. METHODOLOGY

3.2.1 Experimental design

The experimental design involved the initial collection and identification of the plants (*Cleome rutidosperma* and *Senecio biafrae*). They were sequentially extracted with four different solvents; petroleum ether, acetone, ethanol and water. Acute

antihyperglycemic effects of the extracts were evaluated in streptozotocin (STZ)-induced diabetic mice. Based on results of this antihyperglycemic effects of the extracts, the most active extract (aqueous extract) of each plant was used for long term evaluation of the antihyperglycemic effects in STZ-induced diabetic rats. Also, the aqueous extract of each plant was fractionated in stages which were aimed at partially purifying the extracts as shown in Figure 3.1.

3.2.1.1 Segments of Experimental Design

The experiment was divided into three distinct segments with activities as shown in Figure 3.2.

3.2.2. Segment 1

Treatment and Extraction of Plant Samples

The plant samples were washed with distilled water and air-dried at room temperature, cut into small pieces and pulverized into fine powder using pestle and mortar. They were sequentially extracted with solvents of increasing polarity (petroleum ether, acetone, ethanol and water), using the method of Arokiyaraj *et al*., (2009), and Jeyaseelan *et al*., (2011). Measured powder of the plant (50g) was initially soaked in 200 ml of petroleum ether in airtight conical flask with daily shaking for three days at room temperature and were first filtered through doubled layered muslin cloth and then filtered through Whatman No 1filter paper and the filtrates were collected into airtight bottles. The petroleum ether was removed from the filtrate under reduced pressure using rotary evaporator at low temperature. The residue was dried and used for the acetone extraction followed by ethanol and water using procedure similar to the one carried out for the petroleum ether extraction. The crude extracts were stored in refrigerator at 4°C until used.

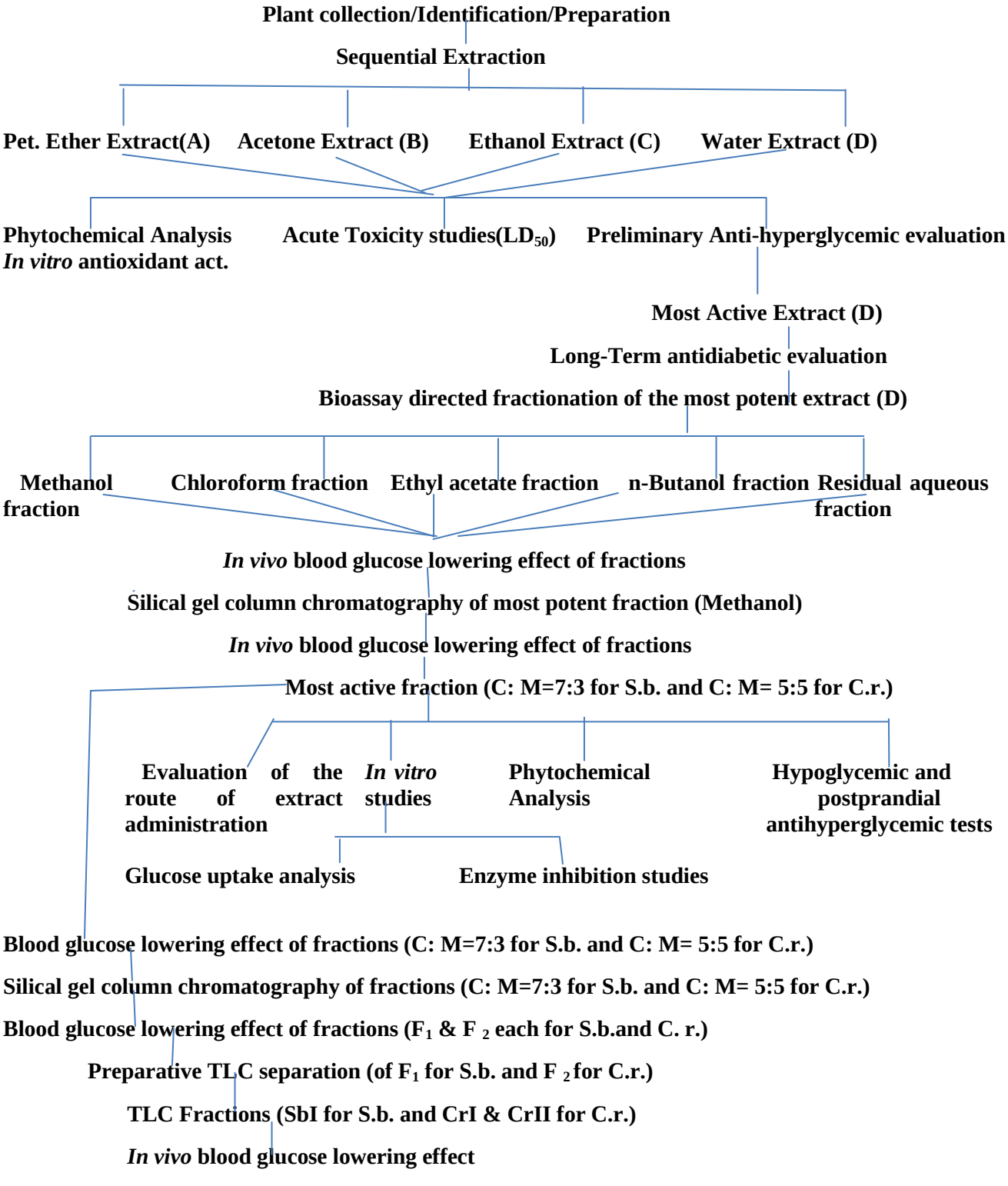

Plant collection/Identification/Preparation
Sequential Extraction
Pet. Ether Extract(A)
Acetone Extract (B)
Ethanol Extract (C)
Water Extract (D)
Phytochemical Analysis
In vitro antioxidant act.
Acute Toxicity studies(LD50)
Preliminary Anti-hyperglycemic evaluation
Most Active Extract (D)
Long-Term antidiabetic evaluation
Bioassay directed fractionation of the most potent extract (D)
Methanol fraction
Chloroform fraction
Ethyl acetate fraction
n-Butanol fraction
Residual aqueous fraction
In vivo blood glucose lowering effect of fractions
Silical gel column chromatography of most potent fraction (Methanol)
In vivo blood glucose lowering effect of fractions
Most active fraction (C: M=7:3 for S.b. and C: M= 5:5 for C.r.)
Evaluation of the route of extract administration
In vitro studies
Phytochemical Analysis
Hypoglycemic and postprandial antihyperglycemic tests
Glucose uptake analysis
Enzyme inhibition studies
Blood glucose lowering effect of fractions (C: M=7:3 for S.b. and C: M= 5:5 for C.r.)
Silical gel column chromatography of fractions (C: M=7:3 for S.b. and C: M= 5:5 for C.r.)
Blood glucose lowering effect of fractions (F1 & F 2 each for S.b.and C. r.)
Preparative TLC separation (of F1 for S.b. and F 2 for C.r.)
TLC Fractions (SbI for S.b. and CrI & CrII for C.r.)
In vivo blood glucose lowering effect

Figure 3.1: Experimental Design

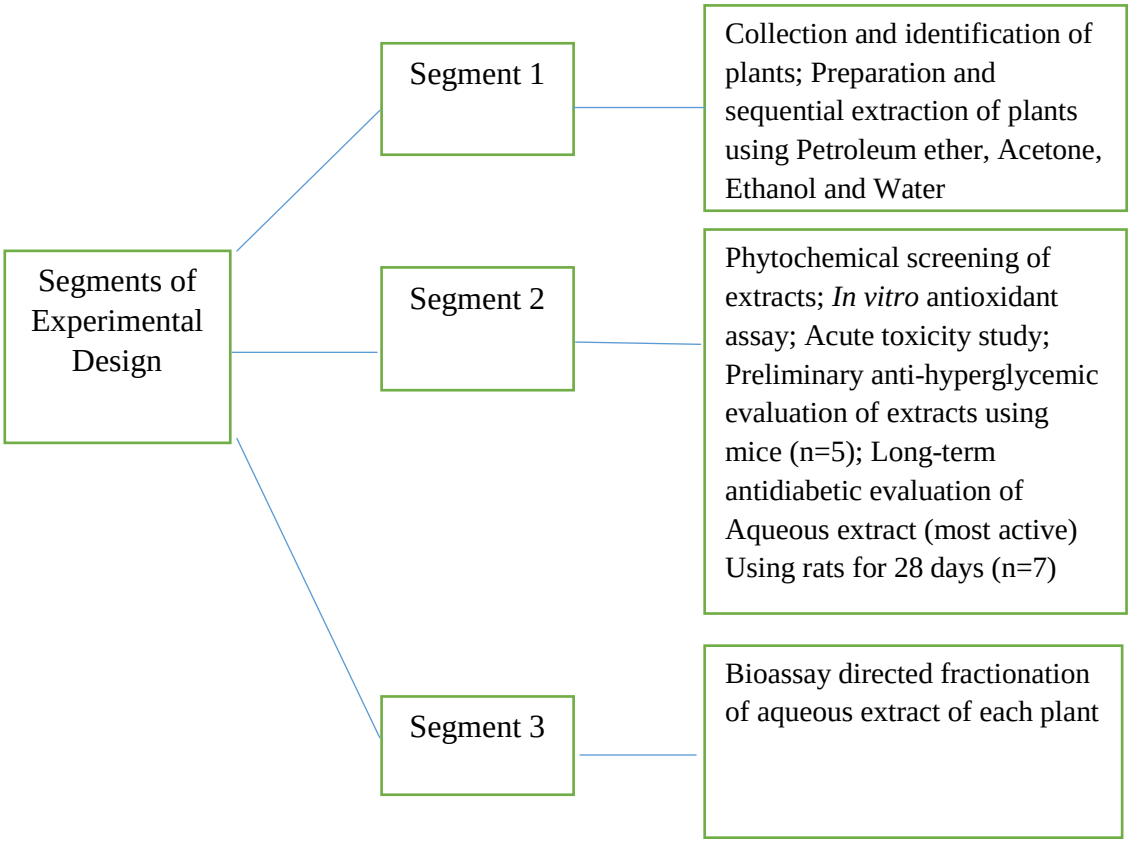


Figure 3.2: Segments of Experimental Design

3.2.3 Segment 2

3.2.3.1 Induction of Experimental Diabetes

Streptozotocin was dissolved in 10 mM citrate buffer, pH 4.5 (Archana *et al*., 2001). The mice/rats (diabetic control and test mice/rats) were injected intra-peritoneally with portions (1μL/g) of this solution at a dose of 60mg per kg body weight after an overnight fast. 3 days after induction of diabetes, the mice/rats with plasma glucose more than 200 mg/dl were considered as diabetic mice/rats and were allowed for 2 weeks diabetic stabilization before being used for the experiment.

3.2.3.2 Oral Acute Toxicity Studies (LD_{50})

Acute toxicity studies of the plants extracts were conducted to ascertain the safe doses of the plant extracts to be administered to animals in this study. LD_{50} determination was done by the method of Lorke (1983) using 13 rats. The evaluation was done in two phases; in phase one, rats were divided into 3 groups of 3 rats each and were treated with 10, 100 and 1000 mg extract/kg body weight orally. The rats were observed for clinical signs and symptoms of toxicity within 24 h. Based on the results of phase one, the second phase was done which consisted of 4 rats divided into 4 groups of 1 rat each and were treated with 1200, 1600, 2900 and 5000 mg extract/kg body weight orally. Clinical signs and symptoms of toxic effects and mortality were then observed for seven days. The median Lethal Dose (LD_{50}) was calculated using the second phase.

3.2.3.3 Intra-Peritoneal Acute Toxicity Studies (LD_{50})

For the intra-peritoneal acute toxicity studies, the procedure discussed in 3.2.3.2 for oral acute toxicity study was used by intra-peritoneally administering fractions of both plants.

3.2.3.4 Quantitative Phytochemical Analysis

The different solvent extracts were subjected to quantitative phytochemical tests to determine the secondary metabolites present therein using standard procedures. Alkaloid and Flavonoids determinations were done using Harborne (1973) method; Tannin determination by Van-Burden and Robinson (1981) method; determination of Saponin by Obdoni and Ochuko (2001) method; evaluation of Glycosides was by the method described by El- Olemy *et al*., (1994) and estimation of Steroids was done by the method of Evans (1996).

3.2.3.5 *In Vitro* Antioxidant Properties of Extracts

The *in vitro* antioxidant activities of the extracts were assayed by standard methods. The ferric thiocyanate (FTC) assay was carried out as described by Osawa and Namiki (1981); Thiobarbituric acid (TBA) assay by the method of Ottolenghi (1959); Reducing power was done according to the method described by Oyaizu (1986), while the DPPH radical scavenging assay was by the method of Liyana-Pathirana and Shahidi (2005).

3.2.3.6 Evaluation of Antihyperglycemic Activity of the Extracts

The assay of the antihyperglycemic activity of the four extracts of plant involved administration of single dose of 500mg/kg body weight of the extracts using mice weighing 25 to 35g. Thirty five (35) mice (30 diabetic mice and 5 normal mice) were randomly allocated to 7 groups of 5 mice each as follows:

Group I: Normal untreated mice.

Group II: Diabetic control mice.

Group III: Diabetic + Glibenclamide (600 µg/kg of body weight).

Group IV- VII: Diabetic mice given 500mg/kg body weight of the different extracts of *Cleome rutidosperma*.

The groupings above were repeated for the extracts of the second plant (*Senecio biafrae*). Fasting blood glucose concentration was first determined in overnight fasted mice by the enzymatic glucose oxidase method using a commercial glucometer (Accu-chek® Active, Roche diagnostic, Mannheim, Germany), following which the plant extracts were administered orally using gastric cannula. Blood glucose values were then estimated hourly for 6 h. The control group (II), received distilled water in place of the extract. Similarly, a standard antidiabetic drug (Glibenclamide 600µg/kg) was administered orally to the group III animals and glucose determined at the same time intervals for the same duration according to the procedure of Aderibigbe *et al*. (2001).

3.2.3.7 Antidiabetic Evaluation of Aqueous Plant Extracts

In order to investigate and compare the antidiabetic potential and sub-chronic toxicity of the two aqueous extracts of the plants, a total of 77 rats (56 diabetic rats and 21 normal rats) were divided into eleven groups of 7 rats each as follows:

Group A: Normal untreated rats

Group B: Normal untreated rats given 500mg/kg body weight of *Cleome rutidosperma* extract

Group C: Normal untreated rats given 500mg/kg body weight of *Senecio biafrae* extract

Group D: Diabetic control rats

Group E-G: Diabetic rats given 125 mg/kg, 250 mg/kg and 500 mg/kg body weight respectively of aqueous extract of *Cleome rutidosperma*.

Group H-J: Diabetic rats given 125 mg/kg, 250 mg/kg and 500 mg/kg body weight respectively of aqueous extracts of *Senecio biafrae*.

Group K: Diabetic rats given reference drug, glibenclamide (600 µg/kg of body weight).

Rats in the control group were given distilled water, while those in the treatment groups were given three different doses as stated above via the oral route for 28 days. Water and feed intake were measured daily while body weights of the rats, FBG and PCV were determined weekly. At the end of the study (day 29), the rats were euthanized in an airtight glass chamber saturated with chloroform and after opening up the rats surgically, blood samples were collected for the analyses. The liver and kidney were carefully excised, blotted and weighed. One gram (1 g) and half of a gram (0.5g) respectively of the liver and kidney were homogenized in 10 ml and 5ml respectively of 0.01M phosphate buffer, pH 7.4, and centrifuged at 3,000xg to collect supernatant; which was used as organ extract.

Glucose Determination (Barham and Trinder, 1972**).**

Principle

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase (GOD). The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form red-violet quinoneimine dye as an indicator

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{POD}} \text{quinoneimine} + 4H_2O$$

Procedure

Sample and standard (40µl each) were placed into a labeled test tube. Buffer and GOD-PAP reagent (400 µl) each was added. It was mixed and incubated for 25 minutes at 25OC. The absorbance was measured against the reagent blank at 500nm.

Estimation of biochemical indices of liver function

i. Assay of Aspartate aminotransferase-AST (Reitman and Frankel, 1957)

Principle:

$$\alpha\text{- oxoglutarate + L- aspartate} \xrightarrow{\text{GOT}} \text{L- glutamate + oxaloacetate}$$

AST is measured by monitoring the concentration of oxaloacetate hydrazone formed with 2,4-dinitrophenylhydrazine at 540 nm and 37^0C.

Procedure

Buffered substrate (0.5ml) was added to 0.1ml of serum and placed in a water bath at 37°C. To the blank tubes, 0.1ml distilled water was added instead of serum. Exactly an hour later, 2 drops of aniline citrate reagent and 0.5ml of DNPH reagent were added and kept at room temperature for 20 min. Finally, 5.0ml 0.4N sodium hydroxide was added. A set of standards was also treated in the same manner and read at 546nm after 5 min.

The results were expressed as IU/L of serum.

ii. Assay of Alanine aminotransferase (ALT)

Serum alanine aminotransferase was assayed by using Randox diagnostic kit based on the method of Reitman and Frankel (1957).

Principle

$$\text{α- oxoglutarate + L- alanine} \xrightarrow{\text{GOT}} \text{L- glutamate + pyruvate}$$

Alanine aminotransferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenylhydrazine at 540 nm and 37^0C.

Procedure

Procedure was same as that used for the assay of aspartate transaminase except the incubation time which was reduced to 30 min (60 min for AST).

The results were expressed as IU/L of serum.

iii. Assay of Alkaline phosphatase (ALP)

Serum alkaline phosphatase was assayed by using Randox diagnostic kit based on Rec GSCC (1972).

Principle

$$\text{p-nitrophenylphosphate + } H_2O \xrightarrow{\text{ALP}} \text{phosphate + p-nitrophenol}$$

Alkaline phosphatase is measured by monitoring the concentration of phosphate hydrazone formed with 2,4-dinitrophenylhydrazine at 405 nm and 37^0C.

Procedure

In this method, the reagent composition was buffer (Diethanolamine buffer = 1 mol/l, pH = 9.8 and MgCI2 = 0.5 mmol/l) and substrate (p-nitrophenylphosphate =10 mmol/l). The reagent was prepared to determine the alkaline phosphatase activity. For this purpose 10 ml of the buffer was mixed with 10 ml of substrate, then 1.0 ml of this reagent and 0.02 ml of sample were mixed thoroughly and the absorbance was taken at 405 nm.

iv. Assay for total protein

The method of Tietz (1995) was used.

Principle

Cupric ions, in an alkaline medium interact with protein peptide bonds resulting in the formation of a coloured complex.

Procedure

Blank reagent, standard and plasma (0.02ml each) were placed into labeled test tubes. Biuret reagent (1.0ml) was added to each. It was mixed and incubated for 30min.at $25^{O}C$. The absorbance was measured against reagent blank.

v. Estimation of albumin (Doumas *et al*., 1971)

Principle

The measurement of plasma albumin is based on the quantitative binding of the indicator 3,3',5,5'- tetrabromo-m cresol sulphonaphthalein (bromocresol green, BCG). The albumin-BCG-complex absorbs maximally at 578nm, the absorbance being directly proportional to the concentration of albumin in the sample.

Procedure

Albumin reagent (Acetate buffer-100mmol / L; Bromocresol green: 0.27mmol / l; pH 4.1), 1ml was added into all tubes. Ten microliter (10 µl) of water was added into the blank tube, then 10 µl of standard / serum was added into the appropriate tubes. The tube were mixed thoroughly and allowed to stand for 1 minute at room temperature. Absorbance of the standard and the sample were read at 630 nm against the blank.

Values were expressed as g/dL of serum.

vi. Estimation of bilirubin (Jendrassik and Grof, 1938)

Principle

Colorimetric method based on that described by Jendrassik and Grof (1938). Direct (Conjugated) bilirubin reacts with diazotized sulphanilic acid in alkaline medium to form a blue coloured complex. Total bilirubin is determined in the presence of caffeine, which releases albumin bound bilirubin, by the reaction with diazotized sulphanilic acid.

Procedure

For total bilirubin; exactly, 0.05ml of reagent 2 made up of sodium nitrate was added into a clean test tube containing 0.2ml of reagent 1 which is made up of sulphanilic acid and hydrochloric acid and 1.0ml of reagent 3 made up of caffeine and sodium benzoate was added followed by 0.2ml of serum, mixed and allowed to stand for 10 minutes at room temperature. 1mL of reagent 4 made up of tartarate, and sodium hydroxide was also added, mixed and allowed to stand for 10 minutes at room temperature. The absorbance of the sample against sample blank (ATB) was read at 560 nm.

For direct Bilirubin; 0.05ml of reagent 2 was added into a clean test tube containing 0.2ml of reagent 1, then 2.0 ml of sodium chloride (9 g/l) was added followed by 0.2ml of serum, mixed and allowed to stand for 5 minutes at room temperature. The absorbance was read against the sample blank (ADB) at 530 nm.

Calculations

Direct bilirubin (mg/dl) = 14.4 × ADB

Total bilirubin (mg/dl) = 10.8 × ATB

ATB = Absorbance of total bilirubin, ADB = Absorbance of direct bilirubin

Estimation of biochemical indices of renal function

i. Estimation of Urea (Weatherburn, 1967)

Principle

Urea in serum is hydrolyzed to ammonia in the presence of urease. The ammonia is then measured photometrically by Berthelot's reaction.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

NH_3 + Hypochlorite + Phenol ⟶ Indophenol (blue compound)

Procedure

Sample, standard and reagent blank (10µl each) were placed into a labeled test tube. One hundred microliter of Reagent R1 (EDTA 116mmol/L, Sodium nitroprusside 6mmol/L and Urease 1g/l) was added to each tube. It was immediately mixed and incubated for 10 minutes at 37°C. 2.50ml of reagents R2 (Phenol, 120mmol/L) and R3 (Sodium hypochlorite, 27 mmol/l and Sodium hydroxide, 0.14N) were added to each test tube and mixed immediately, after which it was incubated at 37 ^{O}C for 15min. The absorbance of sample and standard was measured against reagent blank at 546nm.

Values were expressed as mg/dl

ii. **Estimation of Creatinine (Bartels *et al.*, 1972)**

Principle

Creatinine in alkaline solution reacts with picric acid to form a coloured complex. The amount of complex formed is directly proportional to the creatinine concentration.

Procedure

The sample, standard and blank (0.40ml) each was placed in labeled tests tubes. The reagents (4ml of picric and NaOH), was added to each tube. It was mixed and the absorbance was read at 492nm after 30 secs.

Assessment of lipid profile

i. Estimation of total cholesterol (Trinder, 1969)

Principle

The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.

$$\text{Cholesterol ester} + H_2O \xrightarrow[\text{Esterase}]{\text{Cholesterol}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow[\text{Esterase}]{\text{Cholesterol}} \text{Cholestene-3-one} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4–Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O$$

Procedure

In the assay of total cholesterol, 10µl each of serum and working standard were incubated with 1ml of reagent for 5min at 37°C and the absorbance at 500nm was measured against a reagent blank. For reagent blank, 10µl of distilled water was added to 1ml of working reagent. Concentration of cholesterol in serum samples was calculated as:

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Conc. of standard} = \text{mg/dL}$$

ii. Estimation of triacylglycerols (Tietz, 1990)

Principle

Triacylglycerol is determined after enzymatic hydrolysis with lipases. The indicator quinoneimine is formed from hydrogen-peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.

$$\text{Triacylglycerols} + H_2O \xrightarrow{\text{lipase}} \text{glycerol} + \text{fatty acids.}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol–3-Phosphate} + O_2 \xrightarrow{\text{GPO}} \text{dihydroxyacetone} + \text{phosphate} + H_2O_2$$

$$2H_2O_2 + \text{4–Aminophenazone} + \text{4 chlorophenol} \xrightarrow{\text{POD}} \text{quinoneimine} + HCl + 4H_2O$$

Procedure

For the estimation of triglycerides, 10µl each of serum and working standard were mixed with 1ml of working reagent and kept at room temperature for 15min and the absorbance was read at 500nm against the reagent blank. For reagent blank, 10µl of distilled water was added to 1ml of working reagent. Concentration of triglycerides in serum samples was calculated as:

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Standard Conc.(mg/dl)} = \text{mg/dL}$$

iii. Estimation of serum HDL-cholesterol (Lopes-Virella *et al*., 1977)

Principle

Low density lipoproteins (LDL and VLDL) and chylomicron fractions are precipitated quantitatively by the addition of phosphotungstic acid in the presence of

magnesium ions. After centrifugation, the cholesterol concentration in the HDL (high density lipoprotein) fraction, which remains in the supernatant, is determined.

Procedure

In the assay of HDL-cholesterol, 500µl of serum was added to 50µl of precipitating reagent and kept at room temperature for 10min. Later, it was centrifuged at 3000 rpm for 10 min. The clear supernatant was used for HDL-cholesterol estimation. 20µl of the supernatant and 10µl of standard were incubated with 1ml of the reagent for 5 min at 37°C and the absorbance at 510nm was measured against the reagent blank. For reagent blank, 10µl of distilled water was added to 1ml of the reagent. The amount of HDL-cholesterol in the samples was calculated as:

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{C standard} \times \text{Sample dilution factor} = \text{mg/dL}$$

iv. Estimation of LDL-cholesterol

(Friedwald *et al*., 1972)

This was calculated using the formula:

LDL cholesterol = Total cholesterol – Triacylglycerols/5– HDL cholesterol

The values were expressed as mg/dL of serum

Measurement of carbohydrate metabolizing enzymes

i. Assay of Glucose–6–Phosphatase (Baginsky *et al*., 1992)

Principle

Glucose–6–phosphatase was assayed by estimation of inorganic phosphate liberated from glucose–6–phosphate.

Procedure

On a pre-cooled watch glass, 1g of liver was chopped into small pieces and homogenized in a Potter-Elvehjem homogenizer for 2min with ice-cold sucrose (15ml, 250mM). The homogenate was centrifuged at 2°C for 30 min at 9000 xg.

Four tubes labeled as "sample", "control", "blank" and "standard" were taken. To each tube was added 0.1ml of sucrose/EDTA buffer (0.25M/mM, pH 7), 0.1ml of glucose–6–phosphate (100mM) and 0.1ml of imidazol buffer (100mM, pH 6.5). This was followed by addition of 0.1ml of liver supernatant to the "sample" tube, 0.1ml of distillated water to the "blank" tube and 0.1ml of Na_2HPO_4 (1.5mM) to the "standard" tube. All the tubes were incubated at 37°C for 1min and the enzyme activity was terminated by adding 2ml TCA/ascorbate (10%/2% : w/v). Liver supernatant (0.1 ml) was added to the "control" tube only. It was followed by centrifugation of all tubes at 1000 xg for 10 min. To 1ml of the clear supernatant were added 0.5ml ammonium molybdate (1%) and 1ml of sodium arsenite/sodium citrate (2%/2%: w/v). The tubes were then allowed to stand for a minute at room temperature and absorbance was read at 700nm. The amount of inorganic phosphate liberated by the enzyme was calculated by comparing with the absorbance values of the standard. Enzyme activity was expressed as μ-mole of P_i liberated/min/mg protein. Protein content of the samples was estimated by the method of Lowry *et al*. (1951).

ii. Assay of Hepatic Glucokinase Activity (Brandstrup *et al.*, 1957)

Principle

Glucokinase was assayed by the estimation of glucose-6-phosphate formed from glucose.

Procedure

The liver (1g) was homogenized in 0.1M Tris HCl buffer, pH 7.4, at 4°C in a Potter-Elvehjam homogenizer for 3min and the homogenate was centrifuged at 1000 xg for 10min and the supernatant was used for this assay. The reaction mixture in a total volume of 5.3ml contained 1ml of glucose solution, 0.5ml of ATP solution, 0.1ml of magnesium chloride solution, 0.4ml of potassium dihydrogen phosphate solution, 0.4ml of potassium chloride solution, 0.4ml of sodium fluoride solution and 2.5ml of Tris-HCl buffer. The mixture was pre-incubated at 37°C for 5min. The reaction was initiated by the addition of 2ml of supernatant. The reaction mixture (1ml) was immediately removed to tubes containing 1ml of 10% TCA which was considered as zero time. A second aliquot (1ml) was removed after 30 min incubation at 37°C. The protein precipitate was removed by centrifugation and the residual glucose in the supernatant was estimated. Protein content of the samples was estimated by the method of Lowry *et al*. (1951). The enzyme activity was expressed as μ-moles of glucose phosphorylated/min/g protein.

iii. Estimation of Protein (Lowry *et al*., 1951)

Principle

The blue colour developed by the reduction of phosphomolybdic-phosphotungstic components in Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present

in the protein and the colour developed by biuret reaction of the protein with the alkaline cupric tartarate are measured at 660nm.

Procedure

The enzyme preparation from each assay was used as the protein source for estimation. Standards corresponding to 0.2 to 1.2ml (20-120μg) were pipetted out into a series of test tubes and 10ml of the enzyme extract were used for the estimation. The volume was made up to 1.0ml in all tubes with distilled water. To this, 5.0ml of solution 3 were added, mixed well and incubated at 37°C for 3 minutes. Then 0.5ml of Folin-Ciocalteau reagent was added, mixed well and incubated at 37°C for 3 minutes. The blue colour developed was read at 660nm in a spectrophotometer.

***Assay of oxidative stress markers*:**

Oxidative stress markers were assayed in the supernatant fraction of the liver and kidney homogenates (as prepared in 3.2.3.7.) of each rat according to the following methods:

i. Assay of Glutathione Reductase (GR)

Glutathione reductase was assayed by the procedure adopted by David and Richard (1983).

Principle

Glutathione reductase catalyzes the conversion of oxidized glutathione to reduced glutathione employing, NADPH as substrate. The amount of NADPH utilized is a direct measure of enzyme activity.

Procedure

The assay system contained 1ml of phosphate buffer, 0.1ml of EDTA, 0.1ml of sodium azide, 0.1ml of oxidized glutathione and 0.1ml of enzyme source(as prepared in 3.2.3.7.) and the volume was made up to 2ml with distilled water. The tubes were incubated at 37^0C for 3 minutes and 0.1ml of NADPH was added. The absorbance was read at 340nm in a spectrophotometer (Genesys 10UV) at every 15 seconds interval for 2-3 minutes. For each series of measurements, controls were set up that contained water instead of oxidized glutathione. The enzyme activity was expressed as μmoles of NADPH oxidized/minute/ mg of protein.

ii. Assay of Glutathione S-Transferase (GST)

Glutathione S-transferase was assessed by the method of Habig *et al*. (1974).

Principle

Glutathione S-transferase conjugates GSH with CDNB and the extent of conjugation is used as a measure of enzyme activity from the proportionate change in the absorption at 340 nm.

Procedure

Preparation of Enzyme Extract

The enzyme source (0.5g) was homogenized with 5.0ml of phosphate buffer. The homogenates was centrifuged at 3000 xg for 10 minutes and the supernatants was used for the assay.

Assay

The activity of the enzyme was determined by observing the change in absorbance at 340nm. The reaction mixture contained 0.1ml of GSH, 0.1ml of CDNB and phosphate buffer in a total volume of 2.9ml. The reaction was initiated by the addition of 0.1ml of the

enzyme extract. The readings were recorded every 15 seconds at 340nm against distilled water blank for a minimum of three minutes in a spectrophotometer (JENWAY 6405 UV/Vis). The assay mixture without the extract served as the control to monitor nonspecific binding of the substrates. GST activity was calculated using the extinction co-efficient of the product formed (9.6mM−1cm−1) and was expressed as μmoles of CDNB conjugated/minute/ mg of protein.

iii. Estimation of TBARS (Nichans and Samuelson, 1968)

Principle

Malondialdehyde and other thiobarbituric acid reactive substances (TBARS) react with thiobarbituric acid in an acidic condition to generate a pink color chromophore which was read at 535 nm.

Procedure

The homogenate (0.5 ml, as prepared in 3.2.3.7.) was diluted to 5 ml with double distilled water and mixed well, and then 2.0 ml of TBA-TCA-HCl reagent were added. The mixture was kept in a boiling water bath for 15 min, after cooling, the tubes were centrifuged at 1000 xg for 10 min and the supernatant was used for estimation of the TBARS. A series of standard solution in the concentration of 2-10 nmol was treated in a similar manner. The absorbance of the chromophore was read at 535 nm against reagent blank. The values were expressed as n moles/g tissue.

iv. Determination of Catalase activity (CAT) (Aebi, 1984)

Principle

Catalase exerts a dual function, decomposition of H_2O_2 to give H_2O and O_2, and oxidation of H donors. In the ultraviolet range H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm. The difference in absorbance per unit time is a measure of catalase activity.

Procedure

One gram (1 g) and half of a gram (0.5g) respectively of the liver and kidney were homogenized in 10 ml and 5ml respectively of 0.01M phosphate buffer, pH 7.0. The homogenates were centrifuged at 4^oC for 15 min at 1500×g. The supernatants were removed and kept on ice until the enzyme assay. H_2O_2-phosphate buffer (2.8 ml) was taken in an experimental cuvette, followed by the rapid addition of 0.2 ml of sample and mixed thoroughly. The time required for a decrease in absorbance was recorded at 240nm in a spectrophotometer. The enzyme solution containing H_2O_2–free phosphate buffer served as control. Catalase activity was expressed as units representing the calculated rate constant (K)/mg protein.

Units: µmoles of H_2O_2 consumed/min/mg of protein.

v. **Estimation of Superoxide Dismutase (SOD) (Misra and Fridovich 1972)**

Principle

Superoxide dismutase inhibits the oxidation of epinephrine by superoxide radical.

Procedure

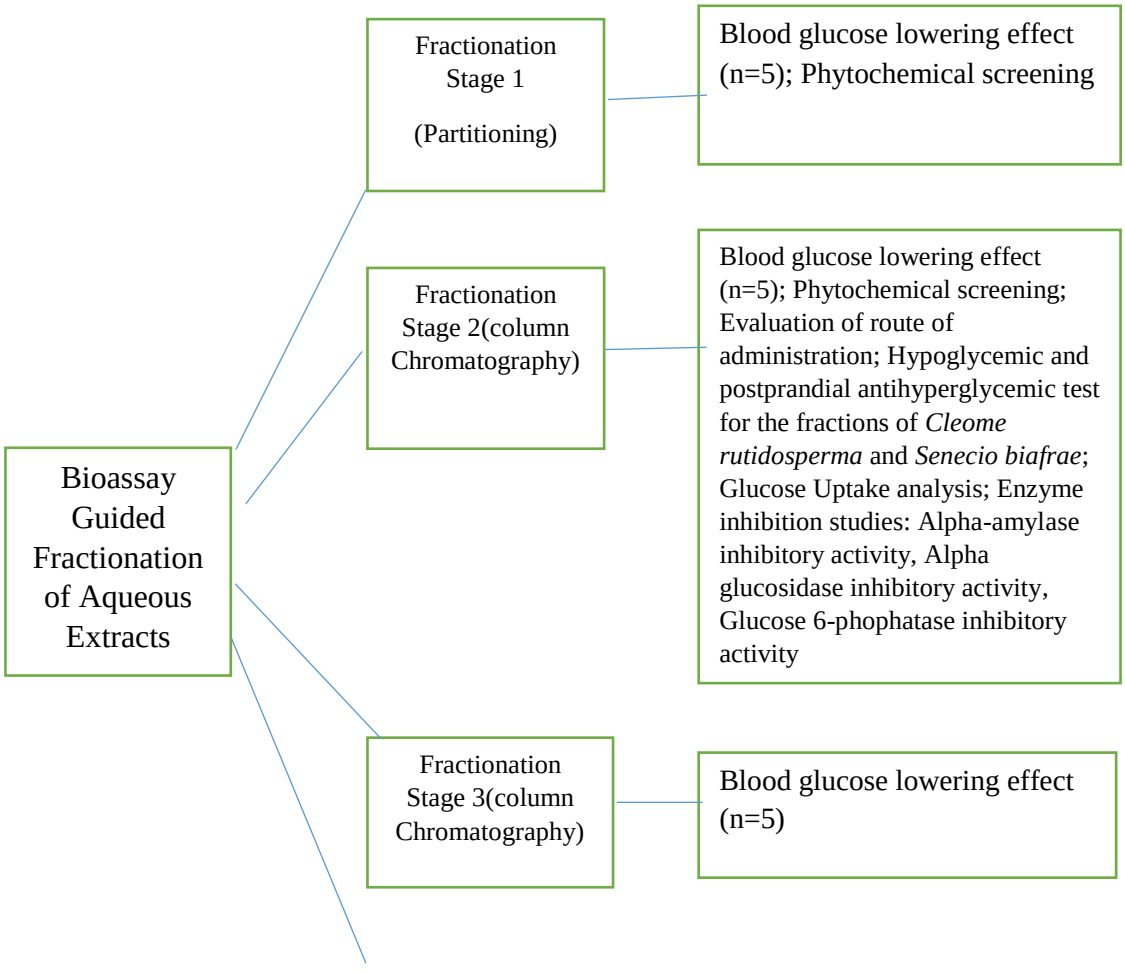
To 0.2ml of the organ extract (as prepared in 3.2.3.7.), 2.5ml of 0.05M phosphate buffer pH 10.2 was added. The reaction was initiated by adding 0.3ml of freshly prepared 0.3mM adrenaline. The mixture was then quickly mixed by inversion. A reference cuvette containing 2.5ml of the buffer and 0.2ml of distilled water and 0.3ml of adrenaline was also prepared. The increase in absorbance for both reference and sample was monitored at 480nm every 30 secs for 150secs. 1 unit of SOD activity was given as the amount of SOD that causes 50% inhibition of the oxidation of adrenaline to adrenochrome in one minute.

3.2.4 Segment 3

Bioassay guided fractionation of the aqueous extracts was done in four stages with the resultant sub fraction of each stage tested for blood glucose lowering effects as shown in Figure 3.3.

3.2.4.1 Preparation and Partitioning of Plant Extract

The crude aqueous extracts of the two plants were fractionated by initially dissolving 110g of the extract in 500 mL of distilled water, mixed in a beaker and then filtered using No.1 What-man filter paper. The filtrate was thereafter transferred into a 1 L separating funnel and extracted with 5 × 250 mL n-butanol. The combined n-butanol fractions were concentrated using water bath at 50^0C. The aqueous layer was sequentially extracted with 3 × 250 mL chloroform, 3 × 250 mL ethyl acetate and 3 × 250 mL methanol. The combined chloroform, ethyl acetate and methanol fractions were separately concentrated using water bath at 50^0C. Finally, the remaining aqueous fraction was also concentrated using water bath at 50^0C.


Bioassay Guided Fractionation of Aqueous Extracts
Fractionation Stage 1 (Partitioning)
Blood glucose lowering effect (n=5); Phytochemical screening
Fractionation Stage 2(column Chromatography)
Blood glucose lowering effect (n=5); Phytochemical screening; Evaluation of route of administration; Hypoglycemic and postprandial antihyperglycemic test for the fractions of Cleome rutidosperma and Senecio biafrae; Glucose Uptake analysis; Enzyme inhibition studies: Alpha-amylase inhibitory activity, Alpha glucosidase inhibitory activity, Glucose 6-phophatase inhibitory activity
Fractionation Stage 3(column Chromatography)
Blood glucose lowering effect (n=5)

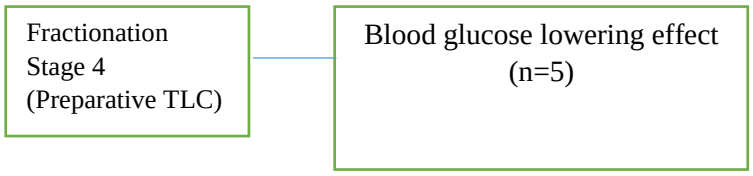


Figure 3.3: Stages of Bioassay Guided Fractionation of Aqueous Extracts

3.2.4.2 Further Fractionation of Active Methanol Fraction Using Silica Gel-column Chromatography

Further fractionation of active methanol fraction (based on *in vivo* antihyperglycemic study of the above fractions) was done using silica gel-column chromatography; by loading it with 100g of silica gel (Qualikems, 60- 120 mesh). Four (4 g) grams of the methanol fraction was pre-adsorbed onto silica gel adsorbent (60–120 mesh) by firstly solubilizing it in little quantity of methanol, followed by addition of the silica gel (3 g) then mixing. The dried fraction-adsorbent mixture was then evenly loaded onto the top of the already packed column. The column was first eluted with 2 × 300 mL 100% chloroform followed successively by 2 × 300 mL chloroform- methanol in graded ratios (9:1), (8:2), (7:3), (6:4), (5:5), (4:6), (3:7), (2:8), (1:9) and finally (0:10). These 1st sub-fractions were collected and concentrated as described earlier. The most active 1st sub-fractions from *in vivo* studies were: chloroform: methanol (5: 5) and chloroform: methanol (7: 3) respectively for *Cleome rutidosperma* and *Senecio biafrae*. These 1st sub-fractions

were further re-fractionated in a column (25 × 3cm) loaded with silica gel (Qualikems, 60-120 mesh) as described above.

The column was first eluted with 2 × 300 mL 100% chloroform followed by 2 × 300 mL chloroform- methanol (5:5) and 2 × 300 mL chloroform- methanol (0:10) for *Cleome rutidosperma*, while the column for *Senecio biafrae* was first eluted with 2 × 300 mL 100% chloroform followed by 2 × 300 mL chloroform- methanol (7:3) and 2 × 300 mL chloroform- methanol (0:10). Second sub-fractions of each plant was collected and on the basis of their R_f values, similar sub-fractions were pooled together to give 2nd sub-fraction C1 (2SFC1) and 2nd sub-fraction C2 (2SFC2) for *Cleome rutidosperma* and 2nd sub-fraction S1 (2SFS1) and 2nd sub-fraction S2 (2SFS2) for *Senecio biafrae*. These sub fractions for each plant were tested for their blood glucose lowering effect in STZ-induced diabetic mice.

The more active of the 2nd sub-fractions for each plant; 2SFS2 for *Senecio biafrae* and 2SFC1 for *Cleome rutidosperma* ,were purified further using preparative thin layer chromatography (TLC) with different solvent systems for each plant. For *Cleome rutidosperma*, Ethyl acetate: Methanol: Acetic acid (7:2:1) solvent system was used, while Chloroform: Methanol (8:2) solvent system was used for *Senecio biafrae*. The resultant spots: spot I for Senecio biafrae (SIS) and spots I and II for *Cleome rutidosperma* (SIC and SIIC) were tested for antihyperglycemic activity *in vivo*.

3.2.4.3 Determination of the Effects of Plant Sub fractions on Glucose Movement

A simple model as described by Gallagher, *et. al.,* (2003) was used to evaluate effects of plant sub fractions on glucose movement *in vitro*. The model involved the use of

a sealed dialysis tube into which 15 ml of a solution of glucose and NaCl (0.15 M) was introduced and the appearance of glucose in the external solution was measured. The model for the present study involved the use of an excised rat ileum (6 cm) into which 2 ml of 0.15 M NaCl containing 0.22 mM D-glucose was added. The ileum was sealed at each end and placed in a 50 ml boiling-tube containing 45 ml of 0.15 M NaCl. The tubes were placed on an orbital shaker (Balart Products, USA) and kept at 37^0C. The movement of glucose into the external solution was monitored at set time intervals. In the first series of experiments, the effects of 50 g/l plant sub fraction on glucose diffusion were compared to control tests conducted in the absence of plant fractions. At the end of the experimental period, the concentrations of glucose within the tube were measured. A second experimental series investigated the concentration-dependent effects (6.25, 12.5, 25 and 50 g/l) of plant sub fractions. All tests were carried out in triplicate. Glucose concentrations were measured using the glucose oxidase method.

3.2.4.4 Hypoglycemic and Postprandial Antihyperglycemic Test

In the hypoglycemic test, mice were fasted 12 h prior to test whereas in postprandial antihyperglycemic test, fed mice were fasted 1 h prior to test. After fasting period, the mice were given orally either plant sub fraction or Glibenclamide using a cannula. Blood was collected from the tail vein just before (0 h) and 2, 4 and 6 h after administration of fraction or Glibenclamide for the measurement of blood glucose concentration, which was done using Acu Check Active glucometer kit (Mohammadi *et. al.*, 2010; Adam, *et. al.*, 2011). Five mice were used in each group.

3.2.4.5 Determination of the Effect of Sub fractions on Oral Glucose Tolerance Test (OGTT)

For OGTT evaluation, the mice were fasted for 12 h and blood was taken 30 min before administration of sub fraction of *C. rutidosperma* and *S. biafrae* or Glibenclamide. Thirty minutes later, the mice from all groups were given glucose (1.5 g/kg) orally. Blood was collected from the tail vein just prior to glucose administration (0 min), and then 30, 60, 120 and 180 min after glucose loading for estimation of blood glucose using glucometer. Also, five mice were used in each group.

3.2.4.6 Comparison of the Effects of Oral and Intra-peritoneal Routes of Administration of Different 1st Sub-fractions of *C. rutidosperma* and *S. biafrae* on Fasting Blood Glucose Level in STZ-induced Diabetic Mice.

In order to compare the effect of oral and intra-peritoneal routes of administration of different 1st Sub-fractions of *C. rutidosperma* and *S. biafrae* on fasting blood glucose level, varied doses of the sub fractions were administered to STZ-induced diabetic mice. Twenty five mice were randomly allocated to negative control and treatment groups of five mice each as follows:

Group I: Diabetic control mice.

Group II: Diabetic mice given 25mg/kg body weight of the sub fraction of *Senecio biafrae* (intraperitoneal).

Group III: Diabetic mice given 125mg/kg body weight of the sub fraction of *Senecio biafrae* (oral).

Group IV: Diabetic mice given 125mg/kg body weight of the sub fraction of *Cleome rutidosperma* (intraperitoneal).

Group V: Diabetic mice given 125mg/kg body weight of the sub fraction of *Cleome rutidosperma* (oral).

Fasting blood glucose concentration was first determined in overnight fasted mice by the enzymatic glucose oxidase method using a commercial glucometer (Accu-chek® Active, Roche diagnostic, Mannheim, Germany), following which the plant sub fractions were administered through oral/ intra-peritoneal routes. Blood glucose values were then estimated hourly for 6 hours. The choice of intra-peritoneal dosage of 25mg/kg body weight for group II above was informed by the results of the intra-peritoneal acute toxicity of the sub fractions.

3.2.4.7 Inhibitory Activity of Sub fractions on Glucose 6-Phosphatase

The effect of plant fractions on glucose-6-phosphatase activity was performed according to the method described by Baginsky *et. al*., (1992); using glucose-6-phosphatase from rabbit liver (Sigma, G5758). Briefly, 0.5 ml (25 units) of glucose 6-phosphatase (E.C. 3.2.3.2) was pre-incubated with 0.25 ml of plant fraction(2.5 – 20 μg/ml) for 15 minutes at 37^0C. The reaction was then started by addition of 0.25 ml of 0.1 M glucose -6-phosphate in citrate buffer, pH 6.5. The reaction mixture was then incubated at 37^0C for 20 minutes. At the end of the incubation period, 2.0 ml of 2% (w/v) ascorbic acid and 10% (w/v)/trichloroacetic acid solution was added to stop the reaction. The inorganic phosphate liberated from the substrate by the enzyme was reacted with 1% ammonium molybdate solution to produce a blue-colored compound whose absorbance was measured at 660nm. A control was prepared using the same procedure except that the extract was replaced with distilled water and the glucose-6-phosphatase inhibitory activity was expressed as percentage inhibition. The glucose-6-phosphatase inhibitory activity was calculated using the equation:

$$\% \text{ Inhibition} = \{(Ac - Ae)/Ac\}\ 100$$

Where; Ac and Ae are the absorbances of the reaction mixture with distilled water (control) and extract, respectively.

3.2.4.8 Inhibitory Activity of Sub fractions on Alpha-Amylase (McCue et. al., 2004)

A total of 250 µL of fractions (2.5 – 20 µg/ml) was placed into tubes and 500 µL of 0.02 M sodium phosphate buffer (pH 6.9) containing porcine α-amylase solution (0.5 mg/ml) was added. The contents of the tubes were pre-incubated at 25^0C for 10 min, after which 250 µL of 1 % starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added. The reaction mixtures were incubated at 25^0C for 10 min. The reaction was terminated by adding 1 ml of dinitrosalicylic acid (DNS) reagent and further incubated in boiling water for 5 min and cooled to room temperature. The content of each test tube was diluted with 5 ml distilled water and the absorbance measured at 540nm in a spectrophotometer. A control was prepared using the same procedure except that the extract was replaced with distilled water. Acarbose was used as reference. The α-amylase inhibitory activity was calculated using the equation:

% Inhibition = {(Ac – Ae)/Ac} 100

Where; Ac and Ae are the absorbances of the reaction mixture with distilled water (control) and extract, respectively.

3.2.4.9. Inhibitory Activity of Sub fractions on Alpha-Glucosidase (Kim et. al., 2005)

The effect of the plant fractions on α-glucosidase activity was determined according to the method described by Kim *et. al.*, (2005); using α-glucosidase from *Bacillus stearothermophilus*. The substrate solution, p-nitrophenyl glucopyranoside (pNPG) (3.0 mM) was prepared in 20 mM phosphate buffer, pH 6.9. Alpha-glucosidase

(100 µL of 1.0 U/ml) was pre-incubated with 50 µL of the different concentrations of the fractions (2.5 – 20 µg/ml) for 10 mins. Then 50 µL of 3.0 mM pNPG (as a substrate) dissolved in 20 mM phosphate buffer (pH 6.9) was added to start the reaction. The reaction mixture was incubated at 37^0C for 20 mins and stopped by adding 2 ml of 0.1 M Na_2CO_3. The α-glucosidase activity was determined by measuring the yellow colored para-nitrophenol released from pNPG at 405nm. Acarbose was used as reference. Also, a control was prepared using the same procedure except that the extract was replaced with distilled water.

The results (% Inhibition) were expressed as percentage of the blank (control) as in the equation below:

% Inhibition = {(Ac – Ae)/Ac} 100.

Where; Ac and Ae are the absorbances of the reaction mixture with distilled water (control) and extract, respectively.

3.2.5 TLC Fractionation of *C. rutidosperma* and *S. biafrae* Active Sub fractions

The most active 2nd Sub-fractions of *C. rutidosperma* and *S. biafrae* obtained from fractionation by column chromatography were re-fractionated by means of a preparative thin layer chromatography (TLC). Briefly, powdered fractions (0.02 mg) were dissolved in methanol (2 ml) and streaked on preparative TLC plates. The plate for *C. rutidosperma* was eluted with a chloroform: methanol (8:2) solvent system, while the plate for *S. biafrae* was eluted with ethyl acetate: methanol: acetic acid (7: 2: 1) solvent system. A small part (part A) of each plate was cut and air-dried. It was then sprayed with 5% sulphuric acid and oven

dried for visualization of the separated fraction(s). The second part of the plates (part B) were air dried without spraying and were crossed matched with the first sprayed part (part A) in order to identify the separated fraction(s). All visible individual fractions in part B were marked, scraped and dissolved in methanol. The separated fractions were centrifuged and decanted.The decanted fractions were concentrated using water bath at 40^0C and tested for their blood glucose lowering effect in streptozotocin-induced diabetic mice to obtain the most active fraction from preparative TLC.

3.3 Statistical Analysis

The results are presented as mean ± SD and were analysed using ANOVA followed by Tukey kramer multiple comparison test on Graphpad Instant Software, version 6.0 (Graph Pad Software, San Diego, CA, USA), and values of $P< 0.05$ were considered significant.

CHAPTER FOUR

RESULTS

The concentration of phytochemicals in different extracts of the plants are shown in Table 4.1. From the Table, the concentrations of alkaloids for the different extracts were in the order: ASB>ESB>AQCR>PSB>ACR>AQSB>ECR>PCR. The highest concentration of saponins was found in ACR (6.01±0.88 %), followed by ECR (4.12±0.81%), AQCR (3.11±0.89%) and the least content was in PSB (0.18±0.02).

Similarly, the lowest (0.04±0.01 %) and highest (14.15±0.73 %) concentrations of tannins were found in ASB and ACR, respectively. The highest concentration of glycosides was found in AQSB (0.113±0.040%), followed by ASB (0.073±0.026%) and the lowest was in PCR (0.002±0.001%). Also, the highest (0.433±0.170%) and lowest (0.011±0.003) concentrations of flavonoids were found in AQCR and ESB, while the lowest (0.003±0.001) and highest (0.021±0.005%) concentrations of steroids were found in ASB and ESB respectively.

Results for the *in vitro* antioxidant activity of the extracts by the FTC method are shown in Figure 4.1. Significantly ($p>0.05$) higher activities were observed in AQCR and ESB compared to ECR, ACR, PCR, ASB and PSB. ACR significantly ($p<0.05$) displayed lower activity compared to all other extracts. The antioxidant activity of AA was significantly ($p<0.05$) higher than all other extracts.

Similarly, results for the antioxidant activity by the TBA method shown in Figure 4.2 reveals significantly ($p<0.05$) higher activity by PSB when compared to all other extracts. Also, ACR was significantly ($p<0.05$) lower in activity compared to all other extracts. Significantly higher activity ($p<0.05$) was observed in AA compared to all extracts.

The reducing power of different extracts of the two plants is shown in Figure 4.3. The petroleum ether extract of *Senecio biafrae* (PSB) had the highest value among the extracts of the plants examined, followed by ASB in the order: PSB>ASB>PCR>ECR>AQSB>AQCR>ACR>

ESB.

To evaluate the antioxidant activity of the different extracts of the plants, the radical scavenging capacity based on DPPH assay was determined and the results are shown in Figure 4.4. The percentage of scavenging effect on the DPPH radical was increased with the increase in the concentrations of the extract from 0.05 -1 mg/mL. The percentage of inhibition of the DPPH radical was varied from 11.61±1.08 % to 58.58±1.58% (in 0.05mg/ml to 1mg/ml of the aqueous extract of *Cleome rutidosperma* - AQCR), 29.23±1.33% to 66.12±1.41% for ECR, 28.75±1.32% to 56.78±1.19% for ACR and 25.4±1.67% to 47.83±1.30% for PCR, while percentage inhibition for *Senecio biafrae* extracts ranged from 14.05±0.78% to 50.88±1.08% for AQSB, 17.72±1.29% to 42.15±1.30% for ESB, 10.43±1.71% to 26.23±0.94% for ASB and 16.09±0.74% to 39.47±0.68% for PSB. The extracts at all concentrations showed lower percentage of inhibition of free radicals than the standard drug, Ascorbic acid (87.21±0.82% - 99.46±2.54% in the concentration of 0.05-1 mg/ml).

Table 4.1: Quantitative Analysis of Phytochemicals in Extracts of *Cleome rutidosperma* and *Senecio biafrae*.

Plant/Extract	Alkaloids(%)	Saponins(%)	Tannins(%)	Glycosides(%)	Flavonoids(%)	Steroids(%)
AQCR	0.29±0.01^{a}	3.11±0.89^{a}	12.03±0.81^{a}	0.033±0.009^{a}	0.433±0.170^{a}	0.004±0.002^{a}
ECR	0.18±0.02^{a}	4.12±0.81^{a}	10.44±0.95^{a}	0.004±0.004^{a}	0.310±0.078^{a}	0.011±0.003^{a}
ACR	0.27±0.01^{a}	6.01±0.88^{b}	14.15±0.73^{b}	0.011±0.004^{a}	0.263±0.070^{ab}	0.003±0.002^{a}
PCR	0.11±0.02^{b}	2.31±0.50^{a}	5.48±0.89^{c}	0.002±0.001^{a}	0.183±0.058^{ab}	0.004±0.002^{a}
AQSB	0.26±0.02^{a}	0.81±0.13^{a}	0.05±0.02^{d}	0.113±0.040^{b}	0.035±0.006^{b}	0.011±0.002^{a}
ESB	0.31±0.04^{ac}	0.22±0.03^{ac}	0.11±0.02^{d}	0.053±0.017^{ab}	0.011±0.003^{b}	0.021±0.005^{b}
ASB	0.34±0.05^{ac}	0.33±0.09^{ac}	0.04±0.01^{d}	0.073±0.026^{ab}	0.021±0.008^{b}	0.003±0.001^{a}
PSB	0.28±0.04^{a}	0.18±0.02^{ac}	0.04±0.02^{d}	0.030±0.008^{ab}	0.012±0.003^{b}	0.004±0.001^{a}

Values are mean of three determinations ± SD.

Values with different superscripts down the column differ significantly ($p<0.05$). Where: AQCR= Aqueous Extract of *Cleome rutidosperma* ECR = Ethanol extract of *Cleome rutidosperma*, ACR= acetone extract of *Cleome rutidosperma*, PCR= Petroleum ether extract of *Cleome rutidosperma* ,AQSB= Aqueous extract of *Senecio biafrae* ,ESB= Ethanol extract of *Senecio biafrae*, ASB= Acetone extract of *Senecio biafrae*, PSB= Petroleum ether extract of *Senecio biafrae*.

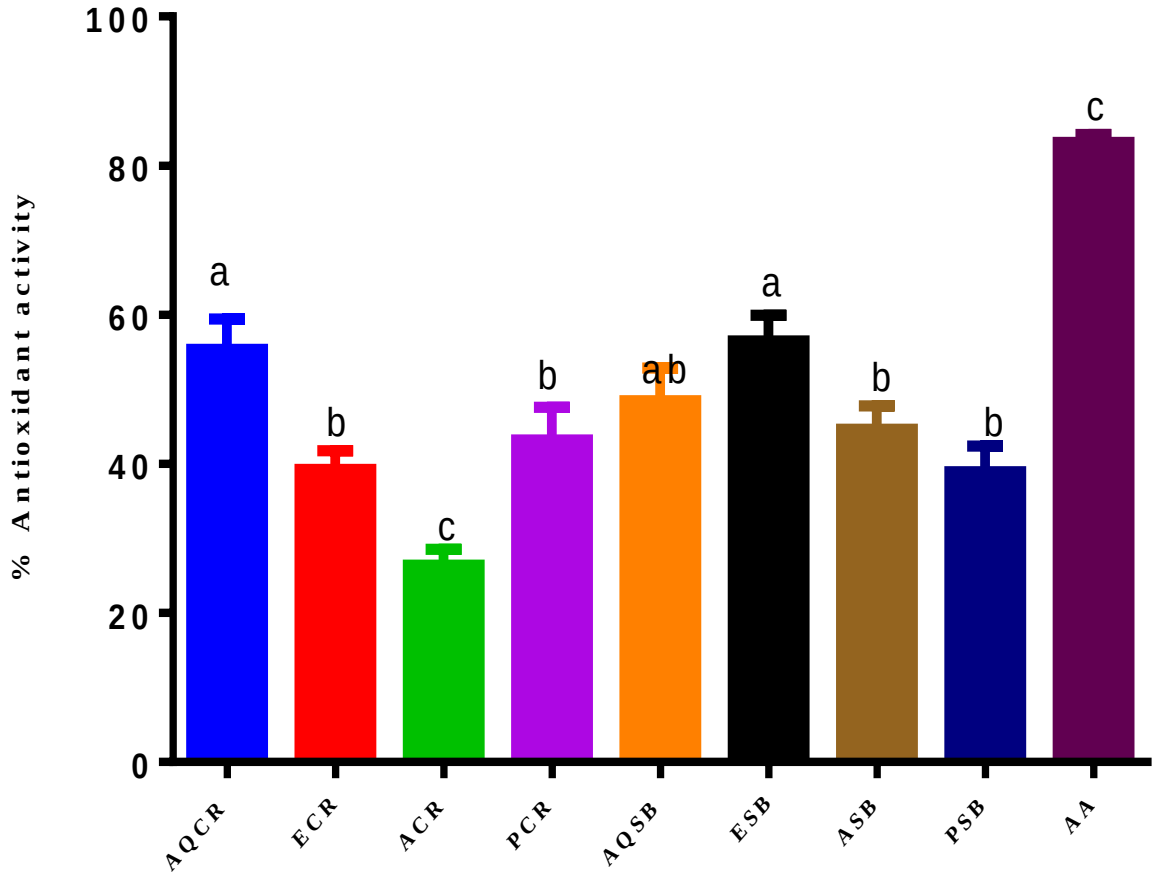


Figure 4.1: *In vitro* Antioxidant Activity of Plant Extracts Using the FTC Method.

Bars with different letters differ significantly ($p<0.05$)

Where: AQCR= Aqueous Extract of *Cleome rutidosperma*,ECR= Ethanol extract of *Cleome rutidosperma*,ACR= acetone extract of *Cleome rutidosperma*, PCR= Petroleum ether extract of *Cleome rutidosperma*,AQSB= Aqueous extract of *Senecio biafrae*,ESB= Ethanol extract of *Senecio biafrae* ,ASB= Acetone extract of *Senecio biafrae*,PSB= Petroleum ether extract of *Senecio biafrae*, AA= Ascorbic acid.

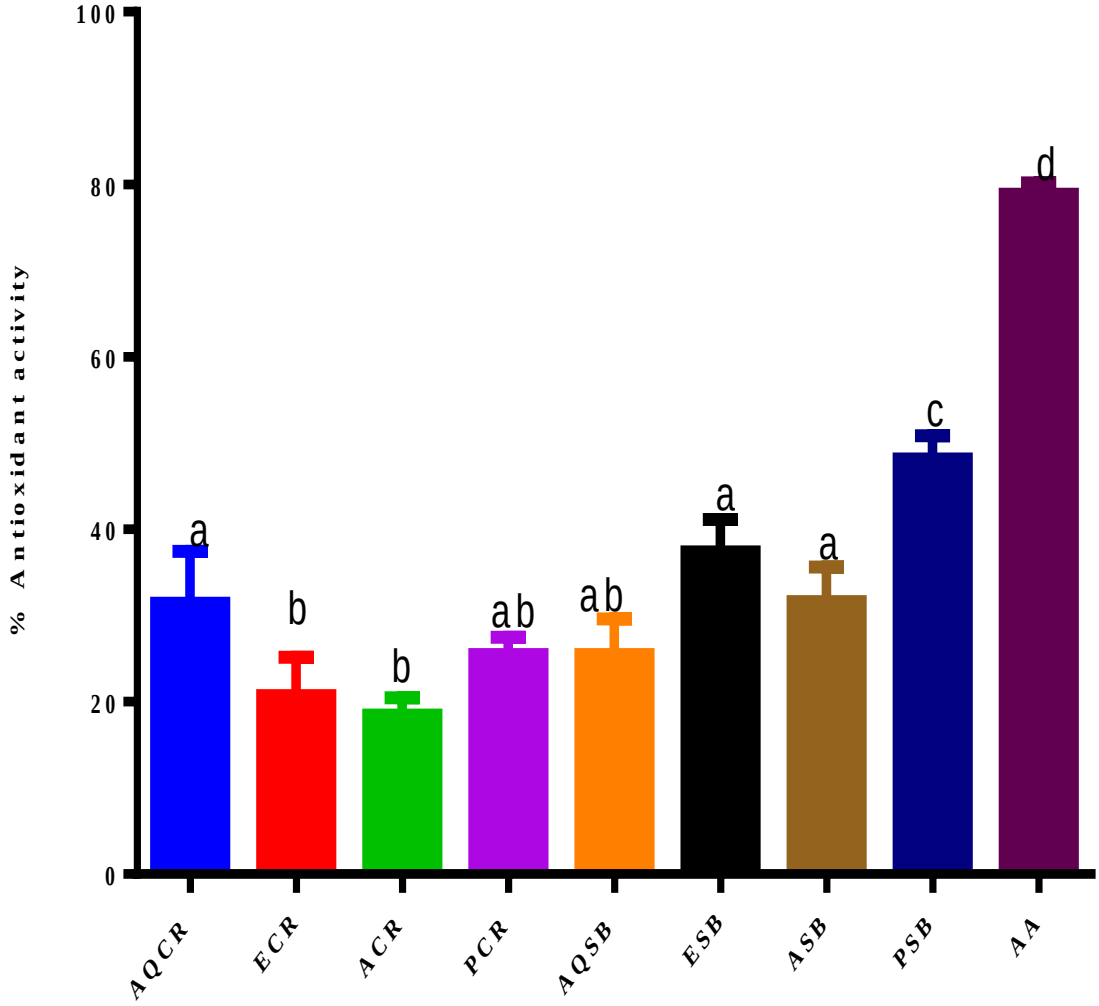


Figure 4.2: *In vitro* Antioxidant Activity of Plants Extracts Using the TBA Method.

Bars with different letters differ significantly ($p<0.05$)

Where: AQCR= Aqueous Extract of *Cleome rutidosperma*, ECR= Ethanol extract of *Cleome rutidosperma*,ACR= acetone extract of *Cleome rutidosperma*, PCR= Petroleum ether extract of *Cleome rutidosperma*,AQSB= Aqueous extract of *Senecio biafrae*,ESB= Ethanol extract of *Senecio biafrae* ,ASB= Acetone extract of *Senecio biafrae*,PSB= Petroleum ether extract of *Senecio biafrae*, AA= Ascorbic acid.

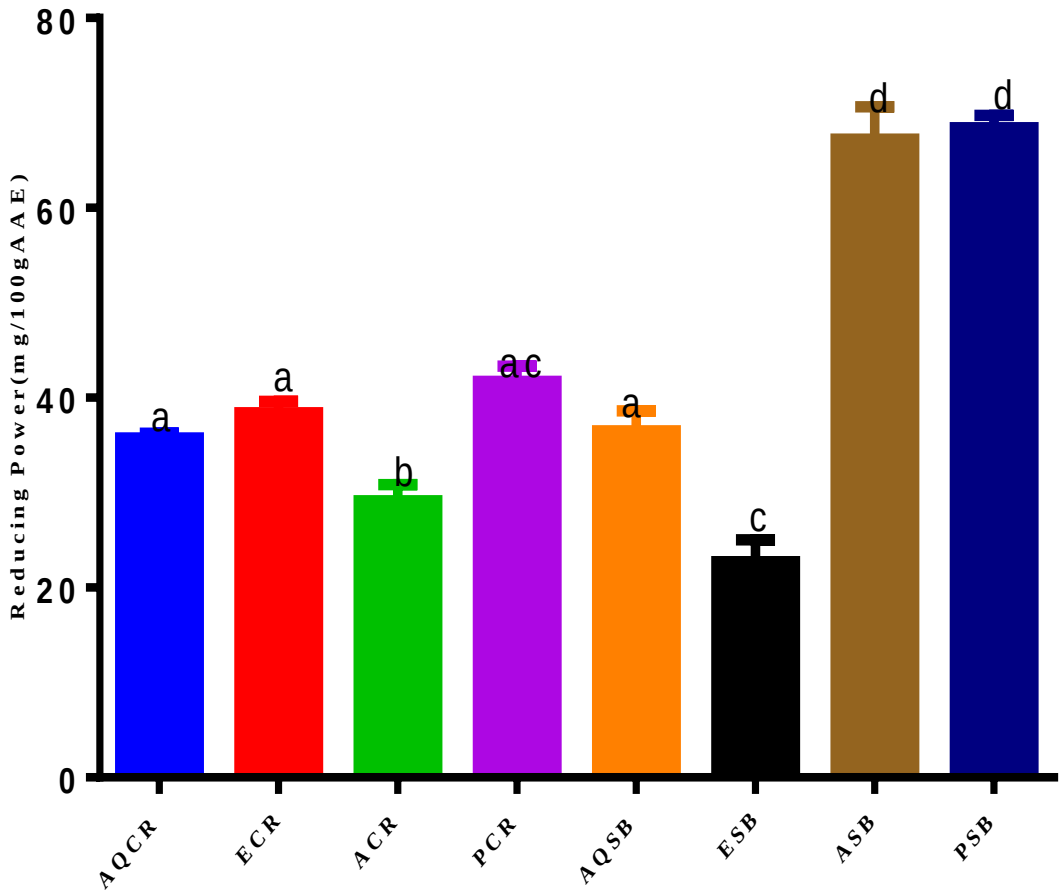


Figure 4.3: *In vitro* Reducing Power of Plant Extracts.

Bars with different letters differ significantly ($p<0.05$)

Where: AQCR= Aqueous Extract of *Cleome rutidosperma*,ECR= Ethanol extract of *Cleome rutidosperma*,ACR= acetone extract of *Cleome rutidosperma*, PCR= Petroleum ether extract of *Cleome rutidosperma*,AQSB= Aqueous extract of *Senecio biafrae*,ESB= Ethanol extract of *Senecio biafrae* ,ASB= Acetone extract of *Senecio biafrae*,PSB= Petroleum ether extract of *Senecio biafrae*, AAE= Ascorbic acid equivalent.

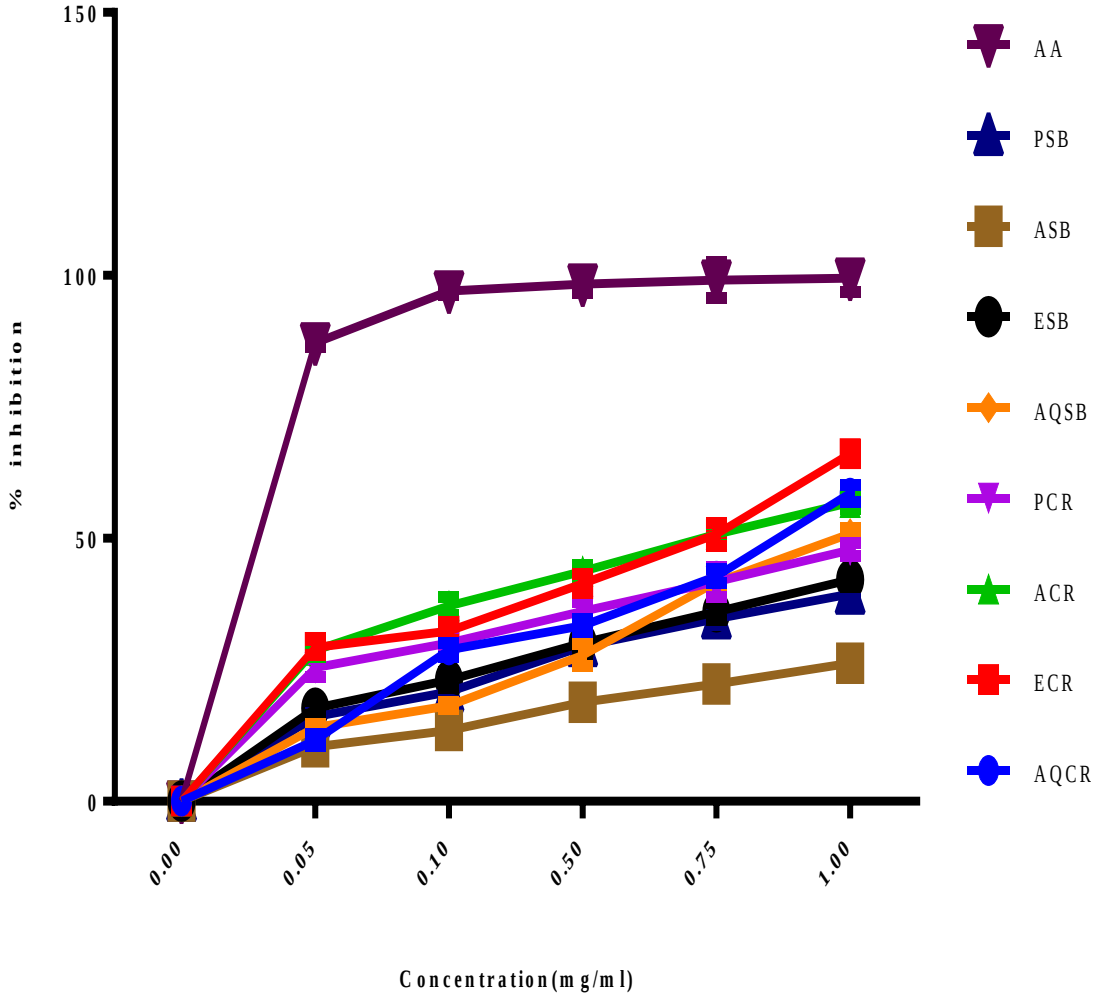


Figure 4.4: *In vitro* DPPH Radical Scavenging Activities of Plants Extracts.

Where: AQCR= Aqueous Extract of *Cleome rutidosperma*,ECR= Ethanol extract of *Cleome rutidosperma*,ACR= acetone extract of *Cleome rutidosperma*, PCR= Petroleum ether extract of *Cleome rutidosperma*,AQSB= Aqueous extract of *Senecio biafrae*,ESB= Ethanol extract of *Senecio biafrae* ,ASB= Acetone extract of *Senecio biafrae*,PSB= Petroleum ether extract of *Senecio biafrae*, AA= Ascorbic acid.

Changes in the levels of blood glucose in normal, diabetic control and experimental groups after oral administration of 500mg/kg body weight of different extracts *of Cleome rutidosperma* are shown in Figure 4.5. Diabetic control mice showed a significant increase ($p<0.05$) in fasting blood glucose at 60 min of the experiment. In animals treated with petroleum ether, acetone, ethanol and water extracts, the fasting blood glucose level were significantly ($P<0.05$) decreased from the 60 min up 360 min post treatment when compared with the FBG at 0 h. The maximum blood glucose lowering effect on diabetic mice, as compared to the diabetic control group was observed with the water extract at the 6th hr. The percentage blood glucose reduction(Table 4.2) of the different extracts at the 6th hr were 29.73%, 28.80%,35.95% and 40.88% for petroleum ether, acetone, ethanol and water respectively, while standard drug, glibenclamide reduced the blood glucose level by 22.68%. The extracts had greater blood glucose lowering effect than the standard drug, glibenclamide

Shown in Figure 4.6 is the acute antihyperglycemic activity of different extracts of *Senecio biafrae* and glibenclamide on blood glucose level of STZ-induced Diabetic Mice. There was significant ($P<0.05$) increase in FBG of the diabetic control group ($P<0.05$) at the 60 min and 120min, while significant decreases ($P<0.05$) were observed in all treated groups when compared with the FBG of each group at 0 h. The percentage blood glucose reduction(Table 4.2) of the different extracts and glibenclamide at the 6th hr were 10.68%, 14.14%,16.64%, 19.90% and 21.39% for petroleum ether, acetone, ethanol, water and glibenclamide, respectively.

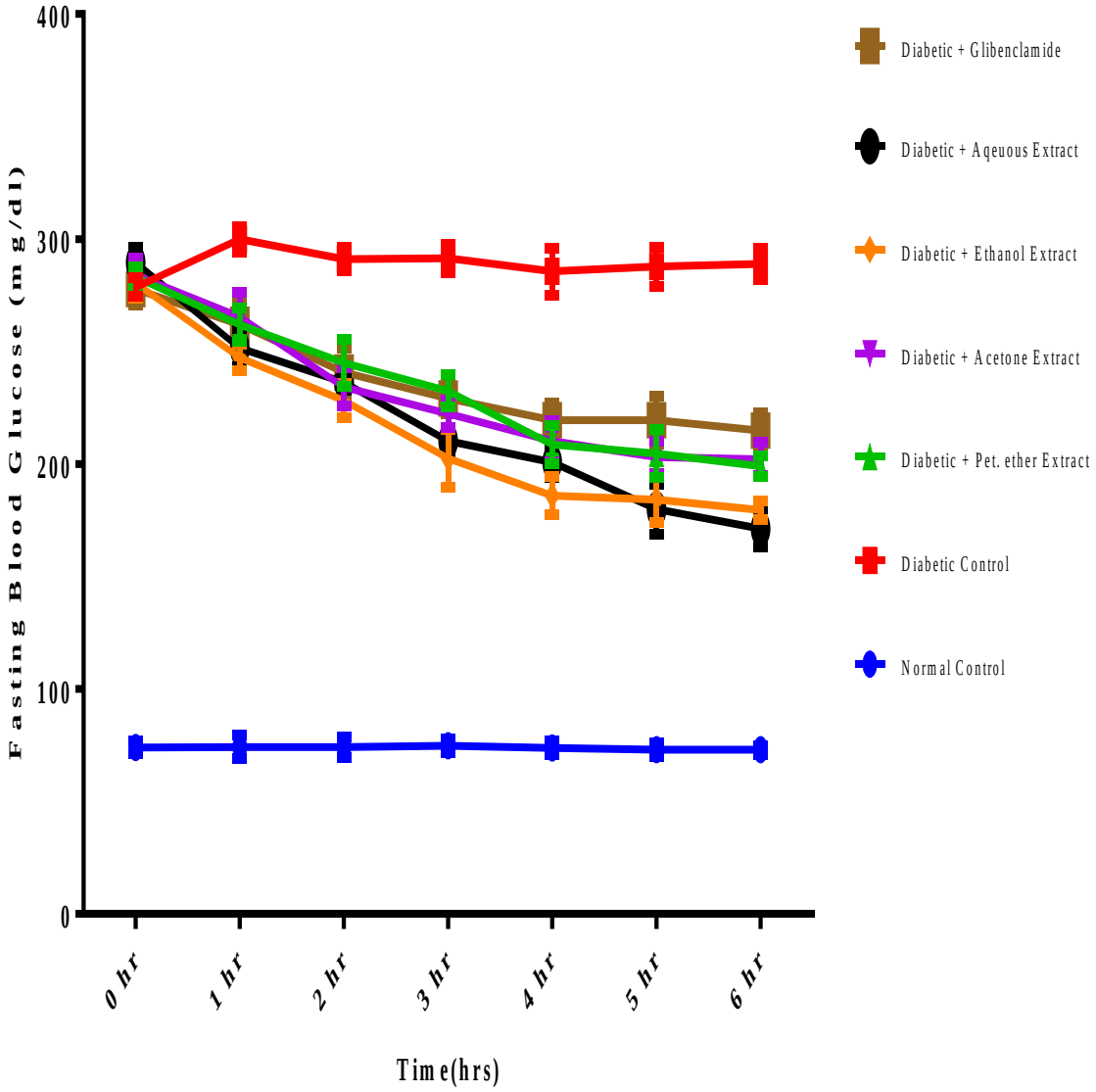


Figure 4.5: Acute Antihyperglycemic Activity of Different Extracts of *Cleome rutidosperma* and glibenclamide on blood glucose level of STZ-induced Diabetic Mice.

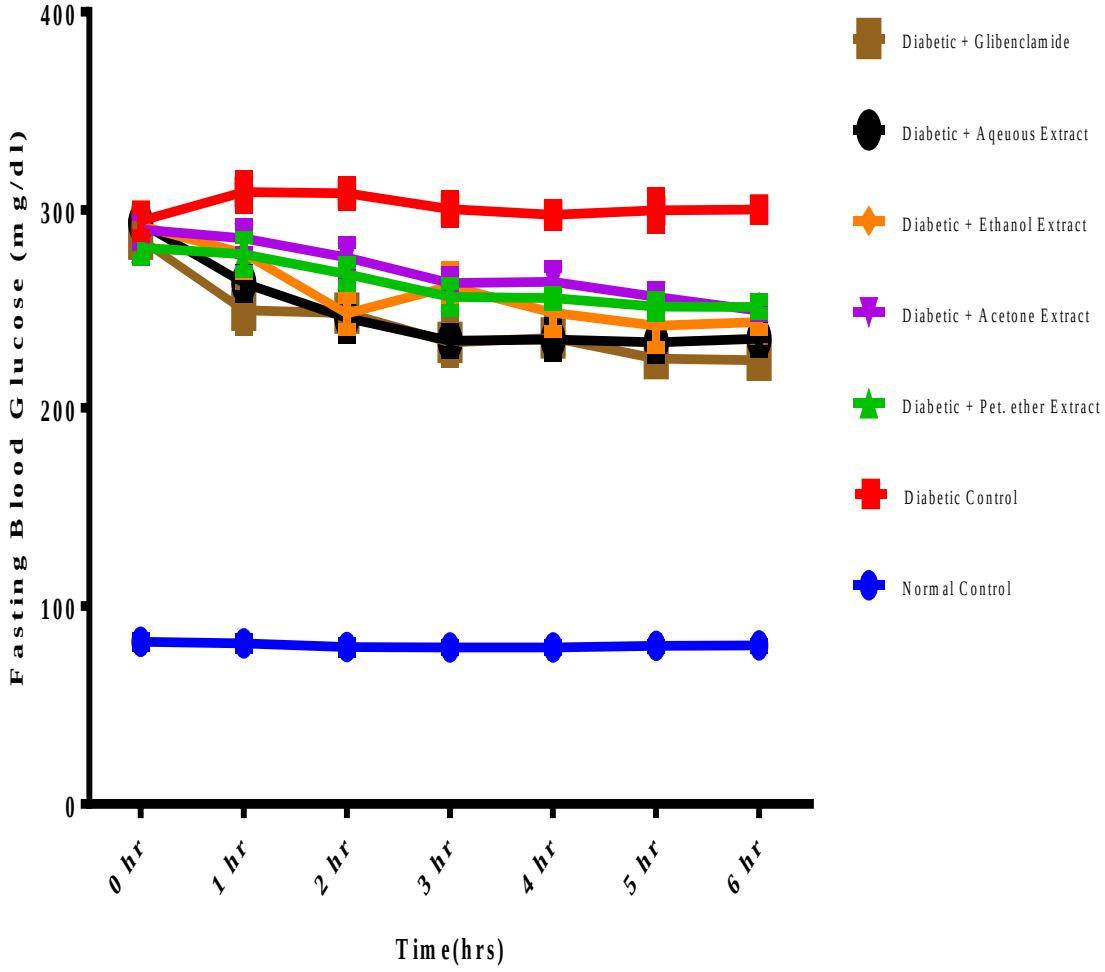


Figure 4.6: Acute Antihyperglycemic Activity of Different Extracts of *Senecio biafrae* and glibenclamide on blood glucose level of STZ-induced Diabetic Mice.

Table 4.2: Percentage Decrease in FBG 6 hours after Treatment with 500mg/kg of Different Extracts and 600µg/kg of Glibenclamide

	% Decrease in FBG at 6th h post-treatment	
GROUP	*Cleome rutidosperma*	*Senecio biafrae*
Normal Control	1.27±3.09[a]	2.406±1.68[a]
Diabetic Control	-3.736±1.82[a]	-2.024±2.62[a]
Pet. Ether Extract	29.706±16.27[b]	10.652±6.36[b]
Acetone Extract	28.784±15.46[b]	14.07±6.94[b]
Ethanol Extract	35.928±15.33[c]	16.568±7.22[bc]
Aqueous Extract	40.866±15.61[c]	19.858±7.65[c]
Glibenclamide	22.646±14.53[d]	21.34±7.95[c]

Values are mean of five determinations ± SD

Values with different superscripts down the column differ significantly ($p<0.05$);

Negative values indicate an increase in FBG.

The effect of aqueous extracts on weekly water intake in both normal and diabetic rats is shown in Figure 4.7. There was significant increase ($p<0.05$) in water intake in diabetic control group, while the extracts of both plants significantly ($p<0.05$) prevented this diabetes- induced polydipsia. Although, there were slight differences in the weekly water intake among the diabetic treated rats and the normal control group, these were not significant ($p<0.05$). Similarly, there was significant increase ($p<0.05$) in the feed intake in diabetic control group, but treatment with both plant extracts and the standard drug, also prevented the diabetes-induced polyphagia significantly ($p<0.05$) as shown in Figure 4.8. Also, no significant ($p<0.05$) difference was observed among the diabetic treated rats and normal control rats.

Although, significant increases were seen in the water and feed intakes for the diabetic control group, these increases did not result in weight gain, rather, a significant ($p<0.05$) weight loss was observed in this group. This weight loss which is usually associated with STZ diabetic rats was nevertheless reversed to a considerable extent by treatment with both plant extracts and the standard drug (Figure 4.9).

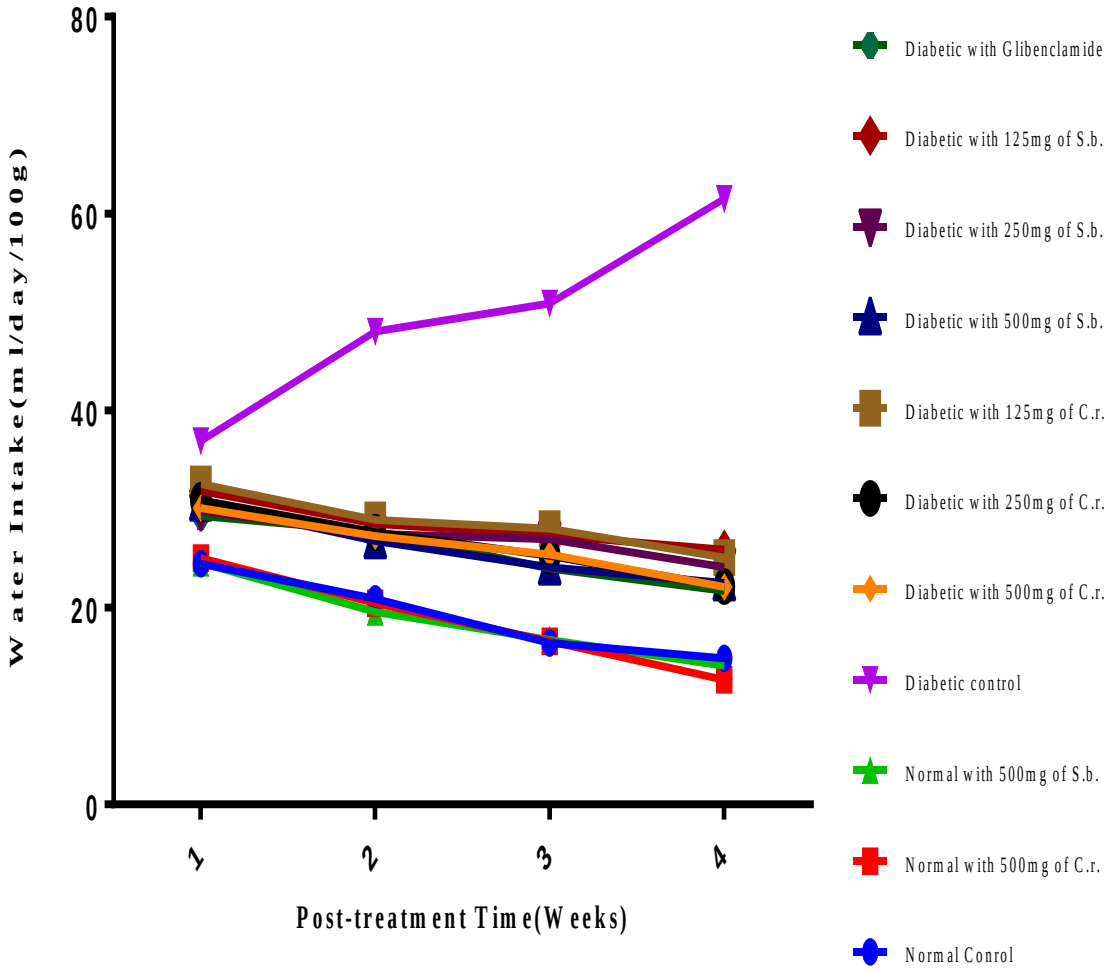


Figure 4.7: Weekly Water intake of STZ-induced Diabetic and Normal Rats Treated with Various Doses of Aqueous Extracts of *Cleome rutidosperma* (C.r.) and *Senecio biafrae* (S.b.)

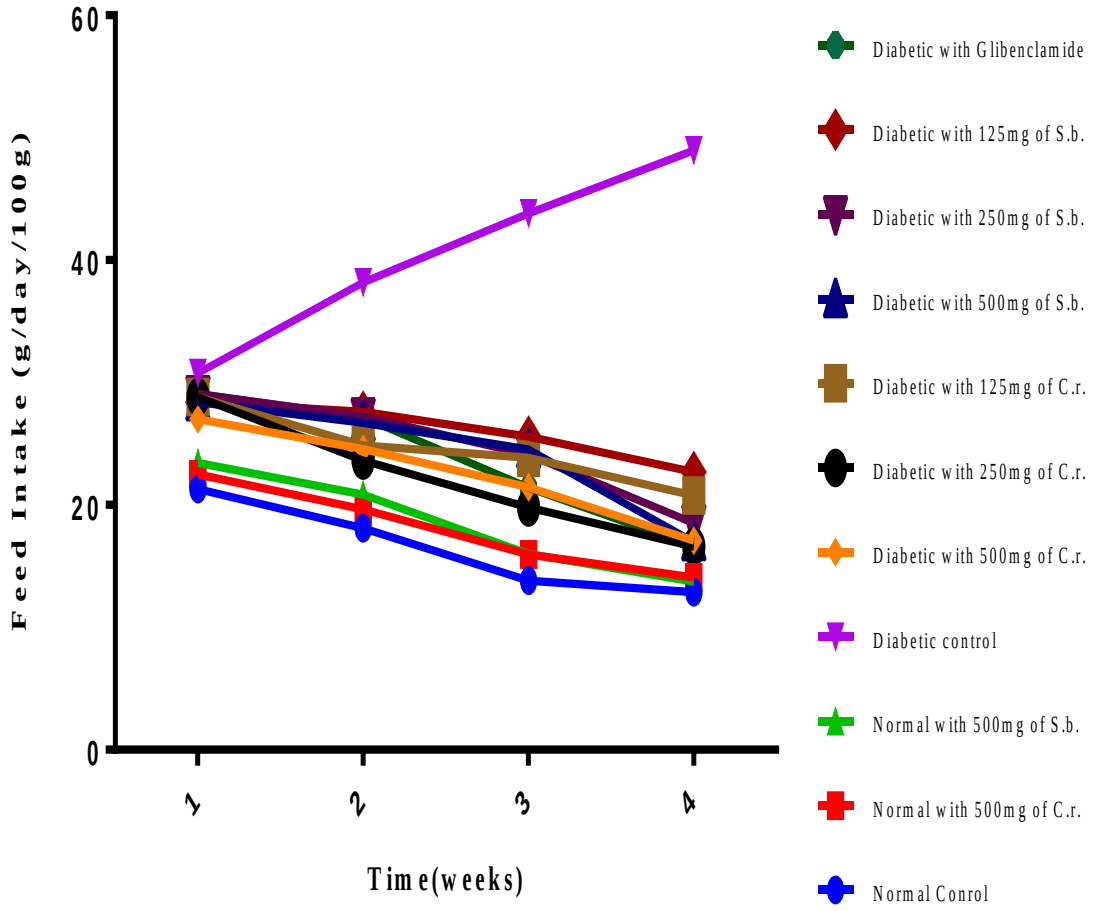


Figure 4.8: Weekly Feed Intake of STZ-induced Diabetic and Normal Rats Treated with Various Doses of Aqueous Extracts of *Cleome rutidosperma* (C.r.) and *Senecio biafrae* (S.b.).

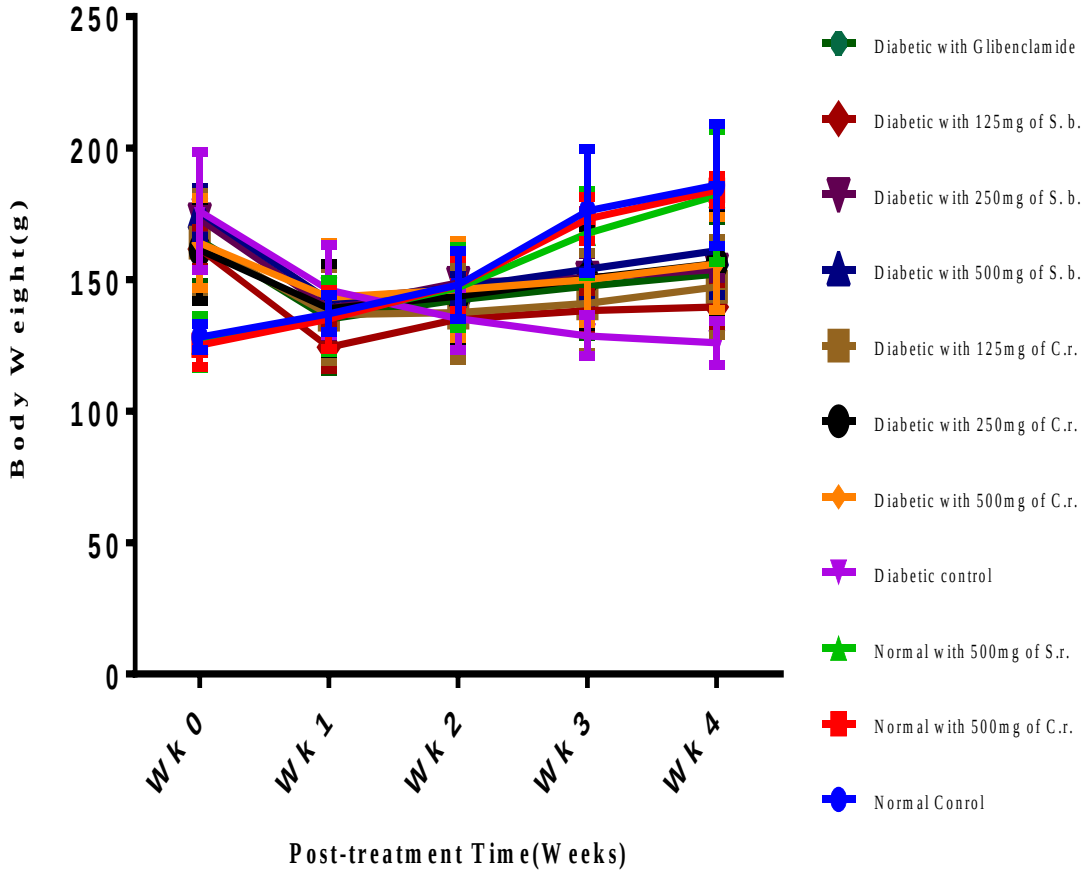


Figure 4.9: Weekly body weights of STZ-induced Diabetic and Normal Rats Treated with Various Doses of Aqueous Extracts of *Cleome rutidosperma* (C.r.) and *Senecio biafrae* (S.b.)

The effect of the aqueous extracts of both plants on fasting blood glucose is shown in Figure 4.10. There was significant ($P<0.05$) increase in fasting blood glucose for the diabetic control group when compared with the normal control. A steady significant ($P<0.05$) decrease in FBG was observed in all treated groups when compared with the diabetic control group, from week one up to week four post treatment . The maximum blood glucose lowering effect of both plant extracts and standard drug on diabetic rats was observed at week four. The percentage blood glucose reduction (Table 4.3) of the different doses of the two plant extracts at week four ranged from 12.28±8.37 to 57.77±5.05(%) for *Cleome rutidosperma* and 3.03±5.19 to 46.43±7.55(%) for *Senecio biafrae* respectively, while the standard drug, glibenclamide reduced the blood glucose level by 44.74±9.24%. On the contrary, there was a steady increase in3 FBG in the diabetic control group throughout the experimental period.

As shown in Figure 4.11, the PCV of the untreated diabetic animals were significantly lower ($P<0.05$) than those of either the normal control group or those given different doses of both plant extracts (*Cleome rutidosperma* and *Senecio biafrae*) and that of diabetic rats treated with the standard drug, glibenclamide. There was however no significant differences among the diabetic rats treated with either plant extracts or the normal control rats.

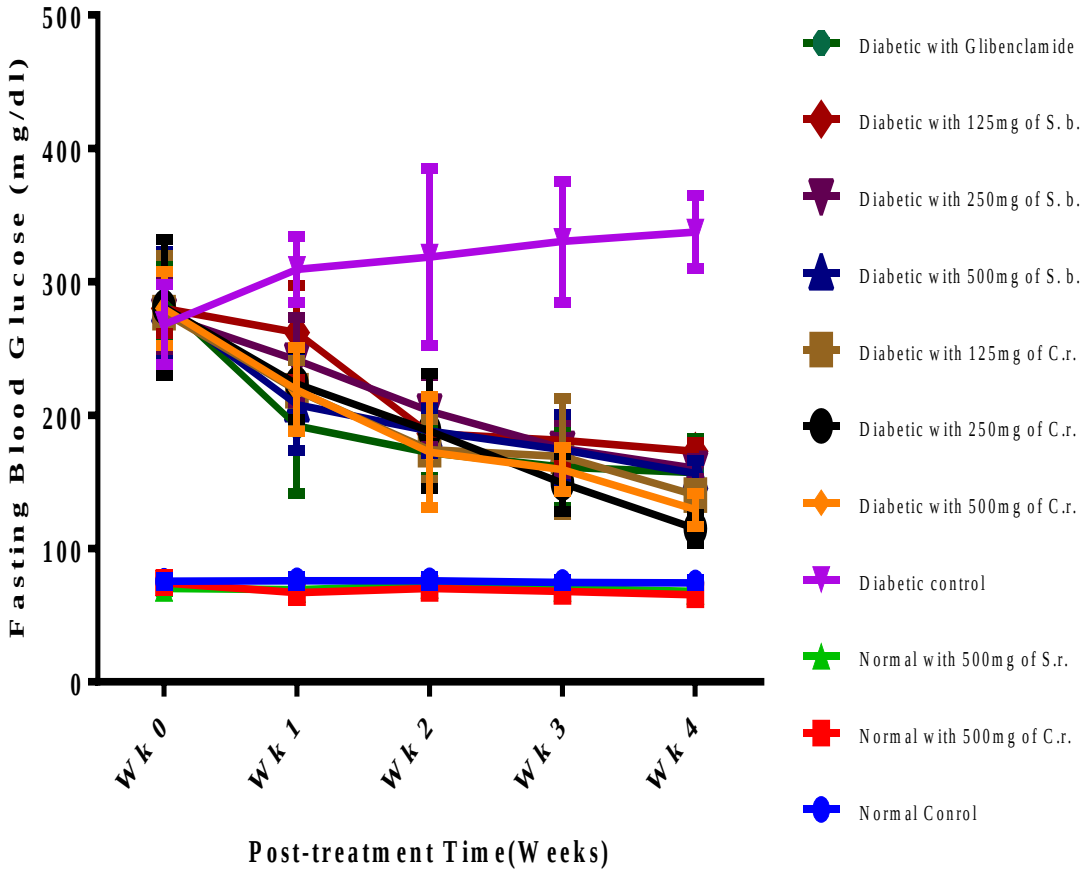


Figure 4.10: Weekly Fasting Blood Glucose levels of STZ-induced Diabetic Rats Treated with Various Doses of Aqueous Extracts of *Cleome rutidosperma* (C.r.) and *Senecio biafrae* (S.b.)

Table 4.3: Percentage Decrease in FBG at Week 4 after Treatment with Various doses of Aqueous Extracts of *Cleome rutidosperma* and *Senecio biafrae* and Glibenclamide

GROUP	% Decrease in FBG at Week 4	GROUP	% Decrease in FBG at Week 4
	Cleome rutidosperma		*Senecio biafrae*
A	1.38±4.64[a]	A	1.38±4.64[a]
B	12.28±8.37[a]	C	3.03±5.19[a]
D	-30.44±17.57[a]	D	-30.44±17.57[a]
E	55.00±3.54[b]	H	46.43±7.55[b]
F	57.77±5.05[b]	I	42.76±5.36[b]
G	47.20±9.24[b]	J	42.76±5.36[b]
K	44.74±9.24[b]	K	44.74±9.24[b]

Values are mean of seven determinations ± SD; Values with different superscripts along a column are statistically different ($p<0.05$). Negative value indicate an increase in FBG.

Where Group A: Normal untreated rats ,Group B: Normal rats given 500mg/kg body weight of *Cleome rutidosperma* extract, Group C: Normal rats given 500mg/kg body weight of *Senecio biafrae* extract, Group D: Diabetic control rats, Group E, F & G: Diabetic rats given 500, 250 and 125 mg/kg body weight respectively of aqueous extracts of *Cleome rutidosperma*.,Group H, I & J: Diabetic rats given 500, 250 and 125 mg/kg body weight respectively of aqueous extracts of *Senecio biafrae* and Group K: Diabetic rats given reference drug, glibenclamide (600 µg/kg of body weight), FBG: fasting blood glucose.

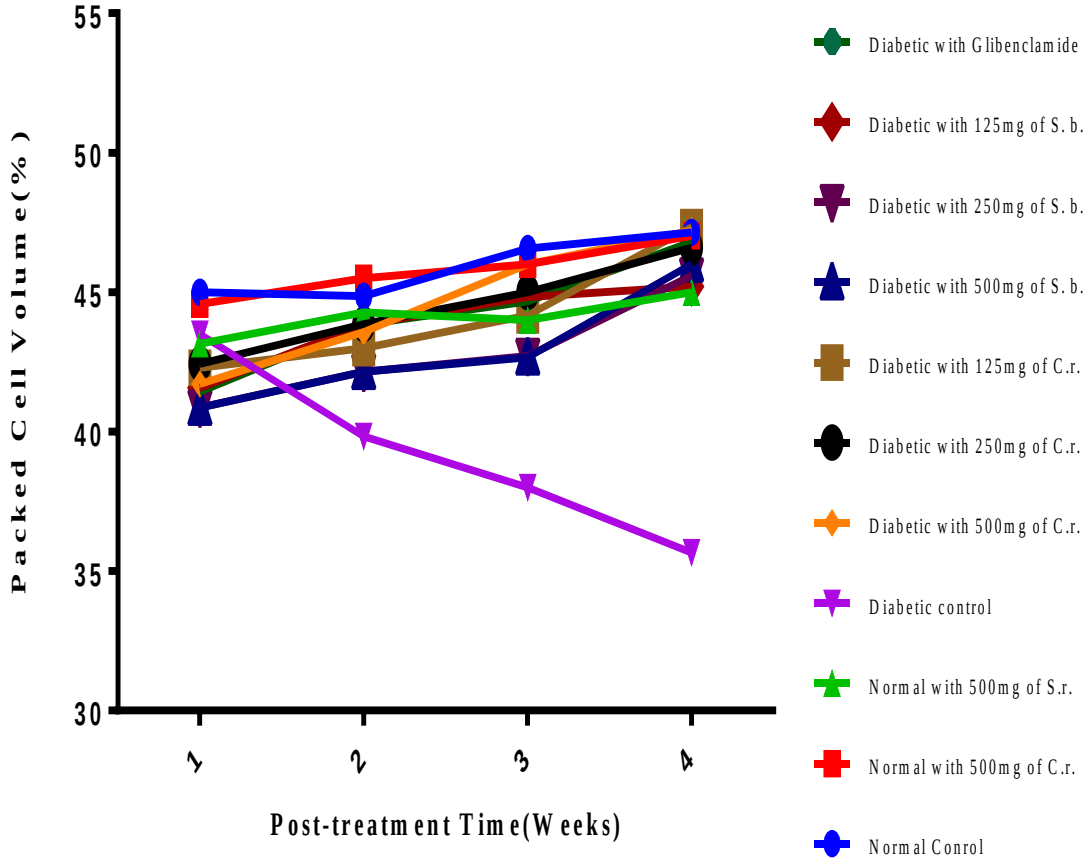


Figure 4.11: Weekly Packed cell volume of STZ-induced Diabetic and Normal Rats Treated with Various Doses of Aqueous Extracts of *Cleome rutidosperma* (C.r.) and *Senecio biafrae* (S.b.)

Data on relative organ weight when aqueous extracts of *Cleome rutidosperma* and *Senecio biafrae* were orally administrated to the STZ-induced diabetic rats for 28 days are shown in Table 4.4. The relative weights of liver and kidney (but not the heart) were significantly increased in diabetic control rats ($p<0.05$) when compared with the normal control rats. Significant decreases ($p<0.05$) were seen in relative weights of liver and kidney for all treated rats when compared with the diabetic control rats. Although decrease in the relative weight of the heart was observed in all treated groups when compared to the diabetic control group, the decreases were not significant. Thus, administration of the extracts reversed the increase in relative weights of liver and kidney seen in the diabetic state.

Table 4.4: Effect of Oral Administration of Different Doses of Aqueous Extracts of *Cleome rutidosperma* and *Senecio biafrae* on Relative Organ Weight (g/Kg body weight) in STZ-induced Diabetic Rats.

GROUP	HEART	KIDNEY	LIVER
A	0.38 ± 0.07^{a}	0.56 ± 0.05^{a}	2.94 ± 0.29^{a}
B	0.34 ± 0.02^{a}	0.55 ± 0.04^{a}	3.03 ± 0.40^{a}
C	0.37 ± 0.07^{a}	0.54 ± 0.09^{a}	2.81 ± 0.42^{a}
D	0.64 ± 0.06^{a}	1.90 ± 0.68^{c}	5.55 ± 0.88^{b}
E	0.37 ± 0.04^{a}	0.74 ± 0.06^{a}	3.71 ± 0.84^{a}
F	0.41 ± 0.08^{a}	0.69 ± 0.11^{a}	4.06 ± 0.98^{a}
G	0.41 ± 0.08^{a}	0.75 ± 0.11^{a}	4.04 ± 0.72^{a}
H	0.36 ± 0.03^{a}	0.80 ± 0.10^{a}	3.63 ± 0.56^{a}
I	0.42 ± 0.12^{a}	0.82 ± 0.05^{a}	4.08 ± 0.40^{a}
J	0.43 ± 0.06^{a}	0.80 ± 0.09^{a}	4.14 ± 0.31^{a}
K	0.42 ± 0.08^{a}	0.77 ± 0.09^{a}	3.78 ± 0.34^{a}

Values are mean of seven determinations ± SD; Values with different superscripts along a column are statistically different ($p<0.05$).

Where Group A: Normal untreated rats ,Group B: Normal rats given 500mg/kg body weight of *Cleome rutidosperma* extract, Group C: Normal rats given 500mg/kg body weight of *Senecio biafrae* extract, Group D: Diabetic control rats, Group E, F & G: Diabetic rats given 500, 250 and 125 mg/kg body weight respectively of aqueous extracts of *Cleome rutidosperma*. Group H, I & J: Diabetic rats given 500, 250 and 125 mg/kg body weight respectively of aqueous extracts of *Senecio biafrae* and Group K: Diabetic rats given reference drug, glibenclamide (600 µg/kg of body weight).

The effects of the plant extracts on biochemical parameters are shown in Table 4.5(a and b). Higher serum Alanine transaminase (ALT) activities were observed in diabetic control rats relative to the normal control ones, while a lower activities of this enzyme were seen in groups E, F and H compared with the diabetic control. Also in serum Aspartate transaminase (AST), lower activities were observed in all treated diabetic groups (except group J) when compared with the diabetic control, while a higher activity was seen in the diabetic group when compared with the normal control group. The serum Alkaline Phosphatase (ALP) was significantly ($p<0.05$) higher in diabetic control group and lower ($p<0.05$) in the treated diabetic groups. Higher levels of serum Total Bilirubin (T.BIL) and Direct Bilirubin (D. BIL) were observed for the diabetic control group while a lower level of the two parameters was observed in all diabetic treated groups. However, the serum Total Protein (TP) and Albumin (Alb) were significantly lower ($p<0.05$) in the diabetic control group compare to all treated diabetic groups. Significantly ($p<0.05$) lower serum creatinine (CREA) levels were observed for groups E, F, H and K when compared with the diabetic control group. Also, lower level of urea was seen in the treated groups compared to the diabetic control group.

Table 4.5a: Serum Biochemical Changes Following Oral Administration of Different Doses of Aqueous Extracts of *C. rutidosperma* and *S. biafrae* in Streptozotocin-induced Diabetic Rats after 28 days.

GROUP	ALT(U/L)	AST(U/L)	ALP(U/L)	D.BIL(mg/dl)	T.BIL(mg/dl)
A	22.38±1.05^{a}	15.14±1.81^{a}	89.11±7.92^{a}	0.12±0.02^{a}	0.63±0.06^{a}
B	23.34±1.84^{a}	17.54±1.92^{a}	90.01±3.74^{a}	0.10±0.01^{a}	0.52±0.08^{a}
C	22.27±1.70^{a}	16.09±1.51^{a}	93.46±5.86^{a}	0.12±0.01^{a}	0.58±0.04^{a}
D	31.91±1.47^{b}	27.40±1.69^{b}	156.91±5.85^{b}	0.23±0.02^{b}	0.89±0.03^{b}
E	23.77±2.05^{a}	18.23±2.97^{a}	118.34±7.11^{c}	0.17±0.01^{c}	0.69±0.07^{a}
F	24.61±1.91^{a}	17.81±2.37^{a}	120.76±10.76^{c}	0.15±0.01^{a}	0.70±0.08^{a}
G	27.47±2.71^{b}	21.44±1.72^{c}	125.80±6.79^{c}	0.18±0.02^{d}	0.71±0.06^{a}
H	23.34±1.10^{a}	20.11±1.99^{c}	110.90±9.33^{c}	0.17±0.01^{d}	0.73±0.05^{a}
I	28.21±1.99^{b}	20.29±5.04^{c}	131.05±10.02^{c}	0.18±0.02^{d}	0.71±0.08^{a}
J	29.40±3.54^{b}	25.11±1.98^{b}	140.37±10.44^{b}	0.21±0.03^{c}	0.75±0.08^{ab}
K	28.02±2.15^{b}	21.73±1.69^{c}	125.39±6.86^{c}	0.17±0.03^{d}	0.73±0.07^{a}

Values are mean of seven determinations ± SD; Values with different superscripts along a column are statistically different ($p<0.05$).

Where Group A: Normal untreated rats ,Group B: Normal rats given 500mg/kg body weight of *Cleome rutidosperma* extract, Group C: Normal rats given 500mg/kg body weight of *Senecio biafrae* extract, Group D: Diabetic control rats, Group E, F & G: Diabetic rats given 500, 250 and 125 mg/kg body weight respectively of aqueous extracts of *Cleome rutidosperma*.,Group H, I & J: Diabetic rats given 500, 250 and 125 mg/kg body weight respectively of aqueous extracts of *Senecio biafrae* and Group K: Diabetic rats given reference drug, glibenclamide (600 µg/kg of body weight).

Table 4.5b: Serum Biochemical Changes Following Oral Administration of Different Doses of Aqueous Extracts of *C. rutidosperma* and *S. biafrae* in Streptozotocin-induced Diabetic Rats after 28 days.

GROUP	TP(mg/dl)	Alb(g/dl)	CREAT(mg/dl)	UREA(mg/dl)
A	10.47±0.98[a]	3.56±0.34[a]	2.35±0.72[a]	29.66±4.90[a]
B	11.08±0.85[a]	3.15±0.55[a]	2.40±0.77[a]	25.81±5.59[a]
C	9.92±0.90[a]	3.11±0.33[a]	2.81±0.72[a]	28.40±3.97[a]
D	5.53±0.73[b]	1.80±0.20[b]	5.19±0.61[b]	50.15±4.76[b]
E	8.14±0.49[a]	3.38±0.39[a]	3.17±0.71[a]	35.83±3.95[a]
F	9.07±0.74[a]	3.17±0.48[a]	3.11±0.61[a]	34.95±6.76[a]
G	8.22±1.78[a]	2.62±0.36[b]	3.78±0.79[ab]	35.50±2.74[a]
H	8.35±0.66[a]	3.07±0.12[a]	3.12±0.94[a]	31.33±3.19[a]
I	7.09±0.49[b]	3.04±0.17[a]	3.68±0.49[ab]	34.24±2.57[a]
J	7.16±0.77[b]	2.85±0.55[a]	3.78±0.51[ab]	42.66±3.45[b]
K	8.23±0.30[c]	3.28±0.78[a]	3.30±0.79[a]	37.24±2.82[a]

Values are mean of seven determinations ± SD; Values with different superscripts along a column are statistically different ($p<0.05$).

Where Group A: Normal untreated rats ,Group B: Normal rats given 500mg/kg body weight of *Cleome rutidosperma* extract, Group C: Normal rats given 500mg/kg body weight of *Senecio biafrae* extract, Group D: Diabetic control rats, Group E, F & G: Diabetic rats given 500, 250 and 125 mg/kg body weight respectively of aqueous extracts of *Cleome rutidosperma*.,Group H, I & J: Diabetic rats given 500, 250 and 125 mg/kg body weight respectively of aqueous extracts of *Senecio biafrae* and Group K: Diabetic rats given reference drug, glibenclamide (600 µg/kg of body weight).

The effect of extracts on serum total cholesterol, total triacylglycerols and low density lipoprotein levels are presented in Table 4.6. The serum triacylglycerols (TRIGS), total cholesterol (CHOL) and low density lipoprotein (LDL)-cholesterol levels were significantly ($P<0.05$) higher in untreated diabetic rats compared with the normal control rats. This dyslipidaemia in the diabetic rats was reduced by administration of both plant extracts and glibenclamide. Thus, there was significant reduction ($p<0.05$) in the levels of serum triacylglycerols, total cholesterol and low density lipoprotein (LDL)-cholesterol in the groups treated with both plant extracts and the standard drug when compared with the diabetic control group. On the other hand, the (HDL)-cholesterol level was significantly ($p<0.05$) lower in the diabetic control group compared with the normal rats. A higher (HDL)-cholesterol level was observed in rats treated with both plant extracts and glibenclamide.

Table 4.6: Effect of Oral Administration of Different Doses of Aqueous Extracts of *C. rutidosperma* and *S. biafrae* on Serum Lipid Profile in Streptozotocin-induced Diabetic Rats after 28 days.

GROUP	CHOL(mg/dl)	C-HDL(mg/dl)	TRIGS(mg/dl)	LDL(mg/dl)
A	76.92±10.34[a]	23.06±1.25[a]	35.71±5.50[a]	46.15±8.10[a]
B	77.99±8.59[a]	23.07±0.92[a]	36.67±6.18[a]	47.58±7.88[a]
C	75.19±6.86[a]	23.79±1.97[a]	34.86±8.03[a]	44.43±5.14[a]
D	162.64±11.30[b]	17.60±0.45[b]	94.00±11.17[b]	126.65±5.46[b]
E	80.53±5.76[a]	23.32±1.07[a]	50.00±7.01[a]	47.61±6.27[a]
F	90.22±5.27[a]	24.00±0.87[a]	52.40±5.85[c]	55.73±6.22[a]
G	95.95±6.45[c]	24.72±1.05[a]	58.00±5.89[c]	59.93±7.14[a]
H	78.84±8.90[a]	23.59±1.68[a]	48.80±6.88[a]	45.09±9.65[a]
I	87.37±8.61[a]	22.27±1.09[a]	53.60±6.37[c]	54.38±4.40[a]
J	85.97±9.96[a]	23.24±0.86[a]	56.00±9.59[c]	51.54±8.75[a]
K	92.21±10.33[a]	22.98±1.77[a]	55.60±8.24[c]	58.47±6.42[a]

Values are mean of seven determinations ± SD; Values with different superscripts along a column are statistically different ($p<0.05$).

Where Group A: Normal untreated rats ,Group B: Normal rats given 500mg/kg body weight of *Cleome rutidosperma* extract, Group C: Normal rats given 500mg/kg body weight of *Senecio biafrae* extract, Group D: Diabetic control rats, Group E, F & G: Diabetic rats given 500, 250 and 125 mg/kg body weight respectively of aqueous extracts of *Cleome rutidosperma*.,Group H, I & J: Diabetic rats given 500, 250 and 125 mg/kg body weight respectively of aqueous extracts of *Senecio biafrae* and Group K: Diabetic rats given reference drug, glibenclamide (600 μg/kg of body weight).

A significantly elevated level of thiobarbituric acid reactive substances (TBARS) was observed in diabetic control rats for both liver and kidney when compared with normal control rats (Table 4.7 and 4.8). However, the TBARS levels in diabetic rats treated with either extracts or glibenclamide were significantly ($p<0.05$) lower than the diabetic control group in both liver and kidney except the glibenclamide group which was not significant in the liver.

The activities of superoxide dismutase (SOD) were significantly higher in diabetic rats treated with both plant extracts or glibenclamide compared to diabetic control rats ($p < 0.05$) in the liver and kidney (Table 4.7 and 4.8). A significantly lower ($p<0.05$) activity was observed in the diabetic group compared to the normal control in both organs.

There were no differences in Catalase (CAT) activity in groups G, I and K (in the liver) and groups G, I, J and K (in the kidney) when compared with the diabetic control group. However, the activities of CAT in diabetic rats treated with both plant extracts and glibenclamide in groups E, F and H (liver) and groups E, F, H and I(kidney) were significantly higher ($p < 0.05$) than the diabetic control rats, while the diabetic control rats were significantly lower in CAT activity when compared with the normal control group.

A significantly lower ($p<0.05$) activity of Glutathione S-transferase (GST) in the diabetic control group when compared with the normal control was observed for both liver and kidney. Also, the GST activity was significantly higher ($p<0.05$) than the diabetic control group in groups E, F, H and I (liver) and groups E, F, G, H and I(kidney). Although the GST activity in groups G, J and K (liver), J and K (kidney) were higher than the diabetic control rats, these were not significant.

The Glutathione reductase (GR) activity was significantly ($p < 0.05$) lower in the diabetic control group than the normal control rats. There was significantly lower ($p < 0.05$)

activity of GR in groups F, H and K when compared with the diabetic control group, while in groups E, G, I and J, though higher values were seen, the values were not significantly different.

Table 4.7: Effect of Oral Administration of Different Doses of Aqueous Extracts of *C. rutidosperma* and *S. biafrae* on Oxidative Stress Markers in Liver of Streptozotocin-induced Diabetic Rats.

GROUP	TBARS (nmoles/g tissue)	SOD (Units/mg protein)	CAT (Units/mg protein)	GST (Units/mg protein)	GR (Units/mg protein)
A	7.17±0.83^{a}	42.80±2.59^{a}	18.89±2.93^{a}	122.82±8.10^{a}	4.04±0.30^{a}
B	8.94±1.00^{a}	40.69±2.13^{a}	17.57±1.64^{a}	116.01±9.74^{a}	3.34±0.71^{a}
C	8.95±1.05^{a}	42.38±3.88^{a}	16.00±3.58^{a}	120.25±9.29^{a}	3.49±0.49^{a}
D	12.66±1.01^{b}	15.98±1.49^{b}	7.72±0.83^{b}	63.67±5.09^{b}	1.08±0.13^{b}
E	8.08±0.76^{a}	28.39±2.15^{c}	13.39±0.98^{c}	97.77±2.43^{c}	2.07±0.36^{bc}
F	6.00±0.35^{a}	31.05±1.56^{c}	14.94±2.21^{c}	92.72±14.27^{c}	2.66±0.54^{c}
G	9.11±0.89^{a}	28.11±9.02^{c}	12.18±1.94^{b}	78.77±8.29^{bc}	2.06±0.34^{bc}
H	9.36±1.18^{c}	28.09±3.10^{c}	13.53±1.01^{c}	92.03±10.94^{c}	2.36±0.49^{c}
I	7.63±0.84^{a}	29.12±4.20^{c}	12.22±1.65^{b}	89.61±11.85^{c}	2.14±0.38^{bc}
J	7.06±1.19^{a}	20.72±3.88^{b}	13.19±2.52^{c}	74.41±4.60^{bc}	2.01±0.50^{bc}
K	10.78±1.41^{a}	24.85±3.81^{b}	12.70±1.40^{b}	77.39±5.48^{bc}	2.85±0.85^{c}

Values are mean of seven determinations ± SD; Values with different superscripts along a column are statistically different ($p<0.05$).

Where One unit was defined for SOD as: the amount of enzyme that causes 50% inhibition of the oxidation of adrenaline to adrenochrome in one minute; CAT as μmol of H_2O_2 consumed per minute; GST as μmol of 1-chloro- 2,4-dinitrobenzene(CDNB) conjugate formed per minute; GR as μmol of CDNB conjugated per minute. Group A: Normal untreated rats ,Group B: Normal rats given 500mg/kg body weight of *Cleome rutidosperma* extract, Group C: Normal rats given 500mg/kg body weight of *Senecio biafrae* extract, Group D: Diabetic control rats, Group E, F & G: Diabetic rats given 500, 250 and 125 mg/kg body weight respectively of aqueous extracts of *Cleome rutidosperma*.,Group H, I & J: Diabetic rats given 500, 250 and 125 mg/kg body weight respectively of aqueous extracts of *Senecio biafrae* and Group K: Diabetic rats given reference drug, glibenclamide (600 μg/kg of body weight).

Table 4.8: Effect of Oral Administration of Different Doses of Aqueous Extracts of *C. rutidosperma* and *S. biafrae* on Oxidative Stress Markers in Kidney of Streptozotocin-induced Diabetic Rats.

GROUP	TBARS (nmoles/g tissue)	SOD (Units/mg protein)	CAT (Units/mg protein)	GST (Units/mg protein)
A	9.64±0.93[a]	18.00±2.13[a]	16.00±2.87[a]	96.67±9.59[a]
B	7.35±0.86[b]	16.45±1.84[a]	15.86±1.74[a]	95.74±13.02[a]
C	9.16±1.25[a]	17.08±1.74[a]	15.06±2.57[a]	91.72±11.87[a]
D	13.59±0.73[c]	6.25±0.74[b]	5.37±0.19[b]	52.55±4.90[b]
E	9.65±0.62 [a]	12.86±1.10[c]	10.25±1.53[c]	80.83±10.86[a]
F	10.68±0.58[a]	13.51±0.96[c]	10.65±1.47[c]	79.34±6.53[a]
G	9.95±0.91[a]	8.80±1.28[b]	9.15±1.35[b]	77.16±12.87[a]
H	10.84±0.52[a]	13.69±1.60[c]	10.87±2.58[c]	103.19±6.32[a]
I	10.22±0.92[a]	11.30±1.51[c]	9.20±1.85[b]	91.24±11.96[a]
J	9.14±1.30[a]	8.79±0.87[b]	8.56±1.38[b]	74.46±13.84[b]
K	8.88±0.61[a]	9.27±0.78[b]	8.77±1.66[b]	77.00±5.09[a]

Values are mean of seven determinations ± SD; Values with different superscripts along a column are statistically different ($p<0.05$).

Where one unit was defined for SOD as: the amount of enzyme that causes 50% inhibition of the oxidation of adrenaline to adrenochrome in one minute; CAT- µmol of H_2O_2 consumed per minute; GST as µmol of 1-chloro- 2,4-dinitrobenzene(CDNB) conjugate formed per minute. Group A: Normal untreated rats ,Group B: Normal rats given 500mg/kg body weight of *Cleome rutidosperma* extract, Group C: Normal rats given 500mg/kg body weight of *Senecio biafrae* extract, Group D: Diabetic control rats, Group E, F & G: Diabetic rats given 500, 250 and 125 mg/kg body weight respectively of aqueous extracts of *Cleome rutidosperma*.,Group H, I & J: Diabetic rats given 500, 250 and 125 mg/kg body weight respectively of aqueous extracts of *Senecio biafrae* and Group K: Diabetic rats given reference drug, glibenclamide (600 µg/kg of body weight).

The effects of *Cleome rutidosperma* and *Senecio biafrae* extracts on carbohydrate metabolizing enzymes in streptozotocin-induced diabetic rats are shown in Table 4.9. Glucose 6 phospatase (G6Pase) activity was significantly higher ($p<0.05$) in diabetic group when compared with normal control group, while G6Pase activity was significantly higher in all treated diabetic groups (except group J) compared with the diabetic control group.

The diabetic rats showed lower activity of glucokinase (GCK).Treatment with the plant extracts and glibenclamide significantly ($P < 0.05$) resulted in higher activity of glucokinase when compared with the control group in a dose-dependent manner in groups E, F, G, H and K (Table 4.9). However, the activity of GCK was significantly higher than the diabetic control in groups I and J.

Table 4.9: Effect of Oral Administration of Different Doses of Aqueous Extracts of *C. rutidosperma* and *S. biafrae* on Liver Glucose 6- phosphatase and Glucokinase in Streptozotocin-induced Diabetic Rats

GROUP	G6Ptase (Units/mg protein)	Glucokinase(Units/mg protein)
A	0.18 ± 0.05^{a}	177.33 ± 13.27^{a}
B	0.20 ± 0.07^{a}	178.24 ± 17.66^{a}
C	0.21 ± 0.05^{a}	173.34 ± 11.75^{a}
D	0.50 ± 0.07^{b}	115.89 ± 11.62^{b}
E	0.27 ± 0.05^{a}	151.15 ± 13.47^{a}
F	0.26 ± 0.08^{a}	163.67 ± 22.86^{a}
G	0.27 ± 0.05^{a}	160.25 ± 11.06^{a}
H	0.31 ± 0.07^{c}	150.20 ± 14.16^{a}
I	0.33 ± 0.06^{c}	140.17 ± 13.13^{ab}
J	0.40 ± 0.05^{bc}	135.60 ± 14.07^{ab}
K	0.31 ± 0.03^{c}	153.80 ± 8.77^{a}

Values are mean of seven determinations ± SD; Values with different superscripts along a column are statistically different ($p<0.05$).

Where one unit for G6Ptase was defined as μmol of P_i liberated per minute and Glucokinase as μmol of glucose-6-phosphate formed per minute. Group A: Normal untreated rats ,Group B: Normal rats given 500mg/kg body weight of *Cleome rutidosperma* extract, Group C: Normal rats given 500mg/kg body weight of *Senecio biafrae* extract, Group D: Diabetic control rats, Group E, F & G: Diabetic rats given 500, 250 and 125 mg/kg body weight respectively of aqueous extracts of *Cleome rutidosperma*.,Group H, I & J: Diabetic rats given 500, 250 and 125 mg/kg body weight respectively of aqueous extracts of *Senecio biafrae* and Group K: Diabetic rats given reference drug, glibenclamide (600 μg/kg of body weight).

The effects of the different fractions of *C. rutidosperma* and *S. biafrae* on fasting blood glucose (FBG) levels of STZ-induced diabetic mice are shown in Figures 4.12 & 4.13. Significantly lower ($p<0.05$) FBG were seen in the diabetic treated mice, particularly the groups treated with methanol fraction of both plants. Thus, the methanol fraction is the most active fraction for both plants. The phytochemical screening of the methanol fraction indicates that the fraction is rich in phytochemicals as shown in Table 4.10.

Shown in Figures 4.14 & 4.15 are the effects of different 1st Sub-fractions of *C. rutidosperma*, *S. biafrae* and glibenclamide on fasting blood glucose level of STZ-induced diabetic mice. A significant lower ($p>0.05$) FBG was seen in the diabetic mice treated with the 1st sub fraction eluted with chloroform: methanol (5: 5) and chloroform: methanol (7: 3) respectively for *Cleome rutidosperma* and *Senecio biafrae*. Phytochemical screening of these sub fractions revealed the presence of all phytoconstituents present in the methanol fractions of both plants except Saponins and Steroids for *Cleome rutidosperma* and Saponins, Steroids and Tannins for *Senecio biafrae* as shown in Table 4.11.

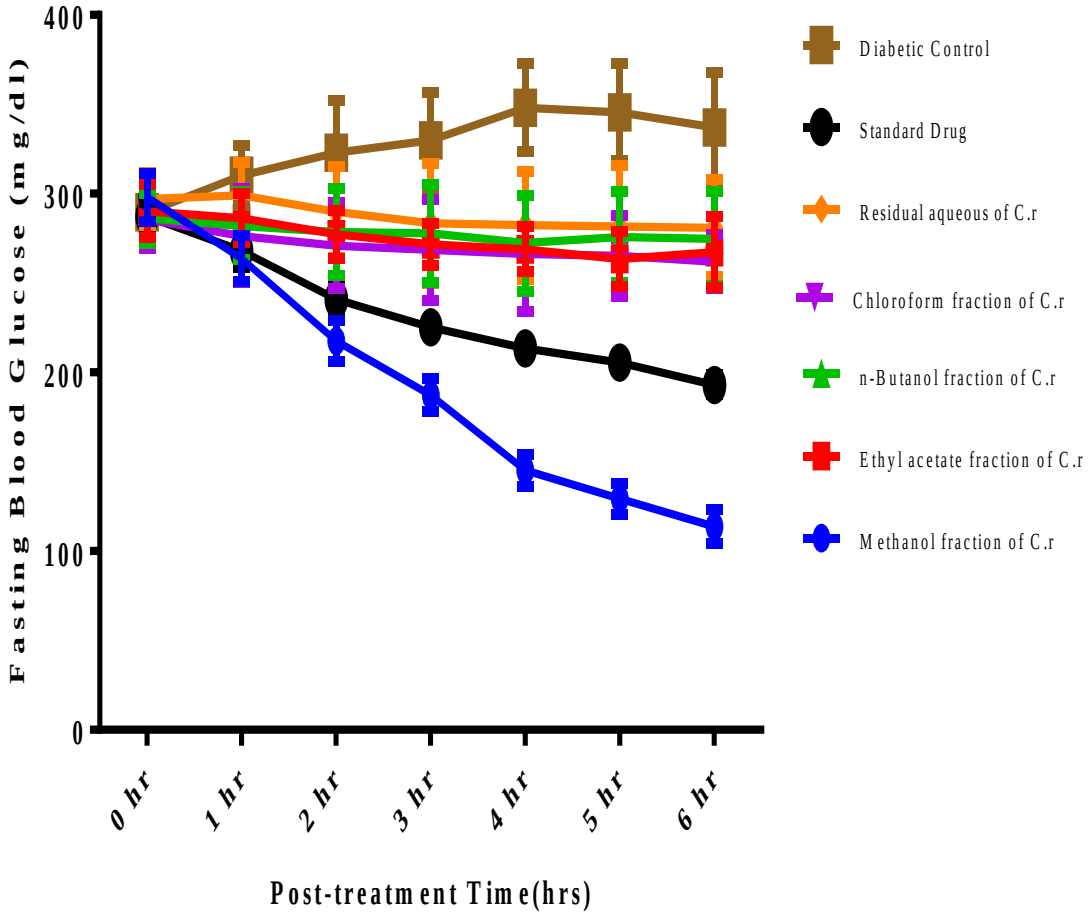


Figure 4.12: Effects of Different Fractions of *C. rutidosperma (*C.r.) and Glibenclamide on Fasting Blood Glucose Level of STZ-induced Diabetic Mice.

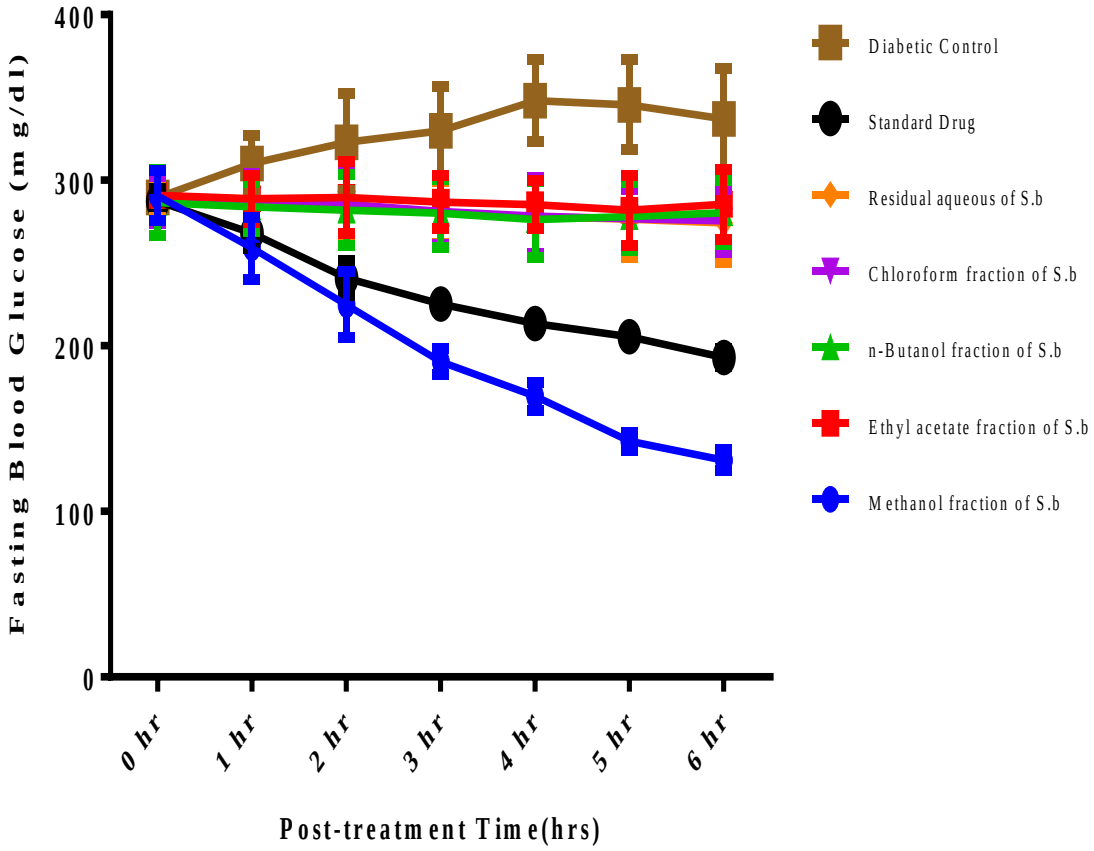


Figure 4.13: Effects of Different Fractions of *S. biafrae (*S.b.) and Glibenclamide on Fasting Blood Glucose Level of STZ-induced Diabetic Mice.

Table 4.10: Phytochemical Constituents of the Methanol Fraction of *C. rutidosperma* and *S. biafrae*

S/N	Constituents	Tests	Results	
			C. rutidosperma	*S. biafrae*
1	Carbohydrates	Molisch	+	+
2	Cardiac glycosides	Kelle Killiam	+	+
3	Saponins	Frothing	+	+
4	Steriod	Liberman Buchard	+	+
5	Triterpene	Liberman Buchard	+	+
6	Tannins	Ferric Chloride	+	+
7	Flavonoid	Sodium hydroxide	+	+
8	Alkaloid	Mayers	+	+
		Dragendorff	+	+
		Wagners	+	+
9	Phenols	Pyridins-ferric chloride	+	+

Where: (+) – Presence of phytochemical compound

(–) – Absence of phytochemical compound

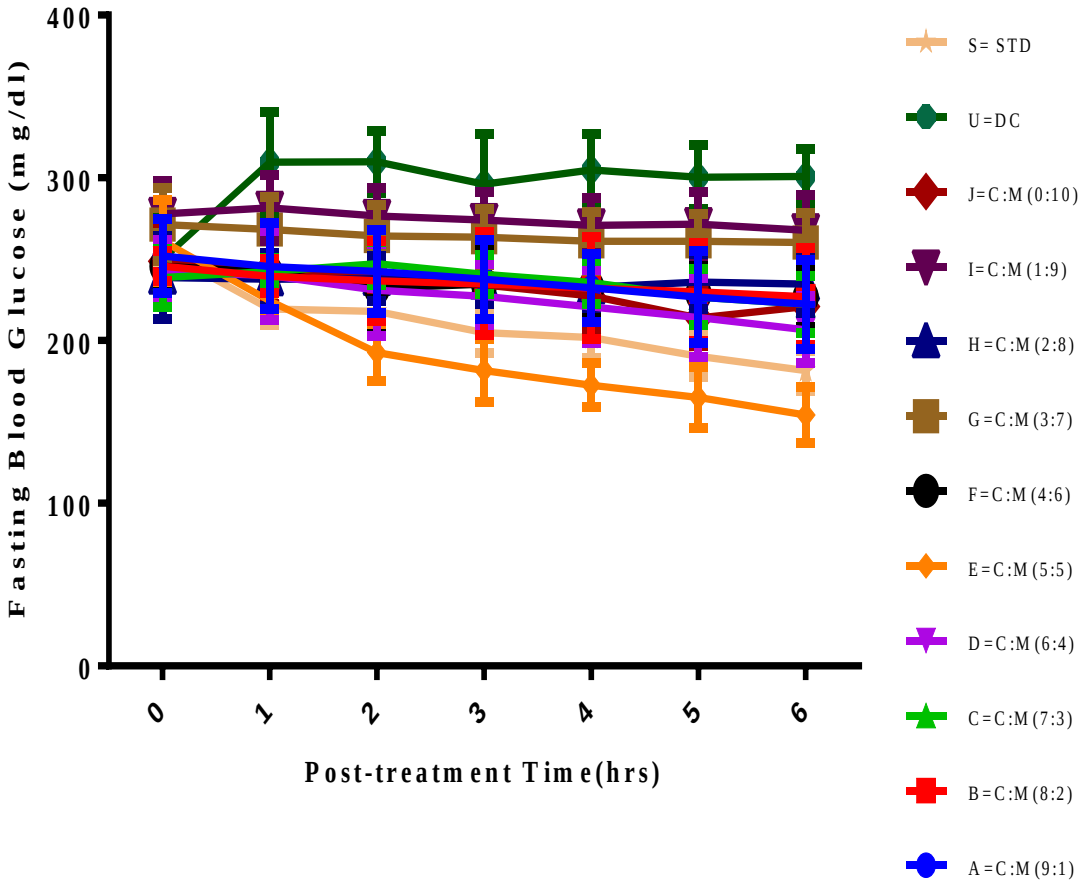


Figure 4.14: Effects of Different 1st Sub-fractions of *C. rutidosperma (*C.r.) and Glibenclamide on Fasting Blood Glucose Level of STZ-induced Diabetic Mice.

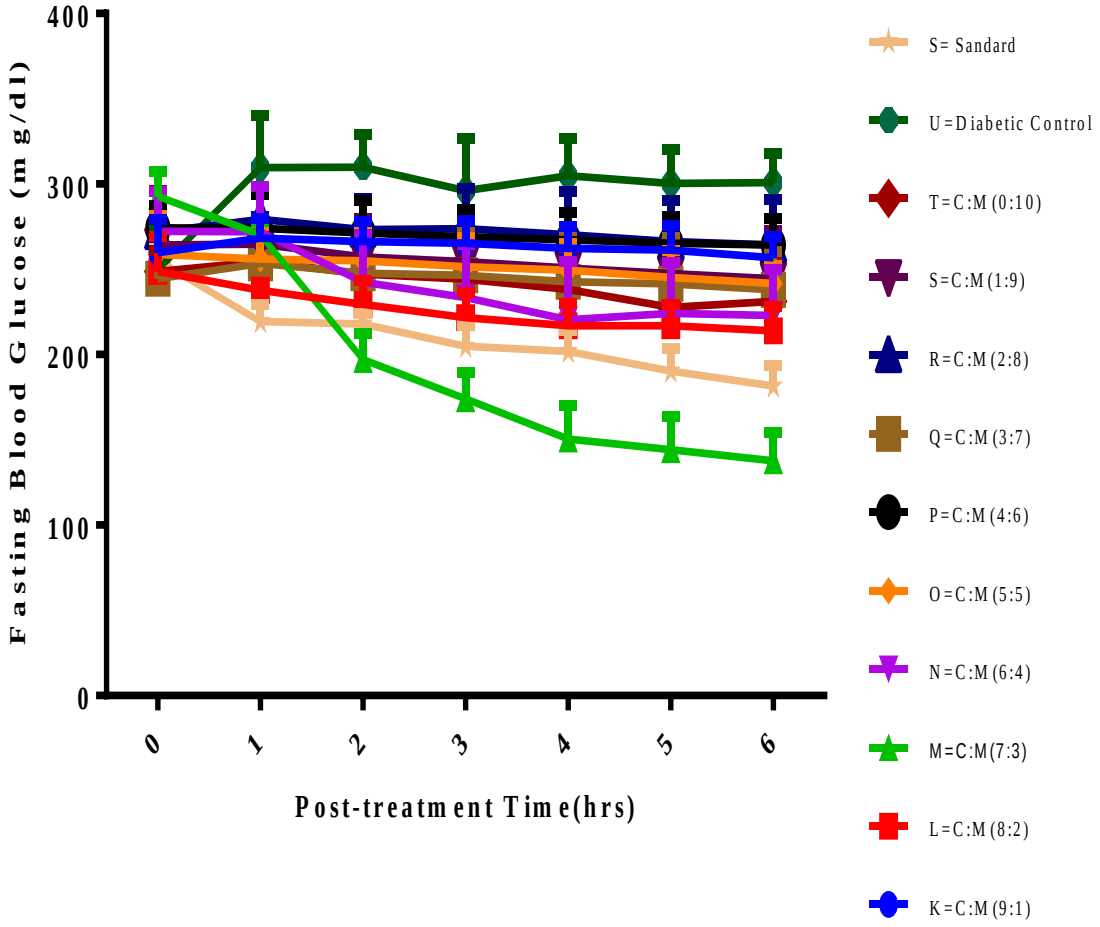


Figure 4.15: Effects of Different 1st Sub-fractions of *S. biafrae* (S.b.) and Glibenclamide on Fasting Blood Glucose Level of STZ-induced Diabetic Mice.

Table 4.11: Phytochemical Constituents of the Most Active 1st Sub-fractions of *C. rutidosperma* and *S. biafrae*

S/N	Constituents	Tests	Results	
			C. rutidosperma	*S. biafrae*
1	Carbohydrates	Molisch	+	+
2	Cardiac glycosides	Kelle Killiam	+	+
3	Saponnins	Frothing	-	-
4	Steriod	Liberman Buchard	-	-
5	Triterpene	Liberman Buchard	+	+
6	Tannins	Ferric Chloride	+	-
7	Flavonoid	Sodium hydroxide	+	+
8	Alkaloid	Mayers	+	+
		Dragendorff	+	+
		Wagners	+	+
9	Phenols	Pyridins-ferric chloride	+	+

Where: (+) – Presence of phytochemical compound

(–) – Absence of phytochemical compound

Comparison of the effects of oral and intraperitoneal routes of administration of different 1st sub-fractions of *C. rutidosperma* (c.r.) and *S. biafrae* (s.b.) on fasting blood glucose level in STZ-induced diabetic mice is shown in Figure 4.16. The intraperitoneal administration of 125 mg/ kg of *C. rutidosperma* sub fraction caused a significant ($p<0.05$) reduction in FBG than the same dose of same sub fraction of the plant administered orally. Comparatively, the intraperitoneal route of administration demonstrated the best effect. The hypoglycemic effects of the 1st sub fraction of both plants were evaluated and the results (Figure 4.17) indicate that the sub fractions of both plant have hypoglycemic effect in a dose dependent manner.

The effects of most active 1st sub-fractions of *C. rutidosperma*, *S. biafrae* and glibenclamide on postprandial glucose concentration in normal mice are shown in Figure 4.18. The results show that sub fractions of both plants at the doses of 125 and 62.5 mg/kg b.w., significantly ($p<0.05$) reduced postprandial hyperglycemia after administration. Also, the administration of both plants 1st sub fraction to normal mice in the glucose tolerance test (Figure 4.19) brought about significant decrease in blood glucose level 180 min after administration of the sub fractions.

Shown in Figure 4.20 are the effects of 1st sub-fractions of *C. rutidosperma* and *S. biafrae* on glucose uptake. The results indicate that only the sub fraction *of C. rutidosperma* at 50g/L significantly decreased glucose movement is this assay.

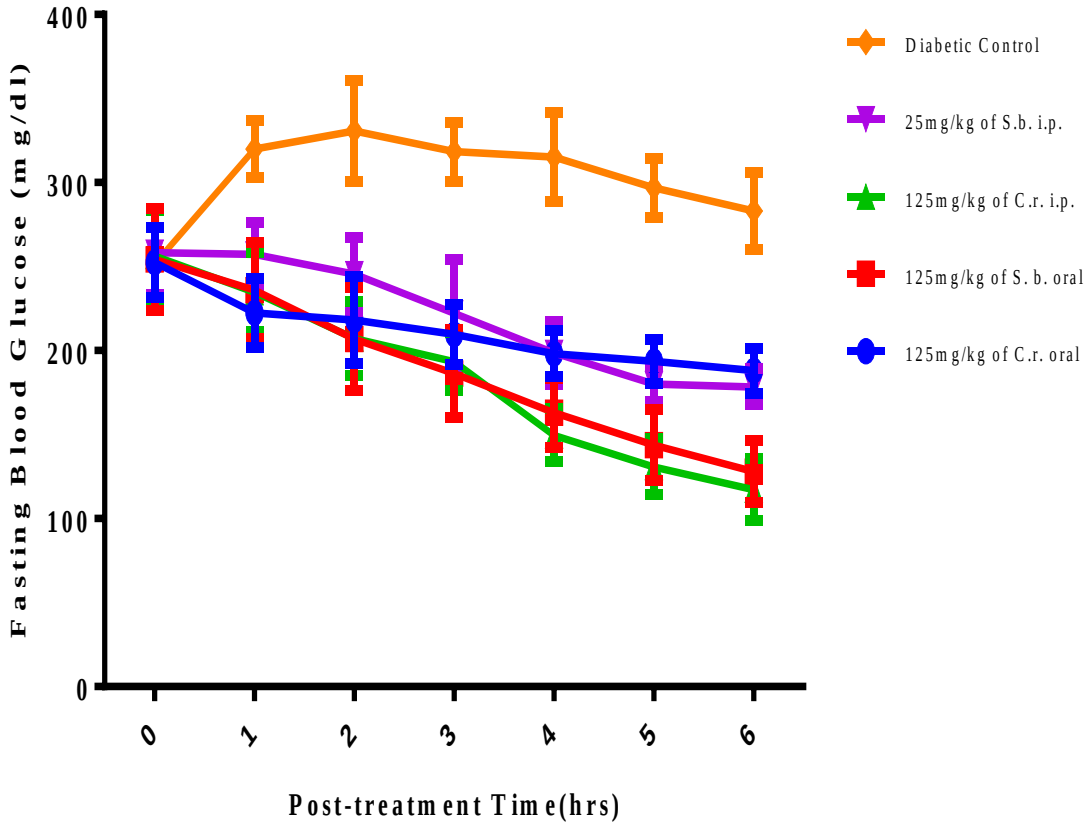


Figure 4.16: Comparison of the Effects of Oral and Intraperitoneal Routes of Administration of Different 1st Sub-fractions of *C. rutidosperma* (C.r.) and *S. biafrae*(S.b.) on Fasting Blood Glucose Level in STZ-induced Diabetic Mice.

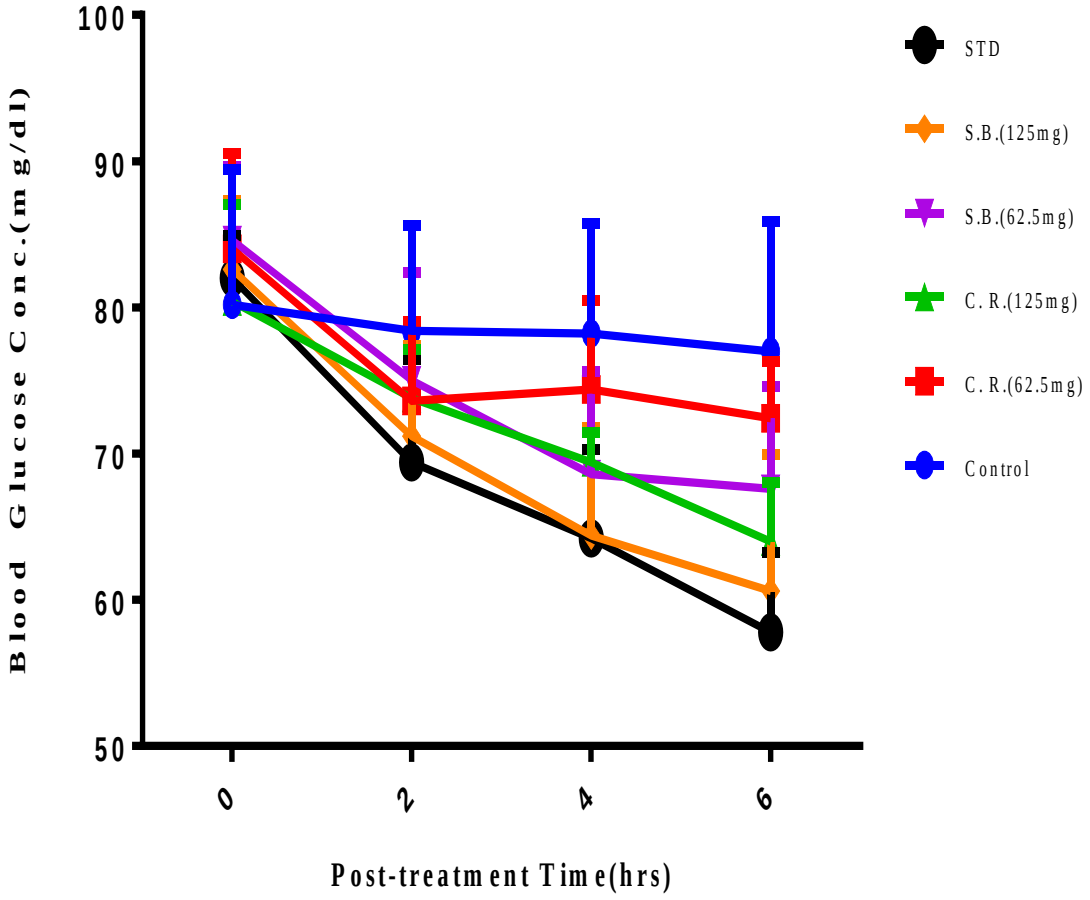


Figure 4.17: Effects of Different 1st Sub-fractions of *C. rutidosperma, S. biafrae* and Glibenclamide (STD) on Blood Glucose Level of Normal Mice.

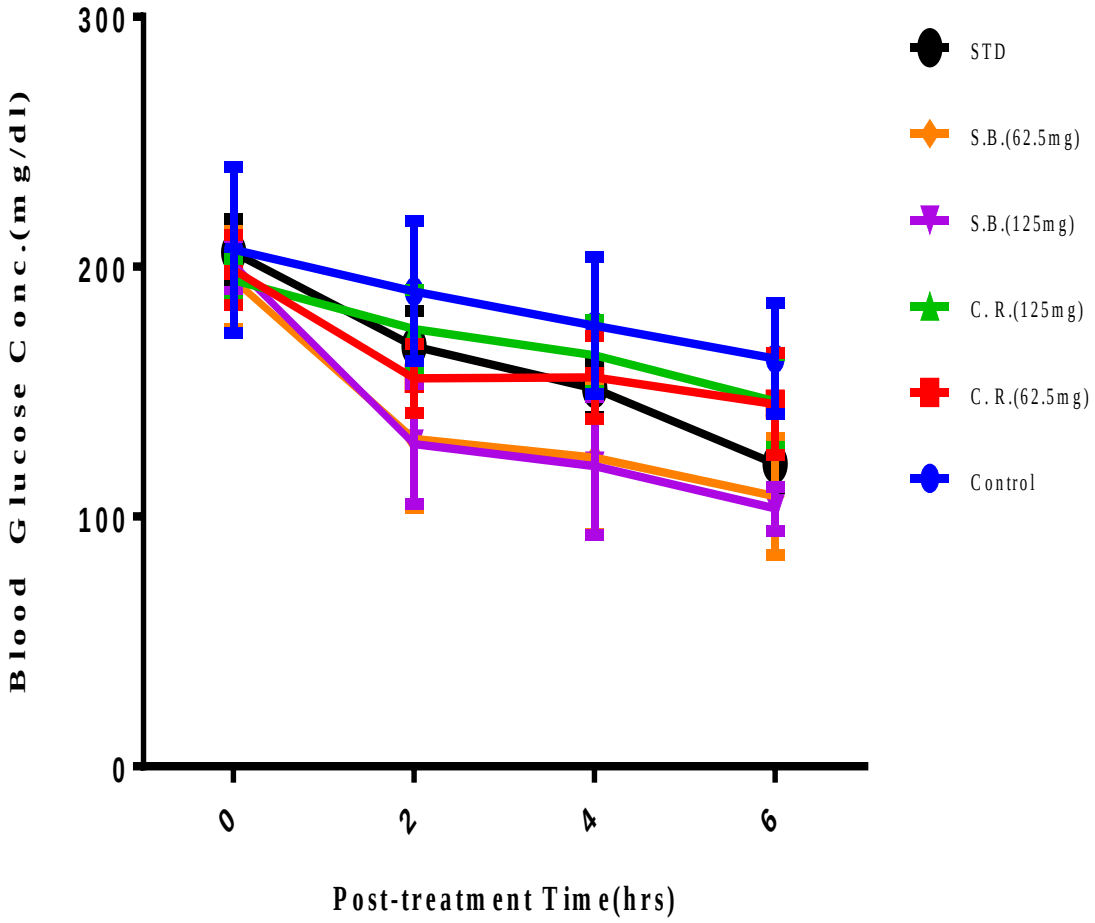


Figure 4.18: Effects of Different 1st Sub-fractions of *C. rutidosperma*, *S. biafrae* and Glibenclamide (STD) on Postprandial Glucose Concentration in Normal Mice.

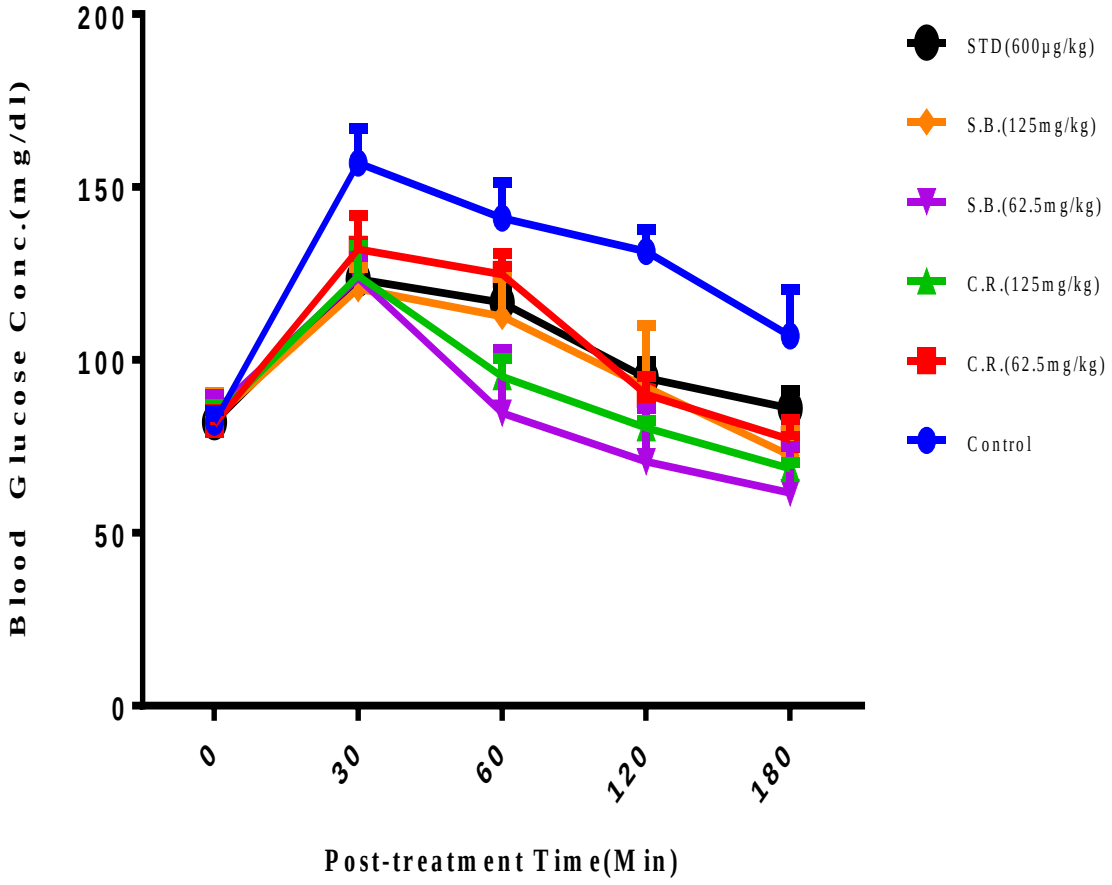


Figure 4.19: Effects of Different 1st Sub-fractions of *C. rutidosperma*, *S. biafrae* and Glibenclamide (STD) on Glucose Tolerance in Normal Mice.

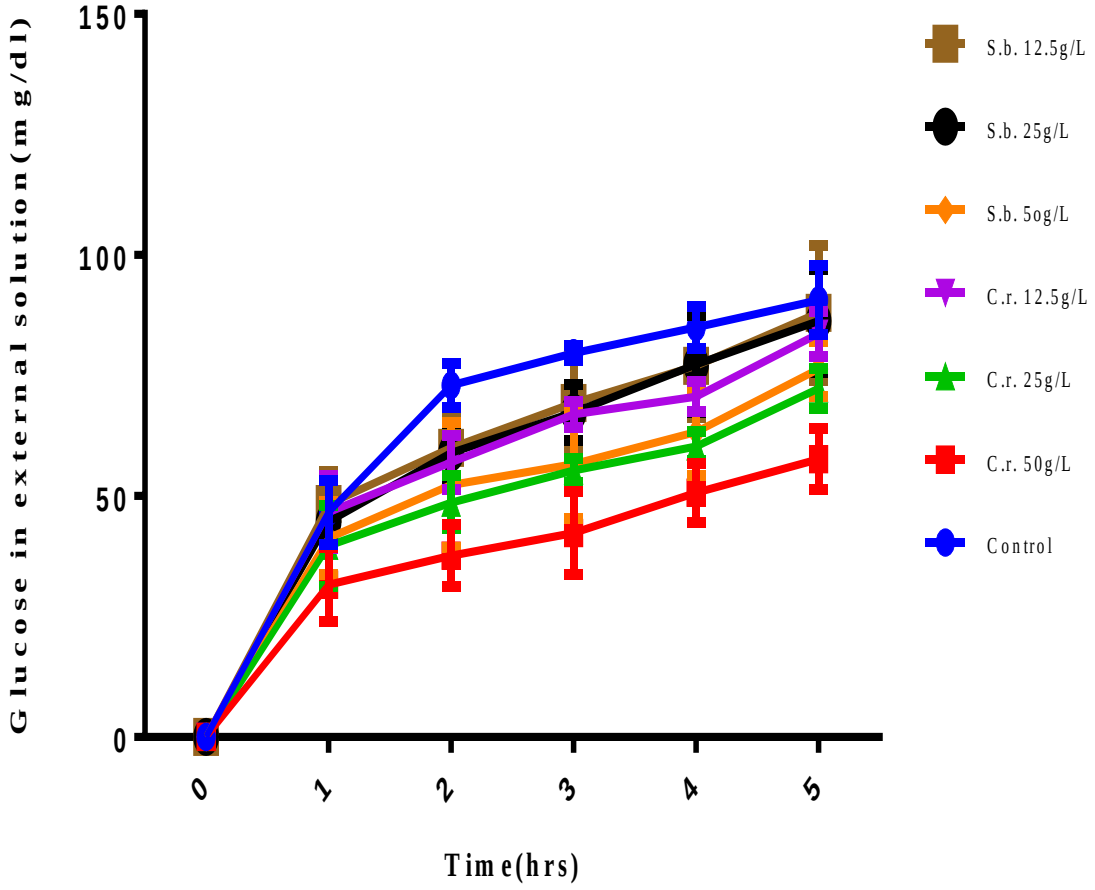


Figure 4.20: Effects of Different 1st Sub-fractions of *C. rutidosperma* (C. r.) and *S. biafrae* (S.b.) on Glucose Absorption by Excised Rat Ileum.

The percentage inhibition of α-amylase by 1st sub-fractions of *C. rutidosperma, S. biafrae* and acerbose is shown in Figure 4.21. There was significant inhibition ($p < 0.05$) displayed by sub fraction of both plants in a dose dependent manner, with sub fraction of *S. biafrae* demonstrating even higher % inhibition than the standard drug, acarbose. The maximum inhibition of this enzyme by sub fractions of *S. biafrae* was 58.22 % and of *C. rutidosperma*, 46.66%, while acarbose was 34.43% at the concentration of 20.00 mg/mL.

Also, the sub fractions of both plants demonstrated a dose dependent inhibition of α-glucosidase as illustrated in Figure 4.22.

Similarly, Figure 4.23 shows the percentage inhibition of glucose 6-phosphatase by 1st sub-fractions of *C. rutidosperma* and *S. biafrae*. Both plant sub fractions demonstrated good glucose 6-phosphatase inhibitory activities with *S. biafrae* giving the highest inhibitions at the lowest sub fraction concentration of 2.50 mg/ml and the highest concentration of 20.00 mg/ml. A dose dependent inhibition pattern of this enzyme was shown by both plants.

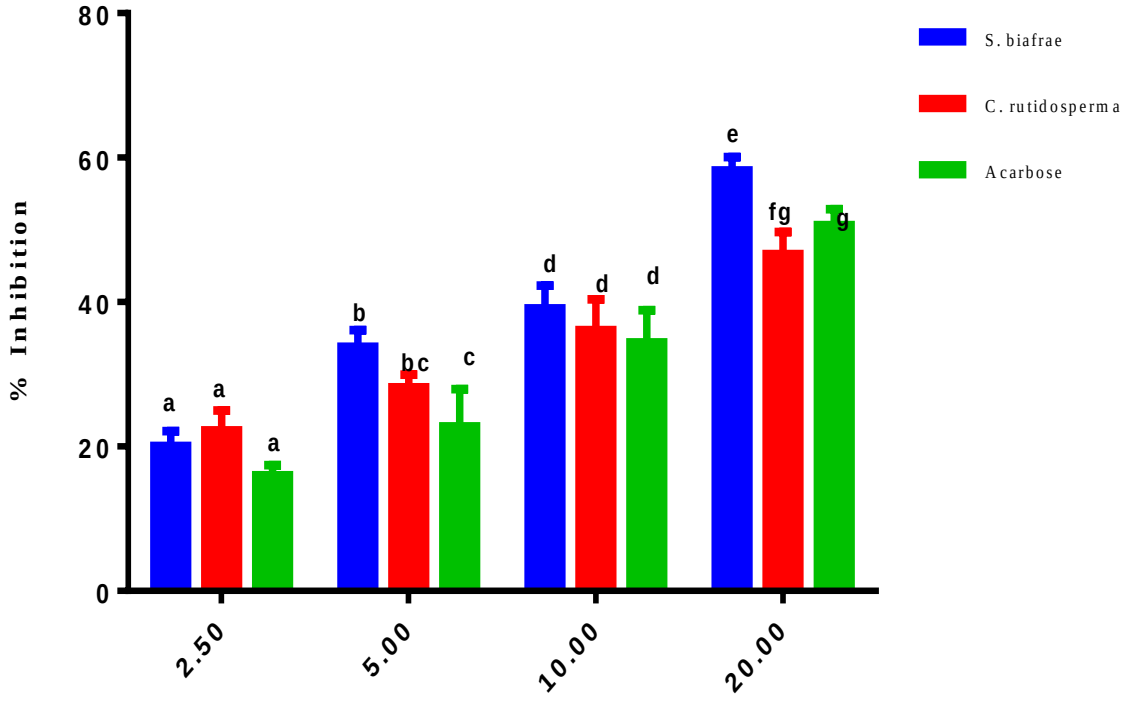


Figure 4.21: Percentage Inhibition of α-amylase by 1st Sub-fractions of *C. rutidosperma*, *S. biafrae* and Acarbose.

Bars with different letters differ significantly ($p<0.05$)

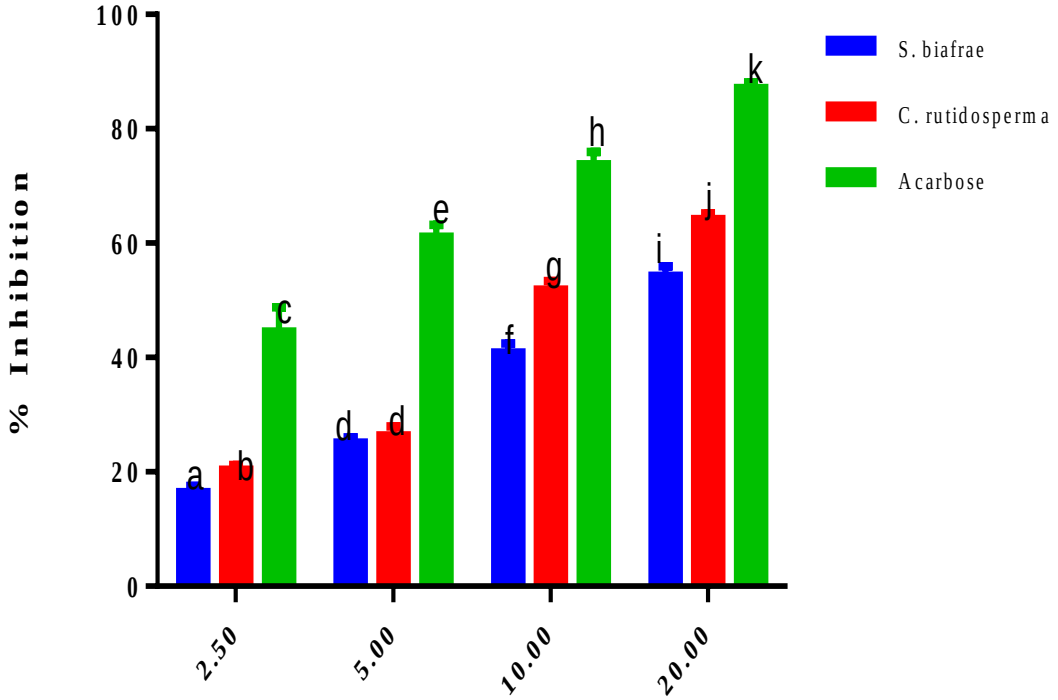


Figure 4.22: Percentage Inhibition of α-glucosidase by 1st Sub-fractions of *C. rutidosperma*, *S. biafrae* and Acarbose.

Bars with different letters differ significantly ($p<0.05$)

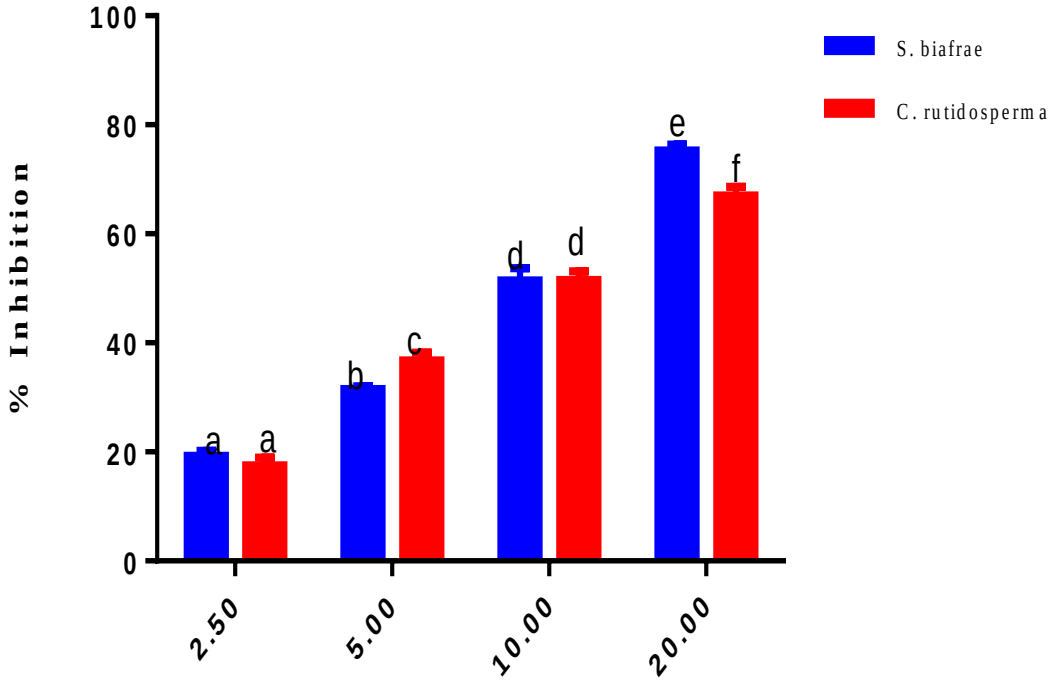


Figure 4.23: Percentage Inhibition of Glucose 6-phosphatase by 1st Sub-fractions of *C. rutidosperma* and *S. biafrae*.

Bars with different letters differ significantly ($p<0.05$)

Shown in Figure 4.24 are the effects of 2nd sub-fractions of *S. biafrae* (S.b.), *C. rutidosperma* (C.r.) and glibenclamide on fasting blood glucose level of STZ-induced diabetic mice. The 2nd sub-fraction of *S. biafrae* demonstrated the highest reduction of FBG which was followed by the 2nd sub-fraction of *C. rutidosperma*.

The effects of constituent of preparative TLC fractions of *C. rutidosperma*, *S. biafrae* and glibenclamide on fasting blood glucose level of STZ-induced diabetic mice are shown in Figure 4.25. Significant ($p < 0.05$) reduction in FBG was demonstrated by the single fraction of *S. biafrae* and was followed by the TLC fraction I of *C. rutidosperma*.

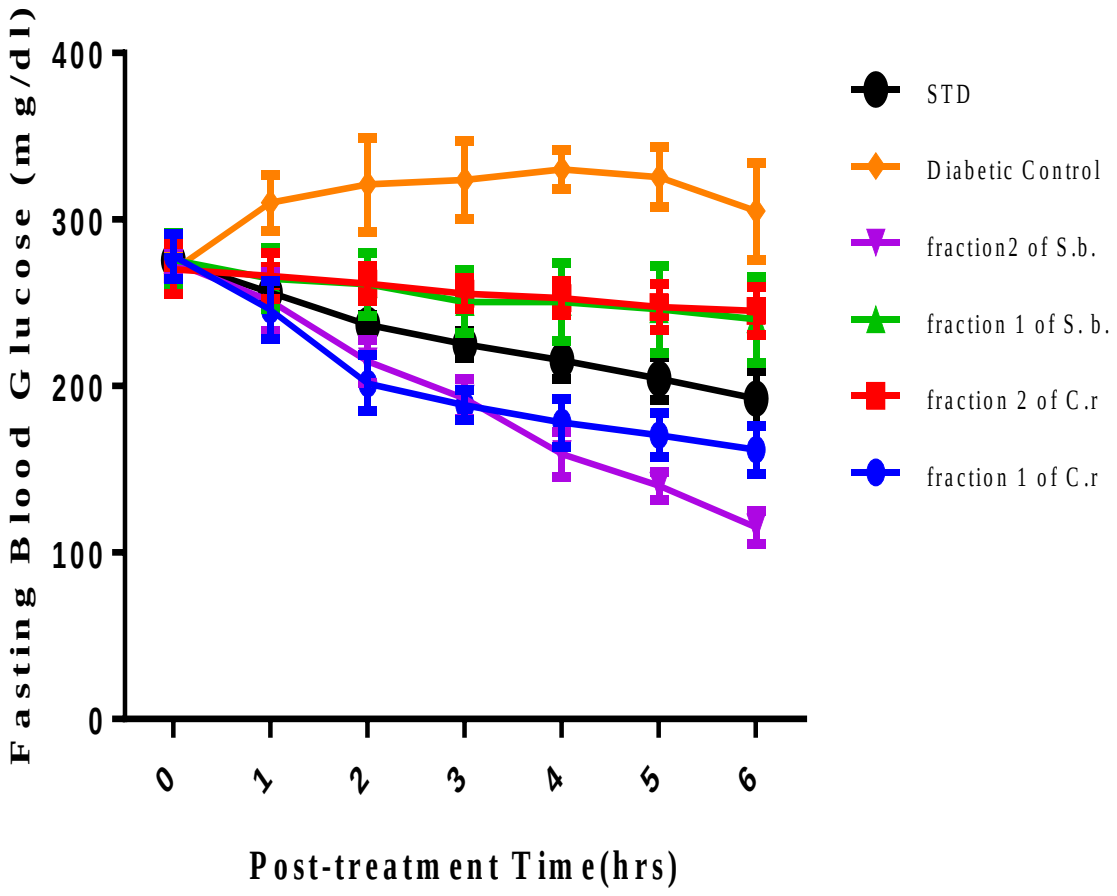


Figure 4.24: Effects of Different 2nd Sub-fractions of *S. biafrae* (S.b.), *C. rutidosperma* (C.r.) and Glibenclamide on Fasting Blood Glucose Level of STZ-induced Diabetic Mice.

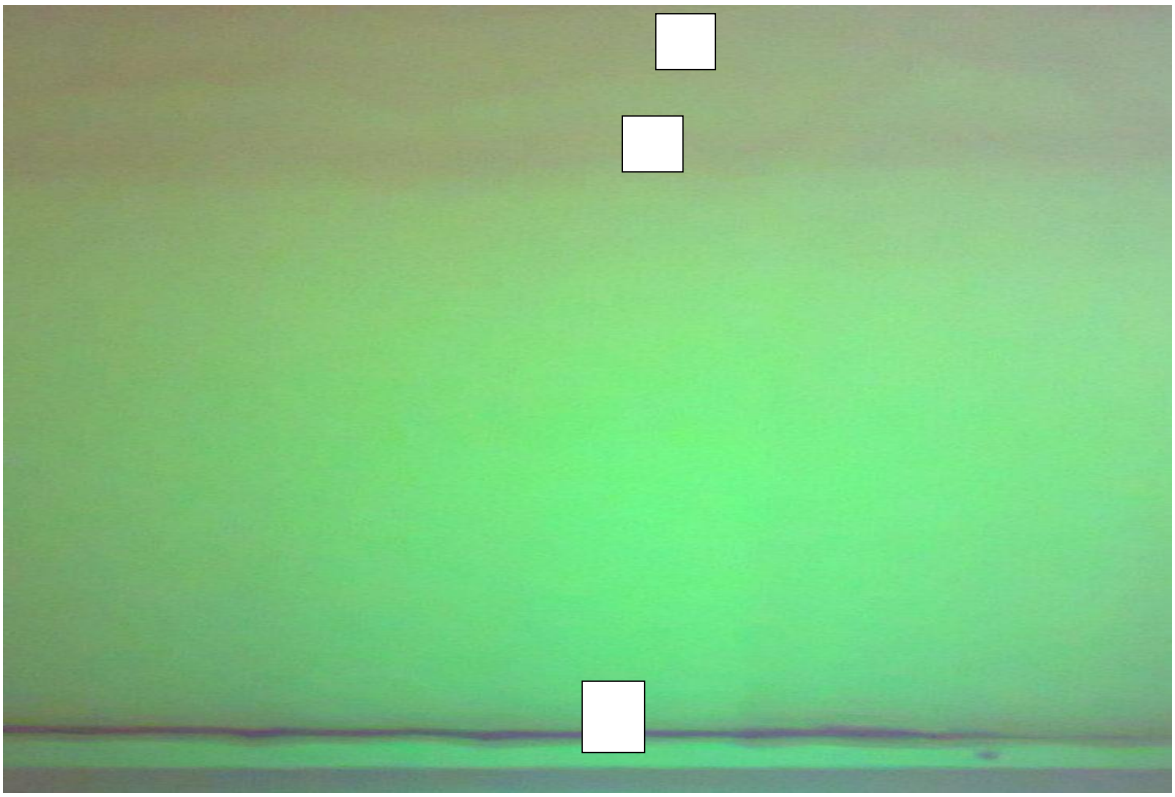


Plate 4.1: Preparative TLC fractionation of Most Active 2nd Sub-fraction of *S. biafrae*.

Where A= Origin, B= TLC fraction Sb I, C= Solvent front.

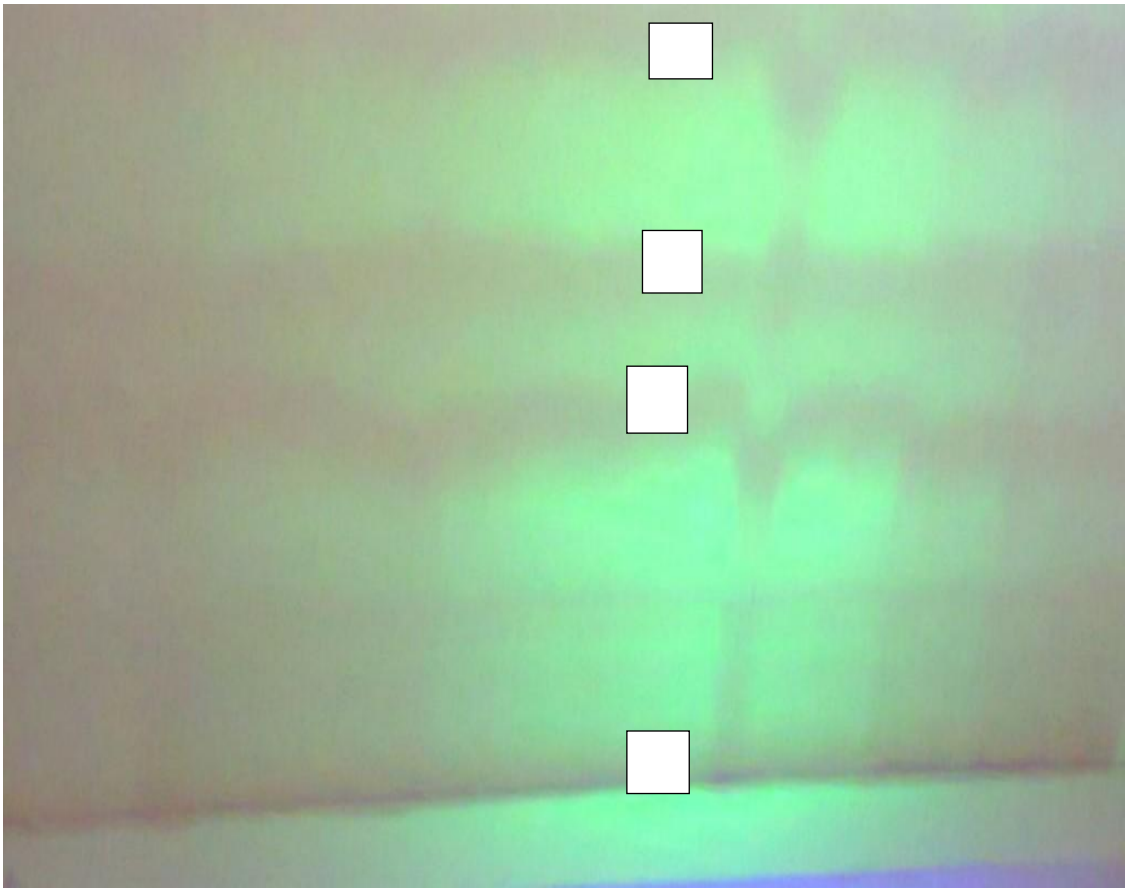


Plate 4.2: Preparative TLC fractionation of Most Active 2nd Sub-fraction of *C. rutidosperma*.

Where A= Origin, B= TLC fraction CrI, C= TLC fraction CrII, D= Solvent front.

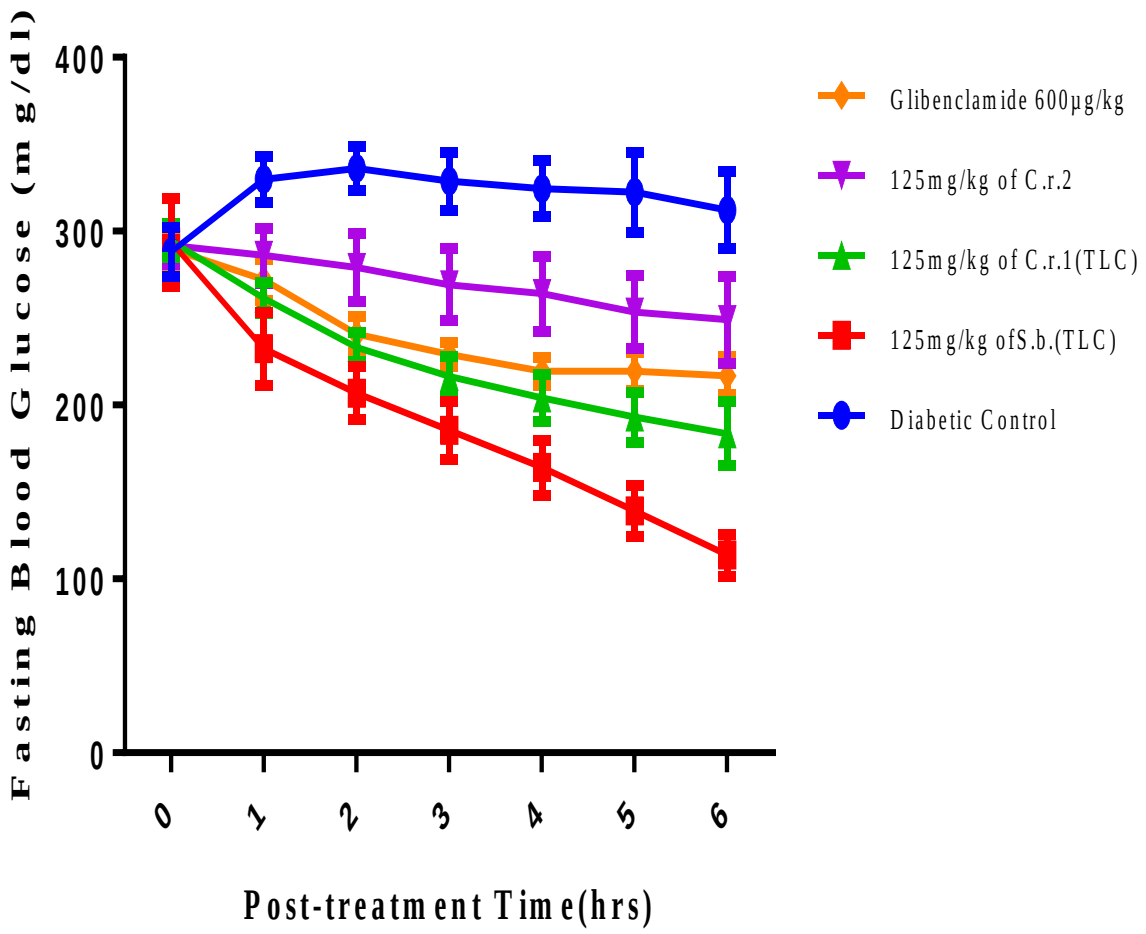


Figure 4.25: Effects of Constituent of Different Preparative TLC fractions of *C. rutidosperma* (C.r.), *S. biafrae (*S.b) and Glibenclamide on Fasting Blood Glucose Level of STZ-induced Diabetic Mice.

A comparative Table of the maximum percentage decrease in FBG of the most active fractions at different fractionation stages in the course of the bioassay guided

fractionation of *C. rutidosperma* and *S. biafrae* in STZ-induced diabetic mice is shown in Table 4.14. There was no significant ($p<0.05$) difference in the fractionation stages of *C. rutidosperma*. However, a significant increase was seen down the fractionation stages of *S. biafrae* from 45.31 ±5.22 at stage one to 61.18 ±3.56 at stage four of the fractionation.

Table 4.12: Maximum Percentage Decrease in FBG of Most Active Fraction at Different Fractionation Stages of *C. rutidosperma* and *S. biafrae* in STZ-induced Diabetic Mice

	Maximum % Decrease in FBG	
Fractionation Stage	*C. rutidosperma*	*S. biafrae*
1	43.49 ±6.33[a]	45.31 ±5.22[a]
2	40.97 ±3.33[a]	53.05 ±4.07[a]
3	41.73 ±4.78[a]	58.12 ±2.87[b]
4	37.76 ±4.08[a]	61.18 ±3.56[c]

Values are mean of five determinations ± SD; Values with different superscripts down the column differ significantly ($p<0.05$).

CHAPTER FIVE

DISCUSSION

The use of herbal medicine is a common practice in many countries, particularly in Asia (Chacko, 2003) and Africa (Shapiro and Gong, 2002). The currently available drug regimens for management of diabetes mellitus have certain drawbacks and therefore, there is a need to find safer and more effective antidiabetic drugs (Grover *et al*., 2002; Rajagopal and Sasikala, 2008).

The quantitative estimation of the percentage crude yields of chemical constituents of the plant extracts showed a good percentage concentration of alkaloids, saponins, tannins, glycosides, flavonoids and steroids in the leaves of *Cleome rutidosperma* and roots of *Senecio biafrae*. Mondal *et al* (2009a) had reported from preliminary phytochemical screening, the presence of terpenoids, flavonoids, tannins and saponins in ethanolic extract of the root of *Cleome rutidosperma.* Also, Edeoga *et. al.* (2005) reported the quantitative phytoconstituents (alkaloids, phenols, tannin, flavonoids and saponins) for aqueous leaves extract of *Cleome rutidosperma*. Similarly, the quantitative phytochemical concentration reported for the ethanolic root extracts for *Senecio biafrae* by Gbadamosi *et. al.* (2012) were alkaloids, tannins, β-Carotene, saponins, flavonoids, steroids, cardenolides, anthraquinones and glycosides. Ajiboye *et. al.* (2013) also found alkaloids, saponin, tannin, phenol, flavonoids and glycosides from quantitative estimation of aqueous leaves extracts of *Senecio biafrae*. There were slight differences in this study from previous reports in the proportion of phytochemicals present in the extracts. This may be due to environmental conditions such as the soil and nutrient level of the plant, and environmental pollutants (Ubani *et al.,* 2012).

Phytochemicals in plants have been reported to be responsible for antidiabetic activities of medicinal plants (Bnouham *et. al*., 2006; Kumar *et. al*., 2011). Flavonoids and

tannins isolated from antidiabetic medicinal plants have been found to stimulate secretion of insulin or possess an insulin like-effect (Marles and Farnsworth, 1995).

Acute toxicity studies are normally conducted to ascertain the safe doses of the plant extracts to be administered to experimental animals. The high oral median lethal dose (LD_{50}) values observed for the aqueous extracts of both plants suggest that the extracts may have low toxicity. It has been established that any substance with LD_{50} estimate greater than 2000 mg/kg body weight by oral route may be considered of low toxicity and safe in humans (Bruce, 1987). Mondal *et al* (2009b) reported the LD_{50} of ethanolic extract of the roots of *Cleome rutidosperma* to be > 3000mg/kg body weight and Bose *et al* (2012) found the LD_{50} of the aqueous extract of the aerial parts of *Cleome rutidosperma* to be >2000 mg/kg. b.w. To our knowledge, there has been no previous report on the LD_{50} of *Senecio biafrae*, so the report of this study is the first.

The results from preliminary study to evaluate the antihyperglycemic activities of the extracts revealed that the leaves extract of *C. rutidosperma* and roots extract of *S. biafrae* caused significant reduction in the fasting blood glucose level of STZ-induced diabetic mice. This observation is in line with the work of Mondal *et al* (2009b) who reported hypoglycaemic activity from the root ethanolic extract of *C. rutidosperma*. To our knowledge, this is the first time the scientific validation of the antihyperglycemic activities of *S. biafrae* (roots) and *C. rutidosperma* (leaves) is being reported.

Comparatively, the most significant reduction of blood glucose level was observed in the aqueous extract of *C. rutidosperma* leaves, which was followed closely by the ethanolic extract. Similar trend was also observed in the extracts of *S.biafrae* roots. Interestingly, the phytoconstituent and *in vitro* antioxidant evaluations revealed higher

concentrations of flavonoids, alkaloids and glycosides for the aqueous extract of *C. rutidosperma*, while glycosides, saponins and flavonoids were highest for the aqueous extract of *S. biafrae*. Also, high antioxidant activities were recorded for the aqueous extracts of both plants compared to other extracts. These variation(s) in concentration and composition of phytochemicals in different extracts of the plants could be responsible for their differences in blood glucose lowering effect. The antihyperglycemic activities of plant extracts have been attributed to the presence of steroids, terpenoids and alkaloids (Manickam *et al*., 1997; Akowuah *et al*., 2002). Based on results of the preliminary study a long term study to further determine the antidiabetic properties of the most active extract of each plant (aqueous extract) was done for 28 days.

Oral administration of both plant extracts and glibenclamide resulted in improvement of the body weight of the animals. The result indicated that both extracts possess the ability of managing glucose level as well as controlling diabetes induced muscle wasting and adipogenesis (Shenoy and Ramesh, 2002). However, a significant decrease in the body weights of the diabetic animals was observed and this agrees with the finding of Oyedemi *et al* (2011) who reported similar effect on diabetic animals induced with streptozotocin. The reduction in weight is caused by the STZ, which causes autoimmune destruction of pancreatic β- cells (Thulesen *et al*., 1997) and it has been linked to degradation of structural proteins and muscle wasting (Rajkumar *et al*., 2005).

The feed and water intake of the diabetic rats were significantly increased compared to the normal rats, while a decrease was seen in the treated groups. These symptoms are well known markers of diabetes in both human and animal models which are direct consequence of insulin deficiency (Swantson-Flatt *et al*., 1990). Increased water

consumption of the diabetic rats could be explained by the fact that the higher sugar level in the blood causes an increase in osmotic diuresis, resulting in large quantities of urine output. Thirst and hunger are compensatory responses to the loss of fluid and the inability to utilize nutrients (Lawrence, 1998). The feed and water intake were significantly reduced after administration of *C. rutidosperma* and *S. biafrae* extracts.

In this study, chronic administration of the plants extracts produced sustained hypoglycemic effects in the treated rats. The antihyperglycemic activities of the plants were comparable with glibenclamide, a standard hypoglycemic drug. Comparatively, *C. rutidosperma* produced the most significant reductions of blood glucose levels in treated diabetic rats. Some plants extracts are reported to exert hypoglycemic action by potentiating the insulin effect, either by stimulating the pancreatic secretion of insulin from the cells of islets of Langerhans (Xu *et al*., 2008) or its release from bound insulin (Pari and Amarnath, 2004); while others act through extra pancreatic mechanisms by inhibition of hepatic glucose production (Edduoks *et al*., 2003) or corrections of insulin resistance (Hux *et al*., 2003). The phytochemicals, alkaloids, steroids, and flavonoids which are present in good proportion in *C. rutidosperma* and *S. biafrae* may be responsible for their antidiabetic activities (Manickam *et al*., 1997; Akowuah *et al*., 2002).

The increased liver weight in STZ-induced diabetic rats as seen indicate the liver probably hypertrophied due to abnormal glycometabolism, accumulated acetyl-CoA, increased fat synthesis and accumulation of fat in the liver due to STZ-mediated inhibition of insulin secretion (Grey *et al*., 1975; Byung-Sung, 2011). When diabetes mellitus (DM) occurs, the free fatty acids produced from body fats due to lack of insulin are transferred into the liver. Triacylglycerides are synthesized and accumulate in the liver and eventually

increase the weight of the liver (Goldberg, 1981). Also, it is known that the reason why the weight of kidney is increased (as observed in this study) is that in diabetes mellitus, the size and volume of kidney are increased together with increase of glomerular filtration rate as the burden of kidney is getting bigger due to increased urine excretion (Mogensen and Andersen, 1973; Gallaher *et al*., 1993).

The elevation of liver biomarker enzymes, such as AST, ALT and ALP in diabetic control rats indicates that diabetes may induce hepatic dysfunction (Rathod *et. al*., 2009). It has been reported by Larcan *et al* (1979) that liver was necrotised in diabetic patients. Therefore, the increase in the activities of AST, ALT and ALP in serum may be mainly due to the leakage of these enzymes from the liver cytosol into the blood stream (Navarro *et al*., 1993). On the other hand, treatment of the diabetic rats with *C. rutidosperma, S. biafrae* or glibenclamide caused reduction in the activity of these enzymes in the serum.

Hyperglycemia increases the generation of free radicals by glucose auto-oxidation and the increase in free radicals may lead to kidney cells damage (Sharma *et al*., 2006). The main function of the kidneys is to excrete the waste products of metabolism and to regulate the body concentration of water and salt (Raghavendra *et al.,* 2013). Diabetic hyperglycemia induces elevation of plasma levels of urea and creatinine which are considered as significant markers of renal dysfunction (Almdal and Vilstrup, 1988). The significant increase in the level of serum urea and creatinine in the diabetic control group is indicative of some degree of damage to renal structures (Kakadiya, 2010). Treatment of STZ-diabetic rats with aqueous extracts of *C. rutidosperma* and *S. biafrae* or glibenclamide, decreased the level of urea significantly in serum (Ashraf *et al.,* 2013).

The significantly higher serum triacylglycerol, total cholesterol and low density lipoprotein (LDL)-cholesterol concentrations and lower HDL-cholesterol in untreated diabetic rats compared with the normal control, were reversed in *C. rutidosperma* and *S. biafrae* treated diabetic rats. Lipids play a vital role in the pathogenesis of diabetes mellitus and the most common lipid abnormalities in diabetes are hypercholesterolemia (Palumbo, 1998). The reductions in serum triacylglycerol, total cholesterol and low density lipoprotein (LDL)-cholesterol concentrations and increase in HDL observed in the treated groups could be beneficial in preventing diabetic complications in diabetics (Cho *et al*., 2002).

Oral administration of *C. rutidosperma* and *S. biafrae* extracts for 28 days caused a significant reduction in TBARS. Rajasekaran *et al* (2005) also reported increased lipid peroxide levels in diabetic rats. Lipid peroxidation of unsaturated fatty acids is a frequently used indicator of increased oxidative stress and subsequent oxidative damage (Hauggard, 1968). Like many chronic diseases, diabetes is widely believed to increase oxidative stress. In diabetes, an increase in oxidative stress arises due to compromise in natural antioxidant mechanisms and an increase in oxygen free radical production (Baynes and Thorpe, 1999). Decreased activities of enzymatic antioxidants such as SOD have been well documented in STZ induced diabetic rats (Sugiura *et al*., 2006). Individuals with reduced CAT activity suffer a heightened risk of developing diabetes (Rajasekharan *et al*., 2005). In this study, oral administration of *Cleome rutidosperma* and *Senecio biafrae* extracts for 28 days significantly increased the SOD and CAT activities in STZ-induced diabetic rats.

Glutathione S-transferases (GSTs) are a family of enzymes that catalyze the addition of the tripeptide glutathione to endogenous and xenobiotic substrates which have electrophilic functional groups (Strange *et al.*, 2000). They play an important role in the

detoxification and metabolism of many xenobiotic and endobiotic compounds (Ji *et al*., 1992). The significant decrease in the GST activity in diabetic control group when compared with the normal control for both liver and kidney was reversed by the administration of the aqueous extracts of *C. rutidosperma* and *S. biafrae* for 28 days in the diabetic rats. The effect was dose-dependent. These observations are in tandem with the reports of previous researchers who have noted a marked decrease in the activity of antioxidant enzymes such as catalase and GST, in liver, kidney and pancreatic tissues from diabetic rats (Anwar and Meki, 2003; Petlevski *et al*., 2003;Yavuz *et al*., 2003; Latha and Pari, 2004). The effects of these antioxidants may be useful in delaying the complications of diabetes such as retinopathy, nephropathy and neuropathy due to imbalance between free radicals and antioxidant systems (Umasankar *et al.,* 2013).

Treatment with extracts of *C. rutidosperma* and *S. biafrae* resulted in significant increase in glutathione reductase (GR) activity in diabetic rats when compared with the diabetic control group which recorded decreased GR activity. GR is a family of homologous proteins whose members are dimeric, NADPH-dependent and FAD containing enzymes. GR maintains the cellular levels of GSH (by the reduction of oxidized glutathione), which protects the cellular membranes from peroxides (Zarida *et al*., 1993). The increase in GR activity in the treated diabetic rats implies that the extracts may protect tissues from oxidative damage occasioned by accumulation of oxidized glutathione.

Blood glucose levels are regulated by the pathways utilizing and generating glucose (Cryer, 2005). Hence the activities of some of the key enzymes of carbohydrate metabolism were estimated in the liver of control and diabetic rats. The activity of hepatic glucokinase was decreased while the activity of hepatic Glucose 6-phosphatase (G6Pase) was increased

in diabetic rats as compared to the normal rats. Oral administration of *Cleome rutidosperma* and *Senecio biafrae* for 28 days caused a significant rise in these enzyme activities in diabetic rats. Thus, administration of both extracts caused a reduction in the G6Pase activity in diabetic rats. This as seen in the treated groups could lead to a reduction in gluconeogenesis and blood glucose concentration (Dijk *et al*., 2001). Glucose-6-phosphatase, a key enzyme in gluconeogenesis or glycogenolysis, plays an important role in glucose homeostasis in the liver and, as such, is a potential target for treatment strategies of diabetes. Zhang *et al* (2009) reported that glucokinase enzyme activity was decreased by more than 90% in the liver of STZ-induced diabetic rats.

In order to investigate the possible mechanism of action of the constituent of the active fraction, *in vivo* and *in vitro* studies were done on 1st sub fractions of both plants.

The antihyperglycemic effect of 1st sub fractions of *C. rutidosperma* and *S. biafrae* was evaluated in different prandial states. Significant reductions in blood glucose level were observed. The control of postprandial hyperglycemia is one of the beneficial therapy for management of type II diabetes mellitus (Kim *et. al.*, 2005) along with nutrition, oral hypoglycaemics and insulin therapies (David, 2005). In addition, controlling postprandial hyperglycemia could also prevent the development of macro-and microvascular complications associated with diabetes (Baron, 1998). The use of plants with postprandial antihyperglycemic property such as *Mucuna pruriens* (Bhaskar *et. al.*, 2008) and *Cynara cardunculus* (Nomikos *et. al.*, 2007) as well as plants with potential of improving glucose tolerance such as *Helicteres ixora* (Venkatesh *et. al.*, 2004) and *Tournefortia hartwegiana* (Ortiz-Andrade *et. al.*, 2007) may benefit the diabetic patient in controlling postprandial hyperglycemia. In the evaluation of postprandial antihyperglycemia, 1st sub fractions at low

and moderate dose, 125 mg/kg b.w. significantly reduced blood glucose after 4 and 6 h of administration suggesting that the mechanism of action of both plant may be mediated through postprandial antihyperglycemic activity.

In the oral glucose tolerance study, all doses of the 1st sub fractions significantly reduced glucose concentration when compared to the control group. At 30 min after glucose administration, blood glucose level increased rapidly from the fasting value to a peak and then subsequently decreased. The sub fractions exhibited remarkable blood glucose lowering effect at 90 min. This suggests that sub fractions of *C. rutidosperma* and *S. biafrae* have the ability to improve glucose tolerance in normal mice. Moreover, these activities were even more effective than glibenclamide as shown in Figure 4.19. This observation indicates that the glucose tolerance activity produced by the sub fractions was more potent than glibenclamide.

Management of blood glucose level is a critical strategy in the control of diabetes and its complications (Dicarli *et. al.*, 2003). Alpha-amylase and α-glucosidase inhibitors have been useful as oral hypoglycemic drugs for the control of hyperglycemia in diabetes mellitus patients. Inhibition of these enzymes delay carbohydrate digestion and prolong overall carbohydrates digestion time causing a reduction in the rate of glucose absorption and consequently reducing postprandial plasma glucose rise (Kimura *et. al.*, 2006).

The moderate inhibition of α-amylase by the 1st sub fractions of *C. rutidosperma* and *S. biafrae* at the highest concentration (20mg/ml) indicates that the plants are mild inhibitors of this enzyme, which is a desirable effect over synthetic drugs with very high inhibitory effects(Kim *et. al.*, 2005). This observation is in agreement with the report of Pinto *et. al.*, (2009) that dietary management of hyperglycemia linked to diabetes can be

targeted through foods or botanical supplements that have moderate α-amylase inhibition. The sub fraction of *C. rutidosperma* at the highest concentration was a better inhibitor of α-amylase than the reference drug acarbose, an indication that this sub fraction at the highest concentration may be more beneficial in preventing postprandial hyperglycemia than acarbose (Kim *et. al.*, 2005).

As for α-glucosidase inhibition, inhibition of the enzyme displayed by the 1st sub fractions of the two plants especially *C. rutidosperma* at the highest concentration (20mg/ml) suggests that these sub fractions contain potent α-glucosidase inhibitor(s). This is a beneficial effect of the plants because any plant or drug which is a strong inhibitor of α-glucosidase could serve as effective therapy for postprandial hyperglycemia with minimal side effects (Kwon *et. al.*, 2007).

The inhibitory effects of 1st sub fractions of *C. rutidosperma* and *S. biafrae* on the α-amlyase and α-glucosidase activities respectively may be attributed to the presence of phytochemicals such as flavonoids, tannins and saponins. The presence of flavonoids have been reported to preserve cell integrity and function by mopping up free radicals in the system and therefore protect against the progression of insulin resistance of type 2 diabetics (Kazeem *et al*., 2013).

Results from the *in vitro* screening of 1st sub fractions of *C. rutidosperma* and *S. biafrae* on glucose-6-phosphatase inhibitory activity indicated that the 1st sub fractions of both plants possess appreciable inhibitory activity towards glucose-6-phosphatase in a dose dependent manner. This activity of the plants may be an advantage over synthetic drugs that inhibit the enzyme, such as sodium orthovanadate and sodium tungstate with their associated toxicity (Dafnis and Sabatini, 1994; Domingo *et al.*, 1995).

Hepatic glycogenolysis and gluconeogenesis are major causes of fasting hyperglycemia in both type 1 and type 2 diabetes mellitus (Smith *et. al.*, 2005). Inhibition of enzymes involved in gluconeogenesis and/or glycogenolysis therefore constitutes an alternative approach to suppress hepatic glucose production and lower fasting plasma glucose (Agius, 2007). Glucose-6-phosphatase (G6Pase) catalyses the final reaction in hepatic glucose production by both gluconeogenesis and glycogenolysis, and has been proposed as a potential target for antihyperglycaemic drugs for type-2 diabetes (Pari and Satheesh, 2006; Agius, 2007).

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

i. The oral median lethal dose (LD_{50}) of the extracts of both *Cleome rutidosperma* and *Senecio biafrae* leaves

and roots respectively were found to be > 5000 mg/kg b.wt., which suggest that the extracts may have low toxicity.

ii. The different solvent extracts are rich in phytochemicals and highly valuable source of natural antioxidants and free radical scavengers. These extracts can be used to prevent damaging effects of free radicals.

iii. Extracts of the two plants achieved reductions in plasma glucose levels especially in STZ induced hyperglycemic mice and rats. This implies that the extracts have anti-diabetic properties and may thus be useful in the management of diabetes.

iv. The aqueous extracts of *Cleome rutidosperma* and *Senecio biafrae* possess *in vivo* antioxidant activity as revealed by increased SOD, CAT, GST and GR activities. Both plant extracts possess hypolipidemic properties as seen from the lipid profile results.

v. Comparatively, the aqueous crude extract of *C. rutidosperma* was more effective than the extract of *S. biafrae* in terms of antidiabetic property. The effective antidiabetic dose was found to be 250mg/kg body weight for *C. rutidosperma* and 500 mg/Kg body weight for *S. biafrae*, but upon fractionation, there was a steady drop in antihyperglycemic activity for *S. biafrae* which might probably be due to loss of synergetic components of the extract.

vi. The interperitoneal median lethal dose (LD_{50}) of the fraction of *Cleome rutidosperma* leaves was

1,095.45mg/Kg while that of *Senecio biafrae* roots was 316.23mg/Kg, which suggests that the fractions may have high toxicity when given interperitoneally.

vii. Fraction of both plants did not produce severe hypoglycemia as shown by their comparable effect with glibenclamide. Likewise, fractions of both plants at the doses of 125 and 62.5 mg/kg b.w., significantly reduced postprandial hyperglycemia particularly after 4 and 6 h of administration. Also, Glucose tolerance activity was also significantly improved in the presence of fractions of *C. rutidosperma* and *S. biafrae*.

6.2 Conclusions

The aqueous extracts/fractions of *Cleome rutidosperma* and *Senecio biafrae* showed significant antidiabetic, antihyperlipidemic activities with minimal toxicity. Therefore, the crude extract or fractionated components could be new sources of development of new plant based therapy for the management of diabetes. The results obtained from the various assays executed on the plants extracts/fractions screened validated the use of both plants for the treatment of diabetes by traditional healers and herbalists.

6.3 Recommendations

Based on results of the present study the following studies are recommended:

The resultant fractions from both plants should further be analyzed using modern liquid chromatographic techniques (LC) coupled with mass spectrometry (LC-MS), nuclear magnetic resonance spectroscopy (LC-NMR), ultra-violet spectroscopy (LC-UV) and infrared spectroscopy (LC-IR) for the specific identification and isolation of novel anti-diabetic component(s) present in the plants.

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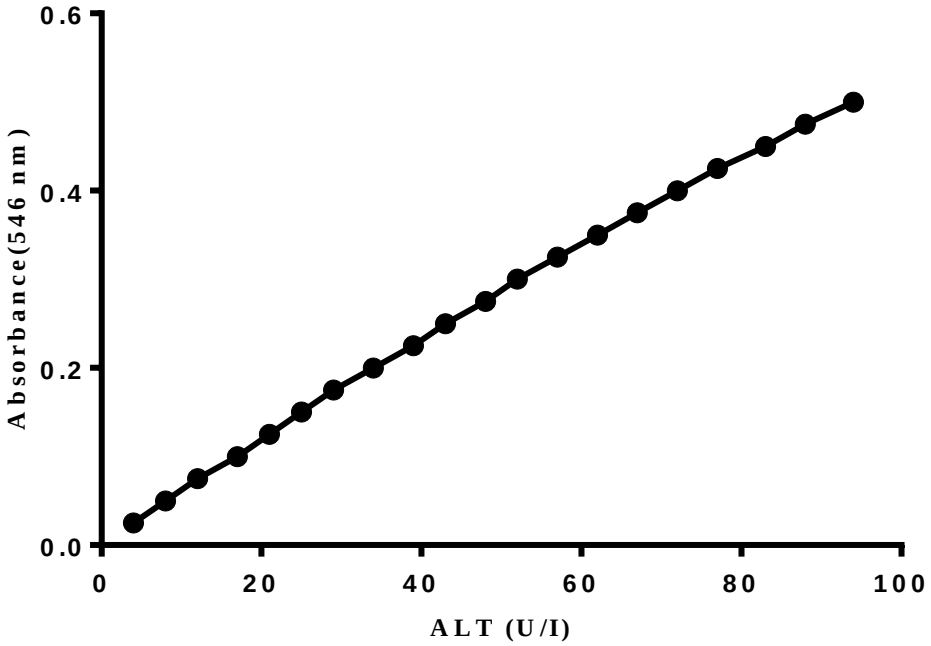
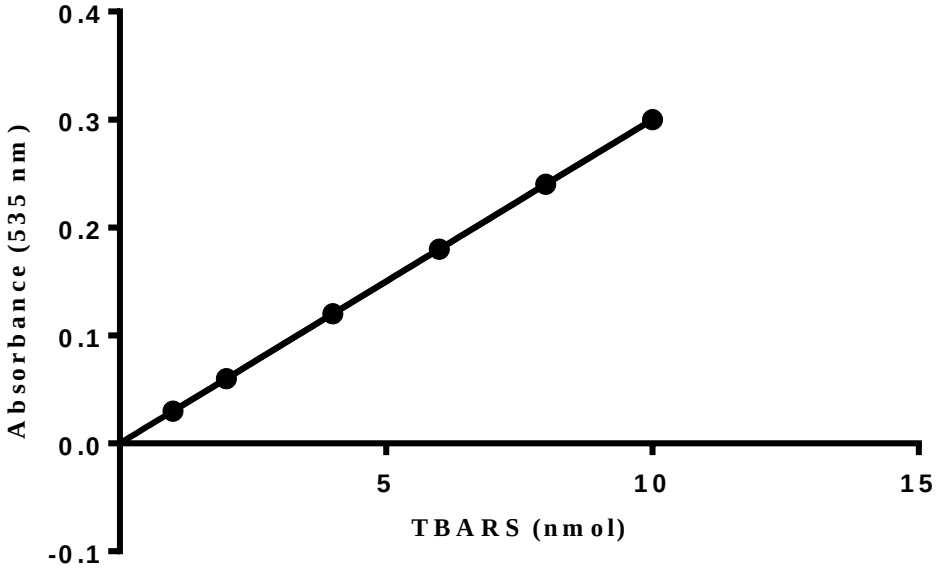
APPENDIX I: Standard Curves

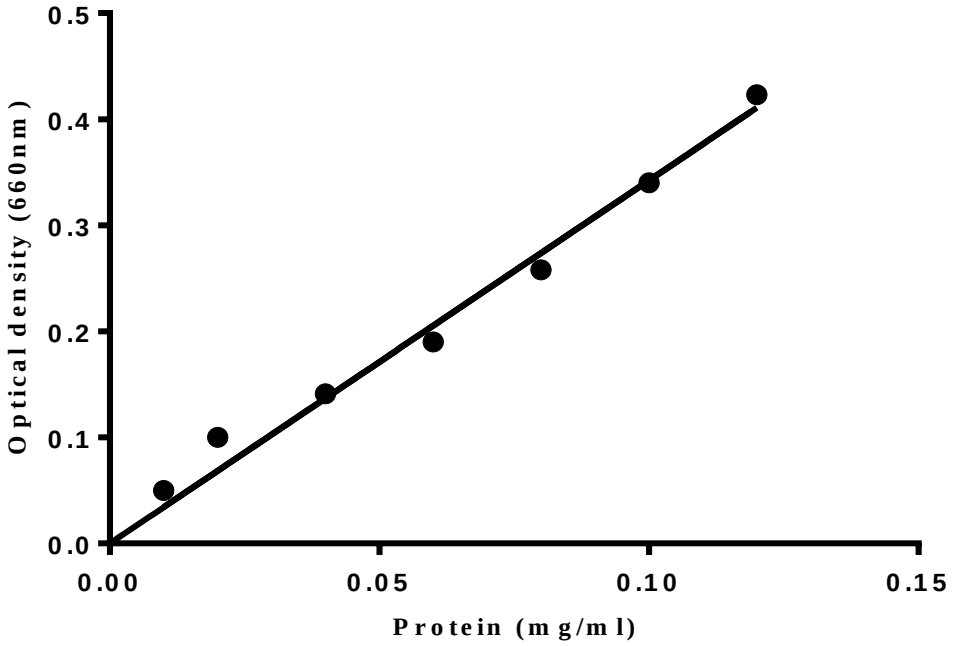
Appendix Ia: ALT Standard Curve



Appendix Ib: TBARS Standard Curve



Appendix Ic: Protein standard curve



APPENDIX II

List of Published Papers from this Dissertation

i. Okoro, I.O., Umar, I. A., Atawodi, S.E. and Anigo, K.M. (2014). Comparative antihyperglycemic effect of petroleum ether, acetone, ethanol and aqueous extracts of *Cleome rutidosperma* DC and *Senecio biafrae* (Oliv. & Hiern) in streptozotocin-induced diabetic mice. *British Journal of Pharmacology and Toxicology*. 5(3): 115-124.

ii. Okoro, I.O., Umar, I. A., Atawodi, S.E. and Anigo, K.M. (2014). Antidiabetic effect of *Cleome rutidosperma* DC and *Senecio biafrae* (Oliv. & Hiern) extracts in streptozotocin-induced diabetic rats. *International Journal of Pharmaceutical Sciences and Research*. 5(6): 2480-97.

iii. Okoro, I.O., Umar, I. A., Atawodi, S.E. and Anigo, K.M. (2015). Bioassay-guided evaluation of the antidiabetic activity of *Cleome rutidosperma* DC. *International Journal of Pharmacy and Pharmaceutical Sciences*. 7(1): 198- 202.

iv. Okoro, I.O., Umar, I. A., Atawodi, S.E. and Anigo, K.M. (2015). *In vitro* and *In vivo* antihyperglycemic effect of active fraction of *Cleome rutidosperma* DC. *International Journal of Pharmacy and Pharmaceutical Sciences*. 7(1): 289- 295.