BLOOD GLUCOSE LOWERING EFFECTS OF LEAF EXTRACTS OF *CLEOME GYNANDRA*, *AMARANTHUS CRUENTUS* AND THEIR MIXTURE IN NORMOGLYCAEMIC AND HYPERGLYCAEMIC RATS

By

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DECLARATION

I, **DONALD ROBERTSON SIWALE**, hereby declare that this dissertation represents my own work and that it has not previously been submitted for a degree, diploma or other qualification at this or another University. All published work or material from other sources incorporated in this report have been specifically acknowledged

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APPROVAL

This dissertation of Donald Robertson Siwale has been approved as fulfilling the requirement or partial fulfilment of the requirements for the award of Masters in Human Physiology by the University of Zambia.

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ABSTRACT

In healthy persons, blood glucose is maintained in the range 3.5 – 8.0 mmol/L. However, in Diabetes Mellitus (DM) chronic hyperglycemia is observed due to defects in insulin secretion, insulin action or both.

This study investigated glucose lowering effects of ethanolic leaf extracts of *Cleome gynandra* (CG), *Amaranthus cruentus* (AC) and their mixture (1:1 ratio) in Normoglycaemic and hyperglycemic rats.

The study was an *in vivo* experimental study conducted in the pharmacology laboratory at the University of Zambia, School of Medicine. The ethanolic leaf extracts was obtained from the leaves of AC and CG by maceration and formulated into 3 dose levels (200, 400 and 600 mg/Kg BW). Normal saline (0.9%) and Glibenclamide (5 mg/kg BW) were used as controls. Male Albino rats (n =55) of species *Rattus norvegicus* weighing 120-300 grams, with Random Blood Glucose (RBG) ranging 4.7 – 7.5 mmol/L were used in the study.

We tested glucose lowering effect of the ethanolic leaf extracts to Normolglycemic and hyperglycemic rats allocated in 11 groups comprising 5 rats per group. After 12 hour fast with access to drinking water, single doses of crude drug of AC, CG and Mixture using a gavage needle were administered to the Rats. We then measured Fasting Blood Glucose (FBG) at times 0, 1, 2, 4, 6, 8, 16 and 24 hours.

Diabetes was induced by a single intraperitoneal injection of 140 mg/kg BW with Alloxan monohydrate to overnight fasted rat. For Sub-acute studies of 10 days, treatments were given orally and we measured RBG every alternate day. Standard euthanasia procedure was performed afterwards. Data was analyzed by Students `t' test and one way ANOVA. Results were expressed as mean ± standard deviation and Statistical significance was set at $p < 0.05$.

Extracts of AC, CG and their mixture produced reduction in FBG of 30% (2.8 ± 0.09 to 1.6 ± 0.03 mmol/L), 35.4% (2.9 ± 0.07 to 2.0 ± 0.05 mmol/L) and 26.1 % (2.9 ± 0.05 to 2.2 ± 0.06 mmol/L) respectively in normolglycemic rats after 6 hours.

Similarly in hyperglycemic rats extracts of AC, CG and their mixture produced maximum reduction in FBG of 23.8% (25.5 ± 1.57 to 20.1 ± 1.66 mmol/L), 31.7 % (25.8± 0.13 to 19.30 ± 1.74 mmol/L) and 26.9 % (24.74 ± 2.26 to 14.2 ± 1.01 mmol/) respectively after 6 hours.Mean RBG was lower in the experimental compared to Hyperglycemic control group at day 10 (17.6±1.61 Vs. 28.8 ± 0.23 mmol/L, p = 0.004). The leaf extracts seem to act via extra-pancreatic mechanisms because there was no statistical difference when the glucose lowering effect of the Extracts on the normolglycemic and hyperglycemic were compared (p > 0.05)

Therefore in normolglycemic and hyperglycemic (diabetic) rats, ethanolic leaf extracts had glucose lowering effects. The Leaf extracts seem to act via extra pancreatic mechanisms.

Key words: Diabetes, Alloxan, Ethanolic Leaf Extracts, Amaranthus cruentus (AC), Cleome gynandra (CG), rats, Glucometer, hypoglycemia, hyperglycemia.

DEDICATION

To: my wife (Dellah), my two girls (Lenganji and Salifya), Brothers and sisters, Dad and Mum.
Your love, patience and support are indescribable.

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ABBREVIATIONS

TIDM	Type 1 diabetes Mellitus.
T2DM	Type 2 Diabetes Mellitus.
WHO	World Health organization
AC	*Amaranthus cruentus*
AC 200	*Amaranthus cruentus* at dose of 200
AC 400	*Amaranthus cruentus* at dose of 400
AC 600	*Amaranthus cruentus* at dose of 600
CG	*Cleome gynandra*
CG 200	*Cleome gynandra* at dose of 200
CG 400	*Cleome gynandra* at dose of 400
CG 600	*Cleome gynandra* at dose of 600
MIX600	Mixture of AC and CG and dose of 600 mg/kg body weight
MIX 400	Mixture of AC and CG and dose of 400 mg/kg body weight.
MIX 200	Mixture of AC and CG and dose of 200 mg/kg body weight.
WHO	World Health organization
IDF	International Diabetes Federation
NIDDK	National institute of Diabetes and digestive and kidney disease
PPAR	Peroxisome proliferator-activated receptor
IC_{50}	Inhibitor Concentration that reduces response by 50%
SGPT	Serum Glutamic Pyruvic Transaminase
SGOT	Serum Glutamic Oxaloacetic Transaminase
ALP	Alkaline Phosphatase Level
GSH	Glutathione Reduction
SH	Sulfhydryl
ROS	Reactive Oxygen Species
DNA	Deoxyribonucleic Acid
GSSH	Glutathion Dissulfide

CHAPTER 1: INTRODUCTION

1.1 Introductions

Blood Glucose is constantly needed as it is an important source of energy for tissues and organs. Despite the varying demands of food, fasting and exercise blood glucose rarely stray outside the range 3.5 – 8.0 mmol/L in healthy persons (Kumar, 2017). Seizures, loss of consciousness and death occurs if blood glucose becomes too Low (hypoglycemia). On the other hand, chronic hyperglycemia can cause blindness, renal failure, vascular disease and many other complications (Barret *et al*, 2014). Regulation of blood glucose is therefore important and this is accomplished by hormone regulation of peripheral glucose uptake, hepatic glucose production, and glucose uptake during carbohydrates ingestion

1.1.1 Normal Blood glucose homeostasis mechanism

Blood glucose Concentration (3.5 – 8.0 mmol/L) is balanced by the interaction of factors that increases and decreases Blood glucose. Factors increasing blood glucose includes; (1) absorption of glucose from the intestines i.e. dietary intake (2) Glycogen breakdown under the influence of Glucagon i.e. glycogenolysis. In contrast, factors decreasing blood glucose include; (1) Glucose uptake by body cells under the influence of hormone insulin and (2) Glomerular filtration of glucose by the kidneys i.e. glucosuria. The interaction of the factors is shown in figure 1.1.

Absorption of glucose from the intestine*:* Firstly, carbohydrates (sucrose, lactose and starch) are digested by enzymes in the mouth and small intestines. Starches are digested by salivary amylase (20-40%) and pancreatic enzymes (50-80%) to form maltose which is further broken down to glucose under the action of maltase and α-dextrinase. Sucrose and lactose are digested to glucose under the influence of sucrase and lactase respectively (Guyton, 2014).

Secondly, Glucose and galactose are absorbed across the apical membrane by secondary active transport mechanisms similar to those found in the early proximal convoluted tubule of the nephrons. Both glucose and galactose move from the intestinal lumen into the cell on the Na^+-glucose cotransporter (SGLT 1), against an electrochemical gradient. The energy for this step does not come directly from ATP but from the Na^+ gradient across the apical

membrane; the Na^+ gradient is created and maintained by the Na^+-K^+ ATPase on the basolateral membrane. Glucose and galactose are extruded from the cell into the blood, across the basolateral membrane, by facilitated diffusion with the help of carrier proteins the GLUT 2 (Costanzo, 2015). The interactions of glucose homeostatic factors are shown in figure 1.1 below.

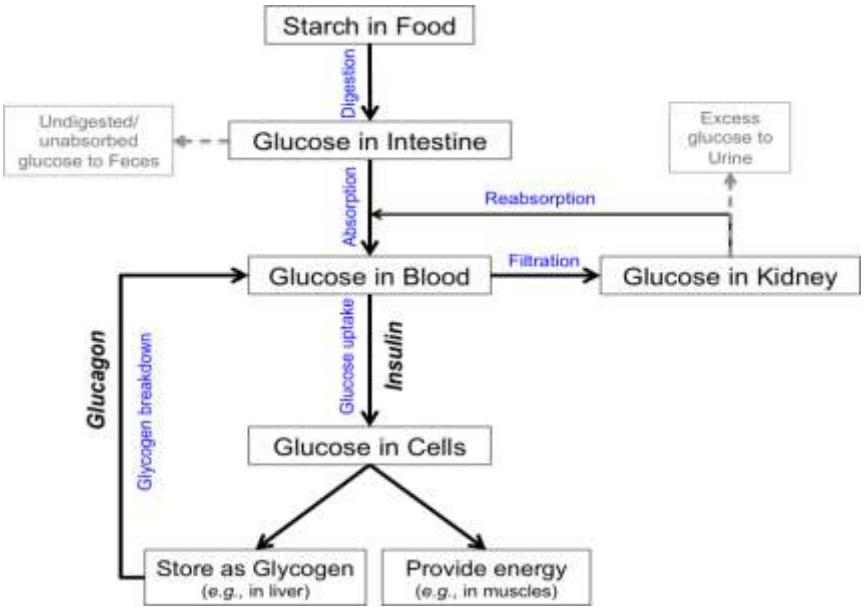

Figure 1.1 Concept map of glucose homeostasis highlighting key steps from food ingestion, digestion, absorption, and use to excretion (Source; Barret *et al*, 2012)

Glycogen breakdown under the influence of Glucagon*:* During periods of starvation, the hormone glucagon triggers conversion of glycogen back to glucose. The major actions of glucagon are on the liver (in contrast to insulin, which acts on liver, adipose, and muscle tissue). Glucagon has the following effects on glucose blood levels;

- Increases blood glucose concentration by stimulating glycogenolysis and simultaneously inhibit glycogen formation from glucose.
- Glucagon increases gluconeogenesis by decreasing the production of fructose 2, 6-bisphosphate, which decreases phosphofructokinase activity. Thus, substrate is directed *toward* the formation of glucose. Amino acids are utilized for gluconeogenesis, and the resulting amino groups are incorporated into urea.

- Increases blood fatty acid and ketoacid concentration.
- Glucagon increases lipolysis and inhibits fatty acid synthesis, which also shunts substrates toward gluconeogenesis. The ketoacids β-hydroxybutyric acid and acetoacetic acid are produced from fatty acids (Baynes, 2009)

Glucose uptake by cells under the influence of insulin*:* Insulin decreases blood glucose concentration through coordinated responses that simultaneously stimulate glucose oxidation and inhibit gluconeogenesis (Figure 1.2):

- Insulin increases glucose transport into target cells such as muscle and adipose by directing the insertion of glucose transporters (GLUT 4) into the cell membranes. As glucose enters the cells, the blood glucose concentration decreases.
- Insulin promotes the formation of glycogen from glucose in the liver and in muscle and, simultaneously, inhibits glycogenolysis (glycogen breakdown).
- Insulin inhibits gluconeogenesis (synthesis of glucose) by increasing the production of fructose 2, 6-bisphosphate (Fru-1, 6-BP), which increases phosphofructokinase activity. In effect, substrates are directed away from the formation of glucose (Baynes, 2009).

Insulin acts on its target cells (as shown in figure 1.2). The steps are as follows;

1. Insulin binds to α subunits of the tetrameric insulin receptor, producing a conformational change in the receptor.
2. The conformational change activates tyrosine kinase in the β subunits, which phosphorylate themselves in the presence of ATP. In other words, the β subunits auto phosphorylates.
3. Activated tyrosine kinase phosphorylates several other proteins or enzymes that are involved in the physiologic actions of insulin including Insulin Receptor Substrate (IRS), Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), 3-Phosphoinositide dependent protein kinase-1 (PDK1), Protein Kinase B (AKT), and Akt substrate of 160 kDa (AS160)
4. Phosphorylation either activates or inhibits these proteins to produce the various metabolic actions of insulin e.g. inducing translocation of the GLUT4 glucose transporter to the cell surface
5. GLUT 4 facilitates the transport of Glucose into cells

Insulin acts on its target cells mainly adipose, hepatocytes and skeletal muscle. The overall function of insulin is increasing glucose uptake by peripheral cells (figure 1.2).

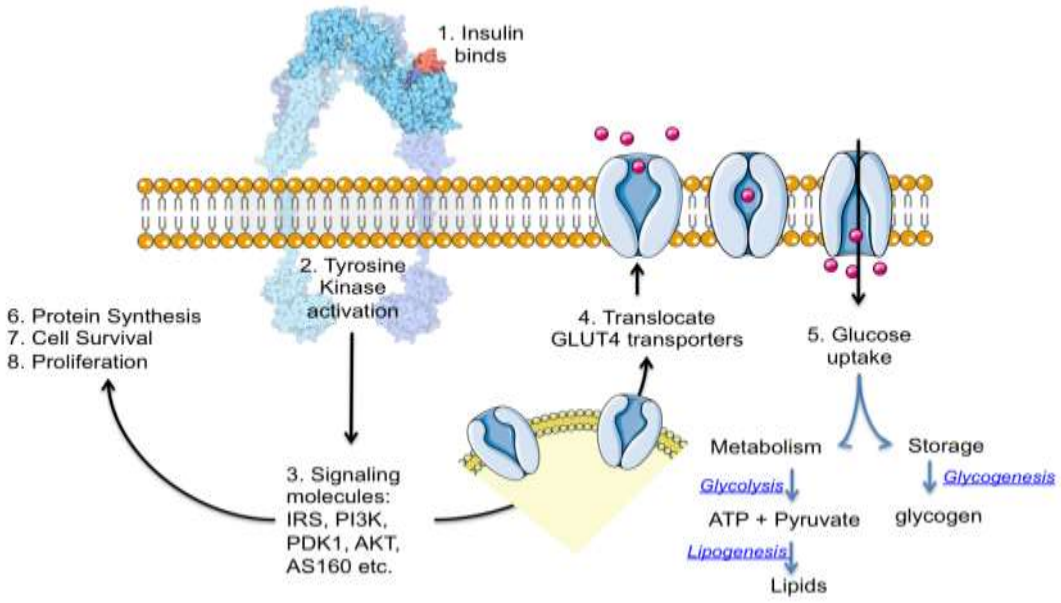

Figure 1.2*:* Insulin directed glucose uptake (Source; Stanford *et al*, 2015)

Glomerular filtration of glucose by the kidney i.e. Glucosuria*:* At normal plasma glucose concentrations (3.5 to 8.0 mmol/L), all of the filtered glucose is reabsorbed and none is excreted. In hyperglycemia, the filtered load of glucose exceeds the reabsorptive capacity (i.e., plasma glucose concentration is above the T_m), and glucose is excreted in the urine (a condition known as glycosuria). Glycosuria occurs in conditions such as Diabetes Mellitus, during Pregnancy and congenital abnormalities of the Na^+-glucose cotransporter (Costanzo, 2015).

Glucosuria causes an osmotic diuresis manifested clinically by polyuria, including nocturia. Dehydration results, stimulating thirst that results in polydipsia. A significant loss of calories can result from glucosuria, because urinary glucose losses can exceed 75 g/d (75 g × 4 kcal/g = 300 kcal/d). Polyphagia also accompanies uncontrolled hyperglycemia. The three "polys" of diabetes—polyuria, polydipsia, and polyphagia—are common presenting symptoms in both type

1 and symptomatic type 2 patients. Weight loss can also occur as a result of both dehydration and loss of calories in the urine. Severe weight loss is most likely to occur in patients with severe insulinopenia (type 1 DM) and is due to both caloric loss and muscle wasting (Barret *et al*., 2010).

Hormones that counter the actions of Insulin are Glucagon, Catecholamines, Glucocorticoids, Growth hormones (Guyton, 2016). They are elevated in blood during Hypoglycemia. Their effect on biochemical processes are shown in table 1.1.

Table 1.1: Anti-insulin hormones that affect Blood sugar levels besides insulin

Hormone	**Effect on Biochemical processes**
Adrenalin	↑Gluconeogenesis ↑Glycogenolysis (↓glycogenesis) ↓Insulin secretion
Glucagon	↑Gluconeogenesis ↑Glycogenolysis (↓glycogenesis)
Glucocorticoid	↑Gluconeogenesis Facilitates the action of glucagon, growth hormone and adrenalin.
Growth hormones	↓Glucose uptake by the tissue. ↑Lipolysis which increase FFA leading to decreased glucose utilization (glucose sparing effect).

Key: Upward and down ward arrows represent "increase" and "decrease" respectively.

1.1.2 Regulation of Blood glucose in Diabetic Mellitus

Diabetes mellitus (DM) is characterized by chronic hyperglycaemic state resulting from defects in insulin secretion, insulin action or both (Ganong, 2016). American Diabetes Associations (ADA) classifies the disease into four categories; (i) Type 1 diabetes, (ii) Type 2 diabetes Mellitus, (iii) Other specific types and (iv) Gestational Diabetes Mellitus (GDM) (diagnosed during pregnancy).

Type 1 DM (Insulin-dependent diabetes mellitus): is caused by destruction of β-cells, often as a result of an autoimmune process. When pancreatic β-cells do not secrete adequate amounts of insulin, Carbohydrate, fat, and protein metabolism all will be disturbed. Type I DM cause increased blood glucose concentration due to decreased uptake of glucose into cells, decreased glucose utilization, and increased gluconeogenesis; increased blood fatty acid and ketoacid concentration from increased lipolysis of fat, increased conversion of fatty acids to ketoacids, and decreased utilization of ketoacids by tissues; and increased blood amino acid concentration from increased breakdown of protein to amino acids. There also is loss of lean body mass (i.e., a catabolic state) and loss of adipose tissue (Costanzo, 2015).

Type 2 DM (Non–insulin-dependent diabetes mellitus): is often associated with obesity. It exhibits some, but not all, of the metabolic derangements seen in type I DM. Type 2 DM is caused by down-regulation of insulin receptors in target tissues and insulin resistance. Insulin is secreted normally by the β- cells, but at normal concentrations, it cannot activate its receptors on muscle, liver, and adipose tissue; thus, insulin is unable to produce its usual metabolic effects. Typically, the blood glucose concentration is elevated in both fasting and postprandial states (Costanzo, 2015). In Type II DM insulin receptors may be less, absent or abnormal, resulting in insulin resistance. Common causes of insulin resistance are Genetic disorders (significant factors causing type 2 DM), Lifestyle changes such as bad eating habits and physical inactivity leading to obesity and Stress (Sembulingam, 2012; Kumar, 2017).

1.1.3 Complication of Diabetes Mellitus

Acute Complication of Diabetes Mellitus

Acute complications include polyuria, polydipsia, polyphagia, diabetic ketoacidosis, Diabetic coma and hypoglycemia. Polyuria is caused by presence of glucose in renal tubular fluids that acts as osmotic diuretics (Barret *et al*., 2011).

Polydipsia is due to the resulting loss of fluids and electrolytes causing reduced ECF volume and increased osmolarity. Increased osmolarity stimulates osmoreceptors in the anterior hypothalamus resulting in increased behavior to drink water (Guyton, 2013).

Polyphagia is due to significant loss of calories as a result of glucosuria. Also, reduced uptake of glucose into cells secondary to insulin deficiency deprives cells of energy from cellular respiration leading to polyphagia. Urinary glucose losses can exceed 75 g/d (75 g X 4 kcal/g = 300 kcal/d). Weight loss also occurs as a result of both dehydration and loss of calories in the urine. Other feature includes Ketonuria, Ketosis, Diabetic ketoacidosis and hyperosmolar coma (Costanzo, 2014).

Long term Complication of Diabetes Mellitus

Macrovascular and microvascular damage, Diabetic eye diseases (such cataracts, diabetic retinopathy, external ocular palsies), diabetic kidney (such as glomerular damage, ischemia due to hypertrophy of afferent and efferent arterioles, ascending infections), Diabetic neuropathy, diabetic foot infections and diabetic cancer (Kumar and Clark, 2017).

1.1.4 Induction of Diabetes mellitus by Alloxan

Alloxan (2,4,5,6-tetraoxypyrimidine;2,4,5,6-pyrimidinetetrone) is a diabetogenic agent that is used to induce diabetes in experimental animals. Alloxan is a toxic glucose analogue, which selectively destroys insulin-producing cells in the pancreas when administered to rodents and many other animal species. This causes an insulin-dependent diabetes mellitus (called "Alloxan Diabetes") in these animals, with characteristics similar to type 1 diabetes in humans (Grover, 2011).

The Underlying mechanism of induction of Diabetes Mellitus, involves the selective uptake of the compound due to its structural similarity to glucose as well as highly efficient uptake mechanism of the pancreatic ß-cells (Rohilla, 2012). This causes selective necrosis of the pancreatic ß-cells.

Alloxan and its reduction product dialuric acid establish a redox cycle with the formation of superoxide radicals. These radicals undergo dismutation to hydrogen peroxide. Thereafter, highly reactive hydroxyl radicals are formed by fenton reaction. The action of reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of *beta* cells (Szkudelski, 2001 cited in Etuk, 2010).

Alloxan induced diabetes is not reversible but Lower doses of alloxan (less than 140 mg/kg) has shown to reverse the diabetic state in animals to normal (Jain, 2013). Hence, with Alloxan, it is possible to produce different grades of severity of the disease by varying the dose of alloxan used (Etuk, 2010).

Similar to Alloxan, Streptozotocin exerts toxicity related to pancreatic beta cells. STZ enters β-cells via GLUT2 glucose transporter. Inside the cell, STZ can induce production of nitric oxide (NO), superoxide anions (O_2^-), hydroxyl radicals ($HO^{\cdot}$), peroxynitrit ($ONOO^-$) and cause DNA alkylation (Figure 1.3).

These processes result in DNA damage and inhibition of mitochondrial function. Massive DNA damage over stimulates poly (ADP-ribose) polymerase, leading to NAD^+ depletion with subsequent ATP deficiency. Alternative mechanism is N-acetyl-β-D-glucosaminidase inhibition characterized with accumulation of irreversible glycosylated proteins. All afore mentioned processes lead to β-cell necrosis and result in condition of insulin-dependent diabetes as shown in figure 1.3 (Soltesova, 2011).

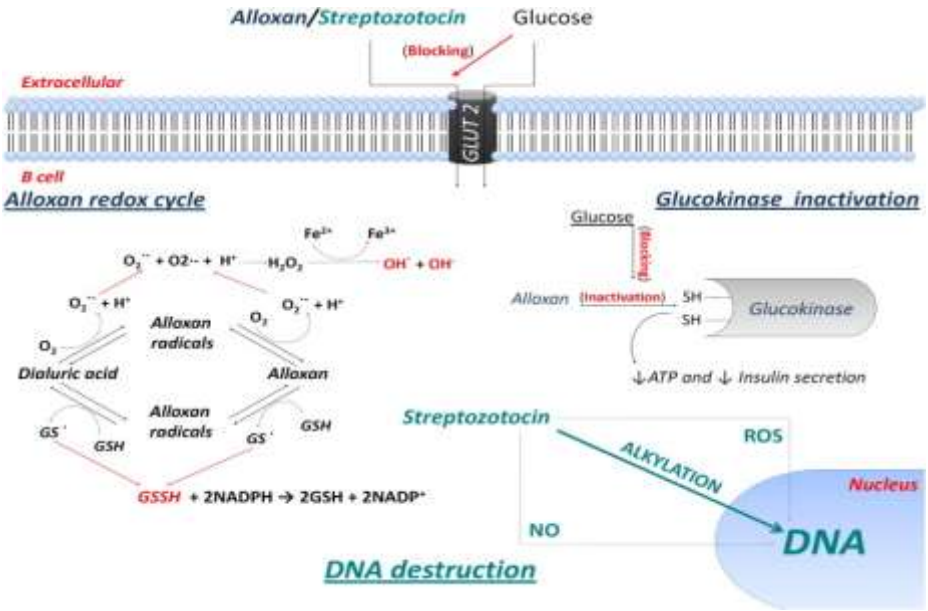

Figure 1.3: Alloxan reduction products that induce diabetes GSH, super-oxide (O_2^-), SH, ROS, DNA, GSSH.Source; Soltesova, 2011.

Thus alloxan induced diabetes mellitus served as a pathological bio model for testing a substance with supposed antioxidant activities *in vivo* (Bartosikova *et al.*, 2003 cited in Etuk, 2010). One of the targets of the reactive oxygen species is DNA of pancreatic islets. Its fragmentation takes place in *β*- cells exposed to alloxan (Takasu *et al.*, 1991 cited in Etuk, 2010). The increase in oxygen free radicals in diabetic conditions is mainly because of the effect of the diabetogenic agent alloxan.

1.1.5 How Anti-diabetic drugs Lowers Blood Glucose levels

Anti-hyperglycemic or anti-diabetic Drugs are used to treat diabetes. They mimic the normal physiological mechanisms involved in glucose homeostasis as explained;

Insulin: it is exogenously administered to Patients with absolute lack of insulin (IDDM). Most patients with NIDDM do not require exogenous insulin but many need exogenous supplementation of their endogenous insulin secretion to achieve optimum health Insulin secretagogue: such as sulfonylureas e.g. Glibenclamide produce their hypoglycemic actions via mechanisms that stimulate insulin secretion from the pancreatic β cells. They also reduce serum glucagon levels possibly contributing to its hypoglycemic effects (Kutzung, 2012).

Biguanides: unlike sulfonylureas, they do not stimulate insulin secretion from pancreatic Beta cells. They do not produce hypoglycaemia and hence are effective in pancreactomized animals. Possible mechanism of action include, reduced glucose absorption from the gut, facilitation of glucose entry into muscle by a non-insulin responsive mechanism, inhibition of gluconeogenesis in the liver, suppression of oxidative glucose metabolism and enhanced anaerobic glycolysis (Graziella, 2010).

Thiazolidinedione (PPARγ agonist) or Glitazones: lower blood glucose and haemoglobin A1C (HbA1c) in type 2 DM patients who are inadequately controlled on di*et al*one or diet and other oral hypoglycaemic drugs. Thiazolidinediones (Tzds) act to decrease insulin resistance. Tzds are ligands of peroxisome proliferator-activated (PPAR-gamma), part of the steroid and thyroid superfamily of nuclear receptors. These PPAR receptors are found in muscle, fat, and liver. PPAR-γ receptors modulate the expression of the genes involved in lipid and glucose

metabolism, insulin signal transduction, and adipocyte and other tissue differentiation (Kutzung, 2014).

α-glycosidase inhibitors (e.g. Acarbose): inhibit intestinal α-glycosidases, delaying the digestion of starch and sucrose. It lowers the postprandial increase of blood glucose. Only monosaccharides, such as glucose and fructose, can be transported out of the intestinal lumen and into the bloodstream. Complex starches, oligosaccharides, and disaccharides must be broken down into individual monosaccharides before being absorbed in the duodenum and upper jejunum. This digestion is facilitated by enteric enzymes, including pancreatic α-amylase and α-glucosidases that are attached to the brush border of the intestinal cells (Rang and Dale, 2012).

The Incretin-based therapies: Incretins are substances that stimulate insulin secretion. Example, after ingestion of a meal, there is an early stimulus to insulin secretion before absorbed glucose reaches the islets cells. This is due to peptide hormones(incretins) released from the gut, mainly Glucagon-like insulinotropic peptide (GIP) and Glucagon-like Peptide – 1 (GLP-1) which stimulates insulin secretion (Rang and Dale, 2012). Therefore, the incretin-based therapies control post-meal glucose excursions by increasing insulin release and decreasing glucagon secretion.

The amylin analog: these decreases post-meal glucose levels and reduces appetite. The bile acid sequestrant's glucose-lowering effect is presumed to be related to a decrease in hepatic glucose output (Kutzung, 2012).

1.1.6 Side effects of synthetic Antidiabetic drugs

Anti-diabetic drugs are without side effects despite them being very useful in treatment and management of DM. Insulin mainly causes hypoglycaemia which can lead to severe brain damage (ADA, 2016).

Sulfonylureas may also cause severe hypoglycemia beside the side effects of weight gain and drug-drug interactions (Sola, 2015).

Metformin cause dose-dependent gastrointestinal disturbances such as anorexia, diarrhea, Nausea, lactic acidosis, pancreatitis, hypoglycaemia and its contra-indicated in people with kidney problems (Rhee, 2017).

Thiazolidinedione also cause weight gain, Edema and Osteoporotic fractures. Other drugs such α- Glucosidase inhibitors may cause flatulence and diarrhea (Kutzung, 2012).

1.1.7 Medicinal plants with Anti-hyperglycemic potentials

More than 80% of the world's population prevents and treat their diseases using traditional medicine. Medicinal plants still represent the largest source of natural antioxidants and antimicrobial components (Usunomena, 2012).
Several phytoconstituents such as alkaloids, glycosides, flavonoids, saponins, dietary fibers, polysaccharides, glycolipids, peptidoglycans, amino acids and others obtained from various plant sources have been reported to have potent hypoglycemic effects (Firdous, 2014).

Despite the availability and accessibility of western medicine in the 21st century, a wide range of cultural communities in Africa still depend on and often prefer traditional medicine (TM) or 'muthi' as an important component of primary health care. It was estimated that 72% of Black africans, even in urban areas, subscribe to traditional health care systems involving the consumption of medicinal plants, and that more than 70,000 tonnes of plant material is consumed in africa each year, with at least 134,000 income-earning opportunities generated by the trade in medicinal plants and related products . Medicinal plants are traded through several large markets throughout South Africa, particularly in the summer-rainfall (Williams, 2013).

About 185 plant species in Africa have been scientifically investigated and shown to possess anti-diabetic properties (Table 1.2). Plants from West Africa sub-region account 51.69% of which 90% are from Nigeria (Mohammed, 2014).

Table 1.2: List of some of scientifically investigated anti-diabetic plants of Africa (Mohammed, 2014)

Scientific name	Common name	Part used	Dose of mg/Kg BW daily
Acacia albida del	Ana Tree	Roots	200
Adansonia digitata	Baobab	Stem/bark	100, 200, and 400
Allium sativum	Garlic	Bulb	200 , 250, 300
Bauhinia refescens		Leaf	200, 300, 400
Carica papaya	Paw paw	Seed	100, 200, 300, 400
Ceiba pentandra	Silk-cotton	Stem bark	250, 400, 800, 1500
Citrus aurantium	Bitter orange	Fruit	400, 800
Citrus paradise	Grape fruit	Seed	100, 300, 600
Curcumin longa	Curcuma	Roots	250
Ficus exasperate	White figure tree	Leaf	100,200 and 300
Gongronema latifolium	Amaranth globe	Leaf	2, 25, 75, 100, 200 and 400
Hibiscus subdariffa	Red sorrel	Calyces	0.5 mg/ml aqueous ethanol
Khaya senegalensis	African mahogany	Stem	50, 100, 150
Mangifera indica	Mango	Leaf	0.5, 1.0 methanol extract
Musa sapientum	Banana	-	5, 10, 250, 500
Persea Americana	Avocado	Seed	450, 900 ethanol extract
Zinger officinale	Ginger	Rhizomes	200. 250, 300
Moringa Stenopetala	Cabbage tree	Leaf	500 butanol extracts
Psidium guajava	Guava	Leaf	250

1.1.8 Botanical identification of *Amaranthus cruentus* and *Cleome gynandra*

Both plants, *Amaranthus Cruentus* (Figure 1.4a) and *Cleome Gynandra* (Figure 1.4b) belong to Domain; Eukaryota, Kingdom; Plantae, subphylum; Angiospermae.

(a) **(b)**

Figure 1.4: Pictures of *Amaranthus Cruentus* (a) and *Cleome Gynandra* (b) plants

The Class for *Amaranthus cruentus* is dicotyledone, Family; Amaranthus, Species; *Amaranthus hybridus*, Subspecies; *Amaranthus cruentus*. While *Cleome gynandra*, the Family is Capparaceae, Species; *Cleome gynandra*, Sub-species; *Gynandropsis gynandra.*

Amaranthus cruentus is a tall annual herb topped with clusters of dark pink flowers. The plant can grow up to 2 m (6 ft.) in height, and blooms in summer to fall. It is believed to have originated from *Amaranthus hybridus*, with which it shares many morphological features. The plant is usually green in color, but a purple variant was once grown for use in Inca rituals (Anshula, 2015). The Local names are *Bondwe* or *Lengalenga* (Bemba), L*ibowa* (lozi), *Bonongwe* (Nyanja).

Cleome gynandra is an annual wildflower native to Africa but has naturalized across tropic and sub-tropical regions across Asia. It grows well in disturbed, well-drained soils, but is also drought-tolerant. It does not tolerate cold temperatures well, and is frost-tender. It is considered an invasive weed in many places. The cat's whiskers (*Cleome gynandra*) grow as a weed in most tropical regions (Chweya, 1997). The genus *Cleome*, with over 200 species, consists of

over 200 species, consists of highly polymorphic herbaceous plants. The local names are *Lubanga* (Bemba), *Shungwa* (Tonga), *Suntha* (Nyanja), *and Sishungwa* (Lozi).

1.1.9 Medicinal use of *Amaranthus cruentus* and *Cleome gynandra*

All parts of *A. Cruentus* are used as medicine to heal many diseases in African communities. The plant is used to cure fever, heamorrhage, anaemia or kidney complaints, laxative for infants, tapeworm expellant, treat inflammation, induce abortion, and treat Gastroenteritis, gall bladder inflammation, abscesses, arthritis, and treatment of malaria and snake bites (Achigan-Dako, 2014).

Rahmatullah *et al* (2013), research findings indicated significant oral hypoglycemic activity of methanolic whole plant extract of *A. tricolor* on glucose loaded Swiss albino mice at all doses of the extracts tested. Maximum antihyperglycaemic activity was shown at 400mg extract/kg BW, which was comparable to glibenclamide (10mg/kg BW). *A.tricolor* has various phytoconstituents such as amino acids, carbohydrates, proteins, cardiac glycosides, steroids, alkaloids, flavonoids and tannins. (Pulipati, 2015). Mechanism of glucose lowering depends on the presence of these phytoconstituents (see Table 2.1)

It is evident from the above studies that the *Amaranthus* family is a potential source of ingredients for management of hyperglycemia, associated lipidaemia, and prevention of diabetic complications and for overall health of diabetic patients. Following clinical trials Amaranthus extracts thus can be used for preparation of prospective Nutraceutical for diabetics.

Cleome gynandra is said to have anti-inflammatory, free-radical scavenging, anti-cancer, immune-modulatory activities and possible anti-diabetic properties (Mishra, 2011). The phytochemical constituents are known to be responsible for the strong anti-oxidant activity exhibited by the plant (Afolayan, 2015; Adams, 2012). *Cleome gynandra* contain anthocyanin, alkaloids, leuco anthocyanin, steroids, mucilage, reducing compounds, anthracene combined with the C-heteroside derivatives and the quinine (Dansi, 2016; Chaya, 2015).

Species of *Amaranthu*s have various phytoconstituents such as amino acids, carbohydrates, proteins, cardiac glycosides, steroids, alkaloids, flavonoids and tannins. (Pulipati, 2015). Mechanism of glucose lowering depends on the presence of these phytoconstituents (see Table 1.3)

Table 1.3: The phytochemical compounds in *Cleome gynandra* (Dansi, 2016) and *Amaranthus cruentus* (Hilou, 2012)

Phytochemical composition	***Cleome Gynandra***	***Amaranthus cruentus***
Alkaloids	Present	Absent
Anthocyanin	Present	-
Anthracene to C- heterosides.	Present	Absent
Anthracene to O- heterosides.	Absent	Absent
Cardenolides	Absent	Preset
Carotenoids	Absent	Present
Catechin Tannins	Present	Present
Coumarins	Present	Absent
Cyanogenic derivatives	Absent	-
Flavanoids	Present	Present
Free anthracene	Absent	-
Gallic tannins	Present	-
Iridoids	-	Present
Leuco-anthocyanins	Present	-
Mucilage	Present	-
Quinone derivatives polyphenols	Present	Present
Reducing compound	Present	-
Saponins	Absent	Present
Steroids	Present	Present
Triterpenoids	Absent	Present

1.2 Statement of the problem

Diabetes mellitus has reached epidemic proportions and continues to be a major burden on society globally. The International Diabetes Federation (IDF) estimated the global burden of diabetes to be 366 million in 2011 and predicted that by 2030 this will have risen to 552 million (Vaz, 2012).

Even with the large number of oral anti-diabetic drugs, patients still develop unwanted side effects such as; severe hypoglycemia, lactic acidosis (English *et al*., 2004), idiosyncratic liver cell injury (Fontana etal.,2014), permanent neurological deficit (Gopal-Kothandapani, 2017), digestive discomfort, headache, dizziness, and even death (ADA, 2016).

Therefore, alternative therapies employing naturally occurring medicinal plants are being encouraged to treat Diabetes mellitus (WHO, 2017). This is because Medicinal plants have reduced risk of side effects, effectives with chronic conditions, lower cost, widespread availability and compatible with the human body (Kavita, 2017). Additionally, medicinal plants have a wide spectrum of medicinal properties due to the presence of many phytochemical compounds in them with different mechanisms of action (Achigan-Dako 2014; Ravichandra 2014; Mishra, 2011).

However, despite wide reported use of herbal medicine to treat diabetic complication within Zambian communities, there is not much documentation reporting the undesirable and disastrous effect of hypoglycemia that can be caused by herbal extracts of AC, CG and their Mixture

Hence this study aimed at assessing hypoglycemic and anti-hyperglycemic effect of AC, CG and their mixture in normolglycemic and hyperglycemic (alloxan-diabetic) rats. Deduction of possible mechanism of action of the leaf extracts was also investigated.

1.3 Research questions

Do leaf extracts of *Cleome gynandra, Amaranthus cruentus* and their mixture (1:1) have blood glucose lowering effects on normolglycemic and hyperglycemic rats? And is the glucose lowering mechanism similar to Glibenclamide (an insulin-secretagogue)?

1.4 General objectives: To assess blood glucose lowering effects of leaf extracts *Cleome gynandra, Amaranthus cruentus and* their Mixture in normoglycaemic and hyperglycemic rats and deduce possible mechanism of action.

1.5 Specific objectives

1. To assess glucose lowering effects of Ethanolic leaf extracts of A*maranthus cruentus, Cleome gynandra* and their mixtures in normolglycemic rats
2. To assess glucose lowering effects of Ethanolic leaf extracts of A*maranthus cruentus, Cleome gynandra* and their mixtures in hyperglycemic rats
3. To deduce possible mechanism of action by which Ethanolic leaf extracts of A*maranthus cruentus* and *Cleome* lower blood glucose in Normolglycemic and hyperglycemic rats

1.6 Study justification

Firstly, information on physiological effect of Ethanolic leaf extracts of AC, CG and their Mixture in lowering blood glucose would be useful in management of Diabetes mellitus associated hyperglycemia. Data so obtained can be useful for the physicians and other health practitioners, and may be a basis for further medical studies in order to explore on the other physiological effects of AC, CG and their mixture.

Secondly, documentation of indigenous knowledge system of medicinal plants is vital, since it provides chemists, pharmacologists, physiologist and other researchers with starting point for "targeted" analysis, discovery of natural drugs for treatment of various pathological conditions including diabetes and related metabolic de-arrangements.

Thirdly, the general public can be educated on the safety measure to be taken when using herbal extracts of AC and CG as these may cause hypoglycemia, an if severe can cause brain damage.

CHAPTER 2: LITERATURE REVIEW

After extensive review of the literature within the specified research objectives, we were able to identify research gaps which marked the basis of our research. These gaps being;

- Lack of published literature describing hypoglycemic effects of ethanolic leaf extracts of *Amaranthus cruentus* or *Cleome gynandra* on blood glucose levels of normoglycaemic and Alloxan-diabetic rats and their possible mechanisms of action.
- Lack of published literature on Synergistic glucose lowering activities of *Amaranthus cruentus* and *Cleome gynandra* and their possible mechanisms of action.

Undoubtedly, *Amaranthus cruentus* and Cleome *gynandra* possess a wide range of medicinal properties and has been used to treat fever, preventing heamorrhage, treating anaemia, anti-inflammatory activities, inducing abortion, anti-cancer, immune-modulatory activities and many others (Achigan-Dako 2014, Mishar 2011).

Our literature analysis of phyto-constituent and the mechanisms were biased towards the families to which the two plants belongs (i.e. Amaranthus and Capparacea). This is because; plants belonging to specific family may have similar type of chemical composition and similar type of mechanism of action (Mishra 2009).

2.1 Glucose lowering effects of a*maranthus cruentus*

Noori (2015) publicized that various *Amaranthus spp* possess Flavonoids which are able to reduce plasma glucose, triglycerides and cholesterol levels. The proposed mechanism of action involved increasing peripheral utilization of glucose and inhibiting the glucose transporter activity from the intestines (Puchchakayala 2012).

Kumar *et al* (2014) reported that ethanolic extract of *Amaranthus spinosus* administered (150, 300 and 450 mg/kg BW) to type-1 and type-2 diabetic rats significantly decreased plasma glucose levels, increased hepatic glucose-6-phophatase activity and increased the hepatic glycogen content with a concurrent increase in hexokinase activity in both type 1 and 2 diabetic rats.

Girija *et al* (2010) investigated anti-hyperglycaemic and Hypolipidemic effect of methanolic leaf extract of *Amaranthus caudatus* in normoglycaemic and Streptozotocin (STZ) induced diabetic rats. The extract was administered at dose of 200 mg/kg and 400 mg/kg p.o. per day for 21 days. Results showed significant increase in the body weight, decrease in the blood glucose, total cholesterol and serum triglycerides, and High density Lipoproteins (HDL) levels was significantly increased when treated with extract. Histologically, focal necrosis was observed in the diabetic rat pancreas but was less obvious in treated groups. This showed that methanolic extract of *Amaranthus Caudatus* had beneficial effects in pancreatic β-cells regeneration resulting into increased insulin synthesis.

Tamilanban *et al* (2014) studied Antidiabetic and Hypolipidaemic activity of ethanolic extracts of *Amaranthus viridis, Ceiba pentadra* and their combination on Dexamethasone induced Type-II diabetic Swiss albino rats. Three test group animals received plant extracts of *Amaranthus viridis, Ceiba pentandra* and their combination respectively at dose levels of 400 mg/kg, 500 mg/kg and 450 mg/kg after simultaneous administration of dexamethasone subcutaneously for ten days. The study showed a significant decrease in serum Tri-glycerides, total cholesterol and blood glucose levels.

The plasma triglyceride-lowering effect of medicinal plant extracts is explained in part by a decrease in hepatic lipogenesis, the moderate fall in total plasma cholesterol is not explained by a reduction of whole-body cholesterol synthesis but may be due to stimulation of reverse cholesterol transport and decrease in blood glucose may be due to increased utilization of glucose by peripheral tissues (Forcheron, 2012).

Desai *et al* (2012) showed that oral administration of aqueous leaf extracts of *A. tricolor* significantly reduced serum glucose, serum TG, total cholesterol, LDL and VLDL, but elevated HDL in alloxan-induced diabetic rats, as compared to diabetic control. The extract prevented decrease in BW of treated diabetic rats and promoted improvement in Red blood cell levels.

In the synthesis of red blood cells, Phytochemical compounds acts synergistically with several hematopoietic growth factors (e.g. interleukin 3, Granulocyte-Megakaryocyte Colony

stimulating Factors) to cause maturation and proliferation of erythroid progenitor cells (primarily colony-forming unit-E) which matures into red blood cells (Fisher, 2013).

Girija etal (2012) studies showed that Methanolic extracts of *A. caudatus*, *A. spinosus* and *A.* viridis leaves showed significant anti-diabetic and anticholesterolemic activity in streptozotocin (STZ) induced diabetic rats. Petroleum-ether, chloroform, methanolic and aqueous extracts of *A. spinosus* was found to exert preventive effect on haemoglobin glycosylation (Kumar *et al*, 2014). Methanolic leaf extracts of *A. caudatus* (Sasikumar, 2015) and *A. spinosus* (Kumar *et al*, 2011) exhibited significant in vitro inhibition of a-amylase enzyme even at very low concentration (IC_{50}- 19.233 mg/ml and IC_{50}-46.02 lg/ml, respectively).

Mishra *et al* (2012) showed that administration of *A. spinosus* ethanolic leaf extract caused significant reduction in blood glucose in STZ induced diabetes in albino mice, with significant increase in activities of both enzymatic and non-enzymatic antioxidants. Also degenerative changes of pancreatic cells in STZ induced diabetic rats were minimized to near normoglycaemic morphology.

Rahmatullah *et al* (2013), research findings indicated significant oral hypoglycemic activity of methanolic whole plant extract of *A. tricolor* on glucose loaded Swiss albino mice at all doses of the extracts tested. Maximum antihyperglycaemic activity was shown at 400mg extract/kg BW, which was comparable to glibenclamide (10mg/kg BW). *A.tricolor* has various phytoconstituents such as amino acids, carbohydrates, proteins, cardiac glycosides, steroids, alkaloids, flavonoids and tannins. (Pulipati, 2015). Mechanism of glucose lowering depends on the presence of these phytoconstituents (see table 2.1).

Biswas *et al* (2013) showed that among the betalains identified from *A. tricolor* leaves, betalamic acid (250 mg/mL) significantly inhibited the porcine pancreatic amylase activity by 22% compared to that of standard acarbose, while amaranthin and betaxanthin did not show any inhibition. This means *A. tricolor* inhibits the activity of pancreatic amylase. Pancreatic amylase digests carbohydrates into monosaccharide such as glucose. Glucose is the form in which

carbohydrates are absorbed into the blood. Hence inhibition of this enzyme reduces blood glucose levels due to reduced activity of carbohydrate digestion (Barret *et al.,* 2014).

Kim *et al* (n.d) studied antioxidant and anti-diabetic effects of *Amaranthus esculentus* in streptozotocin- induced diabetic rats for 3 weeks. It was found that Amaranthus grain and its oil fraction significantly decreased serum glucose and increased serum insulin levels in diabetic rats.

It is evident from the above studies that the *Amaranthus* family is a potential source of ingredients for management of hyperglycemia, associated lipidaemia, and prevention of diabetic complications and for overall health of diabetic patients. Following clinical trials Amaranthus extracts thus can be used for preparation of prospective Nutraceutical for diabetics.

2.2 Glucose lowering effects of cle*ome gynandra*

Chweya *et al* (1997) has reported the use of leaves and seeds of *Cleome gynandra* in indigenous medicine of many countries. Medicinal use includes facilitating childbirth in pregnant women, treating stomach-ache, relieving chest pains and constipation (Eloff, 2017); treating conjunctivitis, treating severe thread-worm infection, dysentery and gonorrhea (Rao, 2017); also used in treatment of Arthritis and as anti-inflammation plant (Fan, 2017).

Ravichandra *et al* (2014) studied on Anti-diabetic and anti-dyslipidemia activity of ethanolic extracts of *Cleome gynandra*. There was some difference between his study and our present research. In his study Metformin served as a reference drug and Alloxan at dose 120 mg/kg BW was used to induce diabetes. Mechanisms of Metfomin action unlike Glibenclamide does not stimulate insulin secretion from pancreatic β- cells instead their actions include, reduced glucose absorption from the gut, facilitation of glucose entry into muscle without insulin requirement, inhibition of gluconeogenesis in the liver, suppression of oxidative glucose metabolism and enhanced anaerobic glycolysis (Graziella, 2010).

Nonetheless, his research findings showed significant reduction in serum glucose and elevated dyslipidemia levels in Alloxan-induced diabetic rats. The extracts were administered over a period of 7 days. The mechanism of Anti-hyperglycemia observed may be due to

antiradical/chelatory properties of flavonoids which are abundant in *cleome gynandra* (Dansi, 2016).

Devi *et al* (2015) reported that methanol extract of *Cleome viscosa* possesses significant ability to reduce the diabetes complications. He administered the extracts to alloxan-induced diabetic rats and then assessed liver and kidney function and lipid profile parameters. A significant elevation of blood glucose, SGPT, SGOT, ALP, urea, uric acid, creatinine and lipid profile was observed in control groups as compared to normoglycaemic groups.

However, there was significant reduction in the tested biochemical parameters in both the groups treated with extract as compared to the control group and the effect was compared with the standard drug, Metformin. Hence, methanol extract of *Cleome viscosa* possesses significant ability to reduce the diabetes complications. Possible mechanism of action include activities of Glucose transporter the intestines, facilitation of glucose entry into muscle without insulin requirement, inhibition of hepatic glucose output, suppression of oxidative glucose metabolism and enhanced anaerobic glycolysis (Graziella, 2010).

Rao *et al* (2016) investigated hypoglycaemic and anti-hyperglycaemic activity of aqueous extract of roots of Gynandropsis gynandra herb in normoglycaemic and diabetic rats. The aqueous extract of root of Gynandra were tested at 3 dose levels (100, 200 and 400 mg/kg each) in rats. In each case the initial test was performed at dose of 100 mg/kg. The actions were compared with standard Tolbutamide drug at a dose of 40 mg/kg. The data obtained revealed that the Gynandropsis gynandra herb have hypoglycemic actions compared with Tolbutamide.

Mohorana *et al* (2011) reported that the leaves and roots of C. gynandra are used by some tribal and traditional healers as an Antidiabetic drug. Although the hypoglycaemic properties are not yet studied or proved, Cleome gynandra is believed to have the efficacy of lowering blood sugar .The possibility of Cleome gynandra to use in Diabetes may be reasoned for its anti-oxidant properties by the phenolic constituents (Dansi, 2016), Immunomodulatory properties and due to its nutritive value(Fan, 2017)

Afolayan *et al* (2015) undertook a study to evaluate quantitatively the compositions of phytochemicals and antioxidant properties of acetone extract of different parts of C. gynandra. Antioxidant activities were assessed against ferric reducing power, ABTS (2, 2'- azino-bis-3-ethyl benzothiazoline-6- sulfonic acid) diammonium salt, DPPH (1, 1- diphenyl-2-picrylhydrazyl) and NO (nitric oxide) radical scavenging activities. Total phenolic, flavonoids, flavanols, proanthocyanidins, tannins, saponins and alkaloids were also investigated.

The reducing power of the extracts was significantly higher than that of the standard drugs used in a concentration dependent manner. The activities of the plant extracts against ABTS, DPPH and NO radicals were dose responsive with IC50 value of 0.2, 0.1 and 0.03 mg/g respectively.

Dansi *et al* (2016) carried out qualitative phytochemical screening on *C. gynandra* and *C. viscosa* using leaves extracts, revealed the presence of tannins, flavonoids, anthocyanin, leuco-anthocyanin, steroids, mucilage, reducing compounds and quinone derivatives which varies according to the plant species. The IC50 of *C. viscosa* and *C. gynandra* noted were respectively 0.78 mg/ml and 3.125mg/ml indicating non toxicity.

From above studies, it is evident that *Cleome spp* are potential source of ingredients for management of hyperglycemia, associated lipidaemia, and prevention of diabetic complications and for overall health of diabetic patients.

2.3 Mixtures of plant herbs

The second objective involved investigating glucose lowering effect of the mixture (1:1) of ethanolic leaf extracts of *A. Cruentus* and *C. gynandra* on alloxan-induced diabetes rats. There is no published research carried on this (*A. Cruentus* and *C. Gynandra* mixture), however an enormous amount of evidence shows that mixing plant extracts (herbs) enhances the performance of herbs in the right combinations and is an important aspect of natural healing (Chukwuedozi, 2014; Keter, 2012).

Shawk *et al* (2012) Investigated the synergism of licorice with two herbs (*Bupleurum chinense* and *Zingiberis officinalis*) commonly used in Classical Chinese Medicine. Each sample was decocted in de-ionized water for one hour at 100 °C. Individual and paired decoctions were

analyzed using High Performance Liquid Chromatography (HPLC) to evaluate differences in chemical signatures between extractions. The results showed that paired herbal decoctions contain increased levels of active components. Furthermore, a decrease in peaks within the paired decoction but not in the individual formulas suggests that herb decoctions generated new chemical structures. This study represents foundational research into synergistic relationships between herbs.

Ebong *et al* (2008) assessed Antidiabetic efficacy of two plants mixture between *Azadiracta indica* (neem) and *Vernonia amygdalina* (African bitter leaf). The results showed that the mixture produced maximum therapeutic efficacy with minimum side effects as compared to individual plants. Leaf Extract mixtures of two plants are traditionally being used in Zambia to treat Diabetes Mellitus. However, to the best of our knowledge, there is no published literature regarding the glucose lowering effects of the mixture (1:1) of AC and CG on Alloxan-induced diabetic rats

2.4 Phytoconstituent and their mechanism of action

Natural products are organic compounds that are formed by living systems. Naturally occurring compounds may be divided into three broad categories. Firstly, there are those compounds which occur in all cells and play a central role in the metabolism and reproduction of those cells. These compounds include the nucleic acids and the common amino acids and sugars. They are known as primary metabolites. Most primary metabolites exert their biological effect within the cell or organism that is responsible for their production. Secondly; there are the high molecular-weight polymeric materials such as cellulose, the lignins and the proteins which form the cellular structures. Finally, there are those compounds that are characteristic of a limited range of species. These are the secondary metabolites. Secondary metabolites on the other hand have often attracted interest because of their biological effect on other organisms (Hanson, 2000).

The Phytochemical contents of *Amaranthus cruentus* and *Cleome Gynandra* are said to be responsible for Medicinal properties exhibited by the plant (Table 2.1, Mishra *et al*, 2010*).*

TABLE 2.1: Phytochemical compounds and their mode of glucose lowering activity (Mishra *et al*, 2010)

CONSTITUENTS	MODE OF ACTIVITY
Alkaloids	• Inhibit alpha-glucosidase and decrease glucose transport through the intestinal epithelium
Catechins	• Inhibition of lipid peroxidation (Sabu *et al*.2002 cited in Mendes, 2015) • Inhibition of intestinal glucose uptake (Kobayashi *et al*. (2000 cited in Mendes, 2015) • Improve of beta cell function and stimulation of insulin secretion • Increased expression level of GLUT4 transporter
Polyphenols	• Regulation of insulin secretion and DPPH free radical scavenging ability (Huang *et al*. 2015 cited in Mendes 2015) • Antioxidant and Antidiabetic properties / increase the number of beta cells
Imidazoline Compounds	• Stimulates insulin secretion in a glucose-dependent manner
Polysaccharides	• Increase the conversion of glucose-6- phosphatase in glycogen • Restore the damaged pancreas to normalcy (Li *et al*. 2015 cited in Mandes, 2015).
Flavonoids	• Increased hepatic glucokinase activity probably by enhancing the insulin release from pancreatic islets. • Inhibitory activity against α-glycosidase
Dietary Fibers	• Effectively adsorbed glucose, retards glucose diffusion and inhibit the activity of alpha-amylase and may be responsible for decreasing the rate of glucose absorption and concentration of postprandial serum glucose
Vitamin C and E	• Synergic effects that reduce the production of reactive oxygen species
Saponins (Triterpenoids, steroidal glycoside	• Stimulates release of insulin and blocks the formation of glucose in the blood stream • Enhancement of antioxidant activity Increase of expression of Glut4 (Elekofehinti.,2015 cited in Mendes, 2015
Ferulic acid	• Stimulatory effect on insulin secretion
limonoids	• Activation of glycogen synthesis(Ovalle-Magallanes *et al*. 2015 cited in Mendes, 2015)

CHAPTER 3: MATERIALS AND METHODS

3.1 Research design

The study was an *in vivo* scientific experimental study using the rat model conducted in the pharmacology laboratory at the University of Zambia, school of medicine

3.2 Chemicals and apparatus

Chemicals and apparatus included; Glibenclamide (Cipla), Alloxan monohydrate (sigma), Solvent (distilled water or ethanol), Lignocaine solution 1% (Ranbaxy), Accu-check glucometer and glucose sticks, Test tubes, Feeding needles and syringes, Lancets, rat cages, Beakers, Buchner's funnel, electronic balance and mouse scale. The materials used and their purpose are listed in Table 3.1.

Table 3.1: Materials required and their purpose

Materials	**Purpose**
Alloxan monohydrate (Sigma Aldrich, St. Louis, MO, USA)	Used to induce Diabetes Mellitus (or Hyperglycemia) in Animals
Ethanol (80%)	Used in the maceration process
Rat cages (standard cages)	Housing of the Rats
Gavage Needles	Used in oral administering of our crude drug (i.e. leaf extracts)
Glibenclamide (Cipla)	Reference drug
Animals (Albino Wister Rats)	Experimental subjects
Glucometer (Roche Diagnostics)	Used to measure blood glucose levels
Normal Saline	Control solution
Lignocaine (Ranbaxy)	Used for local anesthesia
Chloroform	Used in euthanasia

3.3 Collection and identification of plants

Fresh *Amaranthus Cruentus* (AC) and *Cleome gynandra* (CG) plants were collected from Libala south, Lusaka, Zambia during the rainy season (November-December). The plants species were confirmed by a Botanist from University of Zambia (UNZA), Department of Biological Sciences. A herbarium sample, voucher number DS01 (*Cleome gynandra*) and DS02 (*Amaranthus cruentus*) were prepared and deposited in the Department of Biological Sciences, UNZA.

3.4 Experimental Animals

Male Albino rats of Wister strain (*Rattus norvegicus*) weighing 120-300 grams, bred in the central animal house (University of Zambia School of Medicine) and normal blood sugar ranging 4.7 – 7.5 mmol/L were used in the study. The animals were individually housed in standard polypropylene cages (Figure 3.1).

Figure 3.1: picture of standard Rat cages

The cages were placed in well ventilated rooms, under hygienic conditions. Animals were given water ad libitum and allowed to acclimatize. A standard Rat pellet feed was provided and comprised 45% fat, 35% protein and 30% carbohydrate as a percent of total Kilo calories. This feed was given throughout the study except a day prior to induction of Diabetes.

3.5 Extraction of Crude drug by Maceration

The crude drug was extracted from the leaves of *Amaranthus cruentus* and *Cleome gynandra* by maceration. The procedure was based on standard procedure done by other researchers (Muyenga, 2017; Omnkhelin *et al*, 2011; Ezeala *et al*, 2007 etc.), by International center for Science and High technology and United Nation Industrial Development Organization (ICS and UNIDO, 2008).

Principle behind Maceration: It involved soaking plant materials (coarse or powdered) in a stoppered container with a solvent and allowed to stand at room temperature for a period of minimum 3 days with frequent agitation.

Actual Procedure of Maceration

- The leaves of AC and CG *were* washed with water to remove any dust. Then cut into pieces and shade dried at room temperature. Dry sample was preferred considering the time needed for experimental design and do not deteriorate faster as compared to fresh sample, design (Azwanida, 2015).
- The dried plant materials were subjected to size reduction by means of a grinder. The purpose of Size reduction is to maximize the surface area, which in turn enhances the mass transfer of active principle from plant material to the solvent.
- The powdered materials of each plant were socked in 80% alcohol (20% water and 80% ethanol) separately in well labeled rubber corked bottle for 4 days with frequent agitation. 80% hydro-alcohol was used to allow the mass transfer of both water and ethanol soluble active principle (medicinal ingredient) into the solvents by a concentration gradient.
- After 4 days, the solution was filtered with a clean muslin cloth. Filtration allows separation of the marc (exhausted plant material) from the Filtrate thus formed.
- Then filtrate thus formed, was evaporated to dryness in a rotary evaporator at 40 – 50°C to avoid the decomposition of the natural metabolites.
- Finally, then percentage yield was calculated by; % yield $= \frac{m1-m2}{m1} X\ 100$ where m_1 and m_2 are initial mass and final mass respectively.

Preparation of Leaf Extract Doses

The amount of the test drug to be administered was calculated based on the body weight of the rats and the design of the experiment. The 3 dose levels used in this study were 200, 400 and 600 mg/Kg BW. The dose levels were chosen based on the results of the acute tests done by Desai and Ravichandra on *Amaranthus spp* and *Cleome gynandra* leaf extracts respectively (Desai, 2011; Ravichandra, 2014). These doses had Glucose lowering effects on rats.

To calculate the Mass of crude drug (or Test drug) required for dose preparation i.e. 200, 400 or 600 mg/kg BW the formula was used;

$$\textbf{Mass of Crude Drug} = \frac{\boldsymbol{Predetermined\ Dose\ X\ Average\ Mass\ of\ Rats}}{\boldsymbol{1000\ g}}$$

Where Predetermined dose = 200, 400 or 600 mg/kg BW.

Average mass of Rats = 181.7 g

The drug crude drug was then administered orally to each Rat by using a stainless steel feeding-needle on a plastic syringe dissolved in 10 ml distilled water.

3.6 Procedure for Inducing Diabetes in rats

Alloxan was used based on standard procedures (Grover N, 2011; Muyenga T, 2015; Etuk, 2010; Bako, 2014*)* to induced Diabetes Mellitus.

Pre-induction: The Rats were kept in standard rat cages that were well ventilated, had unrestricted access to food, water throughout the study. They fed on standard rat pellets comprising 45% fat, 35% protein and 30% carbohydrate as a percent of total Kilo calories. This feed was given throughout the study except a day before the induction of diabetes since the Rats had to be fasted.

The Rats had access to sunlight and experienced the usual cycle of about 12 hours of light and 12 hours of darkness. The rats were allowed to acclimatize to the new environment for 24 hours before the study could begin and randomly placed into 11 groups (5 rats per group).

The rats were weighed and a baseline glucose level established after 16 hour fasting period, before the Alloxan injection.

Injecting Alloxan: A single intraperitoneally (ip) dose of 140 mg/kg of alloxan monohydrate dissolved 0.9% cold NaCl solution was injected ip to overnight fasted rat. It was administered rapidly and prevented from direct sunlight so as to prevent degradation of alloxan to its toxic secondary metabolite since alloxan is photosensitive.
Mass of alloxan needed to dissolve in 5 ml normal saline and to inject per rat was calculated form the formulae below;

$$\boldsymbol{MAss\ of\ Alloxan\ (in\ mg) to\ dissolve\ in\ 5\ ml\ Normal\ saline} = \frac{\textbf{140 mg Alloxan X weight of Rats in Kg}}{\textbf{1 kg}}$$

An hour after alloxan injection, the injected rats were exposed to 10% dextrose solution to prevent fatal hypoglycemia that often follows alloxan treatment.

FBG was then measured on the 2rd, 3rd and 5th day post-induction to ensure that the desired hyperglycemia (FBG equal or greater than 11 mmol/L) was established and sustained (Ravichandra, 2014). The rats showed FBG level ranging between 12 – 26 mmol/L. Feeding of the diabetic animals continued with the standard diet and water *ad libitum*.

3.7 Experimental procedure and data collection

The experimental procedures focused on obtaining results from the following 3 perspective;

- Acute studies in Normoglycaemic Rats for 24 Hours
- Acute studies in hyperglycemic rats (Alloxan treated Diabetic) for 24 Hours
- Sub-acute studies in hyperglycemic (Alloxan treated Diabetic) rats for 10 days

The treatment option administered to both normolglycemic and hyperglycemic rats is shown in Table 3.2.

Table 3.2: Treatment options administered to each group once per 24 hours

GROUP # (n=5)	**TREATMENT**	
	Before Alloxan (normoglycaemic)	**Alloxan-treated rats (hyperglycaemic)**
1	Saline/distilled water.	Saline/distilled water.
2	Glibenclamide 5 mg/Kg body weight	Glibenclamide 5 mg/Kg body weight
3	200 mg/kg AC leaf extract	200 mg/kg AC leaf extract
4	400 mg/kg AC leaf extract	400 mg/kg AC leaf extract
5	600 mg/kg AC leaf extract.	600 mg/kg AC leaf extract.
6	200 mg/kg CG leaf extract	200 mg/kg CG leaf extract
7	400 mg/kg CG leaf extract	400 mg/kg CG leaf extract
8	600 mg/kg CG leaf extract	600 mg/kg CG leaf extract
9	200 mg/kg AC + CG mixture.	200 mg/kg AC + CG mixture.
10	400 mg/kg AC + CG mixture.	400 mg/kg AC + CG mixture.
11	600 mg/kg AC + CG mixture.	600 mg/kg AC + CG mixture.

CG = *Cleome gynandra*, AC = *Amaranthus cruentus*; AC + CG = mixture of AC and CG leaf extracts

Acute studies in Normoglycaemic Rats for 24 Hours

i Grouped the Normoglycaemic rats in 11 groups comprising 5 rats per group (Table 3.2). Normoglycaemic healthy animals were used for testing potential hypoglycaemic effects of AC and CG. This is a valid screening method and it allows for the effect of the drug to be tested in the animal with an intact pancreatic activity. The comparison with diabetic animals may give some information regarding mechanism of action. Hypoglycaemic agent may be detected at the same time (Etuk, 2010).

ii Fasted the Rats for 12 hours with access to drinking water Prior to administering the leaf extracts

iii After the 12 hour fast, Fasting Blood glucose was measured by Accu-check Glucometer.

iv Then administered various doses of crude drugs of AC, CG and Mixture (200 mg/kg, 400 mg/kg and 600 mg/kg BW) using a gavage needle

v Thereafter, measured FBG for 24 hours at times 0, 1, 2, 4, 6, 8, 16 and 24 hours after administering a single dose of vehicle/extract (Rao, 2017).

vi Lastly discontinued the fasting, Fed the Rats with standard food. Allowed a wash out period of 10 days (Sushruta, 2006, IACUC, 2015).

Acute studies in Diabetic Rats for 24 Hours

i The rats earlier used above were induced with diabetes using alloxan according to standard procedure. Then grouped according to table 3.2

ii After confirmation of diabetes, fasted the rats for 12 hours with access to water

iii After the 12 hour fast, obtained the initial FBG.

iv Then administered various doses of crude drugs of AC, CG and Mixture (200 mg/kg, 400 mg/kg and 600 mg/kg BW) using gavage needles

v Thereafter, Measured FBG for 24 hours at 0, 1, 2, 4, 6, 8, 16 and 24 hours. Lastly discontinued the experiment. Fed the Rats with standard pellets and food, for 3 days.

Sub-acute studies in Diabetic Rats for 10 days

i Grouped the diabetic rats in 5 groups of 5 rats per group and received various treatments as shown in the Table 3.3. Group 1 had normal rats without diabetes.

Table 3.3: Treatment options administered to each group on a Daily basis for 10 days

GROUP # (n=5)	TREATMENT
1	Normoglycaemic Rats + Saline/distilled water.
	Diabetic Rats + Saline/distilled water.
2	Diabetic Rats + Glibenclamide 5 mg/Kg body weight
3	Diabetic Rats + 600 mg/kg AC leaf extract
4	Diabetic Rats + 600 mg/kg CG leaf extract
5	Diabetic Rats + 600 mg/kg Mixture leaf extract.

ii Treatments were given orally once daily for 10 days.

iii Random Blood sugars was measured every alternate day for 10 days (i.e. at Day 1, 3, 5, 7 and 10).

iv Standard Euthanasia (Section 3.9) procedure was performed and ended the experiment.

3.8 Study limitation

We only tested a single dose of Alloxan (140 mg/kg BW) that cause partial destruction of the pancreatic β-cells. Future research should include testing Rats with completely destroyed pancreatic β-cells. Also it would an advantage to monitor glucose levels changes for more than 10 days, example 21 days. This may give us insight to whether Leaf extracts of *A.cruentus* and *C.gynandra* stimulate insulin secretion or regenerate pancreatic Beta cells.

3.9 Ethical Consideration

All animal experiments were conducted in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals (Grover *et al*., 2010). In order to manage animal stress the rats were handled by a shared tunnel and not by standard tail handling. A shared tunnel reduces anxiety and increases willingness of the rat to voluntarily approach the handler when compared with standard tail handling (Hurst, 2013).

When collecting Blood sample, the tail was immersed in 42 °C water for 40-50 sec to dilate blood vessels and dried the tail with a paper towel. This procedure helped in reducing stress in the animals and easy collection of Blood (Goosens 2015). Lignocaine solution 1% for local anesthesia was used before cutting a tip of the tail. This reduces pain and suffering.

The study protocol was observed and approved by University of Zambia Research Ethics Committee (UNZAREC). Ethical clearance reference No. 011-09-16. The animals were housed in standard cages under standard environmental conditions, with a 12 hours light/dark cycle maintained on a regular feed (vital feed) and water *ad libitum*.

3.10 Euthanasia procedure

The procedure was performed according to the Institutional Animal Care and Use Committee (IACUC, 2013). The bell jar was placed in the fume hood located in the laboratory and contained Chloroform soaked absorbent material. The animals from the same group were placed in the bell jar and anesthetized and euthanized together.

3.11 Statistical Analysis

Percent decrease in blood glucose level at any given time,'t' in rats was calculated using formula according to Murthy (2008)

$$\textbf{\% FBG change at time 't'} = [(a - b)/a] \times 100,$$

Where; ***a*** is the initial blood glucose level and ***b*** is the blood glucose level at time**'t'**. Data was expressed as mean ± standard deviation of 5 rats per group. An unpaired student's `t' test was used to compare the effect of the crude drugs with normal saline and also with the reference drug. Statistical significance was set at $p < 0.05$. Graphpad software version 6 was used for all data analysis.

CHAPTER 4: RESULTS

4.1 Preliminary Results

The percentage (%) yield of extracts of *Amaranthus Cruentus* and *Cleome gynandra* was 21.5% and 16.3% respectively. Average weights of Rats was 181.7 ± 6.57 g (n = 55).

The three dose levels of Ethanolic Leaf extracts tested were 200, 400 and 600 mg/kg BW of which 600 mg/kg BW was the most effective.

Random Blood Glucose (RBG) and Fasting Blood Glucose (FBG) levels in the normoglycaemic and Alloxan diabetic rats (n = 55 rats) was in the range 4.7-7.5 mmol/L and 2.46 - 3.28 mmol/L respectively

4.2 Acute studies on Normoglycaemic rats before induction of Diabetes

AC 600 mg/kg BW showed maximum FBG reduction after 6 Hours (from 2.9 ± 0.05 to 2.10 ± 0.11 mmol/L) which represent 30.00 % reduction in Fasting Blood Glucose (Tables 4.1). CG 600 mg/kg BW showed maximum glucose reduction after 6 Hours (from 2.9 ± 0.07 to 2.0 ± 0.05 mmol/L) which represent 35.4% reduction (Tables 4.1). Mix 600 mg/kg BW showed maximum glucose reduction after 8 Hours (from 2.9 ± 0.05 to 2.2 ± 0.06 mmol/L) which represent 26.1 % reduction (Tables 4.1).

The reference drug, Glibenclamide showed maximum FBG reduction after 4 Hours (from 2.8 ± 0.09 to 1.6 ± 0.03 mmol/L) which represent 50.00 % reduction in Fasting Blood Glucose (Tables 4.1, and 4.5). The normal saline showed fluctuation in FBG during 24 hours showing an average increase of 3.05 % and with maximum reduction of 2.0% at 6 hours (Tables 4.1).

Table 4.1: Fasting Blood Glucose (mmol/L) in Normoglycaemic rats administered with ethanolic leaf extracts of AC, CG and Mix at dose 600 mg/kg BW (n=5).

Time (hour)	Normal Saline (0.9%)	Glibenclamide 5mg/kg BW	AC 600	CG 600	Mix 600
0 (Baseline)	2.9±0.09	2.8±0.09	2.9±0.05	2.9 ± 0.07	2.9±0.05
1	3.0±0.09	2.4±0.06	2.5±0.03	2.7 ± 0.10	2.8±0.05
2	3.3±0.07	2.2±0.07*	2.6±0.23	2.5 ± 0.04*	2.7±0.09
4	3.2±0.10	1.6±0.03*	2.4±0.15*	2.20 ± 0.12*	2.3±0.03*
6	2.8±0.10	1.6±0.03*	2.1±0.11*	2.0 ±0.06*	2.3±0.06*
8	2.9±0.10	1.6±0.03*	2.1±0.11*	2.1±0.10*	2.2±0.06*
10	3.00±0.09	1.80 ±0.03*	2.4±0.10*	2.16±0.04*	2.34±0.16*
16	3.48±0.09	2.10 ±0.03*	2.7±0.05*	2.52 ±0.02*	2.74±0.14*
24	3.60 ±0.06	3.20 ±0.1*	2.8±0.06*	2.82 ±0.02*	2.90 ±0.1*

*Values are means ± S.E.M; n=number of animals per group, *p < 0.05 were considered statistically significant compared to Control (Normal Saline). Samples were taken at 0 hr. (just before treatment administration then 1, 2, 4, 6, 8, 10, 16 and 24 hours.*

The ethanolic leaf extracts (at 600 mg/kg BW) showed a glucose lowering pattern which was comparable to the reference drug, Glibenclamide (Figure 4.1).

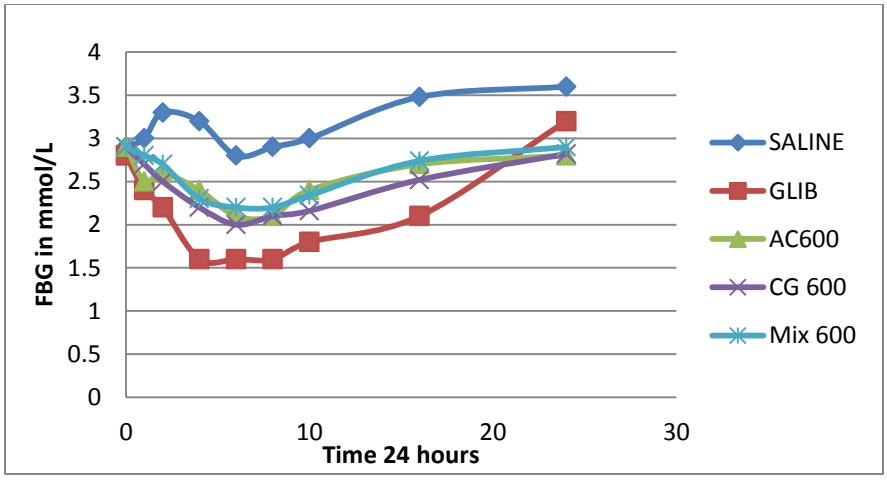

Figure 4.1: The effect of ethanolic leaf extracts of *AC, CG and MIX* (600 mg/kg) on FBG levels in Normoglycaemic rats. Each point is the mean ± SEM

Summary of statistical Analysis

In normal rats, the blood glucose lowering action of the leaf extracts (600 mg/kg BW) was significantly different from that of control (Table 4.2).

Table 4.2 *t*-test results comparing Control and treated groups on the effect of Fasting Blood glucose in Normolglycemic rats

Treatment	n	Mean (mmol/L)	SD	*t*-Cal	*p*	95% CI (mmol/L)		Decision
						Lowest	highest	
Glibeclamide (5 mg/kg BW)	9	2.14	0.57	4.38	0.0006	1.77	2.51	Reject (i.e significantly different)
AC 600 mg/kg BW	9	2.50	0.28	4.49	0.0002	2.32	2.68	Reject (i.e significantly different)
CG 600 mg/kg BW	9	2.43	0.33	4.55	0.0002	2.21	2.65	Reject (i.e significantly different)
Mix 600 mg/kg BW	9	2.56	0.30	3.91	0.0008	2.37	2.75	Reject (i.e significantly different)
Control (0.9% saline)	9	3.13	0.27					

Note; Degree of freedom (Df) =16, t- critical= 2.16, CI= Confidence Interval, SD= Standard Deviation, t-Cal= t-test calculated, p = probabilty of rejecting the Null hypothesis if $p < 0.05$

4.3 Acute studies on Alloxan Diabetic Rats

AC 600 mg/kg BW was the effective dose that showed maximum glucose reduction at 6 th Hour (from 25.5 ± 1.57 to 20.1 ± 1.66 mmol/L) which represent 23.8 % reduction (Tables 4.3). CG 600 mg/kg BW was the effective dose that showed maximum glucose reduction at 6 th Hour (from 25.81± 2.13 to 17.90 ± 1.7 mmol/L) which represent 31.7 % reduction (Tables 4.3). Mix 600 mg/kg BW was the effective dose that showed maximum glucose reduction at 6 th Hour (from 25.8± 0.13 to 19.30 ± 1.74 mmol/L) which represent 26.9 % reduction (Tables 4.3). Normal saline showed fluctuation in FBG during 24 hours showing an average increase of 3.7 % and with maximum reduction of 11.0 % at 6 hours (Tables 4.3).

Table 4.3 Fasting Blood Glucose (mmol/L) of Alloxan diabetic rats administered with ethanolic leaf extract of AC, CG and Mix (n=5)

Time (hours)	**Normal Saline (0.9%)**	**Glibenclamide 5mg/kg**	**AC(600 mg/kg BW)**	**CG (600 mg/kg)**	**Mix 600 mg/kg**
0 (Baseline)	25.4±1.81	24.74±2.26	25.5±1.55	25.81±2.13	25.8±0.13
1	27±1.10	21.04±2.83*	24.8±1.80*	24.1 ±1.1*	24.7±1.08*
2	29.7±0.82	20±2.24*	24±1.61*	22.4 ±1.2*	23.8±1.25*
4	28.2±1.00	14.2±1.01*	21.4±2.09*	20.0 ±1.4*	20.8±1.39*
6	25.4±1.93	15.4±0.50*	20.1±1.66*	17.9 ± 1.70*	19.3±1.74*
8	26.1±1.14	19.36±1.26*	18.6±2.00*	18.4 ±2.09*	19.3±2.09*
10	26.0 ±0.2	17.2 ± 0.04*	20.08 ±0.14*	19.08 ± .08*	20.2 ±0.06*
16	30.58 ±0.3	21.2 ± 0.03*	22.2 ±0.06*	20.5 ± 0.5*	21.3 ±0.09*
24	33.0 ± 0.6	22.06 ±0.18*	24.7 ±0.09*	21.6 ± 0.22*	22.28 ± 0.14*

*Values are means ± S.E.M; n=number of animals per group, *p < 0.05 were considered statistically significant compared to Control (Normal Saline)*

The glucose lowering pattern of the ethanolic leaf extracts was plotted as shown in Figure 4.2. The effect was comparable to the standard diabetic drug, Glibenclamide (5 mg/kg BW).

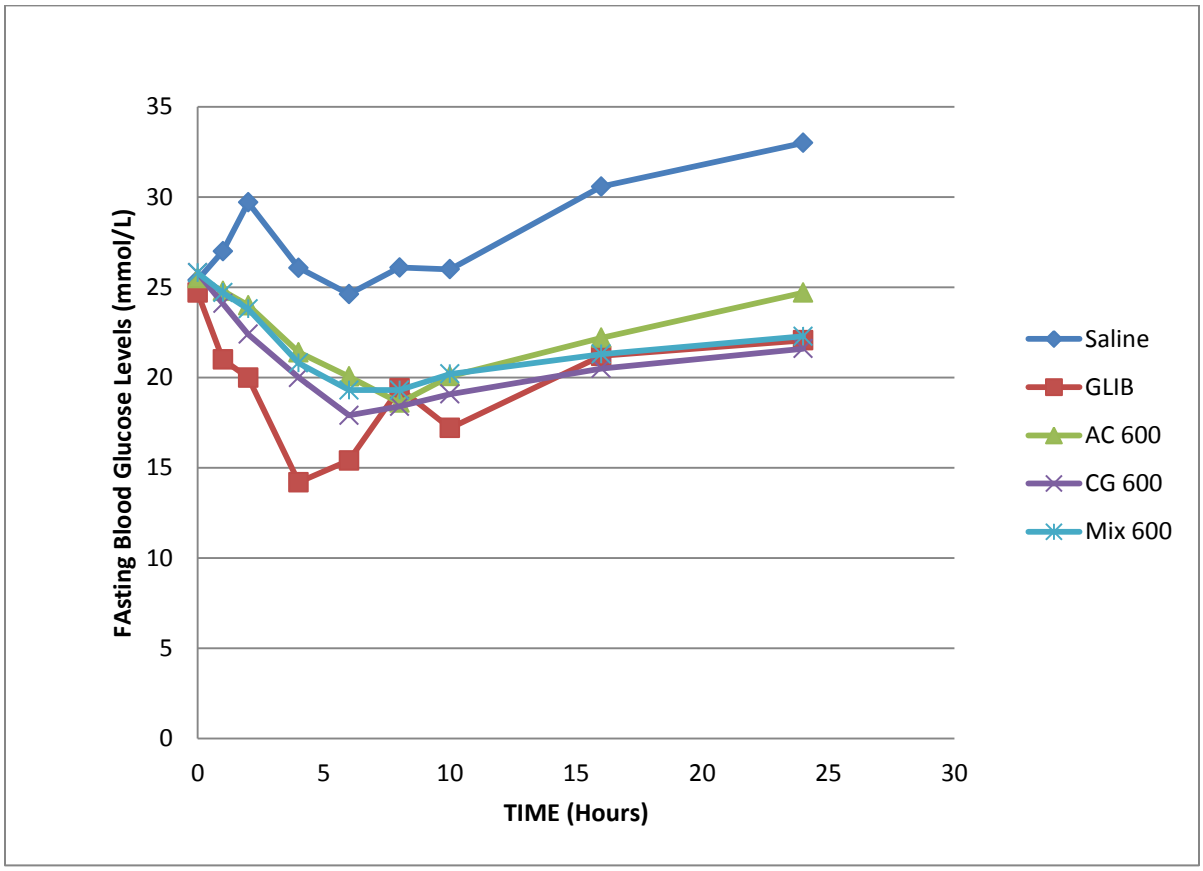

Figure 4.2 The effect of ethanolic leaf extract of *AC, CG and MIX* (600 mg/kg) on FBG levels in Alloxan- diabetic Rats. Each point is the mean ± SEM

Summary of statistical Analysis

In hyperglycmic rats, the blood glucose lowering action of the leaf extracts (600 mg/kg BW) was significantly different from that of control (Table 4.4).

Table 4.4 Unpaired *t*-test results comparing Diabetic Control and treated groups on the effect of Fasting Blood glucose in Alloxan-diabetic Rats

Treatment	**n**	**Mean(mmol/L)**	**SD**	***t*-Cal**	**p**	**95% CI (mmol/L)**		**Decission**
						Lowest	**highest**	
Glibeclamide (5 mg/kg BW)	9	19.46	3.33	5.27	< 0.0001	17.28	21.64	Reject (i.e significantly different)
AC 600 mg/kg BW	9	22.37	2.49	3.94	0.0007	20.74	24	Reject (i.e significantly different)
CG 600 mg/kg BW	9	21.1	2.65	4.77	0.0001	19.37	22.83	Reject (i.e significantly different)
Mix 600 mg/kg BW	9	21.94	2.37	4.35	0.0003	20.39	23.49	Reject (i.e significantly different)
Control (0.9% saline)	9	27.81	0.27					

Note; Degree of freedom (Df) =16, t- critical= 2.16, CI= Confidence Interval, SD= Standard Deviation, t-Cal= t-test calculated, p = probabilty of rejecting the Null hypothesis if p < 0.05

4.4 Deducing possible mechanisms of action

There was no significant difference when we compared the effect the leaf extracts had on normolglycemic and hyperglycemic rats in terms of percentage change in fasting blood glucose levels (Table 4.5)

Table 4.5 Reduction in mean FBG (expressed in percentage) as a result of administration of treatment

	Control		**GLIB**		**AC 600**		**CG 600**		**Mix 600**	
Time	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic
1	-3.45	3.45	14.29	3.45	13.79	3.45	6.90	3.45	3.45	3.45
2	-13.45	-6.55	22.62	8.21	9.79	6.67	14.30	10.50	7.02	7.09
4	-10.42	5.64	49.89	37.21	17.49	17.51	26.30	21.22	21.83	19.70
6	2.08	11.23	49.89	28.76	29.99	23.77	35.39	31.72	26.18	26.91
8	-1.49	5.22	49.89	2.79	29.99	31.05	30.39	28.92	26.18	26.91
10	-4.94	5.61	37.39	13.92	15.70	23.09	27.54	25.23	19.82	22.25
16	-20.94	-12.01	20.73	-8.47	3.20	12.53	10.87	17.79	2.72	16.80
24	-24.39	-19.92	-31.66	-13.02	-0.50	1.27	-1.03	12.42	-3.11	12.20

The results compare percentage changes of FBG between normal and alloxan diabetic groups. *Negative sign (-) = increase in FBG. Positive sign (+) = Decrease, percentage (%) FBG change at time't' = [(a − b)/a] ×100 (Murphy, 2006).*

There was significant difference when we compared the effect Glibenclamide (5 mg/kg BW) had on normolglycemic and hyperglycemic rats in terms of percentage change in fasting blood glucose levels (Figure 4.3).

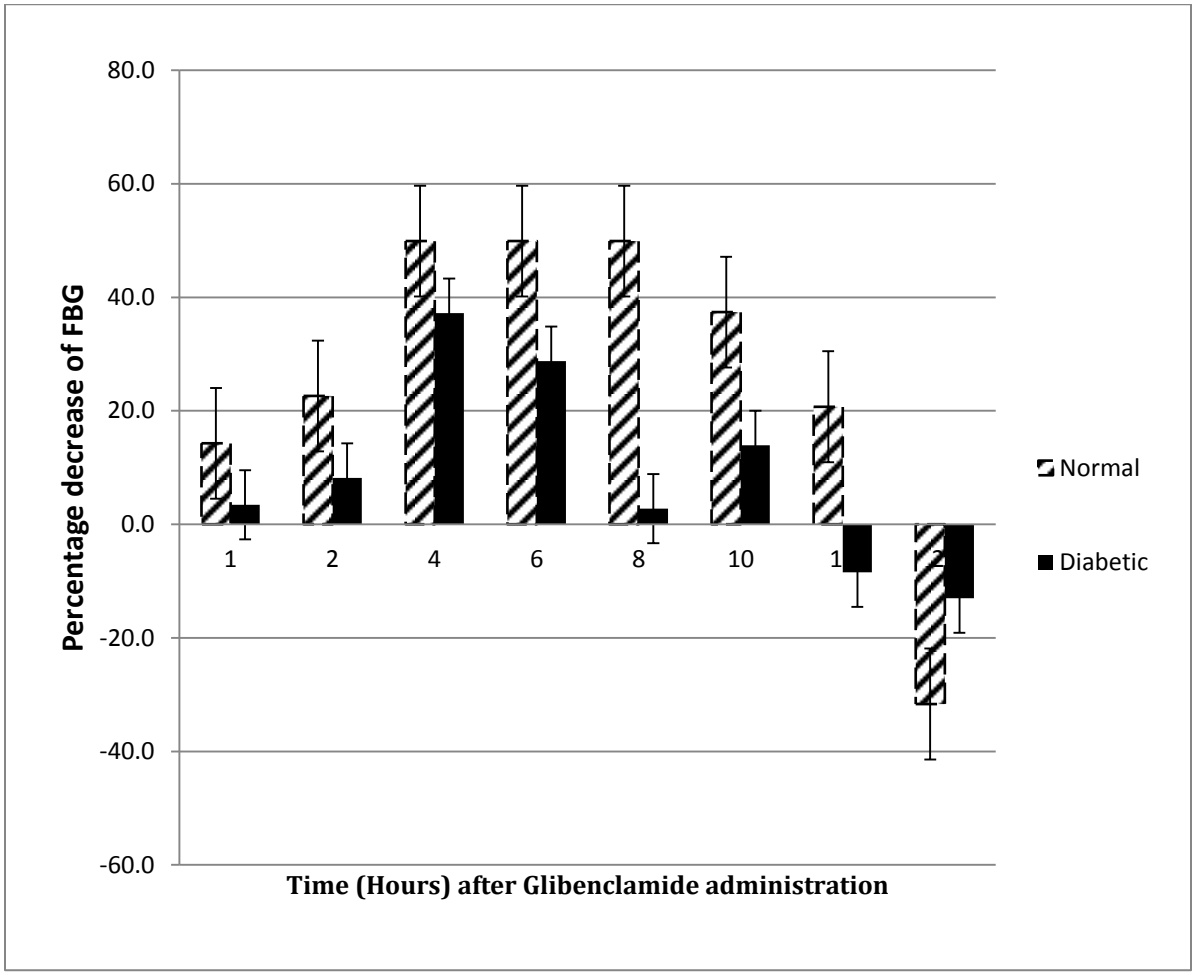

Figure 4.3: The effect of Glibenclamide (5 mg/kg) on Blood Glucose levels in Normal and Alloxan diabetic Rats

There was no significant difference when we compared the effect *Amaranthus cruentus* leaf extracts (600 mg/kg BW) had on normolglycemic and hyperglycemic rats in terms of percentage change in fasting blood glucose levels (Figure 4.4).

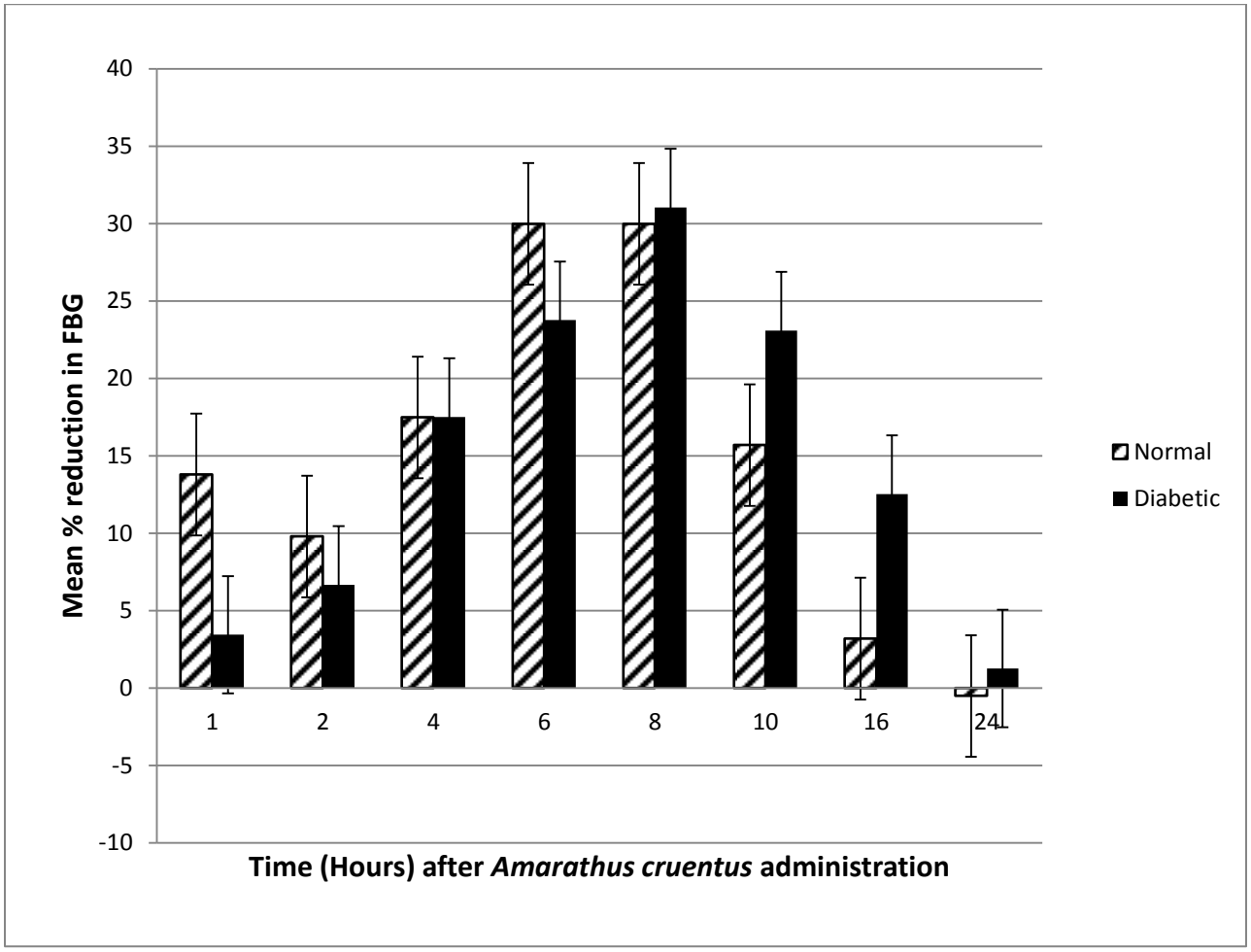

Figure 4.4: The effect of ethanolic leaf extracts of *Amaranthus cruentus* (600 mg/kg) on Blood Glucose levels in Normal and Alloxan diabetic Rats

There was no significant difference when we compared the effect *Cleome gynandra* leaf extracts (600 mg/kg BW) had on normolglycemic and hyperglycemic rats in terms of percentage change in fasting blood glucose levels (Figure 4.5).

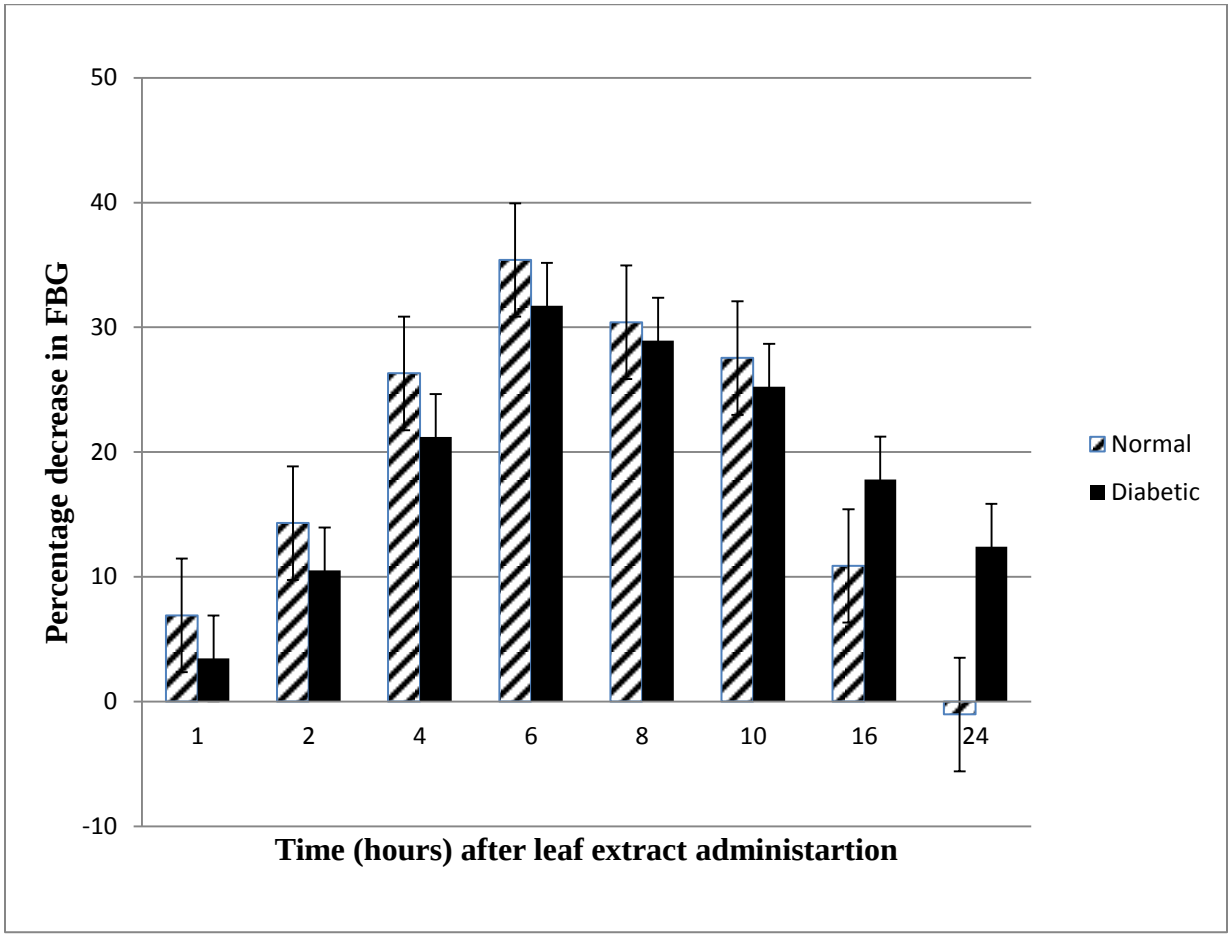

Figure 4.5: The effect of ethanolic leaf extracts of C.gynandra (600 mg/kg) on Blood Glucose levels in Normal and Alloxan diabetic Rats

There was no significant difference when we compared the effect leaf extract Mixtures (600 mg/kg BW) had on normolglycemic and hyperglycemic rats in terms of percentage change in fasting blood glucose levels (Figure 4.6).

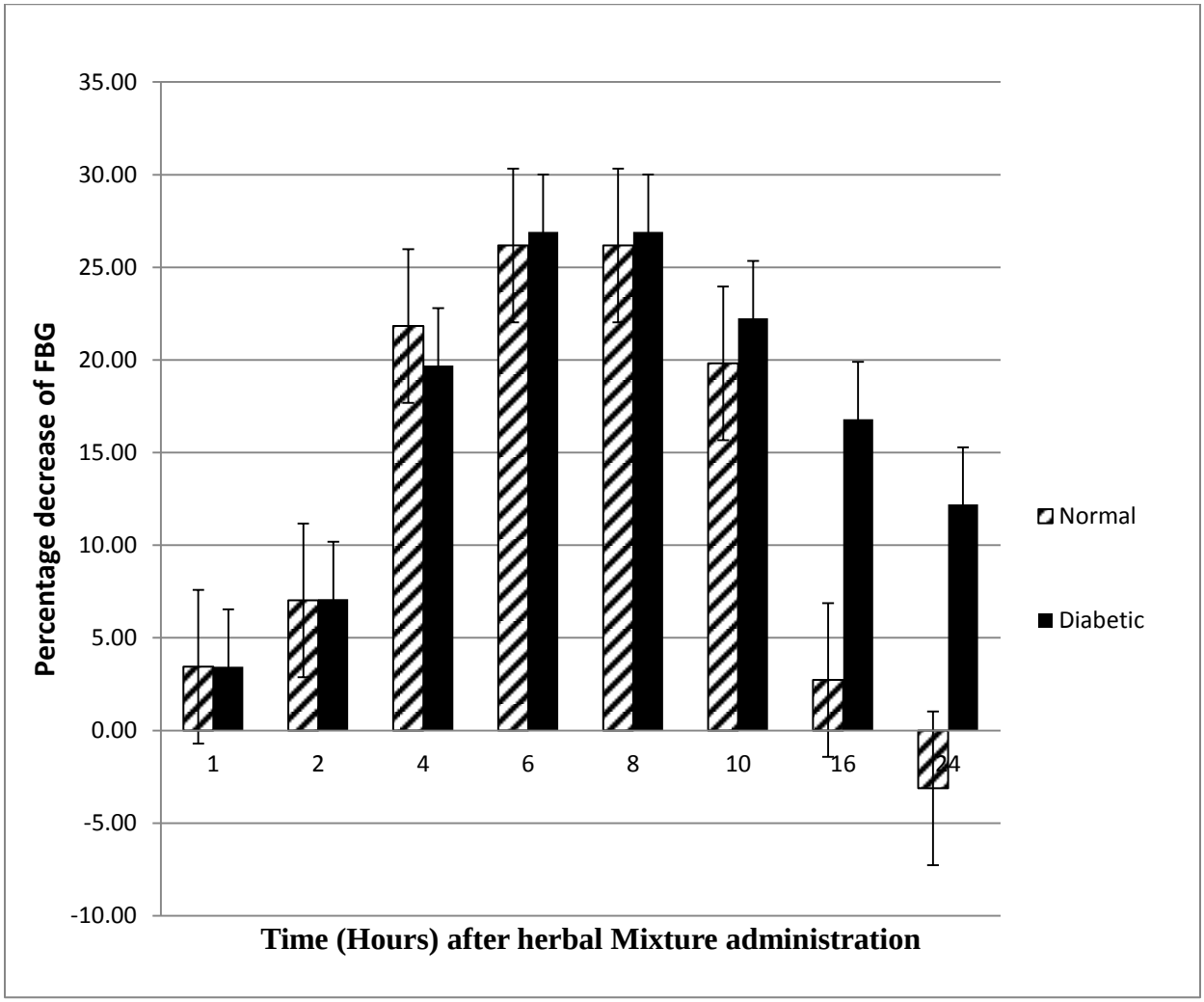

Figure 4.6: The effect of ethanolic leaf extracts Mixture (600 mg/kg) on Blood Glucose levels in Normal and Alloxan diabetic Rats

4.5 Sub-Acute studies for 10 days in Alloxan-diabetic Rats

Daily administering of the leaf extracts to the Alloxan- Diabetic rats showed reduction in blood glucose and arresting of hyperglycemia (Table 4.6).

Table 4.6: Random blood glucose levels during 10 days daily administration of extracts (n=5).

Group	Treatment	Basal Value	1st Day	3rd Day	5 th Day	7 th Day	10 th Day
1	Normal control	5.52±0.4	5.7±0.3	6.0±0.29	5.8±0.25	4.9±0.08	4.7±0.02
2	Diabetic control	25.4±0.22	25.7±0.33	28.4±0.38	28.7±0.22	28.0±0.06	24.8±0.23
3	Diabetic and Glibenclamide	24.7±0.19	22.3±0.08*	17.5± 0.57*	15.2±0.39*	15.8±0.64*	15.3±0.22*
4	Diabetic + AC 600	25.5±0.4	24.1±0.7*	23.2±0.46*	19.9±0.44*	18.7±0.44*	17.8±0.42*
5	Diabetic + CG 600	25.8±0.59	20.1±0.5*	18.8±0.48*	18.0±0.54*	17.5±0.56*	16.8±0.59*
6	Diabetic + Mix 600	25.78±0.23	23.2±0.37*	22.1±0.15*	19.8±0.4*	18.6±0.4*	18.2±0.6*

*Values are means ± S.E.M; n=number of animals per group, *p < 0.05 were considered statistically significant compared to Control (**Diabetic Control)***

AC 600 mg/kg BW showed maximum Random Blood Glucose (RBG) reduction of 14.2 % on day 5 (from 25.5 ± 0.4 to 19.9 ± 0.44 mmol/L). CG 600 mg/kg BW showed maximum Random Blood Glucose (RBG) reduction of 28.56 % on day 3 (from 25.8 ± 0. 59 to 18.8 ± 0.48 mmol/L). Mix 600 mg/kg BW showed maximum Random Blood Glucose (RBG) reduction of 16.47 % on day 5 (from 25.78 ± 0.23 to 18.6 ± 0.4 mmol/L). Refer to Figure 4.7

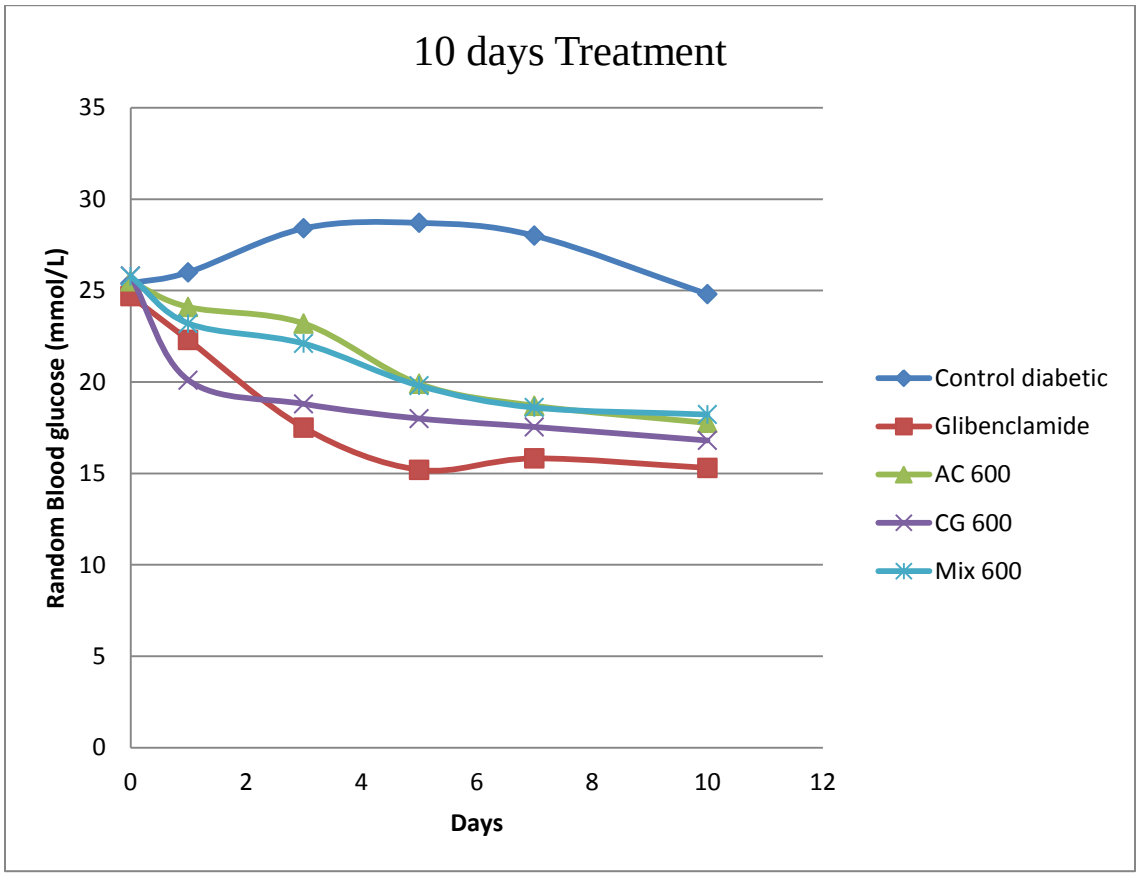

Figure 4.7 10 days Glucose lowering effect of leaf Extracts of AC, CG and Mixture on diabetic rats. . Each point is mean ± SEM

Statistical Analysis

The leaf extracts of AC and CG showed a glucose lowering pattern that was significantly different from that of the control (Table 4.7).

Table 4.7 *t-test* results comparing Diabetic Control to treated groups on the effect of Random Blood glucose in Alloxan-diabetic Rats treated for 10 days

Treatment	n	Mean (mmol/L	SD	*t*-Cal	p	95% CI (mmol/L)		Decission
						Lowest	highest	
Glibeclamide (5 mg/kg BW)	6	18.47	4.05	3.5	0.0025	15.6	21.34	Reject (i.e significantly different)
AC 600 mg/kg BW	6	21.53	3.16	3.34	0.007	19.00	24.06	Reject (i.e significantly different)
CG 600 mg/kg BW	6	19.5	3.28	4.47	0.001	16.90	22.12	Reject (i.e significantly different)
Mix 600 mg/kg BW	6	21.28	2.94	3.69	0.0025	15.6	21.34	Reject (i.e significantly different)
Diabetic Control	6	26.88	1.68					

Note; Degree of freedom (Df) =10, t- critical= 2.16, CI= Confidence Interval, SD= Standard Deviation, t-Cal= t-test calculated, p = probabilty of rejecting the Null hypothesis if p < 0.05

4.6 The Implication of the Results with Regard to Objectives of the Study

1 The ethanolic Leaf extracts of AC, CG and their Mixture at dose level 600 mg/kg BW have; (1) Hypoglycemic effects on FBG of normoglycaemic Rats, (2) Glucose lowering effect on hyperglycemic (Alloxan-diabetic) Rats.

2 The ethanolic leaf extracts of AC, CG and their Mixture at dose 600 mg/kg BW seem to lower blood glucose via extra-pancreatic mechanism (i.e. peripheral action). This is because when we compared the effects of the Extracts on the Normoglycaemic and Alloxan treated groups, there was no significant difference in the percentage reduction of FBG (Figure 4.4, 4.5 and 4.6).

CHAPTER 5: DISCUSSION

The discussion is based on the result of our research objectives.

5.1 Effect of individual ethanolic leaf extracts on Blood glucose levels

Hypoglycemic and glucose lowering properties observed was due to the presence of phytochemical compounds in the plant extracts of *Amaranthus cruentus* (AC) and *Cleome gynandra* (CG*)*. The ethanolic leaf extracts of AC and CG 600 mg/kg BW were comparable to glibenclamide (5 mg/kg BW). The leaf extracts have a wide spectrum of phytochemical compounds (e.g. Alkaloids, anthocyanin, Coumarins, flavonoids, triterpenoids, steroids and many more) that contributes to a variety of mechanisms responsible for hypoglycemic activities (Hilou, 2012; Dansi, 2016).

Similar studies, although differing in some key research methodologies (e.g. mode of extraction, solvent used, and diabetogenic agent used) agrees with our results. For instance, methanolic plant extract of *Amaranthus tricolor* showed maximum anti-hyperglycaemic activity at 400 mg extract/kg BW on glucose loaded Swiss albino mice., which was comparable to glibenclamide of dose 10mg/kg BW (Rahmatullah *et al*., 2013).

Administering *Cleome gynandra* for 7 days caused significant reduction in serum glucose and was comparable with Tolbutamide drug at a dose of 40 mg/kg (Ravichandra, 2014). Similarly when *Amaranthus esculentus* was administered to Streptozotocin- induced diabetic rats for 3 weeks there was significant decrease in serum glucose and increased serum insulin levels in diabetic rats. Possible mechanism of action involved regeneration of pancreatic tissue or increased glucose utilization by peripheral tissues (Rao, 2017; Kim, n.d).

5.2 Effect of Mixtures of ethanolic leaf extracts on Blood glucose levels

Explanation to why mixing of the leaf extracts of AC and CG improved the efficacy of the Leaf extracts could have been due to increased levels of active components and also the formation of new phytochemical structures (Chukwuedozi,2014). Polyherbal mixtures have the synergistic, potentiative, agonistic/antagonistic pharmacological agents within themselves that work together in a dynamic way to produce therapeutic efficacy with minimum side effects (Ebong, 2008).

Although there is no published literature concerning the combination of *A. Cruentus* and *C. Gynandra* and their anti-diabetic action, there is enormous amount of evidence that shows that mixing plant extracts (herbs) enhances the performance of herbs. For instance combination of two herbs (*Bupleurum chinense* and *Zingiberis officinalis*) commonly used in Classical Chinese Medicine showed increased levels of active components and resulted in formation of new phytochemical structures (Shaw *et al*.,2012). Additionally, when two Antidiabetic plants were combined between *Azadiracta indica* (neem) and *Vernonia amygdalina* (African bitter leaf) showed maximum therapeutic efficacy and reduced blood glucose more than the individual plant extracts (Ebong, 2008). Hence, herbal mixtures still remain an important aspect of natural healing (Chukwuedozi, 2014; Ketel, 2012).

5.3. Deducing possible mechanism of Blood glucose lowering of the Ethanolic leaf extracts

We compared the pattern of Blood glucose reduction in Normoglycaemic and Alloxan-diabetic rats after administration of a single dose of Leaf extracts and glibenclamide (Figure 4.3, 4.4, 4.5 and 4.6). This comparison was important so as to gain insight into the possible mechanisms of action of the Leaf extracts. Firstly it's important to understand the physiological status of the pancreas in (1) Normoglycaemic rats, (2) in Alloxan-diabetic rats and (3) also to appreciate the mechanism of action of Glibenclamide

- **Physiological status of the pancreas in Normoglycaemic rats**

No treatment with Alloxan was done to Normoglycaemic rats; hence they had intact pancreas and normal blood glucose regulatory mechanisms. Prior to administering the treatment, baseline FBG was 2.46 - 3.28 mmol/L which was lower than the Random blood glucose (4.7-7.5 mmol/L). This is because during fasting glucose and glycogen stores are depleted resulting into reduced blood glucose (Costanzo, 2015). However, due to intact healthy pancreas, the rats were able to regulate their blood glucose via insulin and glucagon secretion. Insulin stimulate glucose uptake into cells while glucagon inhibits glycogen synthesis and stimulates glycogenolysis and gluconeogenesis (Baynes, 2012, (Mishra, 2014; Bako, 2014; Murthy, 2008).

- **Physiological status of the pancreas in Alloxan-diabetic Rats**

In Alloxan diabetic Rats, blood glucose levels ranged from 12.0 - 26.0 mmol/L due to pancreatic β-cells damage caused by Alloxan monohydrate. Alloxan selectively destroys the pancreatic β-cells resulting in absolute lack of insulin (Figure. 1.3). This results in severe reduction in insulin production, secretion, sensitivity and action, with a consequence of failed glucose homeostasis and onset of hyperglycemia (Rohilla, 2012).

However we did not completely destroy insulin secretory pancreatic β cells, instead we used 140 mg/Kg BW Alloxan that partially destroys β-cells (Etuk, 2010) thereby retaining β-cells activities. Advantageously, this dose (140 mg/kg BW) does not cause Liver or kidney damage that might interfere with our research findings (Ashok, 2017).

- **Mechanism of Action of Glibenclamide**

Glibenclamide stimulates insulin secretion from the pancreatic β-cells by inhibiting the efflux of K^+ from pancreatic ß-cells via a sulfonylurea receptor which may be closely linked to an ATP-sensitive K^+-channel (Figure 5.1).

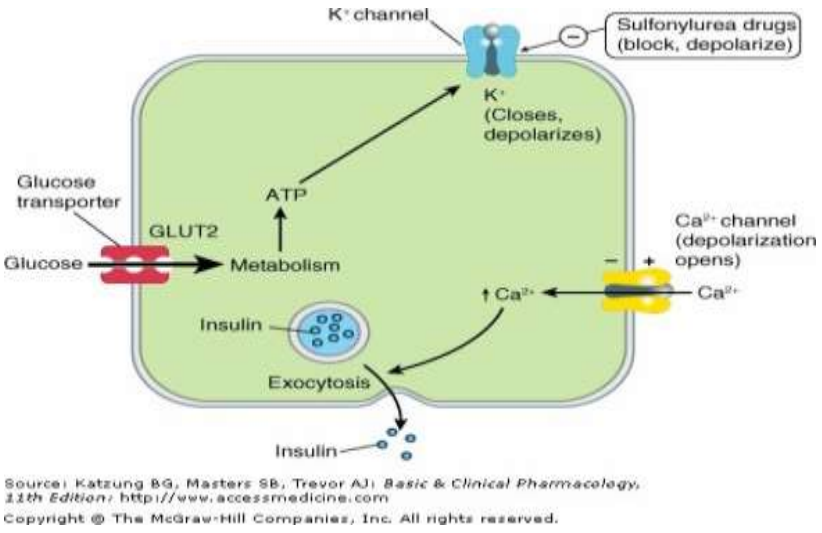

Figure 5.1: Mechanism of action of Glibenclamide

The inhibition of efflux of K^+ leads to depolarization of the ß-cell membrane and, as a consequence, voltage-dependent Ca^{2+} -channels on the ß-cell membrane then open to permit

entry of Ca^{2+}. The resultant increased binding of Ca^{2+} to calmodulin results in activation of kinases associated with endocrine secretory granules thereby promoting the exocytosis of insulin-containing secretory granules (Rang, 2012).

Deducing possible mechanism of Action

In Figure 4.3 percentage decrease of FBG was significantly high in normal rats as compared to Alloxan diabetic rats when Glibenclamide was administered ($p < 0.05$). As earlier mentioned Glibenclamide requires functional pancreatic β-cells to stimulate insulin secretion and exert its physiological action. Hence this agrees with the fact that Glibenclamide is an insulin secretagogue.

In Figure 4.4, 4.5 and 4.6 the percentage decrease of FBG was not significantly different in the normal and Alloxan-diabetic rats when the ethanolic Leaf extracts were administered. Hence, mechanism of action of the leaf extracts seems to differ from that of Glibenclamide. They seem not to depend on functional pancreatic β- cells to cause reduction of blood glucose via insulin secretion. Hence the plants seem to act via extra pancreatic mechanisms in lowering blood glucose. The possible extra pancreatic mechanism of glucose lowering of the leaf extracts could have been;

1. Slowing the absorption of carbohydrates from the gastrointestinal tract (glucose) ingested (especially during the 10 day sub-acute study were hyperglycemia was arrested)
2. Reducing or inhibiting gluconeogenesis and glycogenolysis
3. Increasing insulin sensitivity of body cells and reducing hepatic glucose output
4. Intensifying the effect of intestinal hormones (incretins)involved in the control of blood sugar
5. Mimicking the effects of certain intestinal hormones (incretins) involved in control of blood sugar.
6. Helping in elimination glucose in urine
7. Facilitation of glucose entry into muscle by a non-insulin responsive mechanism. (Mishra, 2014; Bako, 2014; Murthy, 2008).

The leaf extracts have a wide spectrum of phytochemical compounds (e.g. Alkaloids, anthocyanin, Coumarins, flavonoids, triterpenoids, steroids and many more) that contributes to a variety of mechanisms responsible for hypoglycemic activities.

For instance, Phytochemicals such Alkaloids inhibit α-glucosidase. The α-glucosidase is the brush border enzyme of the enterocytes which is responsible for cleavage of single glucose residue from α1, 4-linked oligosaccharide. Hence due to this inhibition there is decreased glucose absorption into blood via the intestinal epithelium (Baynes, 2012).

Flavonoids lowers blood glucose levels by enhancing the insulin release from pancreatic islets and also increasing activity of hepatic glucokinase (Firdous, 2014). Hepatic glucokinase is an enzyme that converts glucose to Glucose-6-phosphate needed for the synthesis of glycogen (Baynes, 2012). Saponins (Triterpenoids, steroidal glycoside) stimulate release of insulin and blocks gluconeogenesis and glycogenolysis (the formation of glucose in the blood stream).

These metabolites are present in *A. Cruentus* (Table 1.3). Dietary fibers effectively adsorb glucose, retards glucose diffusion and inhibit the activity of alpha-amylase and may be responsible for decreasing the rate of glucose absorption and concentration of postprandial serum glucose (Mishra, 2014; Dansi, 2016). Hence, the extracts (AC and CG) due to possession of a wide variety of phytochemical compounds, they are are capable of reducing Blood glucose through a variety of mechanisms (Figure. 5.2). The possible extra-pancreatic mechanism of the ethanolic leaf extracts are summarized in Figure 5.2.

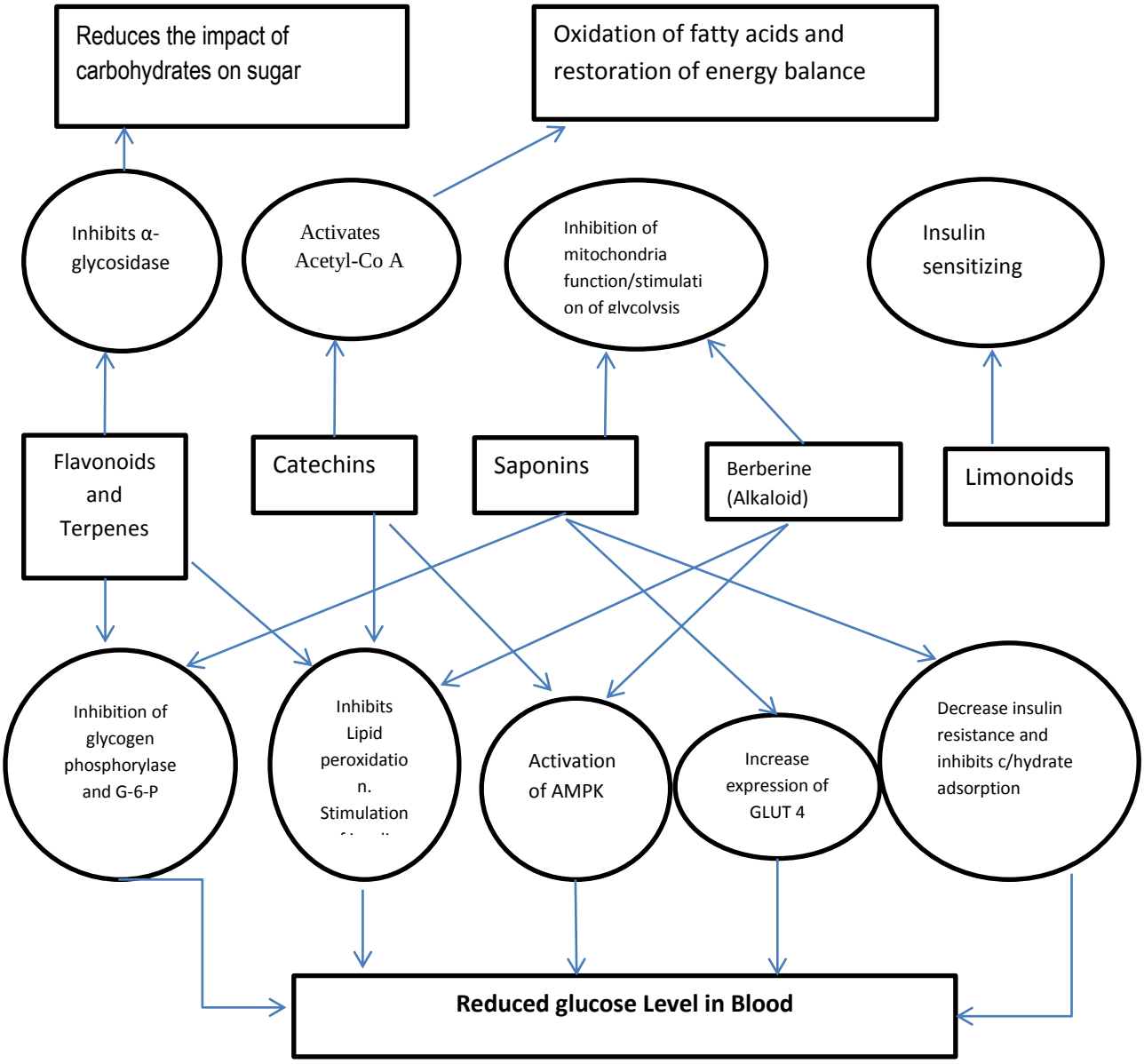

Figure 5.2: Possible active mechanisms of the Bioactive Compounds present in the Medicinal Plants (Source: Mendes, 2015); Glucose 6 phosphate (G-6-P), AMP-activated protein kinase (AMPK)

Sub-acute studies for 10 days in Alloxan-diabetic rats

Sub-acute study was done to strengthen acute study results and the risks of confounders such as stress that could have come about due to repeated hourly collection of blood samples from the animals.

Stress is known to affect blood glucose levels (Guyton, 2014). The Activation of the pituitary-adrenal axis is a prominent neuroendocrine response to stress. Stimulation of this axis results in hypothalamic secretion of corticotrophin-releasing factor (CRF) which stimulates the pituitary to secrete adrenocorticotropic hormone (ACTH). Then ACTH stimulates the adrenal gland to secrete cortisol (Ranbir, 2011). The physiological effect of Cortisol includes increased hepatic glycogenesis, increased gluconeogenesis, increased glucose -6-phosphate and increased plasma glucose levels. Glucorticoids exert an anti-insulin action in peripheral tissues and make diabetes worse (Barret, 2014).

Hence the purpose of Sub-acute study for 10 days was to understand if the glucose lowering effects (observed during Acute study) of the herbs were sustainable over a period of time (10 days). Daily administering of the leaf extracts to the Diabetic rats showed reduction in blood glucose and arresting of hyperglycemia (Table 4.6 and Figure. 4.7). The mechanism for this could probably have been extra-pancreatic mechanism mentioned earlier.

CHAPTER 6: RECOMMENDATION AND CONCLUSION

6.1 Recommendation

A follow up clinical studies with human subject is recommended i.e. diabetic Patients should be administered orally with Ethanolic extracts of *A. cruentus, C.gynandra* and their mixture for a period of time with frequent monitoring of Blood glucose and other Biochemical parameters.

6.2 Conclusion

The results of this research has shown that ethanolic leaf extracts of *A.cruentus, C.gynandra* and their mixtures (at dose 600 mg/kg BW) are able to significantly lower blood glucose levels in both normoglycaemic and hyperglycemic (Alloxan-diabetic) rats.

Unlike Glibenclamide which is an insulin secretagogue, the Leaf extracts seem to act via extra pancreatic mechanisms, however the exact extra-pancreatic mechanism was not established.

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