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Evaluation of hypoglycemic effect of 80% methanol extract and solvent fractions of *Croton macrostachyus leaves* in streptozotocin- induced hyperglycemic mice

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Addis Ababa, Ethiopia

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A thesis submitted to the Addis Ababa University College of health sciences school of pharmacy department of pharmacology and clinical pharmacy in partial fulfillment of the requirements for the Master of Science degree in pharmacology

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School of Graduate Studies

This is to certify that the thesis prepared by Alexander Mersha, entitled "**Evaluation of activity of 80% methanol extract and solvent fractions of *Croton macrostachyus leaves* in streptozotocin- induced hyperglycemic mice**" is original work submitted in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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ABSTRACT

Evaluation of hypoglycemic effect of 80% methanol extract and solvent fractions of *Croton macrostachyus leaves* in streptozotocin- induced hyperglycemic mice

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Diabetes Mellitus (DM) is a metabolic disorder characterized by hyperglycemia, which is usually due to an absence of insulin, impaired effectiveness of insulin action or tissue insensitivity to insulin. It can be classified into type I, type II and gestational DM. Its treatment is based on insulin injection and oral hypoglycemic agents. However, rural parts of worldwide people with DM relay on traditional remedies from plants sources with minimal side effects. This study was conducted to evaluate hypoglycemic effect of 80% methanol extract and solvent fractions of *Croton macrostachyus* leaves in normal, glucose loaded and Streptozotocin (STZ)-induced hyperglycemic mice. 80% methanol extract and different solvent fractions of *Croton macrostachyus* leaves were prepared. Sprague-Dawley mice of either sex were selected for the experiments. In this study mice were grouped into five groups (six mice per group). Hyperglycemia was induced by single intraperitoneal injection of STZ (135mg/kg body weight). Preliminary phytochemical screening was done using standard procedures and acute toxicity study was done as per OECD 425 guidelines. The results were analyzed using one way ANOVA followed by post hoc test at 5% level of significance. 80% methanol and solvent fractions of study plants have blood glucose lowering effect on normoglycemic, glucose loaded and STZ-induced hyperglycemic mice at all doses. However, mice that took chloroform fraction at dose of 100 and 200 mg/kg didn't show any significant reduction in blood glucose level on glucose loaded mice. In addition dose-dependent reductions in blood glucose levels were observed in 80% methanol extract and solvent fractions. Phytochemical screening indicated that alkaloid; flavonoids, tannins, phenols and sapponin were presented in leaves extracts. It was also observed that the extracts didn't have acute toxicity at a single dose of 2 g/kg body weight of mice. So the traditional use of the study plant for the management of DM can be supported as an alternative herbal supplement with further investigation needed to make final conclusion.

Key words: Diabetes mellitus, *Croton macrostachyus*, Streptozotocin, Sprague-Dawley mice normoglycemic, oral glucose tolerance test, hyperglycemic

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LISTS OF ABBREVIATIONS/ACRONOMYS

DM	Diabetes mellitus
GLUT	Glucose transporters
IDF	International diabetic federation
OECD	Organization for economic co-operation and development
OGTT	Oral glucose tolerance test
STZ	Streptozotocin
TIDM	Type I diabetes mellitus
TIIDM	Type II diabetes mellitus
WHO	World health organization

1. INTRODUCTION

1.1 Background

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia, which is usually due to an absence of insulin, impaired effectiveness of insulin action or tissue insensitivity to insulin (Akpaso and Elot, 2017).

It is also an endocrine disorder, which is initially characterized by a loss of glucose homeostasis ensuing from defects in insulin action, insulin secretion which are resulting in impaired metabolism of glucose and other energy-yielding fuels such as lipids and protein and it is a major syndrome which affected near 10% of population all over the world. In spite of the introduction of hypoglycemic agents, related complications continue to be a major medical problem. In the intervening period, variety of plant extracts have been treated orally for patients with non-insulin dependent diabetes mellitus(Choubey et al, 2010).

Diabetic patients, late complications develop alterations and failure of various organs (especially the non-insulin sensitive ones) including the eyes (retinopathy with vision loss), kidneys (nephropathy leading to renal failure), nerves (peripheral and autonomic neuropathy), heart and blood vessels (precocious and severe cardiovascular, cerebrovascular and peripheral vascular atherosclerosis) (Govindappa, 2015).

It is associated with reduced quality of life and increased risk factors for mortality and morbidity. The long-term hyperglycemia is an important factor in the development and progression of micro- and macrovascular complications which include cardiovascular, neuropathy, nephropathy, cerebrovascular diseases (Rao et al, 2010).

DM can be classified into type I and type II, with type I resulting from the body's failure to produce insulin, and requires one to be injected with insulin whereas type II diabetes mellitus is a condition of fasting hyperglycemia that occurs despite the availability of insulin (Asante, 2016).

Table 1: Comparison of type 1 and type 2 diabetes mellitus (Govindappa, 2015)

Feature	Type 1 DM	Type 2 DM
Frequency	10-20%	80-90%
Age at onset	Early (below 35 years)	Late (after 40 years)
Type of onset	Abrupt and severe	Gradual and insidion
Weight	Normal	Obese/non-obese
Family history	<20%	About 60%
Genetic locus	Unknown	Chromosome 6
Pathogenesis	Autoimmune destruction of β-Cells	Insulin resistance, impaired insulin secretion
Islet cell antibodies	Yes	No
Blood insulin level	Decreased insulin	Normal or increased insulin
Islet cell changes	Insulitis, β-cell depletion	No insulitis, later fibrosis of islets.
Clinical management	Insulin and diet	Diet, exercise, oral drugs, insulin
Acute complications	Ketoacidosis	Hyperosmolar coma

1.2. Epidemiology of Diabetes Mellitus

World Health Organization (WHO) reports DM as one of the most common public health problems which will affect a total population of 220 million worldwide in the year 2020. The increasing prevalence of DM worldwide is a major societal issue because diabetes is a complex and multifactorial origin disease. The prevalence of DM is rising all over the world due to population growth, aging, urbanization and an increase of obesity and physical inactivity (Suresh et al., 2012).

WHO in its 2016 global report on DM estimates that, globally, 422 million adults aged over 18 years were living with diabetes in 2014. In African, according to WHO diabetes program on country and regional data on DM 2017, estimated that there will be 18.2 million diabetic patients by 2030 out of which around 4.84 million coming from Nigeria alone. This makes Nigeria the most vulnerable for diabetes in African region (Ejiofo et al, 2017).

It is one of the chronic non communicable diseases (CNCDs) which have emerged as a leading global health problem. It is estimated that developing countries will bear 77% of the global burden of the DM epidemic in the 21st century as a result of population growth, consumption of unhealthy diets, obesity, and sedentary lifestyles. Ethiopia as one of the developing countries which has been showing changes that shifts the lifestyle of the people towards urbanization, particularly in recent decades, 21st century. These rapid changes have led to the emergence of non-communicable

chronic diseases such as diabetes mellitus. According to the international diabetes federation (IDF) atlas guideline report in 2017, there are 352 million adults with impaired glucose tolerance who are at high risk of developing DM in the future. In 2017, it was estimated that 425 million people (20–79 years of age) suffered from DM, and the number is expected to rise to 629 million by 2045. Moreover, in 2017, the projected national diabetes prevalence in Ethiopia estimated by IDF Atlas was 5.2% (Aynalem and Zeleke, 2018).

1.3 Classification of Diabetes Mellitus

1.3.1 Type I diabetes mellitus (TIDM)

TIDM (previously known as insulin-dependent, or childhood-onset diabetes) juvenile is characterized by deficient insulin production in the body with symptoms such as excessive urination and thirst constant hunger, weight loss, vision changes and fatigue. People with TIDM require daily administration of insulin to regulate the amount of glucose in their blood. It's cause is not known and it is not preventable (WHO, 2016).

It accounts for only 5–10% of those with DM and results from a cellular-mediated autoimmune destruction of the b-cells of the pancreas with the presence of markers of the immune destruction of the b-cell in 85–90% of individuals when fasting hyperglycemia is initially detected. It commonly occurs in childhood and adolescence, but it can also occur at any age, even in the 8^{th} and 9^{th} decades of life (American Diabetes, 2012)

1.3.2 Type II diabetes mellitus (TIIDM)

TIIDM (formerly called non-insulin-dependent or adult onset diabetes) results from the body's ineffective use of insulin. It accounts for the vast majority of people with DM around the world which accounts for 90–95% of those with diabetes. Most patients with TIIDM are obese which cause insulin resistance (American Diabetes, 2012).

Symptoms may be similar to those of TIDM, but are often less marked or absent. As a result, the disease may go undiagnosed for several years, until complications have already arisen. For many years, TIIDM was seen only in adults but it has begun to occur in children (WHO, 2016).

1.3.3 Gestational diabetes (GDM)

Gestational diabetes (GDM) is a temporary condition that occurs in pregnancy and carries long term risk of TIIDM. The condition is present when blood glucose values are above normal but still

below those diagnostic of diabetes. Women with gestational diabetes are at increased risk of some complications during pregnancy and delivery. GDM is diagnosed through prenatal screening, rather than reported symptoms(WHO, 2016).

1.3.4 Other specific types of diabetes mellitus

Specific types of diabetes due to other causes, e.g., monogenic diabetes syndromes (such as neonatal diabetes and maturity-onset diabetes of the young [MODY]), diseases of the exocrine pancreas (such as cystic fibrosis and pancreatitis), and drug- or chemical-induced diabetes (such as with glucocorticoid use, in the treatment of HIV/AIDS, or after organ transplantation) (ADA, 2018).

1.4. Pathophysiology of Diabetes Mellitus

Several pathogenic processes are involved in the development of DM such as autoimmune destruction of insulin-producing β-cells in the pancreas with consequent insulin deficiency (TIDM), and others that result in resistance to insulin action or both (TIIDM). The abnormalities of carbohydrate, fat and protein metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin. Insulin resistance and impaired beta cell function, both contribute to gestational diabetes (GDM) (Arika et al, 2016). The major contributors are the placental hormones namely, human placental lactogen, progesterone, cortisol, growth hormone and prolactin. These hormones cause decreased phosphorylation of insulin receptor substrate and thus profound insulin resistance. Cytokines like tissue necrosis factor have also been implicated in pathogenesis of insulin resistance (Anees et al, 2013).

1.4.1 Insulin resistance

The primary events are believed to be an initial deficit in insulin secretion and in many DM patients relative insulin deficiency in association with peripheral insulin resistance. Resistance to the action of insulin will result in impaired insulin mediated glucose uptake in the periphery (by muscle and fat), incomplete suppression of hepatic glucose output and impaired triglyceride uptake by fat. To overcome the insulin resistance, islet cells will increase the amount of insulin secreted. Endogenous glucose production is accelerated in patients with TIIDM or impaired fasting glucose. Because this increase occurs in the presence of hyper insulinemia, at least in the early and intermediate disease stages, hepatic insulin resistance is the driving force of hyperglycemia of TIIDM (Baynest, 2015)

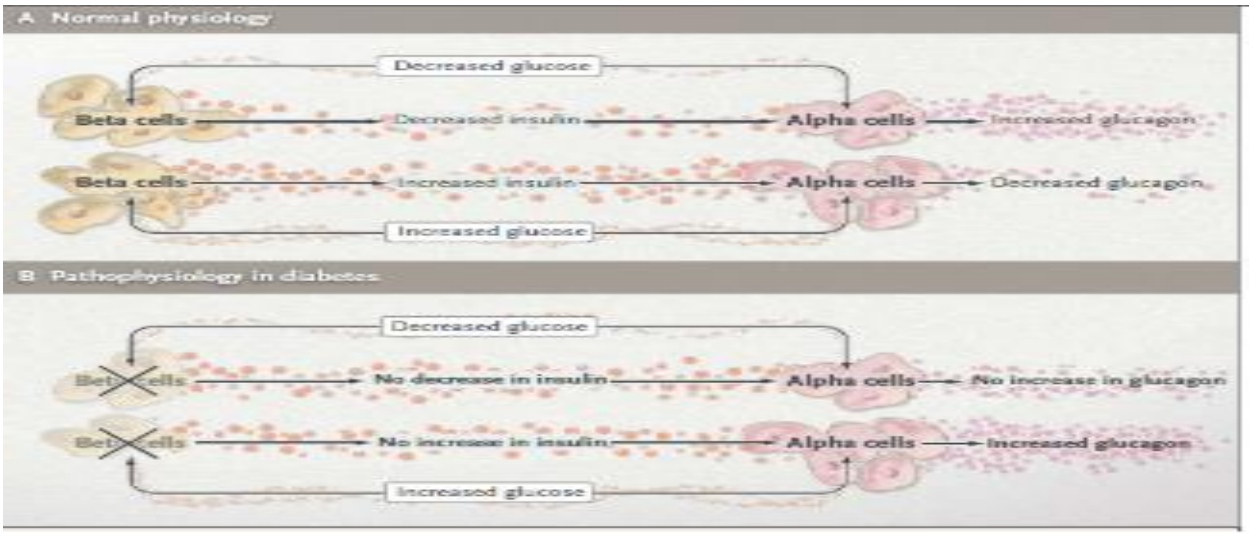

Figure 1: Panel A shows the physiological effect of a decrease in insulin coupled with a low glucose concentration in stimulating alpha-cell glucagon secretion, and Panel B shows the pathophysiological effect of beta-cell failure and the resulting loss of a decrease in insulin secretion and loss of an increase in alpha-cell glucagon secretion, despite a low glucose concentration (Baynest, 2015).

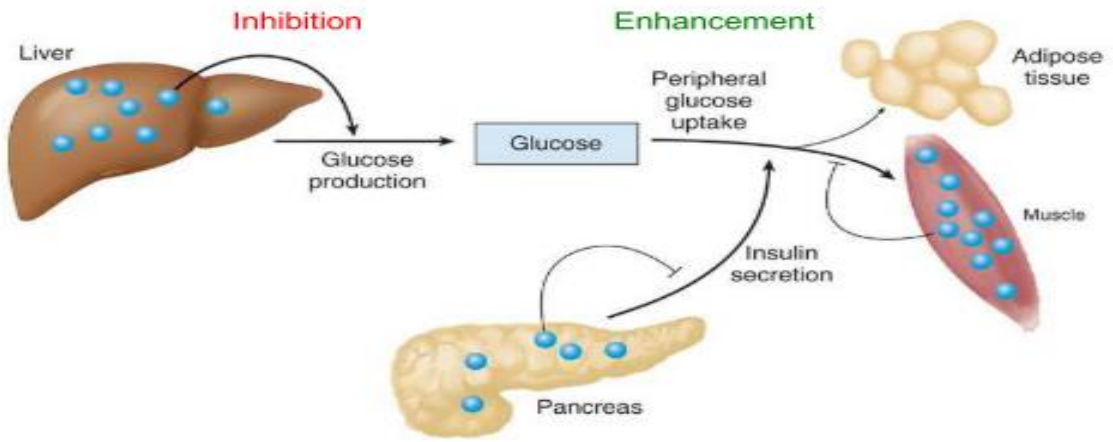

Fig 2. Effects of insulin on blood glucose level (liver, muscle, fat). Metabolic effects of insulin - inhibition of glycogenolysis and gluconeogenesis (liver), enhance peripheral glucose uptake and utilization (muscle, fat) and restain lipolysis and proteolysis (fat, muscle). It also have mitogenic effect (Hosszúfalusi, 2017).

1.5 Clinical Features and Diagnosis of Diabetes Mellitus

Some of the symptoms include weight loss, polyurea, polydipsia, fatigue, cramps, polyyphagia, constipation blurred vision, and candidiasis. Long lasting TIDM patients may be susceptible to microvascular complications; and macrovascular diseases (coronary artery, heart, and peripheral

vascular diseases. Most TIIDM cases are diagnosed because of complications or incidentally. Most patients with TIIDM die from cardiovascular complications and end stage renal disease(Baynest, 2015).

Table 2:Diagnostic criteria of DM (WHO 1998, ADA 2015) adopted from(Hosszúfalusi, 2017)

Diagnosis	Fasting blood glucose (mg/dl, mmol/l)	OGTT 120' (mg/dl, mmol/l)
Normal carbohydrate tolerance	≤ 110, ≤ 6.0 (ADA < 100, 5.6) (HbA1c < 5.7%)	< 140, < 7.8
Impaired fasting glucose (IFG) Prediabetes HbA1c: 5.7-6.4 %	≤ 110 and ≤ 125 6.1-6,9 ➢(ADA 100-125)	< 140, < 7.8
Impaired glucose tolerance (IGT)	< 126 , < 7.0	≥ 140 and < 200 7.8-11.0
Diabetes mellitus HbA1c ≥ 6.5%	≥ 126, ≥ 7.0	≥ 200, ≥ 11.1

1.6 Treatment of Diabetes Mellitus

Pharmacological treatment of DM is based on insulin injection and oral hypoglycemic agents such as metformin, Sulfonylureas, meglitinides (repaglinide and nateglinide), thiazolidinedione's (rosiglitazone and pioglitazone), α-Glucosidase inhibitors etc. However, these have so many side effects, coupled with its high cost which is not affordable in poor economic communities. Consequently, now a days rural parts of worldwide people with DM relay on traditional remedies from plant sources with minimal side effects (Anees et al, 2013).

1.7. Use of Traditional plants

1.7.1 Role of plant extracts for the management of DM

The WHO estimates that around 80% of the world population in developing countries relies on traditional plant medicines for primary healthcare needs, of which a major proportion corresponds to plant extracts or their active principles(Antonio et al, 2007).

Ethnobotanical information indicates that more than 800 plants are used as traditional remedies for the treatment of DM due to their effectiveness, less side effects and low cost. Plant extracts decrease or increase or stimulates the number of reactions to reduce or minimize the risks of the DM in animal experiments through different mechanisms such as regeneration of β-cells of pancreas, initiation of receptor and ligand interactions in productions of insulin, activation of signal transduction for production of insulin and reduction of blood glucose level, initiation of number of liver enzymes for conversion of sugar into various products or limiting the production of byproducts etc. Some of the extracts have insulin like activity or induce the activity of insulin and other extracts may inhibit the activity of enzymes like α-amylase, α-glucosidase (Gavindappa, 2015).

Traditional herbal medicines plays important role in the management of DM. Now a days, there is growing interest in herbal remedies due to the side effects associated with the oral hypoglycemic agents. In recent years, herbal medicines have started to gain importance as a source of hypoglycemic agents. Plant products are rich in phenolic compounds, flavonoids, terpenoids and other constituents which can cause reduction in blood glucose levels (Rao et al, 2010).

1.7.2. Experimental plant (*Croton macrostachyus)*

Croton macrostachyus is a species of the genus Croton L, Euphorbiaceae family, commonly known as the spurge family. It is a medium sized, drought-deciduous pioneer tree which regenerates naturally in less productive sites including forest edges, mountain slopes, and waste grounds under a wide range of ecological conditions. It is regarded as a multipurpose tree by subsistence farmers in Ethiopia, Kenya, and Tanzania, as it is often grown and managed in home gardens for provision of several ecosystem goods and services. In Ethiopia, it is a major tree intercropped in agroecosystems in order to increase soil productivity in mid altitude and semiarid areas. There is also tremendous interest in the medicinal uses and pharmacological properties of *C. macrostachyus* throughout its distributional range in tropical Africa(Maroyi, 2017).

The genus comprising around 1,300 species of trees, shrubs and herbs distributed in tropical and subtropical regions of both hemispheres. It is rich in constituents with biological activities, chiefly diterpenoids such as phorbol esters, clerodane, labdane, kaurane, trachylobane, pimarane etc. Euphorbiaceae, Croton species may contain latex, which is red-colored in some species, a characteristic usually associated with medicinal properties(Antonio et al., 2007).

In south America its leaves and stem bark is used as a medicinal plant in the treatment of DM, high blood cholesterol levels and gastrointestinal disturbances , treatment of inflammatory diseases, leukemia, ulcer and rheumatism as well as hepatic disturbances and weight loss. In north America it is widely used in traditional medicine, including internal use for cough, flu, diarrhea and stomach ulcers, and topically as wound healing for cuts, open sores, herpes, anti-septic after tooth extraction and for oral sores(Antonio et al., 2007)

In Asia it is frequently used in folk medicine; leaves are used as tonic, the flowers against flat worms, the fruits to treat dysmenorrhea, the seeds as purgative to treat dysentery, the bark to treat dyspepsia and the roots and bark is also used to treat chronic enlargement of the liver and remittent fever. It is applied externally to the hepatic region in chronic hepatitis. In India as anti-helminthic and to treat dermatological problems(Antonio et al., 2007).

Its roots are used in Tanzania as antidiabetic and the seeds are widely used in Somalia as purgative. In tropical west and central Africa and it is used to treat fever, dysentery and convulsions. The leaf decoction is used in Benin as anti-hypertensive, anti-microbial (urinary infections) and to treat malaria linked fever(Antonio et al., 2007)

In Ethiopia, ethinopharmacological study have shown that it is used as a traditional medicine in the management of DM (Meresa et al., 2017, Giday et al., 2007) antimicrobial(Gobaw, 2016, Sendeku et al., 2015), antimalarial (Bantie et al., 2014), anti-leishmanial activity(Gelaw et al., 2012).

Figure 3: leaves of *Croton macrostachyus* collected from around Bihardar (Bura), Feb.2018

1.7.2.1 Chemistry of Croton macrostachyus

Croton chemistry is considerably diverse. Terpenoids are the predominant secondary metabolite constituents in the genus, other like diterpenoids and triterpenoids, either pentacyclic or steroidal, have frequently been reported for Croton species. Volatile oils also found in croton species. Several species have been reported as sources of different classes of alkaloids, a fact that enhances considerably the importance of the genus from the medicinal point of view. Phenolic substances have been also frequently reported (Antonio et al, 2007).

1.8. Significance of the study

Despite the introduction of new oral hypoglycemic drugs, many researches have been done on the use of traditional plant in the management of DM. In addition to this, inabilities of the patient to afford the cost of drugs have created a growing public interest in the use of traditional medicines. A lot of plants have been studied for their antidiabetic effect, little is known about the antidiabetic effect of *Croton macrostachyus* in our country.

The traditional claim of the use of *Croton macrostachyus* leaves in treatment of DM has been pharmacologically validated as possessing the potential to lower blood glucose level in experimental diabetic animal models(Arika et al, 2015).

Most studies done on identification of the effect of this medicinal plant through crude extraction. So further investigation of the plant with solvent fractionations is important to identify possible lead compounds for drug development against tested disease (Tesfaye et al, 2016).

Results from evaluating activity of this plant against tested diseases, DM, will provide an avenue for further studies to validate the use of the plant against the disease. Finally, results of the current work will also save as a template for further research on the use of this experimental plant.

1.9. Statement of Problem

Diabetes mellitus is one of the common metabolic disorders with micro-and macrovascular complications that results in significant morbidity and mortality. It is considered as one of the five leading causes of death in the world. In modern medicine no satisfactory effective therapy is still available to cure DM. There is increasing demand by patients to use natural products with antidiabetic activity due to side effects associated with the use of insulin and oral hypoglycemic agents (Ahmed et al., 2010).

DM virtually affects every system of the body mainly due to metabolic disturbances caused by hyperglycemia, especially if diabetes control over a period of time proves to be suboptimal. It is associated with a wide range of circulatory manifestations such as alterations in endothelial function and cardiovascular disease. Most of the complications in DM are due to hyperglycemia and increased generation of oxygen derived free radicals, which may lead to vascular dysfunction (Alemu, 2015).

DM and its associated health problems are the main global heath burdens that challenge mankind globally despite discovery and utilization of hypoglycemic drugs to manage the case. Besides affecting the quality of life, the patients do not perform their daily usual business as they visit heath facilities so many times. This has decreased their income directly and created high economic loss of the country indirectly as it impedes the development of nation at large. It is also expected to happen to be one of the major disablers and killers worldwide within the next 25 years (Meresa et al, 2017).

According to the IDF 2015 estimate, the world wide prevalence is 1 in 11 adults; more than 415 million of people have DM which will rise to 642 million in 2040. Among these Africa account a 14.2 million people with diabetes which is likely to increase to 34.2 million in 2035 and death of 5.0 million from diabetes in 2015. In Ethiopia, the number of people aged 20-79 years living with DM was estimated to be 1.3 million adults with diabetes, and the prevalence was 2.9% which is projected to reach 1.8 million by 2030 (Meresa et al, 2017)

Low adherence to prescribed diabetes medications accounts for 30% to 50% of treatment failures, leading to worse treatment outcomes and which cause damages to vital organs. Treatment failure is in turn associated with reduced treatment benefits and can have a negative financial burden on both individual patients and the society at large. Strict follow-up of prescription of medical treatment by the patient becomes a challenge for the health care deliverer and scientific community. Hence a number of patients do not benefit from medical treatment. This brings a profound negative effect on their quality of life and social impacts in many aspects. In Ethiopia, the anti-diabetic medication adherence is found to be 68.8 to 85 due to poor economic status, low education level and inadequate knowledge about the disease and its medication(Asfaw Meresa et al., 2017). So now a days the use of traditional medicinal plants become more interesting research area to minimize financial burden on diabetic patients due to low adherence and increased cost of currently available antidiabetic medications.

According WHO, in Sub-Saharan Africa, the prevalence diabetes cases in 2013 ranges from 4.5 to 5.0%. This could increase by 98% which is from 12.1 million in 2010 to about 23.1 million in 2030. The impaired glucose tolerance that was reported in 2010 (26.9 million) is also expected to rise to 47.3 million in 2030. It is emerging as one of the major chronic health problems in Ethiopia, although its incidence and prevalence are still unknown in the general population. In Ethiopia,

national data on prevalence and incidence of DM are lacking. However, patient attendance rates and medical admissions in hospitals are rising (Tesfaye et al., 2016).

Ethiopia is the second most populous country in sub-Saharan Africa where more than 80% of the population lives in the country side. The country experiences a heavy burden of disease mainly attributed to communicable infectious diseases and nutritional deficiencies. Currently, Ethiopia is also challenged by the growing magnitude of chronic non communicable diseases. DM is emerging as one of the major chronic health problems in Ethiopia, although its incidence and prevalence are still unknown in the general population (Alemu, 2015).

In addition IDFA reported Ethiopia to be ranked 3rd among the ten top countries in Africa with 1.4 million DM cases and estimated prevalence of 3.32% by year 2012.WHO estimated in 2011 that 34% of Ethiopian population is dying from non-communicable diseases, with a national DM prevalence of 2%. Despite the above estimations for global prevalence of DM, there is no well-documented data in Ethiopia. In addition, accurate information on the prevalence of major public-health importance such as DM is required to have informed health policy decision (Alemu, 2015).

Traditional medicines play a pivotal role among rural communities of developing countries for the provision of health care. Unfortunately, clear documentation of these medicinal plants and traditional remedies is still lacking. Worldwide, several plants used traditionally for the management of diabetes have been studied for their hypoglycemic activity with positive results further strengthening the argument that medicinal plants could play a role in discovery of novel compounds in the management of several diseases including diabetes mellitus. Despite the high reliance on medicinal plants for different therapeutic benefits, little has been done to document herbal medicines. There is consequently an urgent need to document and scientifically prove such plants to provide reference materials for prospective researchers, traditional health practioners as well as the indigenous people, the global community with interest in herbal medicines and the generations to come (Comfort et al, 2015).

Self-care practice using medicinal plants comprises one of the common forms of traditional medicine practices in Ethiopia. Most of the studies reported from Africa, including the one from Ethiopia, focused on the clinical aspects of specific medicinal plants used, but little attention was given to the patient perceptions with regard to their experiences in using (or not using) these medicinal plants. In addition qualitative study were done to explore an in-depth manner the

experiences with medicinal plants of patients with diabetes. Findings from studies done by Bruck et al, 2017 highlight the use of medicinal plants by patients with diabetes in the context of limited information which have suggested the need for the healthcare practitioners in the conventional healthcare system to give more attention to patients' interest in medicinal plants and for providing more evidence-based information about the plants used by these patients so as to improve health outcomes (Bruck et al, 2017). So the present study gives a scientific insight about the use of this traditional plant such as *Croton macrostachyus* leaves using experimental animal models on its role in the management of diabetic mellitus.

Despite the use of medicinal plants in the management of DM, there are few studies on ethnobotanical and ethno pharmacological antidiabetic plants published in the literature. This study aims at scientifically proving traditional medicinal plants particularly Croton macrostachyus which is used for the management of DM in Ethiopia.

2. OBEJECTIVES OF THE STUDY

2.1. General objectives

- To evaluate hypoglycemic effect of 80% methanol extract and solvent fractions of *Croton macrostachyus* leaves in normal, glucose loaded and Streptozotocin-induced hyperglycemic mice.

2.1. Specific objectives

- To determine hypoglycemic effect of 80% methanol extract of *Croton macrostachyus* leaves on blood glucose level in normal, glucose loaded and Streptozotocin-induced hyperglycemic mice
- To determine hypoglycemic effect of each solvent fractions of *Croton macrostachyus* leaves on blood glucose level in in normal, glucose loaded and Streptozotocin-induced hyperglycemic mice
- To evaluate oral acute toxicity of 80% methanol extract *Croton macrostachyus* leave in normal mice
- To evaluate qualitative phytochemical constituents of *Croton macrostachyus* leaves

3. MATERIALS AND METHODS

3.1 Materials

3.1.1. Collection and preparation of plant material

3.1.1.1 Collection of plant material

The plant used in this study was collected around Bihar Dar in February 2018. Identification & authentication of the plant specimens was done by a taxonomist at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University, with voucher specimen (number A001) and deposited for future reference. For this study, the parts of the plants collected were the leaves and the leaves were washed using tap water, dried at room temperature under shade and then pulverized to coarse powder using mortar and pestle. Leaves were collected while green and dried at room temperature away from direct sunlight for different periods of time depending on their succulence and transported after packed with aluminum foil to prevent external contamination. The powdered plant materials were kept at room temperature away from direct sunlight in closed, dry plastic air tight bags ready for extraction.

3.1.1.2. Preparation of extract and fractions of plant material

A. Crude extract

After the leaves were air dried at room temperature under shade and reduced to appropriate size by grinding mill, a total of 950 g powder was used for extraction. The dried leaves was extracted by maceration using 80% methanol (1:5 ratio) for 72 hrs in an Erlenmeyer conical flask with frequent agitation using an orbital shaker adjusting at 120 rpm. The mixture was first filtered using gauze and then with Whatsman filter paper No. 1 using pressurized suction filtration system and the marc was re-macerated twice using the same volume of methanol to exhaustively extract the plant material. Then filtrates from each extraction were combined and methanol was removed from the extract by evaporation under vacuum using rotary evaporator at 40°C.Then the combined filtrates were frozen overnight using deep freezer and water were removed using Lyophilizer and kept in refrigerator until use and fresh stock solution was prepared whenever required for the experiment.

B. Solvent fractionation

The crude extract was subjected to successive extraction using solvents with differing polarity (chloroform, methanol and distilled water). The Chloroform was first added to the flask, and the set up was heated under reflux based on boiling point of the solvent . When a certain level of condensed solvent accumulated in the thimble, it was siphoned into the flask beneath and continued for 3 days to get the chloroform fraction. The residue left in the thimble was next extracted using butanol following the same procedure as described above to get the methanol fraction. The chloroform fraction was air dried at room temperature, while the butanol fraction was removed by using rotary evaporator. The residue left inside the thimble from the two solvent fractions was then macerated in Erlenmeyer flask using distilled water to get the aqueous fraction. The aqueous fraction then concentrated in deep freezer and dried using a lyophilizer. The dried fractions from each solvent were then transferred into separate vials and stored at appropriate temperature until use.

C. Percent yield of plant extracts and fractions

A total yield of 11.87 %(112.77gm) dried crude extract was obtained from 80% methanol extract preparation from the dried leave of *Croton macrostachyus* (950gm). In the case of solvent fractionation of distilled water, chloroform and butanol fraction a total yield of 11.57% (11.27gm), 9.59 %(9.34gm) and 8.02% (7.81gm) of dried powder respectively was obtained from 97.38 gm of crude extract.

3.1.2. Drugs and chemicals

Chemical and reagent that were used in this study includes streptozotocin (sigma Aldrich,USA), glibenclamide (Epharm), 5% glucose (AAU School Pharmacy Department of Pharmacology), distilled water (AAU School of Pharmacy Department of Pharmaceutics), butanol, chloroform and methanol (ZAF pharmaceuticals Plc.), hydrochloric acid and ferric chloride (BDH Laboratory Supplies Poole, England), acetic anhydride and Mayer's reagent (May and Baker LTD Dagenham, England), and sulfuric acid (Fisher Scientific,UK), sodium hydroxide (BDH, chemical lab, England),. All the chemicals were analytical grade.

3.2. Methods

3.2.1. Acute toxicity test

Ten female Sprague-Dawley mice were randomly divided into 2 groups (treatment and control) of 5 mice per group. After being fasted for 2 h, mice in the first group were given 2 g/kg and the second group distilled water orally and mice were observed for any signs of toxicity daily for 14 days to assess safety of the extract. Animals were observed for gross changes such as loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, diarrhea, mortality and other signs of overt toxicity (OECD, 2008).

3.2.2. Induction of hyperglycemia

Hyperglycemia was induced experimentally by a single intraperitoneal administration of streptozotocin (135mg/Kg) (Gebeyehu, 2015) . Seventy two hours after Streptozotocin (STZ) administration, blood samples were taken from tail of mice and measured using a glucometer. Mice with blood glucose levels above 200 mg/dL were considered as diabetic and used in this study. Prior to initiation of this experiment, the animals were fasted for 8-12 hours but allowed free access to water until the end of this experiment (Arika et al, 2015). Besides, to prevent hypoglycemic shock and mortalities during hypoglycemic phase, oral solution of 5% glucose in tap water was provided via water bottle for next 24 hrs (Gidado *et al*., 2005).

3.2.3. Phytochemical screening

Preliminary phytochemical qualitative analysis were performed on 80% methanol extract using standard phytochemical reagents for the presence or absence of secondary metabolites such as alkaloids, steroidal compounds, phenolic compounds, flavonoids, saponins, and tannins following standard procedures (Zohra, 2015, Tensay G. Kiristos et al, 2018, Visweswar, 2013).

A. Test for Flavonoids

0.5g of extract was added into different test tubes. Then, 4 drops of 10% NaOH solution were added and they were heated in water bath for 10 min. The intensity of yellow color which became colorless on addition of 10 drops of 1% hydrochloric acid showed the presence of flavonoids.

B. Test for Alkaloids

1.5 ml of 1% hydrochloric acid was added to 1.2g ml of extracts in test tubes. The mixture was heated for 2 min in a water bath while stirring continuously. It was then cooled and filtered. Then 1 ml of the filtrate was added to 0.4 ml of Mayer's reagent. Formation of cream yellow precipitate indicated the presence of alkaloids.

C. Test for Tannins

1 g of each powdered samples was separately added into 20 ml distilled water test tubes. Then, the mixtures were boiled in a water bath for 7 min and were filtered while hot into Erlenmeyer flask. After cooling, 1 ml of the filtrates was diluted to 5 ml solution with distilled water and then a few drops of 10% ferric chloride were added to it. Formation of blue-black or brownish-green precipitate indicated that the presence of tannins.

D. Test for Saponin (Foam test)

1 g of each powdered sample was placed into test tubes and mixed with 10 ml distilled water. Then, the mixtures were boiled in a water bath for 10 min and were filtered while hot into Erlenmeyer flask. 2.5 ml of filtrate was added to test tube and diluted to 10 ml with distilled water. It was shaken vigorously for 2 min. Formation of froth confirmed the presence of saponin in the filtrate.

E. Test for Phenols

About 0.25 g of extract was treated with few drops of 5% neutral ferric chloride solution; the appearance of a greenish color indicated the presence of phenols.

F. Test for Terpenoids (Salkowski Test)

5 ml of each concentrated extracts was mixed with 2 ml of chloroform in test tubes, and then 2 ml of concentrated sulfuric acid was added carefully and shaken gently to form a layer. A reddish-brown coloration of the interphase confirmed positive results for the presence of terpenoids.

G. Test for Steroids (Lieberman-Bur chard's Test)

2 ml chloroform and 10 drops of acetic acid were placed in test tube; 0.5 extracts was added to the test tube and mixed with the solvents. Then, 1.5 ml of concentrated sulfuric acid was added from

the side of test tube. The change of red color through blue to green indicated the presence of steroids.

3.2.3. Grouping and dosing of experimental animals

3.2.3.1. Experimental animals

Healthy Sprague-Dawley mice of either sex, weighing 20–30 g and aged 6–8 weeks were used for the experiment. The animals were obtained from Addis Ababa University, school of Pharmacy, animal house of Department of Pharmacology and Clinical Pharmacy and were kept in plastic cages at room temperature and on a 12 h light–dark cycle with free access to pellet food and water ad libitum.The animals were acclimatized to laboratory condition for one week prior to the experiments. All studies were conducted in accordance with the guideline for the care and use of laboratory animals (OECD, 2008).

For evaluating the 80% methanol extract, mice were randomly divided into five groups of six mice per group. Group I-III were treated with the extract at different dose (100,200 and 400 mg/kg body weight). Dose selection was based on acute toxicity study. The remaining two groups served as negative and positive controls and were given distilled water (2 ml/100 g body weight) and glibenclamide (5mg/kg) respectively.

The studies on the fractions were also conducted with the same as 80% methanol extract procedure described above for each fraction. Mice were randomly assign into three treatment groups and two controls, six mice per group for each fraction. Negative and positive controls were given distilled water 2ml/100gm of body weight and glibenclamide 5mg/kg respectively for each fraction. Treatment groups were given the fractions at different dose as the same as 80% methanol extract.

3.2.3.2. Normoglycemic Test

Mice were divided into five groups (three treatment groups and two control groups), each group with six mice. The mice were fasted overnight prior to conducting the experiment. The positive control group received glibenclamide (5mg/kg), while the negative control group received distilled water (2ml/100gm body weight), and the test groups were administered 80%methanol extract and each fractions of *C.macrostachyus* (100,200 and 400 mg/kg) orally by using gavage. The effects of the plant extract and fractions were compared with the control groups. Blood sample from the control and test animals were collected after at 0, 1, 2, 3 and 4 hrs following glibenclamide, extracts

and vehicle administration. Blood glucose levels were measured using glucometer on blood drawn from the tail of the mice.

3.2.3.3. Oral Glucose Tolerance Test (OGTT)

Oral glucose tolerance test (OGTT) was performed as per the procedure previously described (Lanjhiyana, 2011). Fasted mice were grouped into five groups of six mice each. Then group I received distilled water 2ml/100g body weight of glucose (negative control); Group II received glibenclamide, 5mg/kg and served as positive control. Group I-III received 80% methanol extracts and fraction at doses of 100mg/kg, 200mg/kg and 400mg/kg of body weight respectively using oral gavage. Following 30 min post extract and fraction administration all the animals were fed with glucose 2 g/kg body weight. Blood samples were collected from tail vein prior to dosing and 30, 60, 90 and 120 minutes after administration of glucose in order to evaluate their blood glucose level. The blood glucose level was measured using glucometer.

3.2.3.4. STZ-induced hyperglycemic test

STZ induced diabetic mice were administered orally 100mg/kg, 200mg/kg and 400mg/kg for test groups; 5mg/kg glibenclamide for positive control group; and distilled water (2ml/100g body weight) for negative control group once daily for 21 days. Blood glucose level was measured using glucometer blood drawn from the tail of the mice. The animal fasting glucose levels were measured on day 0, 7, 14 and 21. Percentage of glucose reduction of blood glucose level was calculated using the following formula: (G0 − G21)*100/ G0, Where G0 is blood glucose level at day 0; G21 is blood glucose level at day 21.

3.2.4. Ethical consideration

The animals was maintain and cared according to the international guidelines for the use and maintenance of experimental animal (Council, 2010). Formal letter was written by School of Pharmacy Department of Pharmacology and Clinical Pharmacy to purchase necessary laboratory reagents.

3.2.5. Data analysis

Results of the study were expressed as mean +/- standard error of mean (SEM). Comparison and statistical significance were determined by one way ANOVA using statistical package for social science (SPSS), windows version 23 followed by Tuckey Post-hoc test. The analysis were performed with 95% confidence interval and the significance level at $p<0.05$.

4. RESULTS

4.1 Acute Toxicity Study

The acute toxicity test was conducted as per the Organization for Economic Co-operation and Development (OECD) guidelines 425. The result revealed that 80% methanol extracts of C*roton macrostachyus* did not produce any morbidity throughout the study period of 14 days at dose of 2g/kg body weight. There were no any behavioral, neurological, autonomic or physical changes such as alertness, motor activity, restlessness, convulsions, coma, diarrhea, lacrimation and appearance of the animals. In addition the extract did not cause mortality in the animals at a dose of 2 mg/kg during the observation time which indicates that the LD50 could be greater than 2g/kg per body weight in mice.

4.2 Hypoglycemic effect of the 80% methanol extract of *Croton macrostachyus* leaves on blood glucose level

4.2.1. Hypoglycemic effect of 80% methanol leaves extract on normoglycemic mice

Hypoglycemic effect 80% methanol leaves extract treatment on the blood glucose level in normal fasted mice was shown in Table 3 below. Orally administered 80% methanol leaves extract at all doses did not significantly reduce the plasma glucose in normal mice one hour after treatment, however, mice treated with glibenclamide (5mg/kg body weight) showed a significant reduction in blood glucose levels when compared with untreated group. The lowest dose taken i.e. 100mg/kg body weight of 80% methanol extract suppressed blood glucose by 12.25 % ($p<0.05$) after 4 hrs of extract administrations, while the dose of 200mg/kg and 400mg/kg body weight was observed to exert glucose lowering effect after 4 hrs of extract administration by 24.16% ($p<0.001$) and 32.67%($p<0.001$) respectively. However, normoglycemic mice treated with standard drug i.e. glibenclamide 5mg/kg body weight suppressed blood glucose level by 57.24 %($p<0.001$) after 4hrs.of administration. In addition, analysis of samples using linear regression ($R^2=0.98$) have shown that dose dependent effect was observed with mice treated with 80% methanol leaves extract.

Table 3: Hypoglycemic effect of 80% methanol *extract* of *Croton macrostachyus leaves* on blood glucose level in normoglycemic mice

Group	**Treatment (dose in mg/kg body weight)**	**Blood glucose level (BGL) at time T. after treatment (mg/dl)**					**%GR**	**R^2**
		0 hrs.	1hrs.	2hrs	3hrs	4hrs		
I	DW (2ml/kg)	116.5±1.54	115.17±1.4	114.83±1.57	114.67±1.7	115.83±1.14		0.98
II	GL 5	114.83±1.58	106.83±2.45*	91.83±2.2***	80.5±1.61***	49.1±1.94***	57.24	
III	ME 100	114.33±1.73	111.17±2.15	106.17±2.5	102.17±2.2*	100.33±2.9*	12.25	
IV	ME 200	113.83±2.2	109±2.4	100.5±3.09*	92±4.2***	86.33±4.75***	24.16	
V	ME400	116.33±2.09	109±2.7	97.67±2.4***	87.17±2.3***	78.33±2.32***	32.67	

Key: DW: distilled water, GL: glibenclamide, ME: Methanol extract, percentage of glucose reduction.

Data are expressed as Mean ± Standard Error of Mean (SEM); n=6 * Significant values at P<0.05 compared to group I ** Significant values at P<0.01 compared to group I *** Significant values at P<0.001 compared to group I

4.2.2. Hypoglycemic effect of 80% methanol leaves extract on oral glucose loaded mice

As shown Table 4 below, 80% methanol leaves extract caused a significant raise of blood glucose at 30 minute time point of the test. After that, mice treated with 200mg/kg and 400mg/kg body weight have shown a significant reduction in blood glucose level 120 minute time point of the test ($p<0.001$) while mice treated with the lowest dose of the extract showed a significant reduction in blood glucose level ($p<0.01$). However, mice treated with the standard drug i.e. glibenclamide 5mg/kg of body weight significantly reduce blood glucose level at 6o minute ($p<0.01$), at 90 minutes and 120 minutes ($p<0.001$) compared to control groups. The overall pattern of glucose tolerance was more improved in extract treated mice than in those untreated.

Table 4. Hypoglycemic effect of 80% methanol extract of *Croton macrostachyus* leave on oral glucose loaded mice

Groups	Treatment (dose in mg/kg body weight)	Blood glucose level (BGL) at time T. after treatment (mg/dl)					%GR	R^2
		0 min	30min	60min	90min	120min		
I	DW(2ml/kg)	107.5±4.31	224.17±8.5	193.2±6.1	172.67±4.61	160.67±5.9		0.94
II	GL5	113±3.59	214.17±7.7	152.2±9.4**	122.83±9.22***	77.17±3.9***	63.97	
III	ME100	115.2±2.21	220±8.5	189.33±6.2	174±6.5	160±7.4*	27.27	
IV	ME 200	114.33±2.4	236.67±5.9	201.33±5.04	169±3.4	135.83±4.3***	42.61	
V	ME 400	109.17±3.05	222.17±4.4	178.7±3.4*	155.33±3.73*	130.17±6.05***	41.41	

Key: DW: distilled water, GL: glibenclamide, ME: Methanol extract GR: percentage of glucose reduction

Data are expressed as Mean ± Standard Error of Mean (SEM); n=6 * Significant values at P<0.05 compared to group I ** Significant values at P<0.01 compared to group I *** Significant values at P<0.001 compared to group I

4.2.3. Hypoglycemic effect 80% methanol leaves extract on blood glucose level in Streptozotocin –induced hyperglycemic mice

Statistical analysis by One-way ANOVA followed by post hoc tukey's test have shown that there were significant difference among diabetic control and the group received the standard drug i.e glibenclamide 5mg/kg of body weight. Post hoc test revealed that all 80% methanol leaves extract and standard drug showed significant reduction in blood glucose level (P< 0.001) compared to diabetic control at 21 days point of test. However, mice treated with the standard drug (glibenclamide 5mg/kg body weight) significantly decreased blood glucose level by 45.86% as compared to mice treated with methanol extract ME 100 (14.22%),ME200 (22.72 %) and ME400 (27.92%) body weight.

Table 5. Hypoglycemic effect of 80% methanol extract of *Croton macrostaycus* leaves on stryptozotocin-induced hyperglycemic mice

Groups	Treatment (dose in mg/kg body weight)	Blood glucose level (BGL) at day D. after treatment (mg/dl)				%GR	R^2
		D0	D7	D14	D21		
I	DW (2ml/kg)	248.83±4.25	260.33±4.33	261.33±5.3	256±.33±3.98		0.981
II	GL 5	267±5.7	238.5±6.41*	205.17± 7.4***	144.55±4.03***	45.86	
III	ME 100	246.17±4.04	233.33±4.35	222±5.01**	211.2±4.65**	14.22	
IV	ME 200	265.5±5.1	249.33±5.6	226.67±6.6**	205.2±6.8***	22.72	
V	ME 400	261.5±4.32	242.67±4.1	217.17±5.33***	188.5± 4.6***	27.92	

Key: DW: distilled water, GL: glibenclamide, ME: Methanol extract.GR: percentage of glucose reduction

Data are expressed as Mean ± Standard Error of Mean (SEM); n=6 * Significant values at P<0.05 compared to group I ** Significant values at P<0.01 compared to group I *** Significant values at P<0.001 compared to group I

4.3 Hypoglycemic effect of solvent fractions on blood glucose level

4.3.1 Hypoglycemic effect of *Croton macrostachyus* leaves solvent fractions on blood glucose level in normoglycemic mice

The effects of solvent fraction treatment with on the blood glucose level in normal fasted mice are shown in Table 6 below. Sample were analyzed using one way ANOVA followed by *post hoc tukey's* test have shown that mice taken water fraction with the lowest dose i.e.100mg/kg body weight did not have significance reduction in blood glucose level as compared to untreated mice. However, mice taken chloroform and butanol fraction treated with 200mg/kg and 400mg/kg body weight significantly reduce blood glucose level after 3hrs.time point of test (p<0.001) .Furthermore the same trend was observed with mice that took glibenclamide 5mg/kg body weight. The result also have shown that mice taken butanol fraction with dose of 400 mg/kg reduce blood glucose level by 58.42% which is comparable to standard drug glibenclamide 5mg/kg (59.83%). However,

mice that took water fraction with the highest dose reduce blood glucose level by 36.71 %. Furthermore, more pronounced dose dependent effect was observed in mice treated with butanol fraction (R^2=0.918) as compared to water and chloroform fraction R^2=0.863, R^2=0.862 respectively.

Table 6: Hypoglycemic effect of solvent fractions of *Croton macrostachyus* leaves on blood glucose level on normoglycemic mice

Group	Treatment (dose in mg/kg body	Blood glucose level at time T after treatment (mg/dl)					%GR	R^2
		0 hrs.	1 hrs.	2 hrs.	3 hrs.	4 hrs.		
I	DW(2ml/g)	99.83±3.93	103.2±3.6	103.2±3.5	102.7±4.6	104.5±4.43		0.863
II	GL5mg/kg	105.8±2.4	91.33±3.7	80±3.1***	67.3±3.3***	42.5±2.72***	59.83	
III	WF100	101.83±4.4	99.31±4.4	92.5±4.5	88±5.1	85.7±5.4*	15.84	
IV	WF200	104.5±2.79	96.2±3.15	86±3.42*	77.7±3.3*	69.5±2.8***	33.5	
V	WF400	110.33±3.01	100.2±3.3	88.7±3.6*	78.5±4.4*	69.53±4.8***	36.71	
VI	CF100	84.83±4.67	80.5±4.51	68.2±3.1***	58.7±2.2***	51.33±1.3***	39.49	0.862
VII	CF200	97.8±3.61	83.7±3.6*	72±5.3***	59.5±6.3***	50.3±4.8***	48.56	
VIII	CF400	98.8±3.74	85.2±4.3*	71.8±5.53***	62.2±5.1***	49.2±4.2***	50.2	
IX	BF100	106±4.04	96.7±3.3	83.8±2.93**	72.83±3.5***	62±3.33***	41.5	0.918
X	BF200	104.5±3.66	87±4.2	73.7±4.2***	61±3.58***	47.7±2.7***	54.35	
XI	BF400	115.83±3.2	92±3.31	79.7±4.25***	61.5±3.7***	46.5±2.4***	58.42	

Key: DW: distilled water, GL: glibenclamide, WF: water fraction, CF: chloroform fraction BF: butanol fraction GR: percentage of glucose reduction. Data are expressed as Mean ± Standard Error of Mean (SEM); n=6 * Significant values at P<0.05 compared to group I ** Significant values at P<0.01 compared to group I *** Significant values at P<0.001 compared to group I

4.3.2 Hypoglycemic effect of solvent fractions on blood glucose level of oral glucose loaded mice

The effect of *solvent* fraction on the blood glucose level in glucose loaded mice is shown in Table 7 below. Increments of blood glucose level have been observed at 30min point of test. Mice administered with standard drug had better glucose tolerance ($p<0.001$) at 120 min point of test as compared to untreated group. However mice administered with the dose of chloroform (100mg/kg and 200mg/kg) and butanol fraction (100mg/kg body weight) did not have any significant association at 120 min point of test but mice administered with water fraction and butanol fraction at dose of 200mg/kg and 400mg/kg have significant association ($p<0.001$) at 120 min time point of test. In addition, more reduction of blood glucose level was observed with mice that took standard drug (by 63.93%) as compared to fraction treated mice. Result from linear regression have shown that dose dependent effect was observed with mice treated with butanol fraction ($R^2=0.933$) as compared to water fraction ($R^2=0.849$).

Table 7: Hypoglycemic effect of *Croton macrostachyus* leaves fractions on blood glucose level on oral glucose loaded mice

Group	**Treatment (dos in mg/kg body weight)**	**Blood glucose level at time T after treatment (mg/dl)**					**%GR**	**R^2**
		0 min	30 min	60 min	90 min	120 min		
I	DW (2ml/kg)	107.5±4.3	224.17±8.3	193.2±6.12	172.7±4.61	160.7±5.9		0.849
II	GL5mg/g	113±3.6	214.2±7.7	152.2±9.3**	122.83±9.22***	77.2±3.9***	63.96	
III	WF100	92.5±2.8	213±7.73	170.2±7.2	141.7±6.86*	135.7±6.6**	36.29	
IV	WF200	99±3.4	222.83±7.2	175±8.11	139.5±8.64*	112.2±1.3***	49.65	
V	WF400	95.83±3.2	218.2±5.95	166.83±4.6	127.3±5.99***	105.3±5.1***	51.74	
VI	CF100	83.7±4.4	219.5±5.1	208.7±4.8	193.7±6.1	178.5±6.4	18.67	0.984
VII	CF200	94.5±2.4	221.8±5.9	202.3±7.4	182.2±5.8	161.8± 5.3	27.05	
VIII	CF400	91.5±4.3	219±6.98	185±5.61	161.83±3.55	131±3.95**	40.18	
IX	BF100	79.7±6.5	224.7±2.74	213.5±2.4	190.3±2.26	168.2±1.3	25.14	0.933
X	BF200	84±4.1	223±2.84	207.5±3.4	168.2±1.88	112.5±6.2***	49.55	
XI	BF400	77.83±3.6	219.83±3.63	187.2±4.64	145.66±4.62**	91.2±1.7***	58.51	

Key: DW: distilled water, GL: glibenclamide, WF: water fraction, CF: chloroform fraction BF: butanol fraction.GR: percentage of glucose reduction Data are expressed as Mean ± Standard Error of Mean (SEM); n=6 * Significant values at P<0.05 compared to group I ** Significant values at P<0.01 compared to group I *** Significant values at P<0.001 compared to group I

4.3.3. Hypoglycemic effect of solvent fractions on blood glucose level in streptozotocin -induced hyperglycemic mice

The activity of solvent fraction on the blood glucose level in streptozotocin-induced diabetic mice is shown in Table 8 below. No significant association was observed with mice treated with all doses of distilled water fraction and the lowest dose of chloroform fraction (100mg/kg body weight) at 7 days' time point of test during treatment. At 14 and 21 days' point of test there was a significant reduction of blood glucose level ($p<0.001$) was observed in mice administered with WF 400, all dose of chloroform and butanol fraction. Overall reduction of blood glucose level was comparable in mice treated with standard drug ($p<0.001$) by 49.2% as compared with fraction treated mice with the highest dose WF, CF and BF with percentage of glucose reduction 47.4%, 51.89% and 47.11 % respectively. In addition, dose dependent effect was observed more pronouncedly in mice treated with butanol fraction (R2=0.961) as compared to water (R^2=0.862) and chloroform (R^2=0.949) fraction.

Table 8: Hypoglycemic effect fractions of *Croton macrosthycus* on blood glucose level in streptozotocin induced hyperglycemic mice

Group	Treatment (dose in mg/kg body weight)	Blood glucose level at day D during treatment (mg/dl)				%GR	R^2
		D0	D7	D14	D21		
I	DW (2ml/kg)	271.3±6.8	276.3±7.7	274.7±5.3	262.7±18.7		
II	GL 5mg/kg	256.3±12.6	215.3±13.3*	175.2±10.4***	130.3±6.6***	49.2	
III	WF100	255.8±13.94	226.8±13.5	202.5± 15.5**	174.2±13***	31.89	0.862
IV	WF200	262.5±10.3	244.5±9.5	214.2±9.9**	144.3±6.1***	45.03	
V	WF400	276.5±10.31	237.8±9.5	204.3±9.2***	145.5±7.6***	47.4	
VI	CF100	261.3±5.2	246.83±5.53	220±4.4***	171±6.03***	34.56	
VII	CF200	252.33±4.7	223.66±2.3***	176.34±1.9***	134.5±3.5***	46.69	
VIII	CF400	258.8±6.76	223.5±6.1***	181.8±4,8***	124.5±2.3***	51.89	0.949
IX	BF100	238.8±4.23	220±6.22***	196.84±6.92***	171.52±6.09***	28.18	
X	BF200	247.53±4.2	213.5±5.15***	183±6.86***	146.16±4,75***	40.95	0.961
XI	BF400	249±5.2	207.2±4.96***	167.5±6.19***	131.7±8.41***	47.11	

Key: DW: distilled water, GL: glibenclamide, WF: water fraction, CF: chloroform fraction BF: butanol fraction GR: percentage of glucose reduction Data are expressed as Mean ± Standard Error of Mean (SEM); n=6 * Significant values at P<0.05 compared to group I ** Significant values at P<0.01 compared to group I *** Significant values at P<0.001 compared to group I

4.4 Preliminary phytochemical screening

Preliminary qualitative phytochemical analysis revealed the presence of different phytochemical constituents such as, alkaloids, flavonoids, saponins and tannins in *Croton macrostachyus* leaves as shown below in Table 9.

Table 9: Results of qualitative phytochemical analysis of 80% methanol crude extract and different solvent fraction of *Croton macrostachyus* leaves

No.	Constituent	80% ME
1	Steroids	X
2	Alkaloids	✓
3	Flavonoids	✓
4	Saponins	✓
5	Tannins	✓
6	Phenol	✓
7	Terpenoids	x

Key: ME: methanol extract, x: absent, √: present

5. DISCUSSION

A recent study has estimated that up to 30% of patients with diabetes mellitus use complementary and alternative medicine. Medicinal plants and its products continue to be an important therapeutic aid for alleviating the ailments of human kind. Developing agents for management of DM that are devoid of adverse effects are still a challenge to the medical care system. This has led to an increased demand for agents known to have fewer side effects than the contemporary oral hypoglycemic agents. Natural products with anti-hyperglycemic activity are stated to have fewer side effects than synthetic oral hypoglycemic agents. Thus, the research on hypoglycemic plants is increasing which might offer a natural key to a better clinical management of DM in the future (Shewamene et al., 2015).

Streptozotocin (STZ) is a widely used chemical for the induction of experimental diabetes. Since the initial report of its diabetogenic properties in 1963. STZ has been used alone or in combination with other chemicals or with dietary manipulations for induction of either type I or type II diabetes(wu and Yan, 2015). Streptozotocin-induced hyperglycemia has been described as a useful experimental model to study the activity of hypoglycemic agents. Streptozotocin exhibits a cytotoxic activity and causes the death of pancreatic β-cell by alkylation of DNA, leaving less active cells and thereby, attenuating insulin synthesis and release. Furthermore, it has been shown to be involved in the fragmentation of DNA by means of production of reactive oxygen species. Due to this it is commonly used to induce diabetes (Shewamene et al., 2015).

In this experiment, streptozotocin was used for induction of DM however, alloxan can also be used for induction, but streptozotocin was preferred due to some advantages over alloxan, like relative long half-life, sustained hyperglycemia and development of well characterized diabetic complications with fewer incidences of ketosis as well as mortality (Ejiofo et al, 2017).

The acute toxicity study indicated that the extract caused no mortality at 2 g/kg within the first 24 hrs as well as for the following 14 days observation. Physical and behavioral observations of the experimental mice also revealed no visible signs of overt toxicity like lacrimation, loss of appetite, tremors, hair erection, salivation, diarrhea and the like. This suggests that LD50 of the extract is greater than 2g/kg which is similar with previous study done (Bantie et al., 2014).

The present results have been shown that significant hypoglycemic effect was observed after the mice were administered with all doses of the extracts. Therefore, this study indicates that 80% methanol extracts of *Croton macrostachyus* leave have hypoglycemic effect on normoglycemic mice and it might be due to hypoglycemic effect of its phytochemical constituents. It is well established that sulphonylureas produce hypoglycemia by increasing the secretion of insulin from pancreas and these compounds have hypoglycemic effect. From the results, glibenclamide produce reduction in blood glucose levels, which validated its activity as a hypoglycemic agent. In case of fractions, all doses also have hypoglycemic effect on normoglycemic mice from 2hrs point of test onward direction ($p<0.01$) but mice administered with chloroform fraction at dose 100mg/kg and 200mg/kg body weight significantly reduced blood glucose level at one hour time point of test. This might suggest that it may have fast onset of action as compared to other fractions. However both extract and fraction significantly reduced blood glucose level in time dependent manner which suggested that this plant extract and fraction is endowed with the ability to enhance regulatory mechanisms, indicating a potential advantage of the extract in minimizing hyperglycemia related complications of diabetes.

The glucose tolerance test (OGTT) measures the clearance of an oral glucose load from the body. It is used to detect disturbances in glucose metabolism that can be linked to diabetes or metabolic syndrome(Louise, 2016). Result from this study might suggest that effect of different phytochemical constituents might be responsible for the effect of extract and each fraction on blood glucose removal from the blood. In addition result from linear regression analysis revealed that there is dose dependent effect were observed from WF (R^2=0.849), and BF (R^2=0.933). Generally, both the crude extract and fraction showed significant reduction in blood glucose level at 120 min. This suggests that the extract and fraction are endowed with the ability to enhance regulatory mechanisms suggesting that both the extract and fraction play a pivotal role in minimizing hyperglycemia related complications of diabetes. The butanol fraction with the highest dose i.e BF400 also brought the hyperglycemic state in glucose loaded mice down to blood glucose level, which was near to the baseline within 120 min like the standard drug. Thus, it is plausible to assume that the plant BF400 and GL5 might produce hypoglycemic and antidiabetic activities by higher percentage of glucose reduction than other fractions. However, chloroform fraction did not show any significant reduction in blood glucose level in glucose loaded mice that could be due to absence or low concentration of active ingredient in this fraction..

The present study also revealed that both extract and fraction have potential anti-hyperglycemic effect on Streptozotocin-induced diabetic mice. Mice treated with standard drug glibenclamide 5mg/kg and extract with 200mg/kg and 400mg/kg significantly reduced blood glucose level from day 14 and similar trend was observed at day 21 ($p<0.01$). Similarly mice treated with fractions also significantly reduced in blood glucose level at 14 and 21 day point of test. This anti-hyperglycemic effect of extract and fraction might be explained by different possible explanation as follows.

Actually, till date there is neither any evidence nor any validation that a natural plant material can serve as a complete substitute for insulin and oral hypoglycemic agents. Though, several plant products have been reported to mimic the effects of insulin partially or enhance the effects of very low endogenous insulin concentrations, but none has sustained life in the total absence of insulin (Eddouks et al., 2012).

It has been suggested that enhanced production of free radicals and oxidative stress are central events to the development of diabetic complications. The cytotoxic action of these diabetogenic agents is mediated by reactive oxygen species (ROS). These radicals undergo dismutation to hydrogen peroxide with a simultaneous massive increase in cytosolic calcium concentration, which causes rapid destruction of pancreatic β-cells. Streptozotocin enters the pancreatic β-cell via a glucose transporter-GLUT2 and causes alkylation of DNA. Furthermore, STZ-induces activation of polyadenosine diphosphate ribosylation and nitric oxide (NO) release. As a result of STZ action, pancreatic β-cells are destroyed by necrosis (Eddouks et al., 2012). There is convincing experimental and clinical evidence that the generation of reactive oxygen species (ROS) increases in both types of diabetes and that the onset of diabetes is closely associated with oxidative stress (Fatmah et al, 2012). Evidence for the protective effect of antioxidants has been presented in experimental, clinical, and epidemiological studies, which have demonstrated that antioxidants might be helpful in treating diabetes and its complications by reducing oxidative stress and alleviates diabetic complications (Koya et al, 2003).This finding suggest that *Croton macrostachyus* leave extract and fraction may have antioxidant or free radical scavenger properties in preventing these changes. The antioxidant activity of present experimental plant might also contribute towards the anti-diabetic effect of the extract and fraction by providing

protection against the cytotoxic effect of free radicals generated by the STZ or diabetes itself and the plant may also have a role in prevention of diabetes.

Secondary metabolites contribute significantly towards the biological activities of different medicinal plants such as hypoglycemic, antidiabetic, antioxidant, etc (Gupta et al., 2018). Preliminary phytochemical screening of the extract of *Croton macrostachyus* revealed the presence of different phytochemicals like saponins, flavonoids, alkaloids, tannins and these secondary metabolites are known to be bioactive antidiabetic principles.

Flavonoids, a group of hydroxylated phenolic substances known to be potent free radical scavengers, have attracted a tremendous interest as possible therapeutics against free radical mediated diseases, particularly diabetes mellitus. The radical derivatives of oxygen (ROS) are the most important free radical in biological systems and harmful byproducts generated during normal cellular functions (Sarian et al., 2017). Increasing intake of natural antioxidants may help to maintain a tolerable antioxidant status, thus preventing the oxidative stress that could lead to pathogenesis of diabetes mellitus. Flavonoids are one of the most important groups of bioactive compounds among secondary metabolites (Testa et al., 2016). Flavonoids are widely recognized for their wide range of biological activities, and many of them have been reported to be beneficial in treating diabetes and its complications. It also has an anti-hyperglycemic effect and a protective effect on pancreatic β-cell destruction in streptozotocin-induced diabetes (Huang et al., 2018).

One of the therapeutic approaches to managing diabetes mellitus is to retard the absorption of glucose via inhibition of digestive enzymes in the digestive organs such as the α-glucosidase and α-amylase. α-Glucosidase is a membrane bound enzyme located at the epithelium of the small intestine that catalyzes the cleavage of glucose from disaccharides to monosaccharides. Inhibitors of α-glucosidase are used to control the blood sugar levels for type 2 diabetes mellitus (Sarian et al., 2017).

Plants like *Croton macrostachyus* which contain saponins have been used to treat diabetes, hepatitis, high blood pressure, high cholesterol, and physical stress, antimicrobial and hypoglycemic. A study on alloxan-induced diabetic mice have shown that it was able to reduce fasting blood glucose and serum insulin levels, which in turn reduce hyperglycemia through different mechanism such as stimulating AMP-activated protein kinase (AMPK) and insulin receptor (IR)/IR substrate-1/P13K/Akt signaling pathways which consequently increases the

activity of the enzymes hexokinase and pyruvate kinase to promote glucose breakdown. AMPK signals act by stimulating glucose synthesis in skeletal muscles, reducing hepatic glucose production, and β-oxidation of fatty acids in adipose tissues (Barroso et al., 2017).

Saponins regulate plasma glucose level and prevent diabetic complications due to their antioxidant activity. The hypotriglyceridemic and hypocholesterolemic actions of saponin will help diabetic patients in reducing the risk of atherosclerosis. Also conventional antidiabetic drugs have side effect like weight gain. Saponin reduces body fat thereby making them excellent for treatment of diabetes. However, more research is needed with respect to toxicological evaluation of these saponins (Elekofehinti, 2015).

Studies also revealed that alkaloids and tannins are reported to have antidiabetic activity that improves the performance of pancreatic tissues by increasing the insulin secretion or decreasing the intestinal absorption of glucose (Elardo et al., 2017; Kooti et al., 2016).

In general, findings from above different research supported that secondary metabolites responsible for its antidiabetic and complication preventive activity of *Croton macrostachyus* plant through different mechanisms. It can then be inferred based on the results that presence of phytochemicals, particularly flavonoids, saponins, alkaloids and tannins may work synergistically or independently to lower blood glucose of mice.

6. CONCLUSION

From findings of this study, it can be concluded that 80 % methanol extract of *Croton macrostachyus* leaves and solvent fractions have significant blood glucose lowering effect on normoglycemic, oral glucose loaded and STZ-induced hyperglycemic mice. Chloroform fraction produce high percentage reduction in blood glucose level in STZ-induced hyperglycemic mice than other fractions. This effect may be through different mechanisms attributed by secondary metabolites such as alkaloids, flavonoids, saponins and other constituents present in the leaves either synergistically or independently. The phytochemical screening indicated the presence of alkaloids, phenols, flavonoids, tannins and saponins. Acute toxicity test of 80% methanol extract was found to be safe at single dose 2g/kg.

Based on findings obtained from present study the traditional use of *Croton macrostachyus* leaves for the management of diabetes mellitus can be supported as an alternative herbal supplement with further investigations needed to make final conclusion.

7. RECOMMENDATION

- Further, comprehensive chemical and pharmacological investigation should be carried out to isolate the active compound responsible for its antidiabetic activity
- In addition, isolation and characterization of the specific compounds are warranted to determine the specific mechanisms of action
- Further histopathological studies to evaluate antidiabetic activity
- Further investigation on chronic toxicity of this plant on different animal species

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