Anti-hyperglycemic activities of hydro-alcoholic, alkaloid and non-alkaloid extracts of*Calpurnia aurea (aiton)* benth*(*Fabacea)against streptozocin induced diabetic mice

A thesis Submitted to the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health sciences in partial fulfillment of the Requirement for the Degree in Master of Science in Pharmacology

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This is to certify that the thesis prepared by: Habtamu Beyene, entitled *Anti-hyperglycemic activities of hydro-alcoholic, alkaloid and non-alkaloid extracts of Calpurnia aurea (aiton) benth(Fabacea)against streptozocin induced diabetic mice*and 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 concerning originality and quality.

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ABSTRACT

Anti-hyperglycemic activities of hydro-alcoholic, alkaloid and non-alkaloid extracts of Calpurnia aurea (aiton) benth(Fabacea)against streptozocin induced diabetic mice

Habtamu Beyene,

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Background: Diabetes mellitus is a metabolic disease with several etiologies that is typified by persistently high blood sugar levels. Given the rising rates of diabetes-related morbidity and death in low- and middle-income countries, it is critical to evaluate the potential pharmacological effects of medicinal plants in order to complement current diabetes treatments. The experimental plant *Calpurinaaurea*(Aiton)Benth is among the Fabaceae species, which is used traditionally for diabetes and other health disorders.

Objectives: The purpose of this study was to assess the effects of crude, alkaloid, and non-alkaloid leaf extractsof *C.aurea* on blood glucose control in streptozotocin-induced diabetic mice and normoglycemic mice.

Methods: The crude, alkaloid, and non-alkaloid extracts were prepared using the proper solvents prior to the commencement of the *in-vivo* investigation. Swiss albino mice, weighing between 20 and 30 grams, were selected for the animal trials. In order to study the hypoglycemic/antihyperglycemic effect of the extract, nine groups of diabetic mice and eleven groups of normal mice were used. Streptozotocin was used to induce diabetes, and blood glucose levels were measured with a glucometer. Doses of the plant alkaloid and non-alkaloid extract (100, 200, and 400 mg/kg) were administered to the test groups in each model while the crude extract was administered at lower doses(50, 100, and 200 mg/kg). Glibenclamide(5 mg/kg) served as a standard drug. The negative control, and the normal control were given 1% of tween 80 (10 mg/kg).

Results: The results demonstrated that crude, alkaloid, and non-alkaloid extracts of *C.aurea* leaves lowered the incidence of hypoglycemia. After the administration of2.5 mg/kg of glucose, the alkaloid extract demonstrated significant lowering of blood glucose levels: 200 mg/kg at 80 minutes ($p<0.05$) and 400 mg/kg at the first and second hour ($p<0.01$). All extract-treated groups of streptozotocin induced diabetic mice had decreased blood glucose levels, besides the mice

administered the alkaloid extract showed a statistically significant drop in blood glucose levels. The alkaloid extract reduced blood glucose levels in the 200 ($P<0.05$) and 400 mg/kg doses (162±5.21, 142±3.51), respectively, were used in a single dose study. Furthermore, the alkaloid extract at the middle dose ($p<0.05$), higher dose ($p<0.01$), and all doses at the third week all significantly decreased the fasting blood glucose level. Furthermore, compared to the negative control group, the groups treated with crude, alkaloid and non-alkaloid extracts experienced a lesser drop in body weight following the onset of diabetes mellitus.**Conclusion:**it can be concluded that the alkaloid extracts of *C.aurea* leaves are effective in lowering blood glucose levels in diabetic mice and lack a hypoglycemic effect in normoglycemic mice. Additionally, the extracts were observed to exhibit no acute toxicity.

Keywords: Hypoglycemic, Ant hyperglycemic, *Calpurnia aurea*, Strepotozotocin, alkaloid extract, non-alkaloid extract, Mice.

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List of abbreviations and Acronyms

AAU: Addis Ababa University

ACA: Alkaloid extract of *Calpurinaaurea*

ADA: American Diabetes Association

AMPK: AMP-activated protein kinase

BGL: Blood glucose level

BMI: Body mass index

DM: Diabetes mellitus

DW: Distilled water

EPHI: Ethiopia Public Health Institute

FBG: Fasting blood glucose

GDM: Gestational Diabetes mellitus

GLP-1: Glucagon-like peptide-1

GLUT: Glucose transporter

HbA1c: Hemoglobin A1C

ICA: Islet cell antibody

IDF: International Diabetes Federation

IsR: Insulin resistance

MCA: Methanol extract of Calpurinaaurea

NACA: Non-alkaloid extracts of Calpurinaaurea

NIDDM: Non-insulin dependent diabetes mellitus

NODM: Non-obese diabetic mice

NO: Nitric oxide

OGTT: Oral glucose tolerance test

STZ: Streptozotocin

SUs: Sulphonylureas

WHO: World Health Organization

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1. Introduction

1.1. Overview of Diabetes mellitus

Diabetes mellitus (DM) is a metabolic disease with multiple etiologies that is characterized by persistent hyperglycemia and changes in carbohydrate, lipid and protein metabolism which is associated with deregulated insulin synthesis, insulin action or both *(Sheleme et al., 2020*). It may manifest with symptoms such as polyuria, polydipsia, blurred vision and weight loss. ketoacidosis or a non ketotic hyperosmolar state may develop in its most severe stages, resulting in stupor ,coma and in the absence of adequate treatment, it results in long-term damage to organs such as the kidneys, liver, eyes, nerves, heart, and blood vessels(Suryasa*et al*., 2021).

Diabetes is divided into two main types: type 1 DM (T1DM), which is characterized by insulin deficiency due to a malfunction of islet-cells, and type 2 DM (T2DM)is primarily characterized by insulin resistance (Oguntibeju, 2019). Although T2DM shows as hyperglycemia, the cause might range from disruptions in insulin secretion, insulin action, insulin resistance, glucose synthesis and absorption, interplay between multiple hormones, and various types of stress (Gomes *et al*., 2019).

1.1.1. Epidemiology of Diabetes Mellitus

According to the International Diabetes Federation(IDF) Diabetes Atlas 2021,approximately 463 million of adult population (20-59 years) has diabetes, with nearly half of those affected are unaware of their illness(Shine *et al.*, 2020). The global diabetes prevalence in 20-79 year olds in 2021 was estimated to be 10.5% (536.6 million people), rising to 12.2% (783.2 million) in 2045. Diabetes prevalence was similar in men and women and was highest in those aged 75-79 years.(https://pubmed.ncbi.nlm.nih.gov

)According to IDF forecasts, one in every eight adults, or roughly 783 million people (46% rise), will have diabetes by 2045 (Monod *et al*., 2023). Moreover,IDF estimates from 2017 indicate that >96 ,000 new cases of type 1 diabetes are diagnosed, globally, per year in children and adolescents aged <15 years (Cho *et al.*, 2018).

In Africa, the frequencies of DM and pre-diabetes are similar for both women and men, with rates of 6.6% and 21.8% respectively. This indicates a significant burden of the disease on the continent (Misganaw *.et al.*, 2014). The prevalence of diabetes (21.8 % versus 13.5%), However, when examining specific age groups, variations in prevalence emerge. Among women aged 45-54 years, the prevalence of diabetes is notably higher compared to their male counterparts, with rates of 21.8% versus 13.5%, respectively. women are also more likely to have pre-diabetes (Koye *et al*., 2022).

Ethiopia is one of the top four African countries with the highest rate of DM and Hospital admissions due to diabetes (Gebre, 2013). However, diabetes remains largely undiagnosed and untreated, mainly in rural areas. According to the 2017 estimate by IDF, Ethiopia has 2.57 million (5.2%) adult people aged 20–79 years with diabetes, making it the largest diabetes population in sub-Saharan Africa (Ogurtsova*et al.*, 2017). Of those, about 1.96 million of them (76%) do not even know they have diabetes or are undiagnosed (Nigatu, 2012).

1.1.2. Pathophysiology

Since DM has a complex pathophysiology and a wide range of presentations, any classification of the disorder is arbitrary but nonetheless helpful, and it is frequently impacted by the physiological parameters that exist at the time of assessment and diagnosis (Banday *et al.*, 2020). The etiology of type 2 diabetes mellitus appears to involve complex interactions between environmental and genetic factors.

The autoimmune destruction of the insulin-producing β-cells in the pancreas, leading to a lack of insulin secretion in T1DM (Poznyak *et al.*, 2020).The histocompatibility antigens ,human leukocyte antigen (HLA-DR3 or HLA-DR4) and the existence of circulating insulin antibodies such as insulin, glutamic acid decarboxylase, islet cell, and ICA 512 (a tyrosine phosphatase antibody) are closely linked to this type of diabetes (Maruhashi and Higashi, 2021).The ability of normal pancreatic β-cells to release insulin is much more than what is required to control the metabolism of lipids, proteins, and carbs (Suryasa*et al.*, 2021).

T1DM is a chronic autoimmune disorder characterized by the selective destruction of insulin-producing pancreatic β-cells. The clinical manifestation of the disease signifies the final stage of β-cell destruction, culminating in type 1 diabetes mellitus. This autoimmune destruction results in an insulin secretion deficiency, leading to the metabolic disturbances associated with T1DM.

Additionally, the function of pancreatic α-cells is impaired, causing excessive glucagon secretion in IDDM patients. Insulin deficiency leads to uncontrolled lipolysis and increased plasma levels of free fatty acids, which inhibit glucose metabolism in peripheral tissues such as skeletal muscle. This condition impairs glucose utilization and reduces the expression of several genes essential for normal insulin response in target tissues, including glucokinase in the liver and the GLUT 4 glucose transporters in adipose tissue (Raju and Raju, 2010).

The three main metabolic disorders that lead to hyperglycemia in T2DMare the inability of insulin to promote glucose absorption in peripheral target tissue, increased hepatic glucose output, and fatty glucose-induced insulin secretion (Kahn *et al*., 2014). These abnormalities also involve the cellular glucose transport in cells, liver, adipose tissue, and skeletal muscle, and they may be the result of alterations in GLUTs (Javeed and Matveyenko, 2018).

Insulin resistance, or the decreased sensitivity of muscle and fat cells to the actions of insulin, may be a major issue in type 2 diabetes (Carlsson, 2019) .Thus, the pancreas may produce normal or even excessive levels of insulin in the early stages of type 2 diabetes and only develop reduced insulin production in the later stages of the disease (Maruhashi and Higashi, 2021) Resistin, a hormone found in adipose tissue, has recently come to light and is thought to be the root cause of many of the disorders that lead to insulin resistance(Ginter *et al.*, 2012).

1.1.3. Complications of Diabetes mellitus

Prolonged elevated blood sugar can damage blood vessels that lead to heart attacks and strokes (Bekele, 2019). Moreover, In the US, retinopathy is a common cause of adult blindness. Retinal capillary micro aneurysms are the initial symptoms, followed by macular edema and neovascularization (proliferative retinopathy) (Harding *et al*., 2019). The disorder can eventually advance at a very variable rate, resulting in focal blurring, vitreous or retinal detachment, and partial or complete vision loss, even when there are no early indications (Papatheodorou *et al.*, 2018a) .

Diabetic nephropathy is one of the main causes of chronic renal disease(Yu *et al.,* 2020).Chronic renal damage might exacerbate the body's inability to expel extra fluid and waste products that result in glomerular filtration rate (GFR) and glomerular hypertension decline gradually as a result of mesangial development, glomerular sclerosis (Nouwen *et al*., 2019). The other complication results from changes in intracellular metabolism that impair neuronal function,

direct hypoglycemic effects on neurons, and microvascular disease-induced nerve ischemia called diabetic neuropathy (Nouwen *et al.*, 2019).

Small fiber neuropathy is characterized by pain ,numbness, and loss of temperature sensation with preserved vibration and position (Worku *et al.*, 2011). According to Papatheodorou *et al*, *patients* have a high incidence of autonomic neuropathy and are susceptible to neuropathic joint deterioration and foot ulcers. Muscle weakness, lack of deep tendon reflexes, and loss of vibration and position (Papatheodorou *et al*., 2018b). In addition Atrophy of intrinsic muscles of the feet and foot drop can occur(Heydari *et al*., 2010).

1.1.4. Diagnosis and treatment

The diagnosis of DM entails evaluating blood glucose levels, there are set of criteria and tests used to produce an accurate diagnosis (Care and Suppl, 2022).The most common diagnostic test for diabetes is the measurement of fasting plasma glucose (FPG) levels (fasting blood glucose level of 126 mg/dL (7.0 mmol/L) or higher on two separate occasions is indicative of diabetes) (Wang *et a*l., 2022). Another method is the oral glucose tolerance test (OGTT), where a glucose load is administered, and blood glucose levels are measured at specific interval (Sugahara *et al.*, 2021).A 2-hour plasma glucose level of 200 mg/dL (11.1 mmol/L) or higher confirms the diagnosis (Petersmann*et al.,* 2019). When diagnosing diabetes, the American Diabetes Association (ADA) advises utilizing any one of these four criteria (Care and Suppl, 2021).

Diabetes management involves a comprehensive strategy involving both pharmaceutical and non-pharmacological therapies (Ginter *et al*., 2012). The goals of these therapies are to manage blood sugar levels, avoid problems, and enhance general health (Odegard *et al.*, 2007). Lowering body weight and increasing daily energy expenditure improve glucose tolerance and reduce insulin resistance (Otto-Buczkowska and Jainta, 2018). Dietary and exercise recommendations are, in fact, a crucial component of T2 DM management (Iyer *et al.*, 2015). It is advised that patients who are overweight consume less calories, eat a diet high in unprocessed carbohydrates, low in total fat, especially saturated fat, and low in calories Patients with insulin-dependent diabetes get subcutaneous insulin injections once a day (Ma *et al.*, 2018). Several class of antidiabetic medications are available for clinical use. Below, some of the main classes of antidiabetic agents are discussed.

Insulin secretagogues: One important aspect of type 2 DM pathogenesis is insulin secretory abnormalities (Scheen, 2016). They are insulin-secreting substances such sulfonylureas, Meglitinides which increase insulin production regardless of glucose. They are primarily used in the treatment of type 2 diabetes mellitus to help manage blood glucose levels. There are two main categories of insulin secretagogues: sulfonylureas and meglitinides.Despite their dissimilar structural characteristics (Seino et al., 2017a) and incretin-related medications like glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase 4 (DPP-4) inhibitors The glucagon-like peptide-1 (GLP-1) and the dipeptidyl peptidase-4 (DPP-4) inhibitors stimulate insulin secretion by a glucose-dependent mechanism, and inhibit glucagon secretion.

(Seino *et al*., 2017b). Depolarization causes the cell's voltage-dependent calcium channels to open, releasing secretory granules containing insulin (Munkboel *et al.*, 2021) .

For patients with moderate-to-severe β-cell failure, secretagogue therapy is the right way to increase circulating insulin levels (Lockyer, 2012). Sulfonylureas have few negative effects and increase insulin production to lower hyperglycemia(Smith and Gerich, 2006). However,prolonged plasma half-lives and long-lasting effects of sulfonylureas raise the risk of hypoglycemia, particularly in older and renally insufficient individuals (Kudo, 2013).

Biguanides :They are class of drugs used to treat T2DM and other disorders function by lowering the amount of glucose produced during digestion (Hotta, 2019).Two biguanide antidiabetics, phenformin and metformin were introduced in the 1950s (Lockwood, 2019) Because of higher risk of lactic acidosis, phenformin was withdrawn in many countries(Hotta, 2019). The exact mechanism of action is unclear but ,explanations offered for their hypoglycemic action are Suppress hepatic gluconeogenesis and glucose output from liver (the major action) ,enhance insulin-mediated glucose disposal in muscle and fat (Di Magno *et al*., 2022) , retard intestinal absorption of glucose, other hexoses, amino acids and vit B12, interfere with mitochondrial respiratory chain and promote peripheral glucose utilization by enhancing anaerobic glycolysis (Grytsai*et al*., 2021). Adverse effects associated with therapeutic use of biguanides include gastrointestinal upset,vitamin B12 deficiency ,and hemolytic anemia .although the incidence is low metformin toxicity can lead to hyperlactatemia and metabolic acidosis(Wang and Hoyte, 2019)

Thiazolidinediones: They function by increasing the body's sensitivity to insulin and decreasing its resistance to it. As specific ligands of the nuclear transcription factor peroxisome-proliferator-activated receptor-g (PPAR-g), the insulin sensitizing thiazolidinediones pioglitazone and rosiglitazone are the first medications to address the fundamental issue of insulin resistance in patients with type 2 diabetes(Lebovitz, 2019). TZDs mainly affect the nuclear receptor known as peroxisome proliferator-activated receptor gamma (PPAR-γ), which is involved in the metabolism of fats and carbohydrates. (Hurren and Dunham, 2021).

Currently, pioglitazone and rosiglitazone are the two thiazolidinediones that are authorized for usage in the United States(Giglio *et al.,* 2022).These drugs improve glucose uptake and utilization by raising insulin sensitivity in adipose and muscle tissues, which lowers blood glucose levels(Arnold *et al.,* 2019). Despite their efficacy in regulating blood glucose levels, thiazolidinediones have been linked to certain adverse effects such as Weight gain, fluid retention, edema, and a higher risk of fractures are typical side effects (Lebovitz, 2019). Furthermore, rosiglitazone's possible cardiovascular hazards have raised concerns, which has resulted in certain nations banning the drug(Lu *et al*., 2015).

Dipeptidyl Peptidase-4 (DPP-4) Inhibitors: are a group of oral drugs that are used to treat type 2 diabetes(Zhao *et al.,* 2021). The several actions of DPP-4 inhibitors contribute to their ability to reduce glucose levels, boost insulin release when blood glucose levels are high and repress it when levels are normal or low means that they promote insulin secretion in a glucose-dependent way (Gilbert and Pratley, 2020). Additionally they lessen the release of glucagon, which lowers blood glucose levels even more, slowing down the emptying of the stomach that can help with better glycemic management (Baetta and Corsini, 2011).

These medications can be taken with insulin or in addition to other oral antidiabetic treatments(Lyseng-Williamson, 2019). Sitagliptin inhibits the action of DPP-4, a cell surface peptidase that cleaves a variety of substrate proteins and peptides(Patel and Ghate, 2014). As a result, endogenous GLP-1 and GIP levels are raised Better glycaemic control results from this as it raises insulin and lowers glucagon secretion(Wang *et al.,* 2018) . In individuals with type 2 diabetes mellitus, it is used as an adjunct monotherapy to enhance glycaemic control in addition to diet and exercise (Abu Khalaf *et al*., 2015).

GLP-1 analogues: are a new class of oral glucose-lowering drugs that mimic the endogenous hormone, glucagon-like peptide(Lau *et al*., 2015).These medications bind to activate the body's

GLP-1 receptors to result in increased insulin production, decreased glucagon secretion, a delayed stomach emptying time, and an increased sensation of fullness (Saraiva and Franco, 2021). Semaglutide, liraglutide, exenatide, and dulaglutide are a few examples of GLP-1 analogues (Gilbert and Pratley, 2020).It increases the secretion of insulin, but only in a glucose-dependent way, meaning that low glucose prevents the release of insulin (Lyseng-Williamson, 2019).

1.1.5. Roles of Medicinal Herbs in Diabetic Mellitus

Plants and herbal remedies have long been utilized for their medicinal, flavoring, aromatic and other properties (Kuhn, 2002). The main benefits of plant extract include minimal toxicity, and environmentally friendly products (Shahid-Ul-Islam *et al.*, 2013) Furthermore, because plant-based medicine is safe and successful, the pharmaceutical community as a whole has refocused on studying medicinal plants (Roodsari*et al.*, 2013). There has been an exponential growth in the field of herbal medicine, and these drugs are gaining popularity both in developing and developed countries because of their natural origin and less side effects (Matheka and Alkizim, 2012).

For ages, traditional herbal remedies have been utilized across diverse cultures to address and cure a range of medical conditions, including DM (Malviya *et al.*, 2010) .*In-vitro* inhibition of α-glucosidase and α-amylase as well as *in- vivo* research on rats and mice have been used to study the potential benefits of some herbs for diabetic activities. Herbals reported for antidiabetic potential include Cinnamon (Zare *et al.*, 2019;Bandara *et al.*, 2012), Bitter Melon (Joseph and Jini, 2013;Leung *et al.*, 2009) , Ginseng (Chen *et al.*, 2019), Centaurea spp (Fattaheian-Dehkordi*et al.*, 2021), leaves extract of *T. browni*istem bark crude extract and its solvent fractions(Alema *et al.*, 2020) ,*Trigonella foenum-graecum (*Neelakantan *et al*., 2014), *Rubus Erlangeri*(Gorems, 2019) etc . In general, utilization of herbal remedies for their possible antidiabetic properties can affect insulin secretion, improve insulin sensitivity, control the metabolism of carbohydrates, prevent the absorption of sugar, and have anti-inflammatory and antioxidant properties (Patel *et al.*, 2012).

1.1.6. The experimental plant

Calpurnia aurea is a plant that is popular member of the Fabaceae family of flowering plants which is found throughout Africa, extending from Eritrea to the Cape Province (Bogale *et al*.,

2022) .The plant has also extra habitat in the southern region of India (Camara, 2022), It is the family of numerous well-known blooming tree species and is renowned for this genus's prodigious blossoming habit, (Figure 1)(Aurea, 1986). Some species of the genus include: *Calpurnia aurea sylvatica, Calpurnia capensis, Calpurnia glabrata, Calpurnia intrusa, Calpurnia robinioides, Calpurnia sericea, Calpurnia villosa and Calpurnia woodii*(Wasihun*et al*., 2023).

In various regions of Ethiopia, it contains shrubs or tiny trees that grow in or near the borders of forests (Ahmed *et al*., 2022). The plant additionally known in the following languages: “Chekata” in Afaan Oromo, “Digita” in Amharic, and “Wild Laburnum” or “Wildegeelkeur” (in Afrikaans) (Eyasu *et al.*, 2013),"Hitsawits" (Tigrigna) (Gebreslassie and Eyasu, 2019),”Yefekmshra” (Guragegna) (Ahmed *et al*., 2022).

Figure 1 :Photo of *Calpurinaaurea*

1.2. Statement of the problem

DM is a chronic metabolic disorder typified by high blood glucose level as a result of insulin resistance or not enough insulin secretion or sometimes it may happen together with insulin resistance and less insulin secretion (Wolosowicz, 2020). The problem for DM current prevalence and burden at the global, regional, and national levels reveal a significant public health challenge. Herbal medicine has been used for years in many nations to treat a wide range of diseases, including diabetes (Yikna and Yehualashet, 2021). The use of particular herbs for glycemic control has been described in a number of traditional medical systems, including Indigenous practices, Traditional Chinese Medicine, and Ayurveda. Examining the scientific underpinnings of these folk remedies can yield important information about the safety and possible effectiveness of herbal remedies for diabetes is crucial(Kumar, 2013).

Secondary metabolites, including alkaloids, tannins, and flavonoids, phenols are abundant in *Calpurnia aurea* leaf extracts (Belayneh *et al*., 2019). Additionally, the findings of this study may facilitate the development of standardized herbal anti-diabetic medications that could supplement or replace contemporary anti-diabetic medications The current antidiabetic medications, while effective for many patients, do have several limitations:

Hypoglycemia Risk: Some medications, particularly insulin and sulfonylureas, can cause hypoglycemia (low blood sugar), especially if not taken or managed properly. This risk can be dangerous and even life-threatening in severe cases.

Weight Gain: Certain antidiabetic medications, such as insulin, sulfonylureas, and thiazolidinediones, may lead to weight gain in some individuals. This can exacerbate other health issues, such as obesity and cardiovascular problems.

Gastrointestinal Side Effects: Some medications, like metformin, can cause gastrointestinal side effects such as nausea, diarrhea, or abdominal discomfort, particularly when treatment is initiated or doses are increased.

Injection Site Reactions: Injectable medications like insulin or GLP-1 receptor agonists may cause injection site reactions, including redness, swelling, or pain, which can affect treatment adherence and quality of life.

Cost: Many newer antidiabetic medications are expensive, which can pose a barrier to access for some patients, particularly those without adequate insurance coverage or financial resources.

Tolerance and Loss of Efficacy Over Time: Some individuals may develop tolerance to certain medications or experience a reduction in their effectiveness over time, necessitating dose adjustments or the addition of other medications.

Limited Long-term Safety Data: For newer antidiabetic medications, there may be limited long-term safety data available, as they have not been on the market as long as some older treatments. This can raise concerns about potential unknown risks with prolonged use.

Individual Variability in Response: Antidiabetic medications may have varying efficacy and side effect profiles among individuals, making it challenging to find the most suitable treatment regimen for each patient.

Addressing these limitations requires ongoing research and development of novel therapies that aim to improve efficacy, safety, tolerability, and accessibility for individuals with diabetes.

1.4. Objectives

1.4.1. General objectives

The main objective of this study is to investigate thehypoglycemia and anti-hyperglycemic properties of crude, alkaloid, or the non-alkaloid leaf extract of *Calpurnia aurea*in mice.

1.4.2. Specific Objectives

- To assess acute toxicity of the crude, alkaloid, and non-alkaloid leaf extracts of *Calpurnia aurea.*
- To investigate hypoglycemic activities of the crude, alkaloid and non-alkaloid leaves extracts of *Calpurneaaureain- vivo* using normal-glycaemic model
- To evaluate antidiabetic activities of the crude, alkaloid and non-alkaloid leaf extracts of *CA in-vivo* using streptozotocin- induction model
- To evaluate anti-hyperglycemic activities of the alkaloid and non-alkaloid CA leaf extractsusing oral glucose loading model

1.5. Significance of the study

In response to the challenges posed by the side effects of prescription medications, traditional herbal medicine has emerged as a promising complementary approach to managing diabetes in various cultures.

There is a growing interest in integrating traditional and complementary medicine into mainstream healthcare, driven by the need for evidence-based research and support. Consequently, the investigation of the antidiabetic activity of extracts derived from *C.aurea* holds significant importance in this context. By exploring the potential of crude, alkaloid and non-alkaloid extracts from leaves of *C.aurea*, valuable insights can be gained. This research endeavor has the potential to contribute to the discovery of novel therapeutic interventions for diabetes management.

By addressing the aforementioned research questions, the current study aims to contribute to the understanding of the pharmacological properties and therapeutic potential of the *Calpurnia* aurea crude, alkaloid leaf extract ,non-alkaloid leaf extract. Such insights can provide a solid foundation for further research and the development of targeted bio active constituents. Ultimately, the outcomes of this investigation have the potential to positively impact public health outcomes related to diabetes, promoting better management and improved overall well-being.

2. Literature review

Calpurnia aurea, commonly known as "*Calpurnia" or" Golden Calpurina,"* is a medicinal plant that holds significant importance in traditional medicine practices (Chemistry, 2014). The genus comprises shrubs or small trees in or along the margin of forests in most part of east Africa including Ethiopia (Beaumont *et al.*, 1999). The popular species of this genus, the most commonly found, is *Calpurnia aurea*(Figure 1) (Aurea, 1986). It grows as a small evergreen tree which is perfectly suitable for small gardens or as hardy street tree. In the forest, it can be a 9 to 15 m tree while in the open it is more often a shrub or small tree 2 - 4 m tall(Korir *et al*., 2014)

Traditional use: The powdered roots and leaves areused as a fish poison or to treat cough, diarrhea, and snake bites, abscesses, lung tuberculosis, fish poisoning, and itching relief (Umer *et a*l., 2013). In addition, it is used as an insecticide and to treat gastrointestinal issues, headaches, eye conditions, and skin infections brought on by ticks (Birhan *et al.*, 2019). Furthermore, the plant has been used to cure eye conditions, coughing, cuts, vomiting, and produce uterine contractions (Mekuriaw*et al.*, 2021)

According to an ethnobotanical survey conducted in the northwest of Ethiopia among the Shenasha, Agew-awi, and Amhara peoples, the plant's seed and leaf are both taken orally to treat diabetes mellitus . Another survey conducted in Nekemte town, east Wollega, Ethiopia, revealed that the plant's leaf decoction is consumed orally to treat diabetes mellitus(Suleman and Alemu, 2012). The plant is traditionally used to treat different types of diseases such as amoebic dysentery, diarrhea, syphilis, tapeworm, scabies (Beyi, 2018).

Previous Phytochemical studies: studies have shown the plant contains alkaloids, terpenoids, flavonoids, tannins, saponins, phenols and cardiac glycosides (Gebreslassie and Eyasu, 2019). From previous literature data s revealed that several alkaloids have been identified in *Calpurinaaurea* which are most abundant and possess various biological activities, such as antimicrobial, anti-inflammatory, and antidiabetic properties (Yikna and Yehualashet, 2021). Flavonoids are second abundant in *Calpurinaaurea*, with compounds such as quercetin, kaempferol, isorhamnetin, and rutin being frequently identified (Gebreslassie and Eyasu, 2019).

Reported Pharmacological activities : Previous studies have provided evidence of the antimicrobial potential of the plant against various bacteria, fungi, and parasites (Melese *et al.*,

2019). This antimicrobial activity is attributed to the presence of bioactive compounds, such as alkaloids, flavonoids, and phenolic acids, which have demonstrated inhibitory effects against pathogens(Umer *et al.*, 2013). In terms of its antidiabetic potential, preclinical investigations have indicated that *C.aurea* shows promise. Studies using animal models of diabetes, including streptozotocin-induced diabetic rats, have been conducted to evaluate the hypoglycemic and antidiabetic activities of *Calpurnia aurea* extracts or isolated compounds (Belayneh *et al.*, 2019). Additionally, both preclinical and clinical studies have suggested that it possesses anti-inflammatory and analgesic properties (Ayal *et al.*, 2019).

Roles of Alkaloidal extracts from *Calpurnia aurea*

Several alkaloidal components have been previously isolated from *C.aurea.* These alkaloids are known for their diverse biological activities and potential therapeutic applications(KORIR, 2012). The alkaloids isolated from *Calpurnia aurea*includes lupinine which possess antimicrobial, anti-inflammatory, and analgesic activities (Shehadeh *et al*., 2021).Several other alkaloids, such as dictyophlebine, 5,6-dehydrolupanine, and lupanidine, have been identified in *C.aurea* (Wiedemann *et al*., 2015) . These alkaloids have exhibited various biological activities, includingantidiabetic, and anti-inflammatory effects (Bobkiewicz-Kozłowska *et al.*, 2007).

3. Materials and Methods

3.1. Instruments, Chemicals, and Drugs

The following drugs, chemicals, and instruments were used during the study. Streptozotocin (Sigma Aldrich) to cause diabetes, A glucometer (Prodigy, America) was used to assess the blood glucose level, and glibenclamide (brand name Daonil, Cyprus) was utilized as a typical hypoglycemic medication. Other chemicals used were normal saline, glucose 40%, trisodium citrate, citric acid, and Tween80 all from the same source (Pspark, Scientific Limited, Northampton, UK), citric acid monohydrate (Lab Tech Chemicals, India), tri-sodium citrate dihydrate (Blulux Laboratories, Faridabad, India), methanol absolute (Nice Chemicals Private Limited, Ernakulam, India), 40% glucose solution (Reyoung Pharmaceuticals, Shandong, China), sterilized water for injections (Nirma Ltd, Ahmedabad, India), analytical balance, blood glucose strips (Alliance International, Taiwan), Every reagent that was utilized was analytical or HPLC grade.

3.2. Plant Material collection

In November 2021, leaves of *Calpurnia aurea* were collected from Ejersalafo Woreda, West Shoa Zone. For identification and authentication purpose, the collected plant material was brought to the Herbarium of College of Natural and Computational Sciences, Addis Ababa University and voucher specimen of the plant (CA001) was deposited at the National Herbarium for future records.

3.3. Experimental Animals

Healthy Swiss albino mice, 20–30 grams in weight, of both sexes were purchased from the Ethiopian Public Health Institute's Experimental Animal Breeding Unit. The animals were kept on a 12-hour light/dark cycle with a regular feed and unlimited access to tap water(Kennard et *al*., 2021). The mice were then allowed to acclimatize for a week before being used in the study (Kim *et al*., 2020)..

3.4. Extraction procedure

3.4.1. Crude extract preparation

When collecting the plant material, latex gloves were used to minimize the risk of contamination. The leaves were dried under shade. After that, the air-dried leaves were hand chopped and then ground using an electrical grinder. An electronic balance was used to weigh a sample of

powdered leaves, which was then subjected to a three-day cold maceration with an equivalent volume of 80% methanol with frequent shaking and stirring using a mechanical shaker. The supernatant was filtered using What Man filter paper no-1 after 72 hours (Belayneh *et al.,* 2019). For thorough extraction, the residue/marc was then re-macerated twice using the same volume of solvent. The resultant filtrates were collected, mixed, and concentrated under decreased pressure using a rotary evaporator at 40°C. After that, the extract was dried in a hot oven at 40°C. Until used in bio-screening investigations, the concentrate was kept in sealed containers at 4°Ctemperature.(Yirga Adugna *et al.*, 2022).

3.4.2. Alkaloid extracts preparation

To remove any moisture, the gathered leaves were thoroughly cleaned and dried (Chemistry, 2014). Low-temperature drying techniques or air drying were used (Aldeen, 2021). The powdered extract was then moistened with lime water (Elisabeth *et al*., 1996,followed by shaking the extract with a chloroform(Yubin *et al.*, 2014).Then after, the organic layer is separated and concentrated ((Kurek,2020).This process separated alkaloids as their salts (in the aqueous layer) from most other impurities, which remain in the organic layer (fraction at the bottom of the funnel) was used as the non-alkaloid extract (Pharmacognosy, 2012).

Calculating the extraction yield: The formula was used to get each extract's percentage yield.

Yield percentage = W2-W1/WO*100

where W1 is the weight of the container alone, W0 is the weight of the dried plant material, and W2 is the weight of the dried extract and the container(Varughese and Tripathi, 2013)

3.5. Acute toxicity studies

The mice were allowed to rest for six days before the experiment began to ensure that they were well-acclimated to their surroundings. They were housed in plastic mice cages with water and pellet meal *ad libtum*, and they were kept in regular light and dark cycles at room temperature (*Wasihun et al.,* 2023).

According to Organization for Economic Cooperation and Development (OECD) standard No. 425, mice aged 6 to 8 weeks were chosen for the acute toxicity test(OECD 425, 2022).For the purpose of conducting an acute toxicity test, the dosage of crude extracts was tapered to lower dosages, with 1000 mg/kg being ingested. However, because the toxicity report for these

extracts had not observed, the acute oral toxicity of the alkaloid and non-alkaloid extract *C.aurea* was assessed in mice at a high dose of 2000 mg/kg.

Five female mice, ages 6 to 8 weeks, were chosen at random, fasted for four hours, and then continued to fast for an additional two hours following the treatment (OECD 425, 2022). One mouse received the initial dose of 1000 mg/kg, and four other mice received the same dose when no mortality was noted within 24 hours Consequently The mice were watched for a full day and then again for a further fourteen days.

Furthermore, ten randomly selected female mice aged 6-8 weeks were divided into two groups (n=5).Group I received 2000 mg/kg alkaloid extract, while group II received the non-alkaloid extract . All mice were fasted for four hours before to extract delivery, and they continued to fast for an additional two hours following the administration. One mouse received the initial dose of 2000 mg/kg, and four other mice received the same amount when no mortality was noted within 24 hours. The mice were examined for four hours straight during the study, with 30-minute breaks during the first twenty-four hours. The main goal of the observations was to discover poisoning signs and symptoms, such as abnormal colors of the skin and fur, tremors, convulsions, salivation, diarrhea, coma, and death. The observation period lasted for 14 days in total(OECD 425, 2022).

3.6. Ant diabetic effect screening

3.6.1. Blood collection and Measurement of blood glucose level

Mice's tail veins were cut for blood samples in each animal model by cutting the tail tip with scissors. In order to avoid infection, cotton was used to apply 70% ethanol onto the tip of the tail. At each measurement, BGL was measured using test strips and a glucometer (Prodigy, America)(Ayele *et al.*, 2021). After taking three measurements, the average was determined. BGL from animals fasting for eight hours was used as the FBG level in the STZ model, particularly for the analysis of the repeated dosing effect (Birhan *et al.*, 2019).

3.6.2. Hypoglycemic effect on Normoglycemic model

Before the experiment, the mice were fasted for the whole night. The test groups were given with crude extract at 50 mg/kg (CA50), 100 mg/kg (CA100), and 200 mg/kg (CA200) doses while100

mg/kg (ACA100), 200 mg/kg (ACA200), and 400 mg/kg(ACA400) of the alkaloid extract as well as 100 mg/kg (NACA 100), 200 mg/kg (NACA200), and 400 mg/kg (NACA400) of the non-alkaloid extract were administered (Yirga Adugna *et al*., 2022). The positive control group was given Glibenclamide, while the negative control group was given distilled water. The plant extract's effects were contrasted with those of the control groups. After administering extract or water for 0, 30, 60, 90, 120, and 180 minutes, blood samples were taken from the test and control animals. Using a glucometer, the blood glucose level in mice obtained from their tails was determined (Du *et al*., 2016). Percent reduction was determined using the formula given below:
Percent Reduction = [(Initial Blood Glucose - Final Blood Glucose) / Initial Blood Glucose] x 100

3.6.3. Assessment of anti-hyperglycemic activity in oral glucose tolerance test

The oral glucose tolerance test (OGTT) was conducted in accordance with the previously mentioned protocol(Ayele *et al.*, 2021). Eleven groups of six mice each were formed from the grouped fed mice. Subsequently, Group I was given distilled water (2 milliliters per 100 grams of body weight) as a negative control, whereas Group II was given glibenclamide (5 milligrams per kg) as a positive control. Group III-XII were given the same doses of the extract as mentioned above. 30 minutes after each treatment, 2 g/kg of glucose solution was administered orally. At 30, 60, and 120 minutes, blood samples were obtained aseptically from the mouse tails.

3.6.4. Streptozotocin-induced model

streptozotocin (STZ) results in full beta cell necrosis and induces diabetes within 48 hours.STZ was given to the mice at a high dose in order to cause diabetes. To ascertain the ideal dosage, a pilot research utilizing three distinct dose levels (150 mg/kg, 180 mg/kg, and 200 mg/kg) was carried out. Following assessment, the dosage of 180 mg/kg was chosen because it produced superior outcomes. The mice were allowed access to water during a 6-hour fast prior to receiving the STZ injection. After that,STZ was dissolved in 2.94% sodium citrate buffer, adjusted at pH 4.5, to a final concentration of 0.1M. Immediately after preparation, the STZ solution was injected i.p. into the mice at a dose of 180 mg/kg.All of the mice were given pellet food and unrestricted access to water after receiving STZ. Following a 4-hour fast, the mice's blood glucose levels were assessed using a glucometer after 72 hours. Diabetes was classified as having blood glucose levels higher than 200 mg/dl in mice. After that, eleven sets of STZ-

induced diabetic mice were created, comprising nine test groups, one positive control group, and one negative control group (Cohrs *et al.*, 2020).

3.7 Single dose study:

Effect of single dose of the extracts was carried out on STZ induced diabetic mice.The experiment was conducted according to the method described in previous study (Ayele *et al*2021).Effect of single dose of all the extracts was carried out on STZ-induced diabetic mice (Ayele *et al*., 2021). After fasting for 14h, blood samples were collected at 0 h(just before treatment) 1, 2, 3 and 4 h after the administration of the extract to the respective groups (Alema *et al.*, 2020).

3.8 Repeated doses study

In this work, diabetic mice induced with STZ were used to assess the antihyperglycemic efficacy of crude, alkaloid, and non-alkaloid extracts. For three weeks, the diabetic mice were split into groups and given distilled water (DW), a common medication, or various dosages of the extracts. A control group without diabetes, however, was given simply DW. Throughout the course of the three weeks, fasting blood glucose (FBG) levels were measured in order to evaluate the extracts' potential to reduce blood glucose.The blood glucose lowering effects of extract was then determined by measuring FBG every seven days for three weeks. After taking a baseline blood glucose level measurement prior to commencing treatment, blood glucose level was determined the first (7^{th} day), second (14^{th} day) and third (21^{st} day) week following fasting for 8 h using aglucometer. . (Ayele *et al*., 2021).

3.9 Determination of body weight

The effects of STZ on body weight reduction and enhancement of body weight change by extract and standard were identified. Before treatment (day 0) and during treatment, that is, the first, second, and third weeks of therapy, the body weights of all treated groups and the mice in the control group were measured. The mice's body weight was measured using an electronic balance, and their weight was given in grams(Tefera *et al.*, 2020).

3.10 Ethical considerations

The experiment was carried out in accordance with ethical principles and approved by Addis Ababa University, college of health sciences, faculty, school of pharmacy ethics committee with Reference number: IRB/347AP/15/2023.

3.11 Statistical analysis

All statistical analyses were performed using the statistical program for the social sciences, (SPSS), version 25. One-way and two-way analysis of variance (ANOVA) was used to examine statistical differences between groups for the *in vivo* antidiabetic effect, and the Tukey post hoc test was then used to assess the results. The purpose of this test was to determine how extract affected body weight and BGL reduction over time. P-values of less than 0.05 were regarded as statistically significant, and all data were reported as mean ± standard error mean (SEM).

4. Results

4.1. Percentage yield

The percentage yield (w/w) of 80% methanol, alkaloid, and non-alkaloid leaf extracts of *C.aurea* was determined to be 18.5%, 17.2% and 8%, correspondingly.

4.2. Acute toxicity study

In the current study, graded doses of both alkaloid and non-alkaloid extracts (up to a dose of 2000 mg/kg) were administered to mice within 24 hours without causing any overt toxicity, as evidenced by no change in autonomic activities such as urination and defecation or in behaviors such as alertness, restlessness, irritability, or fearfulness. In addition, no delayed deaths occurred during the 14-day observation period, suggesting that the medium lethal dose (LD_{50}) in mice is higher than 2g/kg body weight. However, the same dose the crude extract resulted death in 20% of tested female mice. In subsequent test, the crude extract was administered at a dose of 1000 mg/kg and the results showed that it had no effect on mortality during the observation period and the 14-day follow-up

4.3. Hypoglycemic effect of the extracts

4.3.1. The crude extract

There was no noticeable variation in BGL between the groups at zero time, according to the results of the crude extract's effect on BGL in normal mice, which are shown in Table 1 A two-way ANOVA conducted between-group analysis showed a significant difference ($p<0.01$) in the BGL drop pattern among the groups. Every subsequent measurement showed a percentage reduction for all groups, with the negative control group demonstrating smaller drop overall. In addition, the glibenclamidetreated group saw the largest decline from the second hour to the completion of the trial when compared to the control groups. Nevertheless, no significant difference was found on BGL ($p>0.05$) in the study conducted within groups for all time differences (1-4 hours).

Table 1: Hypoglycemic effects of 80% methanol leaves extract of *Calpurnia aurea* on blood glucose levels in normal mice

	Blood glucose level in mg/dl at different time points					
Group	0 h	1h	2 h	3 h	4 h	percent reduction(GR %)
TW 80	82.67±5.93	81.83±4.04	78.33±±4.46	72.67±8.66	80.37±8.05	
GLB5	89.33±5.42	80.67±4.34	65.33±1.08	53.87±2.12$^{a3}_{e3}$	49.50 ± 2.14^{e3d3}	44.57%
MCA50	85.5±3.12	76.5±1.91	70.83±3.47	64.83±2.65	61.54±2.98^{a1}	28.82%
MCA 100	87.83±4.54	80.5±3.21	71±3.30	63.67±3.21^{a1}	70.5±4.98$^{c}_{2a2}$	19.91%

Key Notes: Each value represents mean ± SEM, n=6 for each treatment. a -Compared to the negative control, b-compared GLB5 mg/kg, c- compared to lower dose of MCA, d-compared to middle dose of MCA ,e- compared to high dose of the extract 1p<0.05, 2p<0.01 and 3p<0.001. Abbreviations: MCA; Methanol extract CA; GLB5, glibenclamide, PR (GR %), glucose level reduction

Table 2**:** Hypoglycemic effects alkaloid and non-alkaloid leaf extract of *CA* on blood *Glucose level in normoglycemic mice*

Blood Glucose Level (Mg/dl)

Treatment	0min	30min	60min	90min	120min	180min	P.R
TW 80	182±4.77	187±7.48	190±7.6	195±8.21	192±9.6	142±5.68	
GLB 5	175±5.25	165±4.95	130±6.5^{a3c3e2}	115.4±4.6^{e2h3}	116±5.6^{g3e3}	115.2±6.9^{e3a3}	34.28
ACA100	171±5.13	182±9.1	179±8.95	191±5.73	181±5.43	166±4.98	2.92
ACA200	185±5.55	195±9.75	192±9.6	185±9.3	170±5.1^{a1}	175±5.25^{a2b2}	5.4
ACA400	172±8.45	197±9.85	187±3.74	178±3.5	175±3.51^{c2}	170±5.16^{a3c3}	-4.3
NACA100	184±9.2	192±9.6	191±5.73	185±7.4	162±8.1^{a2}	160±8.4^{a1}	13.05
NACA200	167±8.35	191±9.55	183±9.15	171±6.84	162±3.24	152±3.04^{a3}	9
NACA400	172±3.44	180±5.24	178±5.34	192±3.84^{a1}	185±3.7^{a1}	165±3.43 ^{a3f2e1}	4.06

Key Notes: Each value represents mean ± SEM, n=6 for each treatment. a -Compared to the negative control, b-compared to GLB5 mg/kg, c- compared to 100 ACA, d,compared to 200 ACA, e- compared to 400 ACA f –compared to NACA 100,g-compared to NACA 200 mg/kg , h-compared to NACA 400 mg/kg . 1p<0.05, 2p<0.01 and 3p<0.001. Abbreviations: ACA; Alkaloid extract of CA, NACA; Non alkaloid extract of CA; GLC, glibenclamide

4.3.2. The alkaloid and non-alkaloid extract

One hour after treatment and at zero time, neither the ACA nor the NACA appreciably reduced BGL in normal mice at any dose. The results of the group analysis showed that there was no significant difference across all time points ($p>0.05$), although the middle (200 mg/kg) and higher (400 mg/kg) doses of extracts showed an inconsistent decrease in BGL during the experiment. The 100 mg/kg dose of both extracts did not have any effect. After 60 minutes of administration, BGL decrease in the GLB treated groups (Table 3).

The blood glucose levels of glibenclamide-treated mice were significantly lower (34.28%) than those of the untreated group. After three hours of non-alkaloid leaf extract of CA administration, the lowest dose taken, 100 mg/kg of extract, suppressed blood glucose by 13.05% ($p<0.05$). In contrast, after three hours of exact administration, the doses of NACA 200 mg/kg and NACA 400 mg/kg body weight were found to exert a glucose-lowering effect by 9% ($p<0.05$) and 4.06 % ($p<0.01$), respectively. On the other hand, the evaluation of glucose decrease in the alkaloid extract-treated normal animal groups showed that the extract has a low risk of hypoglycemia effect at 2.92 ($p<0.05$), 5.4% ($p<0.01$), and - 4.3% for 100, 200, and 400 mg/kg of body weight extract, respectively.

4.4. Anti-hyperglycemic effect in glucose loaded mice

4.4.1 Crude extract

After the administration of glucose, all groups experienced hyperglycemia after 30 minutes, with the negative control group showing the highest increase of 145.56%. Although the treatment resulted in a reduction in blood glucose levels at 30 minutes compared to the negative control group, only the standard substance achieved a significant reduction ($p<0.05$)the standard substance also produced significant reductions at 60 minutes ($p<0.01$) and 120 minutes ($p<0.001$). the result from analysis of between group revealed there is no significant difference in BGL across groups and within group analysis showed that no statically measured difference observed across all time points ($p>0.05$) the effect of glucose reduction recorded for MCA 200 and MCA400 sustained up to 120 minutes, whereas the lower dose lost its effect. Result summarized (Table 3)

Table 3: Hypoglycemic effect of *CA* crude leaves extract on Oral glucose loaded mice

Blood glucose level in mg/dl					
Group	**0min**	**30 min.**	**60 min.**	**90 min.**	**120 min.** %BGL30 min
TW 80	73.67±5.71	184± 6.6	111± 5.23a2	90.83± 5.38a1	89.67± 2.8 145.56%
GL5	61.83±4.12	102.5± 5	100.33±3.9	67.17±2.87	45.83±6.26[a3 d3] 66.67%
MCA50	63.67±2.4	134.67± 7.1	98±9.15a1,b 2	93.17± 2.39	82.33± 3.45[a1] 111.63%
MCA100	74.33±10.3	186.83± 6.9	100.83±5.84 b3	73.33±4.1[c1]	72.67± 4.14[a2c1] 114.36%
MCA200	63.17±6.76	115.33±15.07	116.33± 3.5	95.5± 1.45	87.67 ±2.23 [a3b2] 82.5.7%

Key Notes: Each value represents mean ± SEM, n=6 for each treatment. a -Compared to the negative control, b-compared GLB5 mg/kg, c- compared to lower dose of MCA, d-compared to middle dose of MCA ,e- compared to high dose of the extract 1p<0.05, 2p<0.01 and 3p<0.001. Abbreviations: MCA; Methanol extract CA; GLB5, glibenclamide, PR (GR %), glucose level reduction

4.4.2 Alkaloid and non-alkaloid extracts

Table 5 illustrates that, at the 30-minute mark of the test, the alkaloid leaf extract significantly increased blood glucose levels. This increase was comparable to that of the non-alkaloid leaf extract, with the biggest increment with NC (138.5%) occurring after glucose was loaded. An examination of the groups revealed a noteworthy decrease in glucose levels over time ($F(2) = 3.53$, $p<0.01$), primarily following the 120-minute experiment. Additionally, mice administered 200 mg/kg and 400 mg/kg of alkaloid leaf extract demonstrated a significant reduction in blood glucose levels at the 120-minute test time point ($p<0.01$) (82±9.3, 80±4.21) with a dose-dependent effect. In contrast, no significant difference was seen between group analysis ($F(2) = 70.34$, $p>0.05$). Each group's BGL was recorded at the 30-minute time .However, mice treated with the standard drug (GLB5mg/kg) of body weight significantly reduced BGL at 60 and 120 min ($p<0.01$) (114±8.25, 65.4±4.6) respectively, compared with the rest test groups and negative controlled group results revealed that pattern of glucose tolerance was more improved in extract treated mice than the untreated ones.

Table 4: Hypoglycemic effect of *CA* alkaloid and non-alkaloids leaves extract

	Blood Glucose Level (mg/dl)				
Treatment	0 min	30 min	60min	120min	%BGL30 min
TW 80	92.3±5.12	220±9.42	200±13.4	125±6.21	138.35%
GLB (5mg/kg)	90.2±4.13	165±10.65	114±8.25^{a3e3h3}	65.4±4.6^{a3e3}	29.71%
ACA100mg/kg	93±4.52	167±8.01	153±9.15	75±3.73^{a2}	79.56 %
ACA200mg/kg	94.3±3.43	150.2±9.75	140±9.2^{a2}	82±9.3^{a2g1}	59.57%
ACA400mg/kg	97.2±5.35	145±9.85	133±13.74	80±4.21^{a3b2g2h2}	49.48%

NACA100mg/kg	89±1.09	165.12±5.7	175±8.73	105±5.25[a1]	85.39%
NACA200mg/kg	90.1±8.35	191±9.55	162±9.15	85±4.[a2]	88.8%
NACA400mg/kg	101±3.44	180±5.24	217±5.34	89±4.91[a3]	65.16%

Key Notes: Each value represents mean ± SEM, n=6 for each treatment. a -Compared to the negative control, c- compared to 100 ACA, d,compared to 200 ACA, e- compared to 400 ACA f –compared to NACA 100,g-compared to NACA 200mg/kg , h-compared to NACA400mg/kg . 1p<0.05, 2p<0.01 and 3p<0.001. Abbreviations: ACA; Alkaloid extract of CA, NACA; Non alkaloid extract of CA; GLC, glibenclamide

4.5. Anti-hyperglycemic effect of CA

For the purpose of induced diabetes, 150 mice were used; 60 of the mice were used for research involving a single dose, and the remaining 60 for trials involving multiple doses. 86 of these mice (a success percentage of 57.3%) had diabetes successfully produced. Eight of the diabetic mice were removed from the study because their fasting blood glucose (FBG) levels were higher than the glucometer's detection limits (BGL>600 mg/dl).

Furthermore, six mice passed away prior to the induction of diabetic mellitus (DM). At the end of the first week of the trial, two animals from the MCA 200 group and two from the negative control group had perished due to their circumstances. Nevertheless, this loss was offset by inducing more mice in accordance with the prescribed procedure.

4.5.1. Crude extract

There was barely any initial BGL difference between the diabetic groups following the induction of DM. The FBG level was consistently greater in the negative group (mean value >200 mg/dl) than in the normal control group. The study conducted between the groups showed a significant difference ($F(2) = 4.3$, $p<0.05$) in the FBG level at the third and fourth time points when compared to the negative control group.

The extract 200 mg/kg doses considerably lowered the FBG level ($p<0.05$). Similar to the plant extract treated groups, GL5 significantly decreased the FBG level at the 2nd ($p<0.05$), 3rd ($p<0.05$), and 4th ($p<0.01$) hours, but there was no significant difference in BGL at any time

points. However, there was no statistically significant difference in FBG level reduction at all-time points when extract treated groups compared with one another ($p>0.05$). While the negative control group was diabetic at all-time points, GLB5 and MCA200 tended to bring BGL back to the normal control levels at the 4th h. but MCA 50, 100 lost to produce this effect.

Table 5 : Antidiabetic effects of 80% methanol leaf extract of *CA on* blood glucose level in mice

LEVEL IN MG/DL

Group	0 hr.	1 hr.	2 hr.	3 hr.	4 hr.	R^2
TW 80	249.2± 2.87	197.23 ± 4.49	119.32 ± 4.28	186 ± 6.02	190.3 ± 1.92	
Glb5	252.66±3.89	94.16±3.1	97.17±3.12	$104.6\pm3.45^{a3cd3}_{e3}$	$102.5\pm4.13^{d}_{3}$	
CA50	250.33±4.47	96.17±2.3	100.67±3.1	172.1 ± 2.85^{a2}	$162.17\pm3.3^{a}_{1}$	0.68
CA100	256.2±4.83	85.5±1.91	194.17±1.9	181.2 ± 2.52^{a2}	156.17 ± 2^{a2c2}	
CA200	254.3 ±6.18	87.4±1.44	197±3.39	163.8 ± 1.77^{a2c2}	$135\pm4.47^{a3c3}_{d2}$	

Key Notes: Each value represents mean ± SEM, n=6 for each treatment. a -Compared to the negative control, b-compared GLB5 mg/kg, c- compared to lower dose of MCA, d-compared to middle dose of MCA ,e- compared to high dose of the extract 1p<0.05, 2p<0.01 and 3p<0.001. Abbreviations: MCA; Methanol extract CA; GLB5, glibenclamide,

4.5.2. Alkaloid and non-alkaloid leaf extract of *CA*

A. Single dose studies: Group analysis between each group revealed a significant difference in BGL between the diabetic and non-diabetic groups when DM was induced ($F(2)=6.02$, $p< 0.001$). There was no first significant difference in BGL between the diabetic groups after DM was induced. The negative group's FBG level was consistently greater ($p<0.01$) than that of the normal control group, with a mean value of over 200 mg/dl. The extract containing ACA200 and

ACA 400 mg/kg doses considerably ($p<0.05$) decreased the FBG level at 4th hour (192±5.21, 168 ±3.51), according to group analysis. Significant reduction in glucose was achieved with a low dose of alkaloid and non-alkaloid extracts ($F(2) = 3.04$, $p<0.01$).The outcome is compiled in

Table 6: Anti-hyperglycemic effects of single dose of alkaloid and non-alkaloid leaf extract in streptozotocin-induced diabetic mice

	Blood Glucose Level (mg/dl)						
G	Initial	1st hr.	2nd hr.	3rd hr.	4th hr.	%GR	R^2
NOC	273.4±1.431	293±0.93	288.6±0.86	283.3±8.21	282±9.6		
NC	270.4±9.25	275±14.95	268±13.5	263±14.6	267±15.6	1.25	
GLB5mg/kg	285.6±9.13	241.2±12.54	191.2±11.47	180±13.15^{n} 3e3h3	147±8.83^{a3e3}	48	
ACA100mg/kg	272.4±13.55	256.3±12.4	249.3±18.9	232±15.73^{a} 1n1	213.7±5.4^{n2a3}	21.5	0.9
ACA200mg/kg	275.3±13.75	235.3±17.75	215±12.6	186.3±9.3^{a} 2n1	192±5.21^{a2n1}	30	47
ACA400mg/kg	284.3±9.2	235±18.85	187±13.74^{a3}	165.3±3.56 a3n3	168±3.51^{a3n3c3}	40.8	
NACA100mg/kg	287.4±8.35	245.7±11.65	210.4±5.73	190.4±7.4^{a} 1n1	231.5±8.1^{a1}	19.2	0.6
NACA200mg/kg	285.4±14.5	252.17±9.55	240.1±9.15	211±16.84^{a} 1	235±13.24^{n2}	17.5	24
NACA400mg/kg	280.3±13.44	97.26±15.24	242±15.34	230±13.84^{n} 2	200±13.7^{a3n3}	28.67	

Key Notes: Each value represents mean ± SEM, n=6 for each treatment. a -Compared to the negative control n- compared to NOC, c- compared to 100 ACA, d,compared to 200 ACA, e- compared to 400 ACA f – compared to NACA 100,g-compared to NACA 200mg/kg , h-compared to NACA400mg/kg . 1p<0.05, 2p<0.01 and 3p<0.001. Abbreviations: ACA; Alkaloid extract of CA, NACA; Non alkaloid extract of CA;,glibenclamide-GLC

Table 7*:*Anti-hyperglycemic effects of repeated dose of alkaloid and non-alkaloid leaf extract of *Calpurinaaurea* in streptozotocin-induced diabetic mice

	Blood Glucose Level (mg/dl)					
Treatment	Day0	7 days	14 days	21 days	PR	R^2
NOC	267±3.63	242±2.93	235±2.86	231±2.21^{n2}		
TW80(NC)	264.5±10.25	276±11.95	278±13.5	270±10.6	-2.08	
GLB5mg/kg	265±9.13	226±9.26	183±11.47^{e3n3}	176±13.15^{e3n3h3g3}	33.58	
ACA100mg/kg	263±13.55	250.3±12.4	237±9.91	149±15.73^{a3}	16.34	
ACA200mg/kg	270.3±12.7	245.3±17.75	222±11.6	204.5±9.3^{a2n1}	24.2	
ACA400mg/kg	264.3±9.2	227±18.85	206±13.74	189±9.56^{a3n3c3d3}	28.8	.963
NACA100mg/kg	272.4±8.35	260.7±11.65	252.4±5.73	250±17.4^{a3}	8.08	
NACA 200mg/kg	274.4±14.5	245.17±9.55	232±9.15	220±11.84 ^{a2n2}	10.38	
NACA 400mg/kg	275.3±13.44	230.26±15.24	215.1±15.34	184±5.84^{a3n3}	11.18	

Key Notes: Each value represents mean ± SEM, n=6 for each treatment. a -Compared to the

negative control n- compared to NOC, b-compared to GLB5 mg/kg, c- compared to 100 ACA, d, compared to 200 ACA, e- compared to 400 ACA f –compared to NACA 100,g-compared to NACA 200 mg/kg , h-compared to NACA 400 mg/kg . 1p<0.05, 2p<0.01 and 3p<0.001.

Abbreviations: ACA; Alkaloid extract of CA, NACA; Non alkaloid extract of CA; GLC, glibenclamide

Although there was no statistically significant difference in the baseline BGL of the diabetes

groups compared to the normal control, between-group analysis showed that the baseline BGL of the diabetic groups was considerably higher. The effect of the leaf extract was measured by measuring the weekly FBG level after repeated doses. There was no initial FBG difference among the diabetes groups, and between-group analysis revealed no significant difference between the tested groups ($F(2) = 16.62$, $P > 0.05$) (Table 8).

As the effect of extract was compared with the negative control group, the medium dosage and higher dose of alkaloid leaf extracts ($p<0.01$) were found to be considerably higher ($p<0.001$) in the negative group compared to the normal control group, with a mean value >200 mg/dl at all times. Began to dramatically lower the FBG level in the second week; the percent reduction was 24.2, 28.8%. Whereas,GL5 considerably ($p<0.001$) lowered the FBG level at the second and third week, and the percentage of reduction observed was 33.58%, which was the greatest compared to all diabetic control group treated extracts. The reduction was sustained up to the third week with all doses of the extract ($p<0.01$). Analysis of the group results showed that there was a significant change noticed throughout the course of the weeks ($F(2) = 4.21$, $P<0.001$). A dose-dependent impact was observed at all times, indicating a substantial difference between the various alkaloid extract mean dosages.

4.6. Effect of leaf extract on body weight change:

When compared to the normal control group, the diabetic control group showed a statistically significant decrease in body weight on days seven and fourteen of treatment due to STZ-induced diabetes (Table 9). When compared to the diabetic control group treated with DW, the body weight of the diabetic mice increased significantly after 14 days of treatment with all (100,200 and 400) doses of ACA and NACA and GLC. When compared to the baseline body weight, the diabetic control group experienced a significant ($p<0.01$) reduction in body weight on the fourteenth day of treatment, additionally ,the results of the within -group analysis showed that the diabetic control group had a significant ($p<0.01$) reduction in body weight by the 14th day of treatment when compared to the baseline body weight, while the normal control, the extract

treated groups and GLC treated groups did not show any significant changes in body weight when compared to their respective baseline body weight at any point during the treatment .result are summarized (fig2)

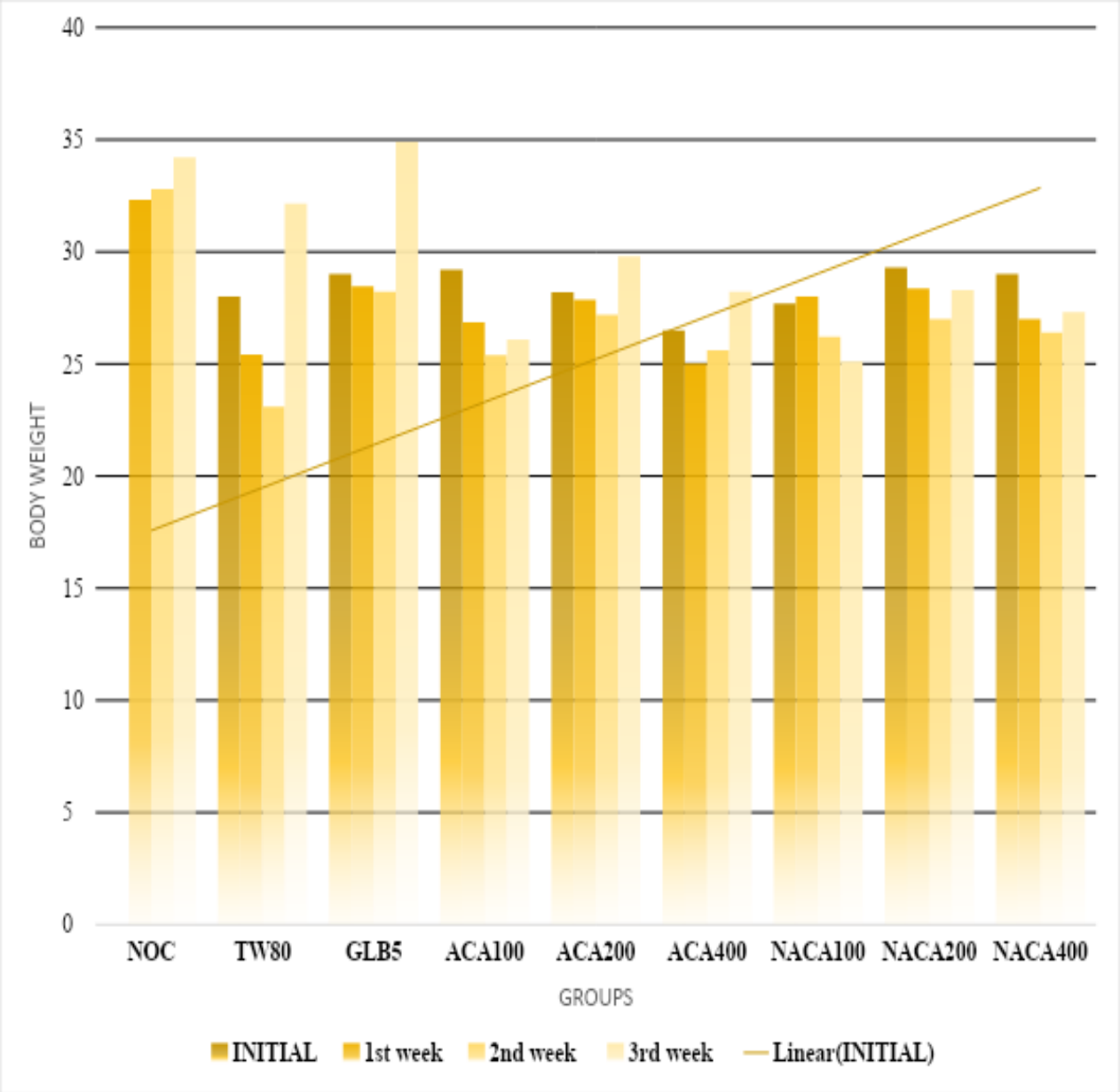

Figure 2: Effect of the alkaloid and non-alkaloid leaf extract of *Calpurnia aurea* on weekly body weight change of diabetic mice

5. Discussion

Diabetes mellitus is defined by a loss of glucose homeostasis brought on by abnormalities in insulin secretion. Insulin action also causes a decrease in the metabolism of glucose as well as other fuels that provide energy, like lipids and proteins (Roep *et al.,* 2021). The major goal of the current study was to identify which extract has a stronger antidiabetic impact. In-depth analyses of each model were conducted, and experiments were conducted to assess the models' effects on blood glucose levels and weight changes. Thus, extracts from powdered plant materials were made using organic solvents prior to the start of each experiment.

In the crude extract preparation methanol 80% was used because methanol is a universal solvent and it has low boiling point ,higher volatility and its higher extraction efficiency (Melese *et al.*, 2019) . The study done by Eyasu*et al* ,belay *et al* was also used similar extraction method (Eyasu *et al*., 2013);(Belayneh *et al.,* 2019) percent yield recorded was 11.7% (from leaf extract) and 13.6% (from seed extract) for eyasu*et al* , Belayneh *et al* respectively but in this study high yield was obtained which is 18.5 % .Alkaloid leaf extract specifically refers to an extract obtained from the leaves of *Calpurinaaurea* that is enriched in alkaloids(Chemistry, 2014).

Non-alkaloid leaf extract refers to an extract obtained from the leaves of *CA* after removing the alkaloids present in the crude extract. Lime water was used in the extraction of alkaloid from the crude extract to liberate the free bases if present as salts and combines with tanins ,acid or other phenolics (Ali Yilmaz *et al.*, 2012) also the study of Alias *et al* suggested that lime water used to facilitate maximum effective contact of the solvent with the ruptured alkaloid bearing tissues and cell (Alias *et al.,* 2010). The acid extract underwent agitation with chloroform to eliminate pigments and other unwanted impurities (Djilani*et al*., 2006).

There were no outward indications of extreme toxicity in the experimental mice's appearance or behavior, such as lacrimation, appetite loss, tremors, hair erection, salivation, or diarrhea. These findings are in line with the recommendations made by the OECD after administration of the Alkaloid and non- alkaloid extract at a dose of 2g/kg did not lead to any mortality within the first 24 hours and during the subsequent 14-day observation period. The physical and behavioral observations of the experimental mice also showed no visible signs of excessive toxicity, such as

lacrimation, loss of appetite, tremors, hair erection, salivation, or diarrhea. These observations align with the guidelines provided by the OECD (OECD 425, 2022).

These findings suggest that the LD_{50} of the leaf extract is higher than 2g/kg, which is in contradiction with previous study conducted by Belayneh that reported mortality in mice exposed to seed extracts and suggested an LD_{50} greater than 2000mg. However, the safety of the leaf extract is supported by earlier research conducted by *Ayal, Desse, Umer, and Birhanu*. These studies investigated the wound healing activity (Ayal *et al.,* 2019), Antioxidant and Antimicrobial Activity of Solvent Fractions of *Calpurnia aurea*(Belay *et al*., 2021) , Antimalarial activity (Z. *et al*., 2015) , Antidiarrheal and antimicrobial activity(Umer *et al.,* 2013) respectively .

The first model used to test hypoglycemic activity was the hypoglycemic effects of crude, alkaloid, and non-alkaloid leaf extract of CA in normal mice. This model evaluates a test substance's potential to lower glucose levels (Du *et al*., 2016). The primary parameters were found to be blood glucose levels. Three measurements were made, and the average result was recorded.(Khan *et al.,* 2023). The mice given the crude, alkaloid, and non-alkaloid extracts of CA leaves did not exhibit any significant hypoglycemic impact, indicating a low risk of hypoglycemia. The methanolic extract of the *Calpurina aura* seed was used in a prior investigation on normoglycemic mice, but no discermible hypoglycemic effect was seen. previous study done ,*(Belayneh et al.,2019*) supports this study. In a clinical setting utilizing mice, the OGTT model which gauges the body's capacity to utilize glucose was used as a routine diagnostic technique to identify patients who may be borderline diabetic (Care and Suppl, 2022).

In the current study mice were fasted before glucose was administered in order to provide a stable baseline glucose level and remove variations brought on by food intake. Additionally, fasting increases insulin sensitivity induced by glucose (Marium *et al.*, n.d.). Oral loading was used to administer glucose. Although, intraperitoneal IP injection and oral glucose loading were both viable approaches, the oral approach was chosen because intestinal L-cells release gastrointestinal hormones{Formatting Citation}, namely GLP-1, in response to glucose absorption from the stomach(Lopes *et al*., 2021). In comparison to intraperitoneal injection, this improves glucose-induced insulin release and permits lower blood glucose levels (Andrikopoulos *et al.*, 2008). The extract's reported antidiabetic effect in the OGTT can be linked to its reduction

of the absorption of glucose. (Kokil *et al.*, 2010). While it took more than two hours for BGL to return to normal in NC, the extract and GL5 in this investigation brought BGL down to baseline in less than two hours. In animal models of OGTT, released insulin takes more than two hours to return the glucose level to normal. With an onset of about 80 and 120 minutes, respectively, GL5 and all doses of the alkaloid extract began to reduce BGL. With GLB5, the effect persisted for at least 90 minutes, while with moderate and high doses of the alkaloid extract, it remained for at least 1 hour. However, the effects of ACA100 and NACA BGL decreased were only felt for 30 minutes. Given that the alkaloid extract reduced blood glucose levels after glucose loading and that glucose causes the release of insulin, the extract's anti-hyperglycemic action may be due to an insulin-like action that either increases peripheral glucose uptake or increases β-cell sensitivity to glucose, which in turn increases insulin release.

The extract's OGTT antidiabetic effect may be related to its suppression of glucose absorption (Jacob, 2019).The ability to improve glucose tolerance might be due to other possible mechanism like stimulation of glycogenesis in liver, enhanced tissue glucose utilization, and decreased gluconeogenesis (Matheka and Alkizim, 2012).

The purpose of the other model was to evaluate the extract's anti-hyperglycemic effect on diabetes induced by streptozotocin. In order to examine the result of each extract and the glucose decrease pattern over time The results of the two-way ANOVA and turkey post hoc test (tables 5, 6, and 7) indicated that there was a significant difference between the groups and within the groups ($F(2)=6.02$ and 4,02 $p<0.05$, 0.01). This suggests that the concentration and time (minutes) of the alkaloid extract of *CA* may interact with mice's BGL levels, which may be one of the factors affecting glucose levels.

In the current work, STZ was used to induce diabetes .It is one of the most widely used chemicals to create diabetic mice. Prior to administering STZ, the mice were fasted. Since glucose and STZ can compete in a fed condition, animals that have fasted are typically more vulnerable to this chemical (King, 2012). STZ (2-deoxy-2-3-methyl -3 nitrosourea) is a broad antibiotic isolated in 1959 from *Streptomyces achromogenes* bacteria (Furman, 2021).It is the most common method of chemically inducing diabetes in animal models today(Al-Awar *et al.*, 2016).

Its mechanism is by DNA alkylating agent and is often used as both an antimicrobial and an anticancer drug (Wszola*et al.*, 2021). The chemical's harmful effects arise from its metabolic byproducts and the free radicals they produce, which ultimately lead to the death of pancreatic β-cells through DNA alkylation, mitochondrial system impairment, and O-GlcNA case inhibitor. It is a provider of nitric oxide (NO), and research suggests that NO plays a role in the deterioration of the beta cells in the Langerhans islets (Priya *et al.*, 2021).

The results of this study, which were in line with many previous studies, demonstrated that STZ resulted in chronic hyperglycemia and significant weight loss in experimental mice(Valayapathi*et al.*, 2019). The loss in body weight is due to the fact that polyphagic condition and loss of weight tends to promote excessive catabolism of fats and tissue proteins as an alternative energy source because of unavailability of carbohydrates(Furman, 2021). Mice with diabetes caused by modest doses of STZ showed enhanced body weight loss after oral administration of CA extract. Reduced body weight in diabetic mice may result from excessive tissue protein and fat breakdown brought on by insufficient insulin, which prevents carbohydrates from being available for utilization as an energy source(Cao *et al*., 2022).

CA extract-treated diabetic mice showed an improvement in body weight, which may have been attributed to increased metabolic activity and increased ability of the organism to maintain blood glucose homeostasis. Nevertheless, after three weeks of treatment, mice given 5 mg/kg of glibenclamide gained weight relative to the diabetic group, with *Calpurnia aurea* having a greater effect on normalizing weight. (Geremew *et al.*, 2022). This could be the result of glb slightly increasing mice's weight more than *Calpurnia aurea* alkaloid extract. Glb ability to reduce insulin resistance and hyperinsulinemia is what causes this weight gain impact (Balsells*et al.*, 2015).The drug's mode of action involves inhibiting ATP-sensitive K+ channels, which causes cell depolarization and insulin release, increased pancreatic activity in the liver, skeletal muscle, heart musclanism., and smooth muscle sites is likewise based on the same mechanism (Munkboel*et al.*, 2021).

In the current investigation, mice treated with Glb (5 mg/kg) showed effects on normal and diabetic mice caused by streptozotocin (STZ) that were both hypoglycemic (dropping blood sugar) and anti-hyperglycemic (reducing high blood sugar). By reducing inflammation and

neutralizing free radicals, non-alkaloidal extracts can protect pancreatic beta cells, improve insulin sensitivity, and mitigate complications associated with diabetes.

The previous literature highlights the potential of alkaloids found in medicinal plants as promising avenues for developing novel treatments for diabetes mellitus, through their ability to enhance glucose uptake by Inhibition of digestive enzymes (Adhikari, 2021) . The drug's mode of action involves inhibiting ATP-sensitive K+ channels, which causes cell depolarization and insulin release(Hui *et al*., 2009). According to a research by Behl and Adhikari, based on prior studies on plant alkaloids, Quinazoline alkaloids, notably vasicine and vasicinol from *Justicashimperiana*, are believed to block digestive enzymes, which explains the alkaloid extract's effectiveness in treating diabetes (Tiong *et al*., 2015).

Certain alkaloids have been discovered to inhibit the production of advanced glycation end products (AGEs), which are substances that arise from the non-enzymatic reaction between sugars and proteins, as well as digestive enzymes involved in the breakdown of carbohydrates, preventing sharp spikes in blood sugar levels, the production of oxidative stress and inflammation, they have a role in the development of diabetes complications (Shehadeh *et al.*, 2021). Alkaloids have the ability to slow the advancement of diabetes problems and enhance insulin release by preventing the creation of AGEs (Behl *et al.*, 2022).

From study of Asres *et al*the chemical investigations of *C. aurea* alkaloid extracts isolation resulted a series of quinolizidine alkaloids (two novel alkaloids 13a-trihydroxylupanine(digittine),4 adihydroxy 13a-O-(2’-pyrrolykarbonyl)-lupanine (calpaurine) leaves , lupanine which may be useful for supportive treatment of T2DM (Wiedemann *et al*., 2015) which might support our result the alkaloid extract might possessed dose dependent antihyperglycemic activity .According to Wiedemann *et al*.'s study, lupanine enhances glycemic management by lowering plasma glucose concentration. According to Korr the alkaloidal extract effect was dose-dependent, and potential mechanisms include lupanine directly modifies KATP channels to enhance glucose-stimulated insulin release , raises the expression of the insulin gene(Korir, 2012).in the study of Sukhih he tried to mention that alkaloids make up the majority of the responsible phytochemicals in medicinal plants with antidiabetic potential (Sukhikh *et al.*, 2023) .

6. Conclusion

The findings of the current study suggest that *Calpurnia aurea* crude, alkaloid and non-*alkaloid* extracts might contain bioactive chemicals with anti-hyperglycemic activity. The extracts can lower blood glucose level in chemical induced diabetes which upholds the traditional claim of the plant in the management of DM.

7. Recommendations

- Purification is recommended using different advanced technologies like HPLC to isolate and identify the exact molecules which are responsible for antidiabetic effect.
- To determine which extract has a greater antidiabetic effect, further comprehensive studies would be needed that involve evaluating their effects on blood glucose levels, insulin secretion, glucose uptake, and other relevant parameters in both in vitro and in vivo models.
- Additionally, identification and quantification of the specific active compounds present in each extract could provide insights into their contributions to the observed antidiabetic effects.
- Further research is needed to elucidate the precise mechanisms of action and identify the specific bioactive compounds responsible

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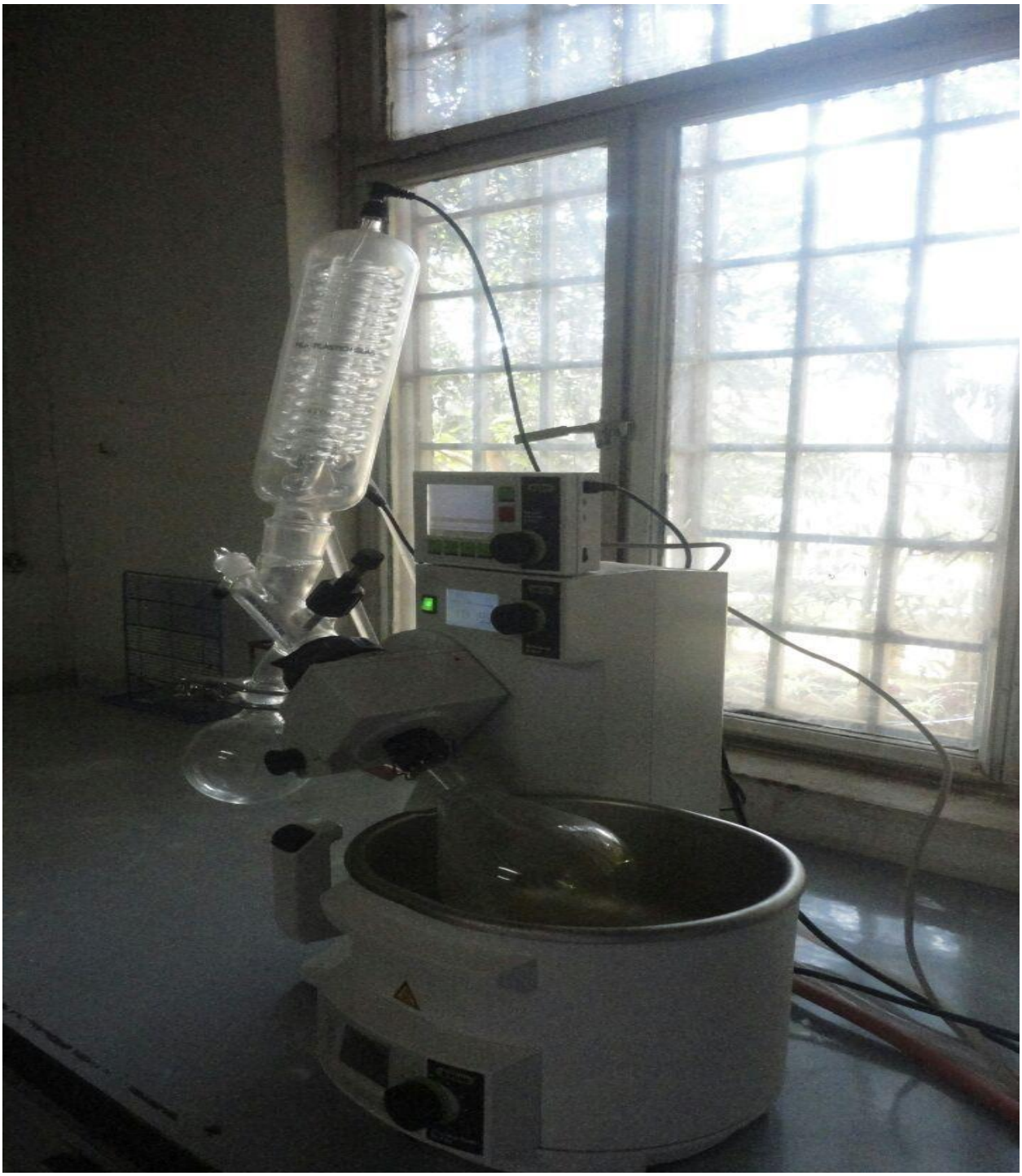
9. ANNEXES

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