Therapeutic Evaluation of *Cestrum diurnum* Extract for the Treatment of Diabetes Mellitus Type 2

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A thesis submitted in the partial fulfillment of the requirement for the degree of

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Ayesha Nawaz

Hafiza Ayesha Nawaz

Master of Science in Healthcare Biotechnology

Registration No. 00000330319

DEDICATION

All my effort is dedicated to
"My beloved family"
Especially my Father and Mother

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I would like to express my gratitude and admiration to all those who offered me the opportunity to complete this thesis. Bounteous praise for **Allah Almighty**, the Magnificent and Supreme, Whose Blessing, Greatness and Magnificence flourish my thoughts and all the Praise and Gratitude for the **Holy Prophet (PBUH)**, who is the thriving city of knowledge.

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Hafiza Ayesha Nawaz

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List of Acronyms

ADA	American Diabetes Association
DM	Diabetes Mellitus
FFA	Free Fatty Acids
GLUT	Glucose Transporter
IDF	International Diabetes Foundation
INSR	Insulin Receptors
IRSs	Insulin Receptor Substrates
MGWAS	Metagenome-Wide Association Study
mL	Milliliter
OGTT	Oral Glucose Tolerance Test
PI3K	Phosphatidylinositol 3-Kinase
T1DM	Type-1 diabetes Mellitus
T2DM	Type-2 Diabetes Mellitus
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
UPR	Unfolded Protein Response
VDR	Vitamin D Receptor
WHO	World Health Organization
µL	Microlite

Abstract

Type 2 Diabetes Mellitus (T2DM), a kind of Diabetes Mellitus (DM), is emerging as a challenging global epidemic. Hyperglycemia is the hallmark of T2DM and the major factors regulating the metabolism of glucose in the body are Insulin Production from pancreatic β-cells and Insulin Resistance (IR) of target tissues. Current therapeutic options pose certain side effects. After the ineffectiveness of chemically synthesized compounds, the world has come to explore natural resources for the purpose of treatment and pain alleviation once again. *Cestrum diurnum* is a shrub grown in several parts of the world for ornamental purposes and it is being in use since ages in traditional medicine. To investigate the anti-diabetic property of *Cestrum diurnum*, this study is had been directed. Phytochemical and biochemical assays were performed which reveals the in-vitro biochemical activities and phytochemical constituents of ethanolic extract of *Cestrum diurnum* leaves. To check the restorative impact of *Cestrum diurnum*, in-vivo study on BALB/c mice models was conducted. Mice were administered different doses of plant extract after diabetes induction. Diabetes was induced by combination of high fat diet and streptozotocin. Treatment with *Cestrum diurnum* was found to be significantly effective in managing hyperglycemia and improving glucose in mice models.

1 Introduction

1.1 Type-2 Diabetes Mellitus

Diabetes Mellitus (DM) is a chronic metabolic disorder associated with endocrine functioning and primarily characterized by hyperglycemia. It is emerging as one of the fastest spreading diseases worldwide affecting adults as well as children. Diabetes Mellitus is categorized into Type-1 diabetes Mellitus (T1DM), Type-2 Diabetes Mellitus (T2DM), and Gestational Diabetes (Cole & Florez, 2020).

T1DM is an early early-onset autoimmune form of DM characterized by destruction of beta-cells resulting in insufficient production of insulin from pancreatic beta-cells (Cole & Florez, 2020). Gestational diabetes is termed as glucose tolerance disorder which occurs during pregnancy. South Asian women are at higher risk of developing T2DM, and it is also associated with maternal obesity (Hills et al., 2018). T2DM is the major form of diabetes mellitus characterized by insulin resistance. T2DM is the most serious public health concern which is emerging as challenging epidemic globally. 90 percent of the cases of diabetes mellitus are of T2DM (El-Kebbi et al., 2021).

T2DM is associated with inefficient response of target tissues toward insulin which leads to insulin deficiency in body and hence, high blood glucose level. Various genetic, environmental, and metabolic factors are involved in pathophysiology of T2DM. These risk factors are also responsible for early onset of disease among adults and children (Galicia-Garcia et al., 2020). Multiple genes play role in dysfunctioning of pancreatic beta-cells and insulin resistance while, environmental conditions include sedentary lifestyle, improper diet, and obesity. Various other symptoms are associated with T2DM which involves polydipsia (thirst), polyphagia (overeating) and

polyuria (frequent urination) and weight loss. T2DM leads to several complications which mainly includes cardiovascular diseases, kidney malfunctioning, diabetic retinopathy, and nephropathy. Due to emerging epidemic of T2DM, the financial burden is increased specifically on developing countries like Pakistan affecting the quality of life and productivity of youth (El-Kebbi et al., 2021).

1.2 Epidemiology

T2DM is alarming and fastest growing public health concern specifically in underdeveloped countries it is highly associated with mortality and other health complications. According to IDF report of 2021, diabetes has affected 537 million people worldwide and it is predicted that by 2030, 643 million people and by 2045, 783 million people will be affected with diabetes mellitus. Most of the people affecting with diabetes belong to low-income and middle-income countries. Diabetes is also associated with higher mortality rate as 6.7 million deaths were caused be diabetes in 2021. T2DM is responsible for up to 90% of the total cases of diabetes. In the region of South-East Asia, 90 million are suffering from diabetes and around USD 10 billion has been spent on this disease in 2021 increasing the financial burden on these countries (Edward J Boyko, 2021).

South Asian countries like Pakistan, India, Bangladesh, Nepal, Bhutan, and Sri Lanka are at higher risk of developing T2DM. It has been demonstrated by several studies that South-Asians are more susceptible to T2DM than European and American population due to multifactorial, genetic, epigenetic, pathophysiological, and different lifestyle factors (Narayan et al., 2021). The first national diabetes survey in Pakistan was held in 1999 which reported the incidence of T2DM to be 11%. The diagnostic criterion of this survey was oral glucose tolerance test (OGTT) (Aamir et al., 2019).

The second national diabetes survey was carried out in 2018 by Aamir et al., 2019 which reported 16.98% prevalence of T2DM in Pakistan. The diagnostic criteria for this survey were based on hbA1c which is an applicable test accepted by WHO due to its accurate measurement. This survey indicates a huge population of diabetic and pre-diabetic group which is alarmingly rising in Pakistan. The development of T2DM is associated by various genetic and environmental factors including elevated blood pressure, BMI, waist circumference and smoking.

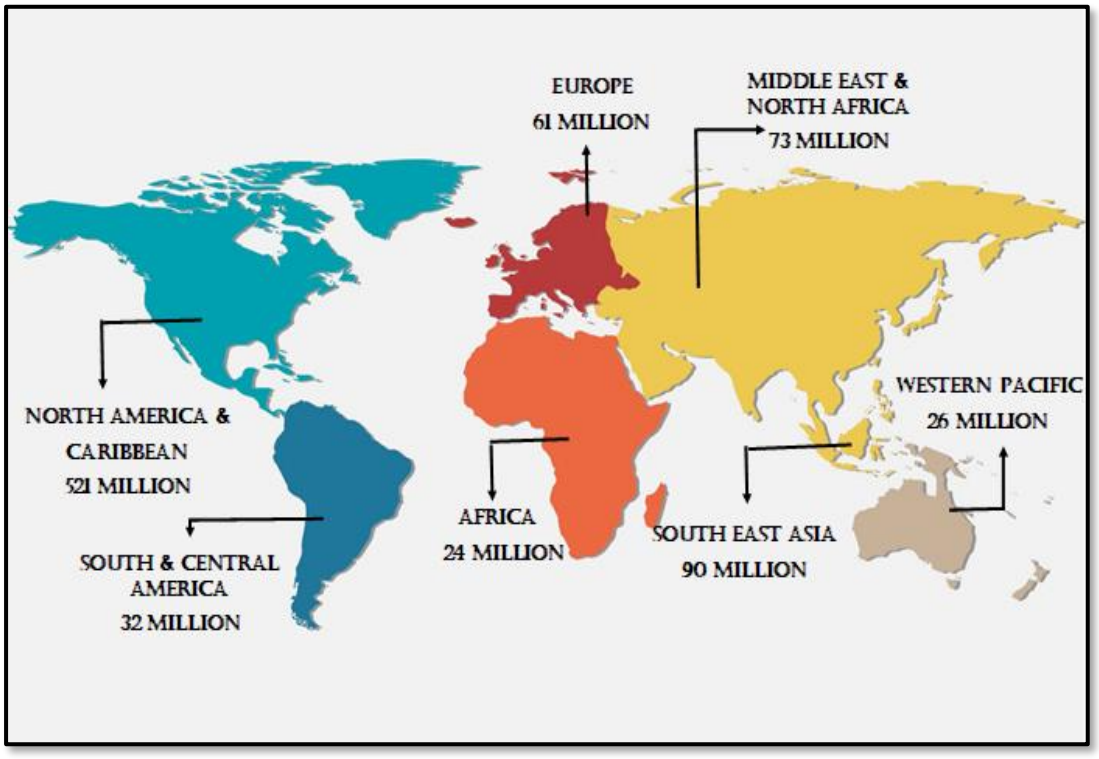


Figure 1.1 *Prevalence of Diabetes around the Globe*

1.3 Pathophysiology of T2DM

The precise synchronization of glucose consumption and glucose production is the key for the regulation of normal plasma glucose concentration. Glucose absorption from intestine takes place from three sources i.e., digestion of carbohydrates, gluconeogenesis and glycogenolysis. During fasting, the rate of glucose production equals the rate of glucose utilization resulting in the stable concentration of plasma

glucose. After meal, glucose absorption from intestine increases depending on the carbohydrate content present in the meal. This result in the suppression of endogenous glucose synthesis maintains the glucose concentration in plasma (Giugliano et al., 2008).

Insulin and glucagon are the mainstream glucoregulatory hormones responsible for the regulation of glucose in blood. Both of these hormones are produced from pancreas. Glucagon is produced from α-cells and insulin is produced by β-cells of pancreas. These hormones work opposite to each other maintaining the regulation of glucose in plasma. In the fasting condition, insulin prevents synthesis of glucose from liver and activates the utilization of glucose. On the other hand, glucagon increases the production of glucose from liver as it is hyperglycemic hormone. The intake of carbohydrate increases concentration of insulin and decreases the concentration of glucagon. The release of insulin prevents hyperglycemia as it acts on liver and inhibit the production of glucose (Giugliano et al., 2008). In T2DM, these pathways and mechanism become defective either by abnormal functioning of β-cells or by increased resistance of target tissues like liver, adipose and skeletal tissues, against insulin.

1.3.1 Normal physiology of β-cells

For the normal secretion of insulin, the integrity of β-cells must be assured by constantly regulating the cellular pathways and mechanisms responsible for the normal functioning of pancreatic β-cells. Under normal conditions, 30-70 units of insulin is released from β -cells per day and the main role is played by glucose in the release and enhanced production of insulin (Marchetti et al., 2020). Inulin is first produced in an inactive form, pre-proinsulin, which go through conformational changes and converted into proinsulin. It is then transported to Golgi apparatus where

it is cleaved into c-peptide and insulin. This matured insulin is then stored into granules, and it is primarily released in response to higher concentration of glucose. Amino-acids, hormones and fatty acids are also responsible for the excretion of insulin from granules. The rise of glucose concentration in blood triggers the intake of glucose by glucose transporter 2 (GLUT 2) located on the membrane of β-cells. This glucose is catalyzed in the cell which results in membrane depolarization due to the increased concentration of ATP in cell. The calcium ions start entering the cells from voltage dependent Ca^{2+} channels. These Ca^{2+} ions fused with insulin granules releasing insulin from cells. The insulin then acts on liver and other body tissues for the regulation of plasma glucose (Galicia-Garcia et al., 2020).

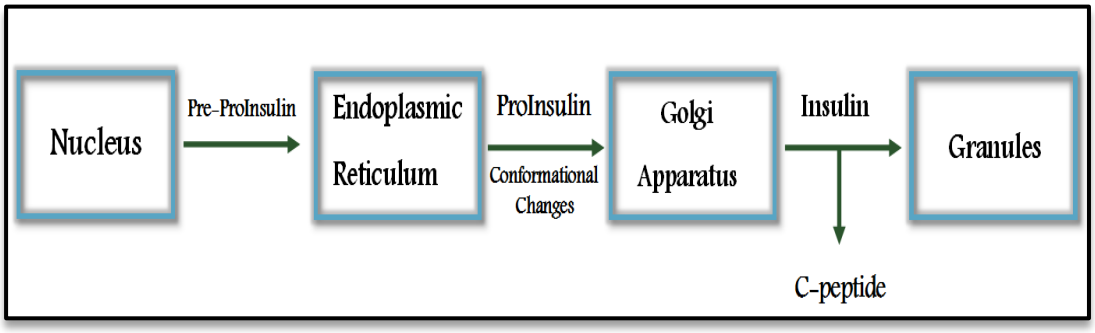


Figure 1.2 *Pictorial representation on insulin synthesis in normal conditions*

1.3.2 β-cells dysfunction

The failure of β-cells in the production of insulin plays major role in development of T2DM. β-cells dysfunction is thought to be resulted from the degeneration and death of β-cells by apoptosis leading to reduction in the production and stimulation of insulin thus, increasing glucose concentration in blood plasma (Marchetti et al., 2020). The downstream signaling pathways for insulin secretion can also be affected by the reduced gene expression of glucose transporter and glucokinase in T2DM (Taneera et al., 2012).

The uncontrolled nutrition state i.e., obesity, hyperlipidemia and hyperglycemia, results in insulin resistance and chronic inflammation in pancreas. The pancreatic β-cells are then subjected to toxic environmental conditions including inflammation, oxidative stress, endoplasmic reticulum stress, and amyloid stress (Halban et al., 2014). Endoplasmic reticulum stress is generated by the presence of excessive free fatty acids (FFA) and higher glucose concentration. These FFA activates apoptotic unfolded protein response (UPR) pathways leading to apoptosis of β-cells (Yamamoto et al., 2019). Inflammation in β-cells occurs when macrophages are inducted by interleukin-1β due to the altered mobilization of calcium ions from endoplasmic reticulum (Galicia-Garcia et al., 2020).

1.3.3 Insulin Resistance

Insulin resistance is mainly the ineffective response of target tissues towards insulin hormone, and it is thought to be the major hallmark of T2DM as it is the first detectable defect. It is present in prediabetic condition in individuals with family history of type 2 diabetes (Goldstein, n.d.). It can occur due to suppressed secretion of insulin from β-cells, presence of insulin antagonist in blood and ineffective response of target tissues. Skeletal muscles, adipose tissue and liver are the three main targets of insulin (Galicia-Garcia et al., 2020). The synchronization of glucose levels in the blood is controlled by the insulin and glucagon ratio. Insulin breaks down the glucose or converts it into glycogen for storage and lowers the glucose levels in the blood. On the other hand, glucagon prevents insulin-based hypoglycemia by converting glycogen into glucose (Galicia-Garcia et al., 2020).

Due to the impaired insulin action, β-cells are forced to increase the synthesis of insulin which leads to functional defects in β-cells. Insulin resistance is the leading cause hyperinsulinemia, hyperglycemia, cardiovascular disease, and other metabolic

disorders. Hyperinsulinemia results due to the over secretion of insulin from pancreas in order to overcome the defects of insulin resistance. In patients with T2DM, increased secretion of insulin helps to sustain euglycemia in body. Insulin resistance has an impact on a wide range of tissue functions and metabolic processes in the body. Exaggerated responses in insulin-sensitive tissues are generated by hyperinsulinemia. It stimulates the sympathetic nervous system, which can lead to hypertension, and it also boosts ovarian androgen production, which contributes to the hormonal abnormalities (Goldstein, n.d.). Regarding impaired response from insulin-responsive cells, skeletal muscles, adipose tissue, and liver plays important role in the development of T2DM (Wilcox, 2005).

1.3.4 Skeletal Muscle

The most essential factor responsible for T2DM besides the pancreas is skeletal muscles. Under a normal environment, insulin increases glucose uptake and enhances the synthesis of glycogen from glucose. Three factors are crucial in this conversion: hexokinase, glucose transporter 4 (GLUT 4), and glycogen synthase. Muscles cells contain Insulin receptors (INSR) to which insulin binds which results in the translocation of GLUT 4 from the intracellular chamber to the plasma membrane which initiates glucose uptake lowering the levels of circulating glucose in the blood (Satoh, 2014).

Insulin binds to the α-subunit of INSR which results in the phosphorylation of the β-subunit on tyrosine residues, activating the INSR tyrosine kinase for glucose intake. Any mutation in these subunits results in the inactivation of INSR tyrosine kinase that leads to impaired glucose intake by skeletal muscles. Environmental or nutritional factors are also responsible for IR besides genetic factors. A sedentary lifestyle and excessive nutritional conditions lead to chronic inflammation in skeletal muscles.

Obesity contributes to the infiltration of macrophages and proinflammatory cytokines which eventually results in muscle inflammation and IR (Wu & Ballantyne, 2017).

1.3.5 Adipose Tissue

Adipose tissue is involved in a wide range of metabolic processes occurring in the body including the production of bioactive compounds, coagulation, angiogenesis, reproduction, immunity, appetite regulation, and glucose and lipid regulation (Rosen & Spiegelman, 2006). Two major mechanisms are involved in insulin action on adipose tissue: (i) increasing glucose intake and triglycerides (TGA) production, and (ii) mitigating hydrolysis of TGA and increasing FFA and glycerol uptake from blood circulation (Gastaldelli, Gaggini, & DeFronzo, 2017).

In normal conditions, circulating blood glucose is taken into the cell by GLUT-4. Once inside the cell, glycolysis is activated, and glucose-3-phosphate (G3P) is synthesized from glucose which is then utilized in lipogenic pathways. G3P then contributes to the formation of TGA by combining with fatty acids (esterification). TGA is then stored in lipid droplets. In metabolic stress conditions, TGA is broken down into free fatty acids that can be used as an alternative energy source for the body (Unai, et al., 2020). Any abnormality in the above-mentioned process can lead to reduced glucose uptake, increased secretion of FFA and glycerol resulting in hyperglycemia and IR (Czech, 2020).

1.3.6 Liver

The liver is responsible for glucose synthesis, utilization, and lipid metabolism. Under normal circumstances, glucagon, and Insulin both play a vital role in the regulation of hepatic glucose. Glucagon activates glucose synthesis while insulin lowers the level of glucose by converting it into glycogen for storage. With the rise in the level of

circulating blood glucose, insulin is released from pancreatic β-cells. Insulin binds to INSR resulting in the autophosphorylation of the receptor. Insulin receptor substrates (IRSs) are thereby recruited and phosphorylated. After that, phosphatidylinositol 3-kinase (PI3K) is activated by IRSs which produce phosphatidylinositol (3,4,5)-triphosphate (PIP3) by phosphorylating phosphatidylinositol (4,5)-bisphosphate (PIP2). PIP3 then stimulates pyruvate dehydrogenase kinase-1 (PDK1), causing protein kinase B (AKT) to phosphorylate. AKT is also phosphorylated by mTOR Complex 2 (mTORC2). This completed activation of AKT performs several vital roles in metabolic pathways like glycogenolysis, gluconeogenesis, lipid production, and glycolysis (Unai, et al., 2020).

The other mechanism by which insulin reduces glucose production in the liver is activating fork head box O1 protein (FOXO1), a transcription factor. This factor identifies an insulin response element (IRE), a specific regulatory element, on the promoters of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) genes, which both play critical roles in maintaining glucose levels in starving situations. By inhibiting FOXO1, glucose storage in the form of glycogen is elevated and glucose production is stopped (Montal, et al., 2015). Under pathogenic conditions such as IR, glycogen synthesis is reduced, gluconeogenesis and lipogenesis are increased. The production of proinflammatory C-reactive protein (CRP) is also elevated (Leclercq, Da Silva Morais, Schroyen, Van Hul, & Geerts, 2007).

1.4 Etiology of T2DM

1.4.1 Genetic Factors

The pathophysiology of insulin resistance has not been clear yet, but it is investigated that genetic factors play role in onset of T2DM (Wu et al., 2014a)**.** T2DM, a

polygenic disorder proved by segregation analysis, is termed to have several susceptible loci located by genome wide association studies (GWAS) in 2007. After GWAS studies conducted on people belong to different countries and ethnicities has discovered more than 80 susceptible loci connected to T2DM **3.** These particular genes include KCNJ11, TCF7L2, IRS1, MTNR1B, PPARG2, IGF2BP2, CDKN2A, HHEX and FTO are found to correlated with development of T2DM. There are still a lot of unsorted loci responsible for pathogenesis of T2DM. To fully comprehend and manage T2DM, we must thus enhance our present biological understanding (Wu et al., 2014a)**.**

1.4.2 Environmental Factors

Sedentary lifestyles, unbalanced diet, physical inactivity, smoking, alcohol consumption, and obesity are the major environmental risk factors associated with T2DM pathogenesis. Other environmental factors like pollution, unbalanced sleep cycle and effect of different chemical has not clearly understood yet (Hills et al., 2018)**.** Obesity is a strongly inherited trait, and it is highly associated with onset of T2DM. According to WHO report 2011, 90 percent of patients develop T2DM due to excess body weight. Obesity is the major source of several other complications and metabolic disorders which ultimately results in insulin resistance, glucose intolerance and also development of prediabetes (Wu et al., 2014a)**.** Lifestyle management and proper diet is considered as the safest method for the regulation of T2DM. Now-a-days, the dietary patterns have been changed dramatically. The different nutrition transitions i.e., high fiber with low fat diet has been replaced by high fat and low fiber diet along with improper exercise routine and excessive use of sugar has increase the ratio of obese and diabetic people (Hills et al., 2018)**.** Physical inactivity is linked to energy imbalance, body mass gain, inflammatory response, and insulin resistance.

These features accumulate over time and dramatically raise the chance of developing type 2 diabetes, cardiovascular disease, and several malignancies, resulting in a shorter lifespan.

Data shows that the healthy practices, lower chance of noncommunicable diseases, and better prognosis associated with a healthier and more active lifestyle are similar in high-, middle-, and low-income settings (Hills et al., 2018).

1.4.3 Epigenetic Factors

Epigenetic factors contributed to the development of T2DM as much as the genetic factors are involved. It is the study of any modification in gene function but not in the sequence of DNA. These modifications include histone modification, acetylation, and DNA methylation. Several environmental components such as diet, physical activity, exposure to chemicals and age have been known to influence the epigenetic changes affecting susceptibility and gene expression. It's likely that maternal diet, metabolism, and other undiscovered factors influence the south Asian phenotype epigenetically (Hills et al., 2018)**.**

1.4.4 Gut Metagenome Interrelation

Gut metagenome has shown to influence the development of T2DM as different types of bacteria located in gut interacts with environment. T2DM patients have a significant degree of gut microbial dysbiosis, as shown in a two-stage metagenome-wide association study (MGWAS), with certain butyrate-producing bacteria being reduced and other opportunistic infections being elevated.

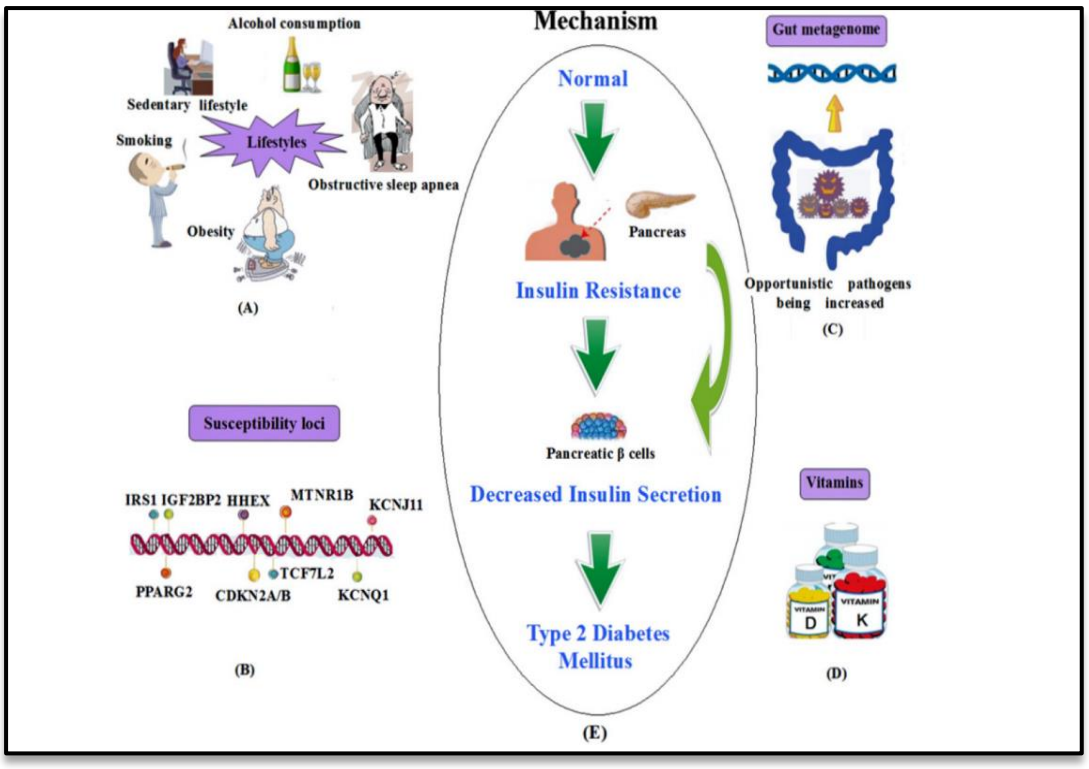


Figure 1.3 *An overview of factors influencing development of T2DM. (A) Lifestyle management (B) Genetic factors (C) Gut metagenome interrelation (D) Vitamins (E) Overall mechanism of T2DM (Wu et al., 2014b)*

1.4.5 Vitamin D

Growing data suggests that vitamin D may have a role in the regulation of T2DM, as glycemic status of T2DM patients varies periodically, and hypovitaminosis D, which occurs commonly in the winter, is believed to be linked to T2DM severity. Vitamin D insufficiency has been shown to have deleterious effects on hyperglycemia, insulin secretion, and T2DM, either primarily through vitamin D receptor (VDR) activation or secondarily through calcemic hormones and inflammation, according to new research. Vitamin D plays an important role in the manufacture and release of insulin since both 1-hydroxylase and VDR are present in pancreatic cells. It also affects insulin sensitivity by modulating calcium flow across the membrane in both cells and target organs. Furthermore, vitamin D supplementation is considered as a potential and low-cost therapy that may reduce the risk of T2DM and improve T2DM patients'

glycemic indices (Wu et al., 2014a). As a result, Vitamin D has been proposed as a diabetes mellitus preventive component. Several pathways connecting vitamin D to immune response control suggest that vitamin D has a role in the etiology of autoimmune diabetes (Maddaloni et al., 2018).

1.5 Diagnostic Criteria

American Diabetes Association (ADA) and World Health Organization (WHO) has given the diagnostic criteria for diabetes. Different tests are being used for this purpose:

1.5.1 HbA1C Test

This test measures the amount of blood glucose attached to hemoglobin. The average quantity of glucose connected to hemoglobin over the previous three months is determined by HbA1c test. It's a three-month average because that's the usual lifespan of a red blood cell. The normal HbA1C levels are below 5.7% but for a diabetic patient it is greater than 6.5% and it is between 5.7 and 6.4% for prediabetes. It reflects the long-term glucose concentration, but it is not for diagnostic use in pregnant women and children (Punthakee et al., 2018).

1.5.2 Fasting Blood Sugar test

Glucose is the main source of energy for the body. Fasting blood glucose test measures measure the levels of glucose in blood. Any disturbance in the level of glucose can results in various medical complications. The normal concentration of blood sugar is 99mg/dL and if it is higher than 126 mg/dL, it represents diabetes (Punthakee et al., 2018).

1.5.3 Oral Glucose Tolerance Test

This test measures glucose concentration before and after taking a glucose containing liquid. After drinking liquid, the blood sugar levels are checked 1, 2, and 3 hours afterwards. The normal levels are represented by 140 mg/dL and is it is greater than 200 mg/dL then it represents diabetes (Punthakee et al., 2018).

1.6 Treatment Options

A combination of lifestyle adjustments and pharmaceutical treatment is required to establish and maintain optimal metabolic control in diabetes throughout time. For the treatment of T2DM, various oral and injectable therapies are currently available. Glycemic targets and glucose-lowering medication selection should be tailored to the needs and circumstances of individual patients, which could be aided by future pharmacogenomics advances (Tahrani, Barnett, & Bailey, 2016). Cost, probable side effects, potential benefits, glucose-lowering efficacy, and dosing schedule are all factors to consider before choosing a medicine (Tan, et al., 2019).

1.6.1 Insulin Therapy

Insulin is the primary choice of treatment for T1DM but in the case of T2DM, it is encouraged to use when other drugs become incapable of regulating glucose levels in the blood. It can be utilized in combination with other oral drugs. Insulin's limitation is that it must be supplied by injections (Tan, et al., 2019).

1.6.2 Biguanides

The commonly used biguanides are metformin. Other biguanides like phenformin and buformin are withdrawn due to the risk of lactic acidosis (Tahrani, Barnett, & Bailey, 2016). Metformin is the most prescribed diabetes medication, especially among obese and overweight people. For monotherapy, this medicine is still the best option. It

works by improving insulin sensitivity, enhancing glucose uptake by phosphorylating GLUT enhancer factors, and inhibiting hepatic gluconeogenesis. Metformin helps in weight loss by lowering triglycerides and LDL cholesterol levels (Tan, et al., 2019). Abdominal discomfort and other gastrointestinal problems, such as diarrhea, are the most common side effects of metformin medication (Tahrani, Barnett, & Bailey, 2016).

1.6.3 Sulfonylureas

Sulfonylureas are secretagogues that act by causing pancreatic β-cells to secrete natural insulin. It primarily targets β-cells' ATP-sensitive potassium channels and is only effective when residual pancreatic b-cells are present. Sulfonylureas have no long-term protective effects on β-cell activities, and they may hasten β-cell death. There have been numerous reports of sulfonylureas causing hypoglycemia, particularly with earlier generation medications (Tan, et al., 2019).

1.6.4 Thiazolidinediones

Peroxisome proliferator-activated receptor g activators, such as thiazolidinediones (TZDs), improve insulin sensitivity in adipocytes, cardiac muscles, and the liver (Eldor, DeFronzo, & Abdul-Ghani, 2013). They work on b-cells to regulate insulin secretion. As a result, it's being utilized as a part of an insulin resistance therapy plan for T2DM patients, with results lasting up to 5 years (DeFronzo, et al., 2013). Increased body weight is a typical TZD adverse effect.

1.6.5 Dipeptidyl peptidase-4 (DPP4) inhibitors

Gliptins, commonly known as DPP4 inhibitors, are a novel class of therapeutic agents that function by blocking the enzyme dipeptidyl peptidase 4. Inhibition of dipeptidyl peptidase 4 delays the inactivation of incretin hormones including glucagonlike

peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP), which are involved in physiologically maintaining glucose homeostasis (Singh, 2014). These medications have fewer reported side effects, reduced risk of hypoglycemia, and are weight-neutral (Brunton, 2014).

1.6.6 Glucagon-like peptide 1 (GLP-1) analogues

GLP-1 analogs are primarily incretin-based medicines that boost insulin release in a glucose-dependent manner, suppress glucagon secretion, and eventually inhibit hepatic glucose synthesis (Bunck, et al., 2011). HbA1c levels have been seen to drop for up to three years. Even though these medicines are not as well handled as DPP4 inhibitors, they result in higher HbA1c reduction and weight loss (Stonehouse, Darsow, & Maggs, 2012). There is some evidence that incretin-based medications have beneficial effects on sleep, inflammation (via lowering reactive protein levels), the central nervous system, liver, and cardiovascular health (Inzucchi, et al., 2015).

1.6.7 Sodium-glucose co-transporter-2 (SGLT2) inhibitors

By inhibiting glucose absorption in proximal renal tubules, SGLT2 inhibitors, also known as gliflozins, reduce sodium transport and promote glucose excretion via the kidneys, decreasing plasma blood glucose concentrations (Kalra, 2014). Canagliflozin, dapagliflozin, and empagliflozin are examples of pharmacological drugs in this class. Because they act independently of insulin, they can be utilized in individuals at any stage of diabetes (Inzucchi, et al., 2015). These medications can help to improve b-cell activity, insulin sensitivity, and glucotoxicity caused by glucosuria. They can cut HbA1c levels by 0.5%, lose weight, and control blood pressure (Abdul-Ghani, Norton, & DeFronzo, 2011). Urinary tract infections, vaginal mycotic infections, particularly in females, and volume depletion-related symptoms

have all been reported (Cherney, et al., 2014). When prescribing this medication to the elderly and patients on diuretics, extra caution is required.

1.6.8 Combination Therapy

Combination therapy is initiated for more effective blood glucose control and dose reductions in individual drugs. When monotherapy fails to keep blood glucose levels under control, it is usually started. Exogenous insulin can be coupled with a variety of oral antidiabetic medications to reduce insulin dosage. Glycemic control is improved when insulin is combined with metformin or TZD. HbA1c levels are reduced along with weight loss when basal insulin is coupled with GLP-1 receptor agonists. SGLT2 inhibitors are commonly used in combination with metformin or other drugs (Eng, Kramer, Zinman, & Retnakaran, 2014). It can also be used with DPP4 inhibitors to improve glycemic management and weight loss while lowering the risk of hypoglycemia (Min, Yoon, Moon, Hahn, & Cho, 2018).

1.7 Ethno-botanical Medicines

Plants are the rich source of phytochemical such as phenols, alkaloids, terpenoids, flavonoids and other secondary metabolites. These phytochemicals possess antioxidant, anti-inflammatory, antimicrobial, and antidiabetic properties. Some phenolic compounds have shown to alter inflammatory activity, genes involved in the pathogenies of T2DM and transcriptional factor enzymes. Antioxidants found in phytochemicals have attained greater attention as they have potential for mopping up reactive oxygen species and lowering the oxidative stress. The vast therapeutic potential of plants regarding antidiabetic is yet to be explored in detail (Unuofin & Lebelo, 2020).

1.8 Cestrum diurnum

Cestrum diurnum belongs to family *Solanaceae*. It is native to west Indies and blooms during daylight. It is also tropically and sub-tropically distributed throughout the world. It is commonly known as day-blooming jasmine or din ka raja (Naz & Bano, 2013). It is an evergreen woody shrub containing various leafy branches and leaves. The fruit of *C. diurnum* looks like black, small, and berry-shaped (Khatun et al., 2022). Leaves of *Cestrum diurnum* has shown to possess bactericidal and analgesic activities. it is also rich source of vitamin D which is why it is the current focus of research for the therapeutics of T2DM. The oil of *Cestrum diurnum* is being used in the prevention of malaria in African region (Naz & Bano, 2013).

1.9 Aims and Objective

1 Phytochemical screening of ethanolic extract of *Cestrum diurnum*

2 Evaluation of antioxidant, anti-inflammatory, and anti-diabetic effect of extract by various biochemical assays.

3 Investigation of in-vivo anti diabetic effect of extract in streptozotocin induced diabetic mice models.

2 Literature Review

Type 2 diabetes mellitus remains to be major and highly pervasive public health disorder. It is highly associated with morbidity and mortality in diabetic individuals worldwide. Genetic predisposition, obesity, sedentary lifestyle, urbanization, and poor dietary habits are the leading causes of T2DM. These risk factors are associated with earlier development of T2DM (El-Kebbi et al., 2021). T2DM is a multifactorial disease of endocrine system characterized by hyperglycemia and insulin insensitivity. It is diagnosed by the abnormal levels of blood glucose and leads to various complications such as cardiovascular disease, diabetic retinopathy, diabetic nephropathy, and neuropathy which in some cases proved to be fatal. The clinically distinct and differentially related subtypes of T2DM have been identified by using clustering approaches through genetic biomarkers (Cole & Florez, 2020).

2.1 Glucose Metabolism

Glucose is the main source of energy for the body. It is precisely regulated in a relatively narrow range for proper functioning of body organs. Blood plasma glucose concentration is determined by the rate of glucose entering bloodstream countered by the rate of glucose eliminated from bloodstream. Glucose is procured from three main sources: gluconeogenesis, glycogenolysis and the intestinal absorption. Gluconeogenesis and glycogenolysis take place in liver and they are partly under control of pancreatic hormones. Glycogenolysis is the breakdown of glycogen into glucose while gluconeogenesis is the synthesis of glucose from amino acids and lactate. The primary mechanism for the production of glucose during first hours of fasting is glycogenolysis and it is facilitated by glucagon. After several hours of fasting, glucose is produced from liver by gluconeogenesis (Aronoff et al., 2004). The

synchronization of glucose levels in the blood is controlled by the insulin and glucagon ratio. Insulin is produced from β-cells of pancreas and breaks down the glucose or converts it into glycogen for storage or lowers the glucose levels in the blood. On the other hand, glucagon, produced from α-cells of pancreas prevents insulin-based hypoglycemia by converting glycogen into glucose (Galicia-Garcia et al., 2020).

Insulin, a dominant glucoregulatory pancreatic hormone, is comprised of two polypeptide chain with 51 amino acids. It is secreted in response to the higher level of glucose in circulation after taking a meal. It regulates the level of glucose primarily by signaling the cells like skeletal muscles to increase their uptake of glucose, by promoting glycogenesis and by inhibiting the secretion of glucagon from pancreas (Aronoff et al., 2004).

The higher level of circulating glucose is the primary risk factor of T2DM. One of the most suitable therapeutic methods for T2DM is to lowers the levels of glucose in circulation by inhibiting the enzymes responsible for the hydrolysis of carbohydrates. α-amylase and α-glycosidase are the main digestive enzymes responsible for the production of glucose from the hydrolysis of carbohydrates in digestive tract. α-amylase is secreted from pancreas and salivary glands, and it hydrolyzes oligosaccharides and starch. On the other hand, α- glucosidase is secreted from intestinal cell membrane and it hydrolyzes polysaccharides. Inhibition of these enzymes lowers the production of glucose by retarding the hydrolysis of carbohydrates and thus prevents hyperglycemia (Kalita et al., 2018).

2.2 Oxidative Stress

T2DM is highly related with non-enzymatic protein glycation and oxidative stress. Due to hyperglycemia, level of advanced glycation end products (AGEs) increases effecting the function and conformation of proteins. It has been suggested that not only the acceleration in the level of advance end glycation product is due to oxidative stress in the cell but also AGEs are reason for the formation of reactive oxygen species and other reactive intermediates. The rate of antioxidant activity in the cell is also related to the development of T2DM as they protect cells from ROS as well as AGEs. One of the most suitable therapeutic methods is through the compounds that offer antiglycation and antioxidant properties. The ongoing research and emergence of new compounds with antioxidant and antiglycation capabilities would help in the treatment of T2DM (Ramkissoon et al., 2013).

2.3 Therapeutic Potential of Medicinal Plants:

It is a well-known fact that plants possess pharmacological properties as they are being in use since centuries as traditional medicine. After the ineffectiveness of chemically synthesized compounds, the world has come to explore natural resources for the purpose of treatment and pain alleviation once again. Plants are natural source of secondary metabolites such as phenolic compounds which are possess strong antioxidant properties. Phenolic compounds are found in number of plants. Recently, the researchers are focused on the exploration and extraction of secondary metabolites from plants that has become a trend in scientific community. Antioxidants substances are being in use and commercially available. Polyphenols have been reported by many researchers to play a role in the prevention of obesity, coronary heart disease, colon cancer, gastrointestinal disorders, and diabetes by preventing AGEs formation.

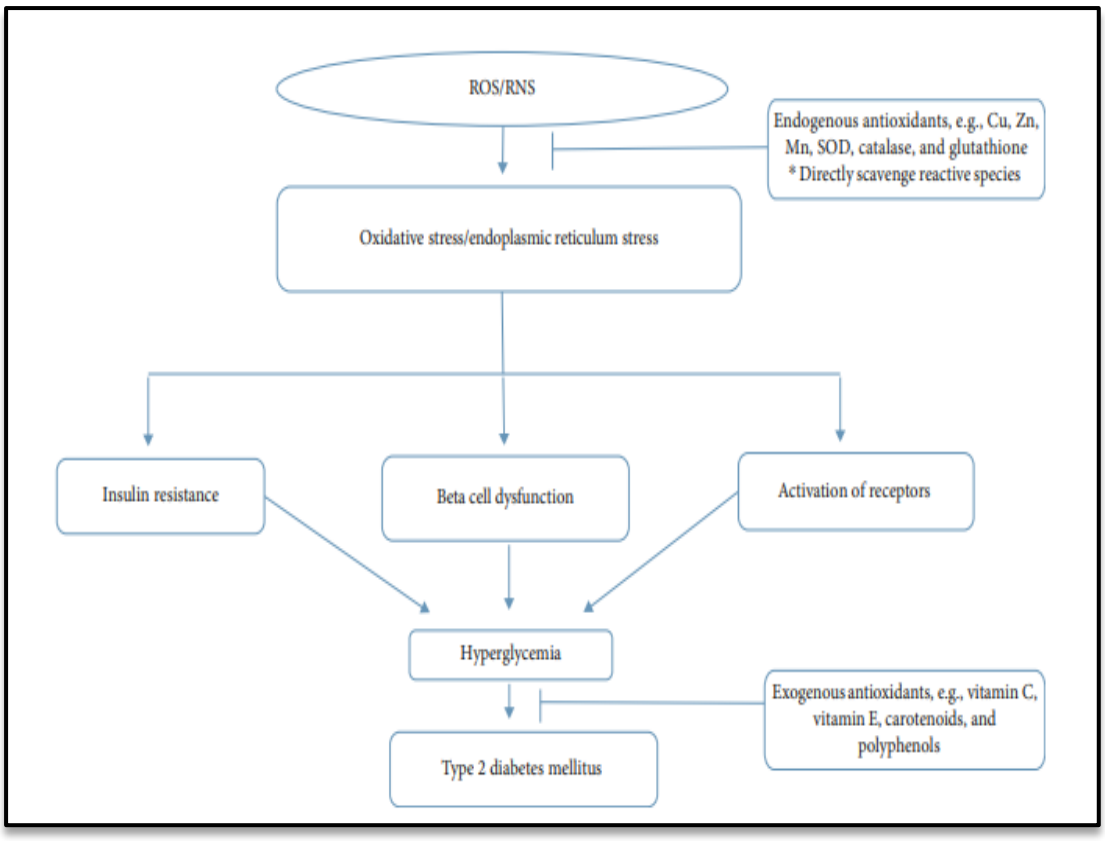


Figure 2.1 *Targets of antioxidative compounds (Unuofin & Lebelo, 2020)*

2.4 Medicinal Plants in Pakistan

Pakistan has an incredible reputation in terms of plant diversification among underdeveloped countries, due to variations in physiographic and climatic conditions. This valuable treasure can be found all around the country. It is estimated that there are around 6000 species of flowering plants throughout the country, including Kashmir. Nearly 600 to 1000 plants have pharmacological potential, according to ethnobotanical study. Only 12% is employed in the treatment of certain medical disorders. Almost 350 to 400 species are revived and employed by various manufacturers in the development of herbal remedies in the local drug markets (SADIQ, 2020).

2.5 Family Solanaceae

Family *Solanaceae* comprises of 83 genera and more than 3000 species. This family possess a number of pharmaceutical properties. Potato, tomato, eggplant, and pepper are the major members of this family. The main morphological characteristics of this family is the presence of alternate opposite leaves, hypogynous, and cymose flowers. The fruit is a berry-shaped (capsule). The hereditary material is composed of 12 chromosomes. Family *Solanaceae* is one of the important plant families as it is source of vegetable crops. It has economical as well as commercial values because it contains plants of spices, drugs, and food (SADIQ, 2020).

2.5.1 Genus Cestrum

Genus *Cestrum* was thought to have two species *Cestrum diurnum* and *Cestrum nocturnum* in 1753. But now this genus contains over 300 species distributed all around the world and native to warm area. Many species of this genus are being in use for the treatment of burn and swelling in Chinese traditional medicine (Mohamed et al., 2007a). *Cestrum* is the second largest genus of family *Solanaceae* after genus *Solanum*. The main morphological characteristics of this family are ring-shaped nectaries, floral tube, dorsifixed anthers, hinged-base flowers, fruits having angled seeds, and berry-shaped fruits. The genus *Cestrum* is found to have therapeutic compounds like flavonoids, Vitamin-D3, saponins and lignans (SADIQ, 2020).

2.6 Botanical characterization of *Cestrum diurnum*

Cestrum diurnum is commonly known as Chinese inkberry, Day Blooming Jasmine, Dina Mallika, or Din Ka Raja as their flowering time is daylight hours. It is an erect, evergreen perennial, seed propagated, woody shrub, can possess single or multiple stems with several leafy branches. The flowers of *Cestrum Diurnum* are white in color with sweet smell and fruits are small berry shaped in black color (Khatun et al., 2022).

The plant is native to west indies, but it can be found all over the world, mainly Asia (Pakistan, Singapore, and India), Australia, China, South Africa, Oceania, and Southern region of United States (Jawale, 2014a).

2.6.1 Taxonomic Tree

Domain	Eukaryota
Kingdom	Plantae
Phylum	Spermatophyta
Subphylum	Angiospermae
Class	Dicotyledonae
Order	Solanales
Family	Solanaceae
Genus	*Cestrum*
Species	*Cestrum diurnum*

Table 2.1 *Taxonomic tree of Cestrum diurnum*

2.6.2 Ethno-Pharmacology of *Cestrum diurnum*

Cestrum diurnum is widely traded as an ornamental plant because of its sweet-smelling flowers and pleasing appearance. *Cestrum diurnum* is being in use since ages in Chinese traditional medicine. It is traditionally being in use to treat several disease conditions such as burn, swelling, weeping illness in children and joint pain in different regions of the world (Acharya & Pokhrel, 2006). The inhibitory effect of *C. diurnum* on central nervous system has also been reported. It is an ingredient in cosmetic products that are used to prevent or treat skin conditions that result in wrinkly, fragile, aged, and depigmented skin due to a lack of vitamin D (Khatun et al., 2022). It is also reported to possess hyperlipidemic and hepatoprotective activity

(Khatun et al., 2014). *Cestrum diurnum* is also a rich source of free and glycosidic vitamin D3 and its metabolites. The active principal compound is a glycoside of 1,25-dihydroxy vitamin D3 (Prema & Raghuramulu, 1994).

2.6.3 Anti-psoriatic activity

Anti-psoriatic activity of *Cestrum diurnum* has been reported. The external application of leaves pastes has been in traditional use in Andhra Pradesh for the treatment of psoriasis. The oil prepared from *Cestrum diurnum* has proved to be clinically effective in case of psoriasis (Prasanna Bandi et al., 2018). The ointment prepared from the extract of *Cestrum diurnum* is commercially available under name of Psoriaban Natural by Aurochem Laboratories Pvt. Ltd. This ointment is proved to be effective in the treatment of scalp and face psoriasis (Dabholkar et al., 2021) .

2.6.4 Larvicidal Activity

Due to its sweet and strong smelling flowers, *Cestrum diurnum* is being in use against mosquitoes' larvae (Ghosh et al., 2021). *Cestrum diurnum* is also one of the components of some mosquito repellents. It is proved to possess biocontrol efficacy against larvae of *Aedes Aegypti* and *Culex.* The methanolic extract of *Cestrum diurnum* is rich source of secondary metabolites that possess insecticidal and larvicidal activities (Jawale, 2014b). *C. diurnum* is used in Africa for the prevention of malaria. The extract of *C. diurnum* proves the promising activity against larvae of *Culex quinquefasciatus* and *Anopheles stephensi* (Prasad et al., 2013) (Ghosh & Chandra, 2006).

2.6.5 Anti-microbial Activity

Anti-bacterial activity of *Cestrum diurnum* have been reported such as the aqueous, n-hexane and ethanolic extract demonstrated anti-microbial activity against *S. aureus,*

B. subtilis, E.coli and *P. aeruginosa* (Bhattacharjee et al., 2011). Leaves of *C. diurnum* have shown toxicity against the mycelial growth of *Rhizoctonia solani* confirming its fungitoxic nature. Fungitoxic nature of *Cestrum diurnum* oil has been reported against a wide range of fungi (Renu et al., 1980).

2.6.6 Anti-inflammatory activity

Cestrum diurnum is reported to possess anti-inflammatory and analgesic activities. In a study by Khatun et al, the anti-inflammatory activity of aerial parts of *Cestrum diurnum* and its role on the inflammatory mediator NF-kB was investigated. The study demonstrated the anti-inflammatory nature of *C. diurnum* (Khatun et al., 2022). The plant proved to be the rich source of natural anti-inflammatory agents.

2.6.7 Antioxidant activity

The antioxidant activity of *C. diurnum* has also been reported in several studies. Phytochemical screening showed the presence of alkaloids, phenols, and flavonoids. Antioxidant testing demonstrated high activity against free radicals confirming higher antioxidant potential and thus preventing cellular damage by free radical species (Prasad et al., 2013) (SADIQ, 2020).

2.6.8 Cytotoxic and thrombolytic activity

Cytotoxic and thrombolytic activity of aerial parts of *Cestrum diurnum* is reported in many studies. This plant is mainly known for its toxicity due to presence of high concentration of vitamin D3 causing calcinosis in grazing animals (Khatun et al., 2014) (Singh Brar & Angad, 2011). *C. diurnum* causes cardiac tissues, arteries, and tendons to develop dystrophic calcinosis. This specie has been linked to numerous poisonous alkaloids. It also includes a synthetic glycoside that hydrolyzes to produce

vitamin D3 and increases intestinal calcium absorption far beyond levels that can be tolerated physiologically (Shadab et al., 2020) (Khatun et al., 2022).

2.7 Bioactive compounds in *Cestrum diurnum*

Cestrum diurnum is reported to possess wide range of secondary metabolites, alkaloids, phenols, anthraquinones, steroids, saponins, glucosides, and flavonoids which proves to be effective. The main compound found in *C. diurnum* is vitamin D3 and its analogues. It was reported that the leaves of *C. diurnum* are a good source of vitamin D3, calcium, lutein, and alpha-carotene. Other fatty acids found in *C. diurnum* include myristic, palmitic, stearic, oleic, and linoleic acid (Khatun et al., 2022).

When compared to other investigated *Cestrum* species, the *C. diurnum* showed a various metabolic framework from the GC-MS technique, assuring the complexity of the plant with various metabolites. A study comprising GCMS based evaluation of *C. diurnum* has revealed a number of active compounds found in the plant i.e., 15-methyltricyclo 6.5.2-pentadeca-1,3,5,7,9, 11,13-heptene and 2,2′,6,6′-tetra-tert-butyl-4,4′-methylenediphenol (El-Demerdash et al., 2021).

Muhamed et al., have isolated a new 9-norlignan glucoside along with six known glucosides from *C. diurnum* by utilizing NMR and MS spectral analysis technique (Mohamed et al., 2007b). Following phytochemical analysis of a methanolic extract of leaves from *Cestrum diurnum* L. (Solanaceae), multiple furstanol steroidal saponins known as cesdiurins I–III (1-3) were extracted. Spectroscopy examinations, including the use of 1D and 2D NMR spectroscopic techniques as well as by mass spectrum analyses, were used to determine the structures of the isolated compounds (Fouad et al., 2008).

2.7.1 Vitamin D3

Cestrum diurnum is previously reported as one of the toxic plants for grazing animals as the toxicity was reported in Florida cattle. It has been shown that *C. diurnum* can counteract the impact of a stable strontium feed in chicks, proving that the active principle in *Cestrum* species has biological characteristics with 1,25-(OH)2D3 (Wasserman et al., 1975). The toxicity od *Cestrum diurnum* is compared with *Solanum Malacoxylon* as these both species are responsible for calcinosis in cattle. According to reports, these plants contain a glycoside of 1,25-dihydroxy vitamin D, or 1,2S-(OH),D, as their active ingredient. The most effective hormonal form of vitamin D, known as 1,25-(OH), D, is what keeps the balance of calcium and phosphorus in higher organisms. Therefore, calcinogenic plants can be considered a significant and affordable source of 1,25-(OH)2D for both human and veterinary usage (Prema & Raghuramulu, 1994).

3 Methodology

3.1 Plant Collection

Cestrum diurnum was purchased from nursery located in H-9 and the accession number was retrieved from National History Museum.

3.2 Plant Extract Preparation

The leaves were washed, shade dried and then grinded to form fine powder. Extract was prepared in ethanol in the ratio of 1:10 by the help of Soxhlet apparatus. 10 gram of leave powder was subjected to Soxhlet extraction with 100mL of ethanol. After the extract formation, it was filtered by taking precautions of light and temperature to avoid adulteration of extract.

The extract was shifted into petri plates for evaporation of solvent and placed into incubator. The temperature of incubator was kept 60ºC to save the bioactive components from any harm. After the evaporation of solvent, the extract was scratched out from petri plates in the form of crystallized powder and was stored at 4ºC for further use.

3.3 Extract Characterization

3.3.1 Preliminary Phytochemical Screening

Alkaloids

Hager's test method was used for the confirmation of presence of alkaloids in plant extract. Few drops of Hager's reagent were added into 2 mL of extract. Appearance of yellow precipitates confirms the presence of alkaloids.

Phenols

Few drops of FeCl3 were added into 1 mL of extract. Appearance of bluish black color confirms the presence of phenols.

Flavonoids

1ml Pb(C2H3O2)4 (10%) was added into 1mL of extract. Appearance of yellow precipitates confirms the presence of flavonoids.

Tannins

Braymer's test method was used for the confirmation of presence of tannins in plant extract 2ml H2O and Few drops of FeCl3 (5%) were added into 2mL extract. Transient greenish to black color indicates the presence of tannins.

Steroids

Salkowski's test method was used for the confirmation of presence of steroids in plant extract 2ml CHCl3 and 2ml cH2SO4 were added into 2mL extract. Appearance of reddish-brown coloration at interface indicates the presence of steroids.

Saponins

5mL water was added into 5 mL extract and it was placed on heat. Appearance of froth indicates the presence of saponins.

Glycosides

Liebermann's test method was used for the confirmation of presence of glycosides in plant extract 2ml CHCl3 and 2ml CH3 COOH were added into 2mL extract. Violet to blue to green coloration indicates the presence of glycosides.

Cardiac Glycosides

Few drops of FeCl3, 1 mL cH2SO4, and 2 mL acetic acid was added into 2mL extract. Violet coloration below brown color indicates presence of cardiac glycosides.

Carbohydrates

Fehling's test was used to check the presence of carbohydrates in extract. 2 mL of extract was added into 1mL of Fehling's solution A and B and the reaction mixture was heated. Formation of red precipitates indicates the presence of carbohydrates.

Amino acids

Ninhydrin test was used to confirm the presence of amino acid in extract. Few drops of ninhydrin solution were added into 1 mL of extract. Violet coloration indicates the presence of amino acids.

Deoxy Sugars

Few drops of FeCl3, 1 mL cH2SO4, and 2 mL acetic acid was added into 2mL extract. Formation of brown ring indicates presence of cardiac glycosides.

3.3.2 Quantitative Phytochemical Screening

Total Phenolic Content

The total phenolic content of plant was determined by Folin-Ciocalteu reagent method with slight modifications in (Beseni et al., 2017). 1mg/mL stock solution was prepared in distilled water. 1mL of plant extract or gallic acid concentrations (25, 50, 75, 100ug/mL) was added into 0.4mL of 100% FC reagent. 4 mL of 75% sodium carbonate was added after 5 min wait. Volume was made up to 10mL and was incubated for 2 hours in dark at room temperature. The absorption was check at

765nm by spectrophotometer and the standard curve was drawn from gallic acid and for sample, measurement of just 1 concentration was taken. For determination of total phenolic content (TPC) linear regression is used from a standard calibration curve of gallic acid. To calculate concentration in mg gallic acid / gram of extract, following formulae were used:

Y=slope x intercept (standard=gallic acid)

X= (y-intercept)/slope

C= c(V/M)

Where c=x, V=vol, M=mass

Total Flavonoid Content

Total flavonoid content of plant extract was determined by using Aluminum chloride method with slight modifications in (Beseni et al., 2017). Stock solution was prepared in 1mg/ 1mL of methanol. The concentrations (25, 50, 75, 100ug/mL) was prepared for rutin as a standard. 100uL of sample or standard concentration was added into 100uL of 10% aluminum chloride. Then 100uL of 1M potassium acetate and 2800uL of distilled water was added. Then mixture was kept at room temperature for 30 minutes. Absorbance was measured at 415 nm by spectrophotometer and the standard curve was drawn from rutin and for sample, measurement of just 1 concentration was taken. For determination of total flavonoid content (TFC) linear regression is used from a standard calibration curve of rutin. To calculate concentration in mg rutin / gram of extract, following formulae were used:

Y=slope x intercept (standard=rutin)

X= (y-intercept)/slope

$$C = c(V/M)$$

Where c=x, V=vol, M=mass

3.3.3 Antioxidant Test

The antioxidant activity of extract was determined quantitatively by using DPPH free radical scavenging assay (Beseni et al., 2017).Stock solutions (1mg/1mL methanol) of extract and standard (L-ascorbic acid) and (1mg/25mL methanol) of DPPH (standard) were prepared. Concentrations (10, 20, 30, 40, 50, 60 ug/mL) of extract, methanol and equal volume of DPPH were added into 96-well plate and was incubated at dark for 1 hour. The absorption was taken from spectrophotometer at 550nm. Methanol was taken as blank, and DPPH was taken as negative control. Percentage inhibition was calculated from following formula:

% inhibition= (absorption of blank- absorption of sample) / absorption of blank x 100

3.3.4 Anti-inflammatory Test

Anti-inflammatory activity of extract was determined by using inhibition of albumin denaturation assay with slight modifications in (Leelaprakash & Dass, n.d.). Stock solutions (1mg/1mL water) of extract and standard (Ansaid) were prepared. Concentrations 0f 1, 10, 100, 1000 ug/mL were prepared from stock solution and added into 200uL of 1% BSA, 2.8mL of 0.2M PBS. Total volume was made up to 5mL and the reaction mixture was kept in water bath 37C for 15 min and in incubator 70C for 5 min. absorption was checked by using spectrophotometer at 620nm. Percentage inhibition was calculated from following formula:

% inhibition= (absorption of control- absorption of sample) / absorption of control x 100

3.3.5 Alpha Amylase Inhibitory Test

Alpha amylase inhibitory activity was performed by slight modifications in (Fatima et al., 2019). Concentrations (2,4,6,8,10 ug/mL) of extract and standard (metformin) was prepared in 1mL of DMSO and added into 1mL of alpha amylase (1 mg/ml (9 unit) α-Amylase was prepared and finally diluted to prepare 8 unit by dissolving in 20 mM phosphate buffer (pH 6.9, containing 6.7mM NaCl)). The reaction mixture was incubated at 25C for 30 min. then, 1 mL was taken from this reaction mixture was taken and mixed with 1mL of starch and incubated for 10 min at 37C. after incubation, 1mL of DNSA was added and placed in water bath at 90C for 5min. The dinitro salicylic acid (DNSA) color reagent was prepared using 96 mM 3,5-dinitrosalicylic acid of 0.438g dissolved in deionized water (20 mL) with the help of magnetic stirrer. The reaction mixture was kept at room temperature to lower its temperature and volume was made up to 10mL with distilled water. Absorption was taken at 540nm by using spectrophotometer. Percentage inhibition was calculated by using the formula:

% inhibition= (absorption of control- absorption of sample) / absorption of control x 100.

3.3.6 Hemolytic Toxicity Assay

Anti-hemolysis assay was performed for the ethanolic extract of *C. diurnum* following the protocol of (Fazel Nabavi et al., 2012) with certain modifications. Different concentrations (5000, 500, 50, 5, and 0.5 ug/mL) of extract were prepared by serial dilution. 0.2M PBS with pH 7.5 was prepared in water. Fresh blood was used for this assay. Plasma was removed by centrifugation at 4000 rpm. RMS red pallet was taken, and it was washed 3 -5 times with equal volume of 0.8% saline solution. With the help of PBS, 2% RBC solution was prepared. 190uL of RBC solution was taken and

added into 100uL of different concentrations of extract prepared. For negative control, PBS was added in place of sample and for positive control, triton X was added. The reaction mixtures were incubated for 30 minutes at 37C. incubation was followed by centrifugation for 6 min at 2500xg. Absorbance was measured at 520nm with spectrophotometer. Percentage inhibition was calculated by following formula:

(Sample-negative control)/(positive control-negative control)*100

With the help of linear regression equation %H50 (hemolysis percentage) was calculated to determine the concentration required for 50% hemolysis.

3.3.7 Oral Acute Toxicity Assay

Acute toxicity of extract was determined orally in normal mice. The protocol was followed from (Hooda et al., 2014). In first phase, 3 groups of mice having 2 mice per group were given plant extract 100, 500, 1000 mg/kg of body weight respectively. The animals were observed for the signs of toxicity and possible death 24 hrs., 72 hrs. and 10 days. In the second phase, 2 groups of mice having 2 mice per group were again given plant extract 2500, 5000 mg/kg of body weight respectively. The animals were observed for the signs of toxicity and possible death 24 hrs. 72 hrs. and 10 days.

3.4 Animal Model Construction

6–8-week-old BALB/c mice were purchased from National Institute of Health, Islamabad. All of the mice were housed in Laboratory Animal house, ASAB, NUST. The experimental procedures and protocols were approved by institutional review board of ASAB. Animals were screened for any physiological and pathological abnormalities. Animals were acclimatized for a week and were given normal basic feed and distilled water.

3.4.1 Diabetes Induction

Diabetic mouse model was constructed by combination of high fat diet and streptozotocin. 20 mice (male and female) were taken and divided into 5 groups (n=4, half male, and half female). 16 mice were given high fat diet (75% milk fat and 25% normal feed) and 4 mice were given normal feed as they were control. The methodology for induction of diabetes was adopted from (Parveen et al., 2018). High fat diet was given for 3 weeks. At the end of 3rd week 4 intraperitoneal injection of streptozotocin (40mg/kg) were given for 4 consecutive days and high fat diet was continued for 4th week. After giving streptozotocin injection mice were given 10% sucrose water. At the end of 4th week, fasting blood glucose levels were checked by using On-Call blood glucose meter. Mice showing fasting blood glucose above the level of 200 mg/dl were taken as diabetic.

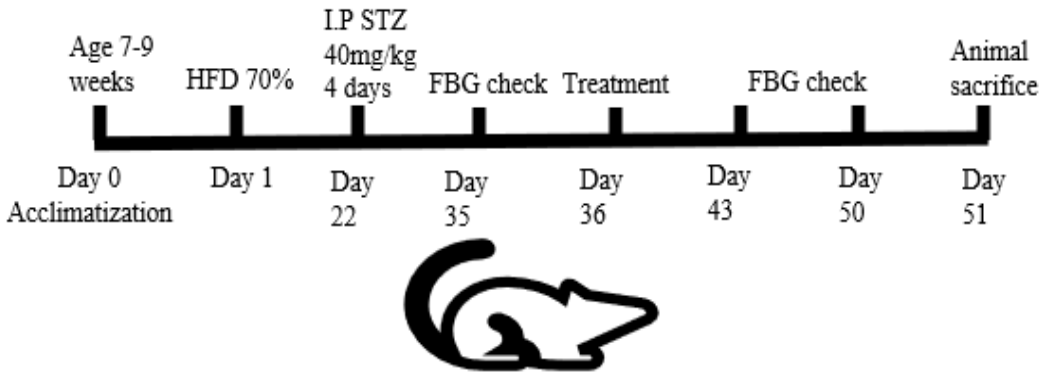


Figure 3.1 *Schematic representation of diabetic model construction*

3.4.2 Treatment of Mice Models

Mice were divided into five groups and each group was given different kind of treatment. 4 groups were of diabetic, and the last group consist of normal mice. Negative control group was given no treatment, positive control group was given standard treatment of metformin 250mg/kg. Experimental group 1 was given plant

extract of 250mg/kg of body weight while experimental group 2 was given plant extract of 500mg/kg of body weight. Doses were selected after oral acute toxicity assay. Treatment was given for two weeks, and fasting glucose was check d after 4 days. Weight of mice were measured after intervals.

3.4.3 Fasting Blood Glucose Test

Fasting blood glucose was measured after every 4 days. For this purpose, mice were kept on fasting for overnight. Fasting blood glucose was checked by pricking vein from the tail and using On-Call blood glucometer. Values were recorded for further analyses.

3.4.4 Oral Glucose Tolerance Test

Oral glucose tolerance test was performed on overnight fasted normal mice following protocol of (Hooda et al., 2014). Mice were given 10% glucose orally and then their blood glucose levels were checked and recorded after 60 min. then extract of 250mg/kg and 500mg/kg with metformin as standard was given and then glucose level was checked after 30 min to evaluate the glucose reduction potential in normal glucose loaded mice. Blood glucose was measured using On-Call Blood glucose meter.

3.4.5 Histopathology

Histopathological analysis of liver kidney and pancreas was performed to check the therapeutic efficacy of extract. For this purpose, mice were dissected, and their liver, kidney and pancreas were taken and stored in 10% formalin. Hematoxylin and eosin slides were prepared which were observed under light microscope at 4X, 10X and 40X to observe any variation.

3.5 Statistical analysis

Statistical analysis was performed by using GraphPad Prism software. Unpaired student T test was used to compare means and obtain p-values in comparison to standard. P-value of 0.05 and 0.1 was considered as significant. Two way ANOVA was used for comparison among groups. Graphs were made by using GraphPad Prism software.

4 Results

4.1 Plant Collection

Plants were purchased from nursery located in H-9 Islamabad. Documentation, identification, and accession number of plant was retrieved from Herbarium of Pakistan Museum of Natural History, Islamabad.

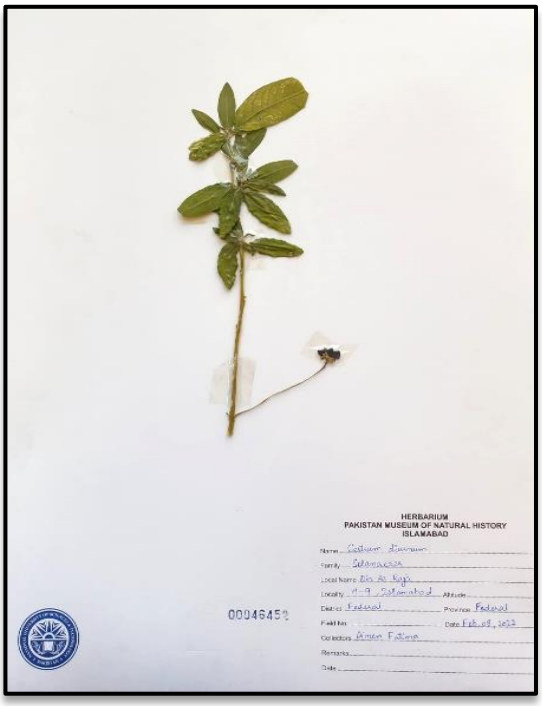


Figure 4.1 *Herbarium sheet*

Figure 4.2 *Cestrum diurnum*

4.2 Plant Extract Characterization

4.2.1 Qualitative Phytochemical Tests

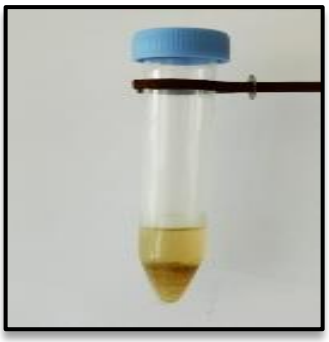
Qualitative phytochemical tests were performed to determine the phytochemical constituents of ethanolic extract of *Cestrum diurnum*. Presence of alkaloids, phenols, flavonoids, tannins, steroids, saponins, glycosides, cardiac glycosides, amino acids and deoxy sugars was confirmed y using respective tests. The tests for glycosides and tannins were negative confirming the absence of these phytochemicals in ethanolic extract of *C. diurnum*.

Serial No.	Phytochemical Tests	*C. diurnum*
1	Alkaloid	+
2	Phenols	+
3	Flavonoids	+
4	Tannins	-
5	Steroids	+
6	Saponins	+
7	Glycosides	-
8	Cardiac glycosides	+
9	Carbohydrates	+
10	Amino acid	+
11	Deoxy sugars	+

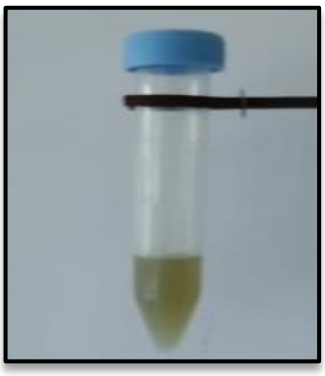
Table 4.1 *Results of the phytochemical testing of Cestrum diurnum*

a- Before

b- Alkaloid



c- Flavonoid



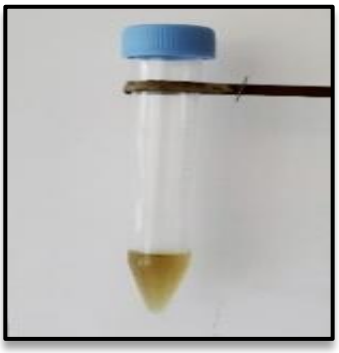
d- Tannins

e- Steroids

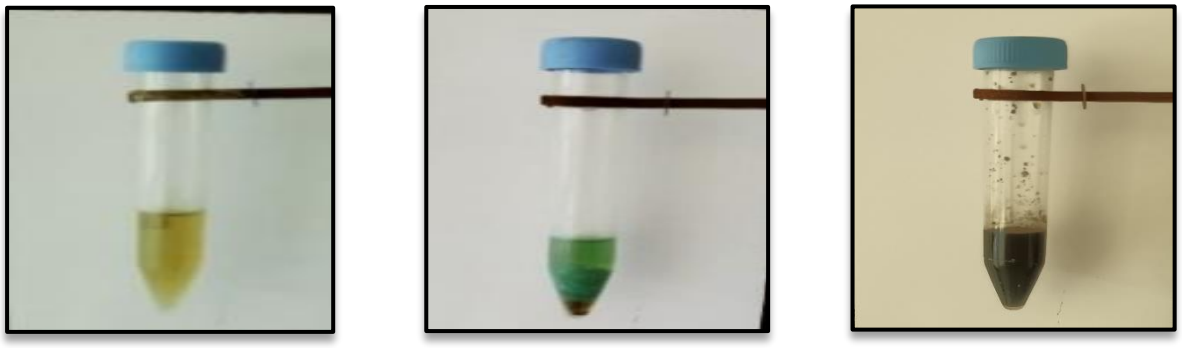
f- Saponins

g- Glycoside

h- Cardiac Glycoside



i- Amino Acid



j- Deoxy sugar **k-** Carbohydrate **l-** Phenol

Figure 4.3 *Results of preliminary phytochemical analysis*

4.2.2 Quantitative Phytochemical Tests:

Total Phenolic Test:

The phenolic content of ethanolic extract of *C. diurnum* was determined as gallic acid equivalent using linear regression from its standard curve. It is expressed as mg of gallic acid equivalent (GAE)/ g of extract. Figure 5 shows the standard curve of gallic acid. Total phenolic content was calculated by using slope of the standard curve. Data shows that 1105 mg of phenolic content is present in per gram of plant extract. The significance of data was proved as the regression analysis gives p-value of 0.0093.

$$C = x\ (V/m)$$

Where,

X = concentration of extract

V = volume of extract

m =mass of extract

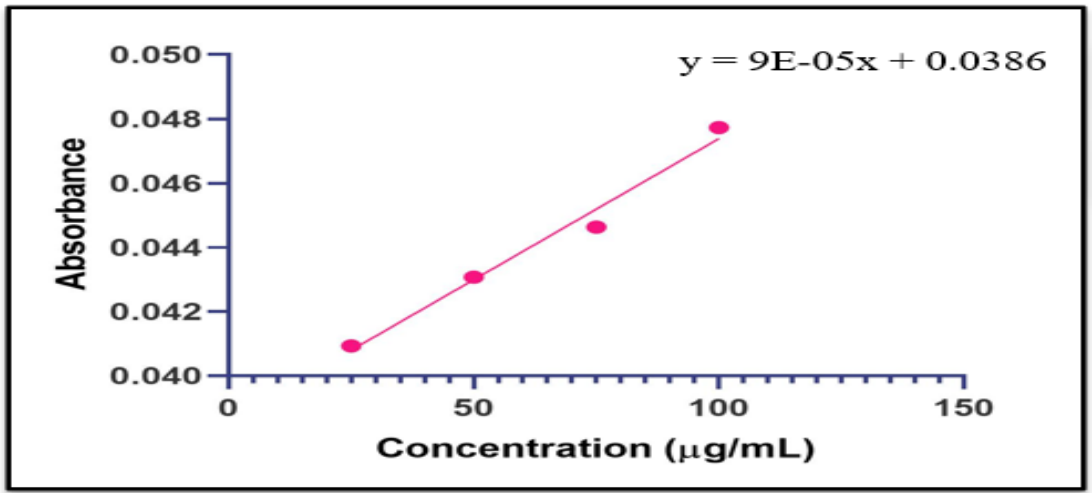


Figure 4.4 *Standard Curve of Gallic Acid*

Total Flavonoid Content:

The total flavonoid content of ethanolic extract of *C. diurnum* was determined as rutin equivalent using linear regression from its standard curve. It is expressed as mg of rutin equivalent / g of extract. Figure 6 shows the standard curve of rutin. Total flavonoid content was calculated by using slope of the standard curve. This data shows that 181.62 mg of flavonoid content is present in per gram of plant extract. The significance of data was proved as the regression analysis gives p-value of 0.0072.

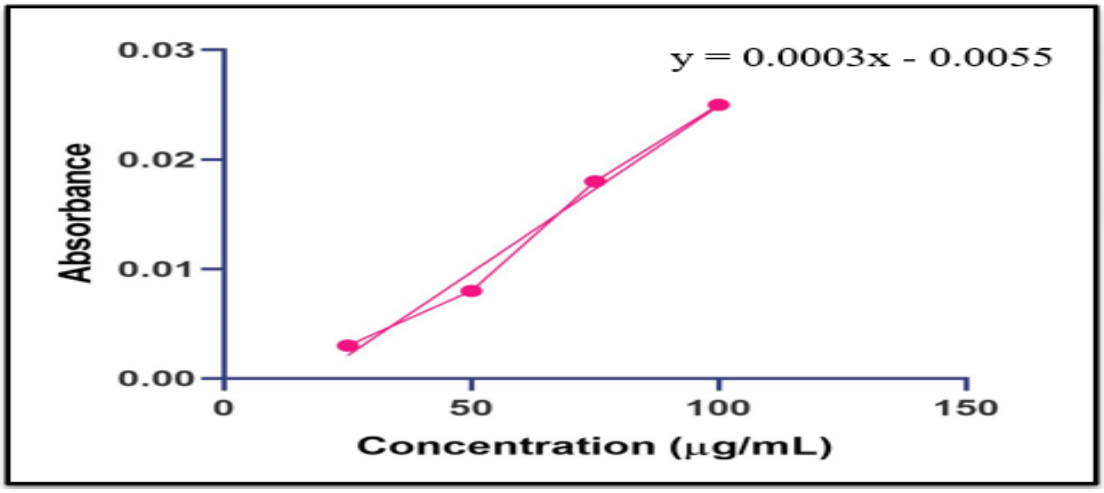


Figure 4.5 *Standard Curve of Rutin*

C= x (V/m)

Where,

X = concentration of extract

V = volume of extract

m =mass of extract

4.3 Antioxidant Test:

Antioxidant activity of *C. diurnum* ethanolic extract was evaluated by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. By observing free radical scavenging activity of ascorbic acid compared to extract, antioxidant activities of both were determined. A direct linear relationship was observed between concentrations and percentage inhibitions for both standard and sample (figure 6). Unpaired T-test was used to compare the mean between the two groups. P-value of 0.0001 was observed which proves that ethanolic extract of *C. diurnum* shows significantly higher antioxidant activity than ascorbic acid.

Figure 4.6 *DPPH assay of standard and plant*

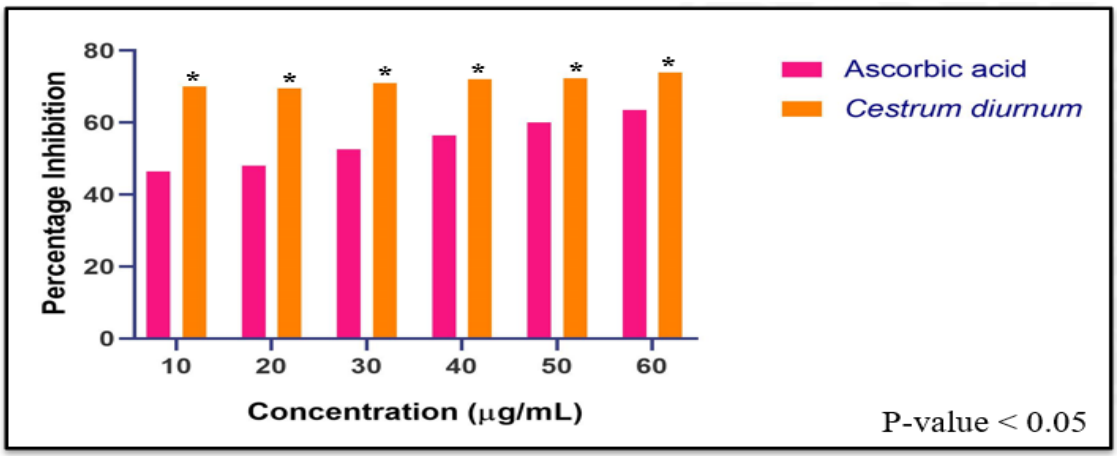


Figure 4.8 *Antioxidant activity of standard and plant extract*

4.4 Anti-inflammatory Test:

Anti-inflammatory activity of ethanolic extract of *C. diurnum* was evaluated by using albumin denaturation assay. Continuous trend was observed in case of standard and extract. Data shows that the protein denaturation activity of extract at the concentrations of 1, 10, and 100 ug/mL was lower as compared to standard but at the concentration of 1000ug/mL, it is greater than the standard. P-value of 0.8880 was observed which shows that the anti-inflammatory activity of extract is significantly lower than the standard.

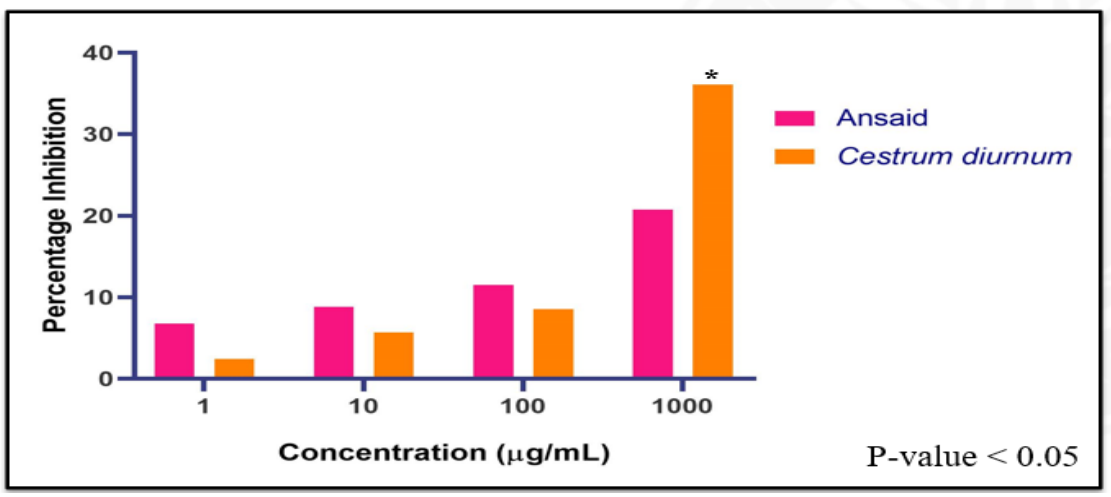


Figure 4.9 *Albumin denaturation activity of standard and plant extract*

4.5 Alpha Amylase Inhibition Assay:

Alpha amylase inhibition assay was performed to evaluate in-vitro anti-diabetic activity of ethanolic extract of *C. diurnum.* A direct linear relationship was observed between concentrations and percentage inhibitions for both standard and sample. P value of 0.0071 was observed by applying unpaired T-test which proves that the alpha amylase inhibition activity of extract is significantly higher than the standard.

Figure 4.10 *Alpha amylase inhibition test for plant extract*

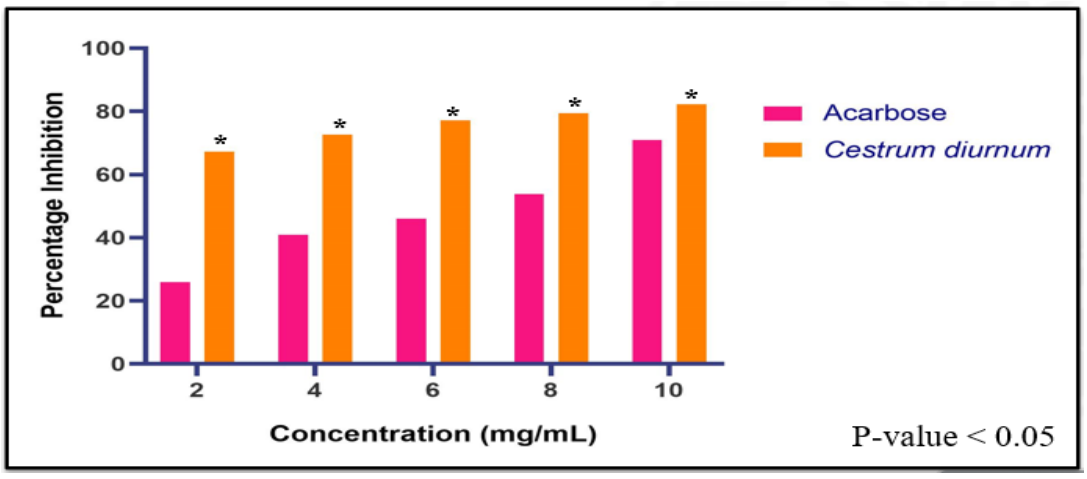


Figure 4.11 *Alpha amylase inhibition activity of standard and plant extract*

4.6 Hemolytic Toxicity Test:

Anti-hemolysis activity of ethanolic extract of *C. diurnum* was evaluated by taking triton X as positive control. The effect of extract on human RBCs were evaluated and the extract was proved to be harmless to RBCs as the maximum hemolysis percentage of 30.3 was observed at 1000 ug/mL. Figure 10 shows that the extract shows satisfactory inhibitory activity against hemolysis even at low concentrations. The significance of data was proved as the regression analysis gives p-value < 0.0001.

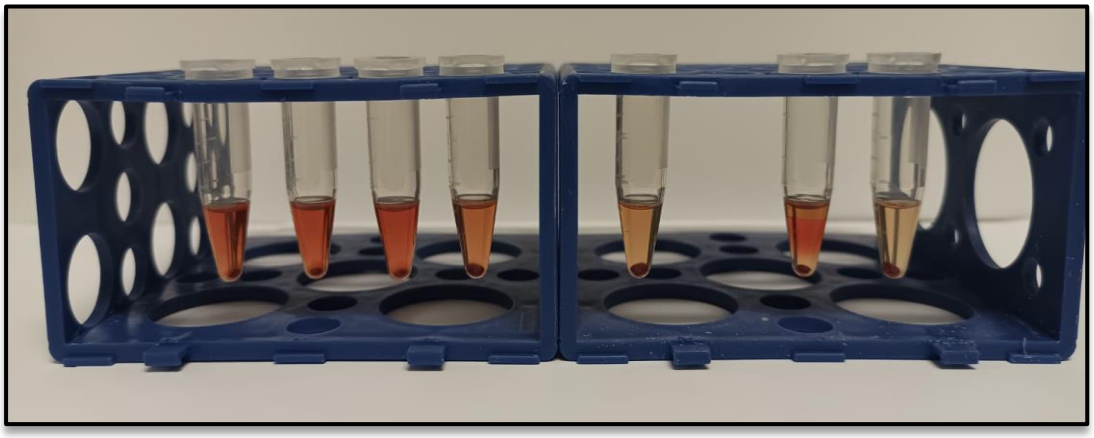


Figure 4.12 *Hemolytic toxicity assay for plant extract*

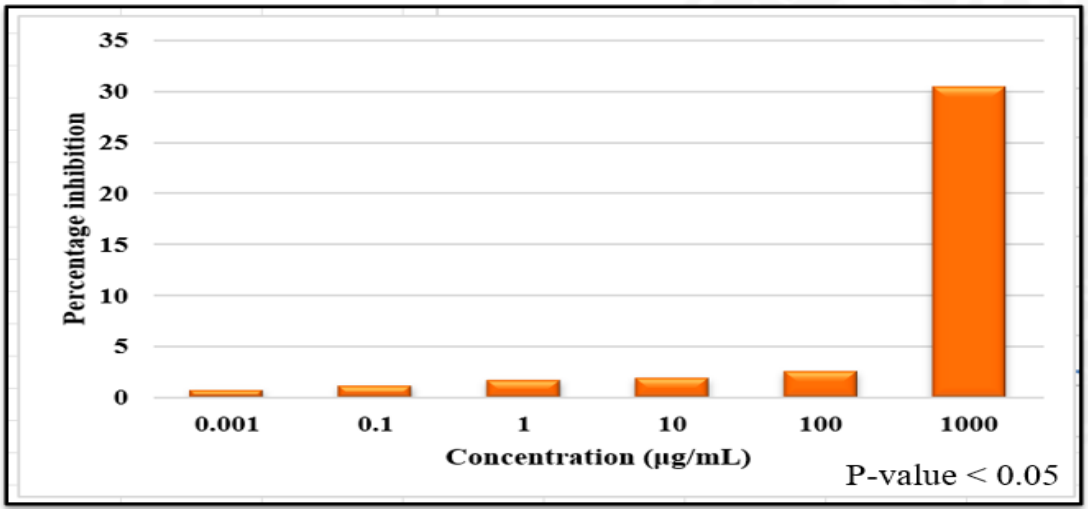


Figure 4.13 *Hemolytic toxic activity of plant extract*

4.7 Oral Acute Toxicity Assay:

Oral acute toxicity assay was carried out in two phases to check out the median lethal dose of ethanolic extract of *Cestrum diurnum*. In first phase, 3 groups of mice (n=2) were taken and administered plant extract of 100, 500, 1000 mg/kg of body weight. Mice were observed for any sign of toxicity and possible death for 24h, 72hr, and 7 days. No mortality and toxicity were observed in first phase. In second phase, two group of mice (n=2) were taken and administered plant extract of 2500 and 5000 mg/kg of body weight. Mice were monitored for toxicity and possible death as in phase one. 1 death was observed in group 5 which were given 5000mg plant extract /kg of body weight.

Extract dose (mg/kg body weight)	Mortality
100	0/2
500	0/2
1000	0/2

Table 4.2 *Record of mortality in phase 1*

Extract dose (mg/kg body weight)	Mortality
2500	0/2
5000	1/2

Table 4.3 *Record of mortality in phase 2*

Median lethal dose (LD50) of plant extract was calculated by using Korbe's method:

$$LD50 = \sqrt{Highest dose\ that\ recorded\ no\ death \times Lowest\ dose\ that\ recorded\ death}$$

$$= \sqrt{2500} \times 5000$$

= 3535.53 mg/kg bwt

It shows that ethanolic extract of *Cestrum diurnum* leaves is toxic above the dose of 3535.53 mg/kg of body weight.

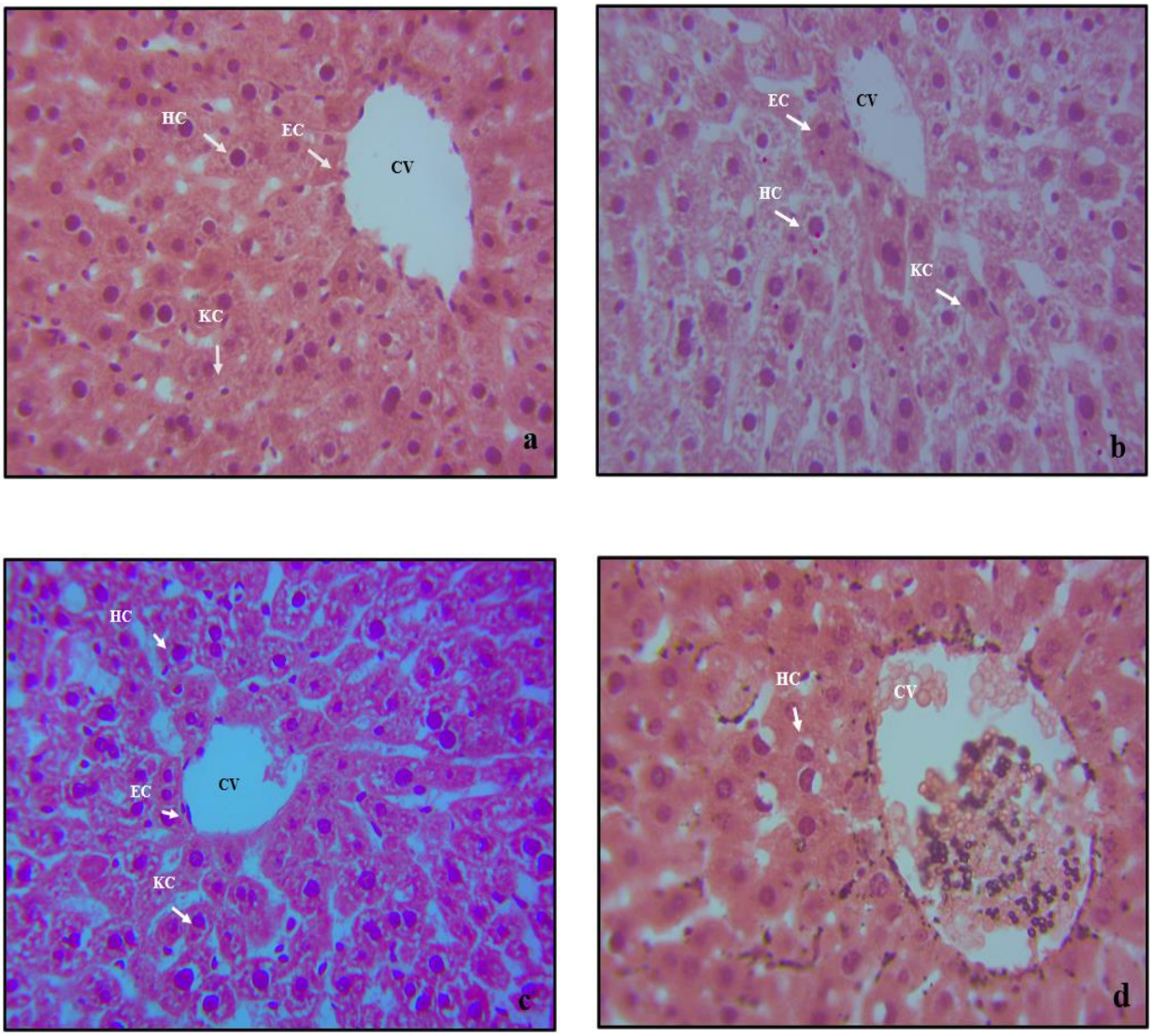


Figure 4.14 *Figure Photomicrographs of mice liver. (a) Represents the liver section of normal mouse in normal physiological state. (b) And (c) represents liver section of mice given 500 and 2500 mg/kg of plant extract respectively with no sign of any structural and physiological abnormality. (d) Represents liver section for mouse given 5000mg/kg of plant extract. This slide shows that the lumen of central vein has blood showing necrosis. The surrounding hepatocytes show infarction and is infiltrated by macrophages and lymphocytes showing signs toxicity.*

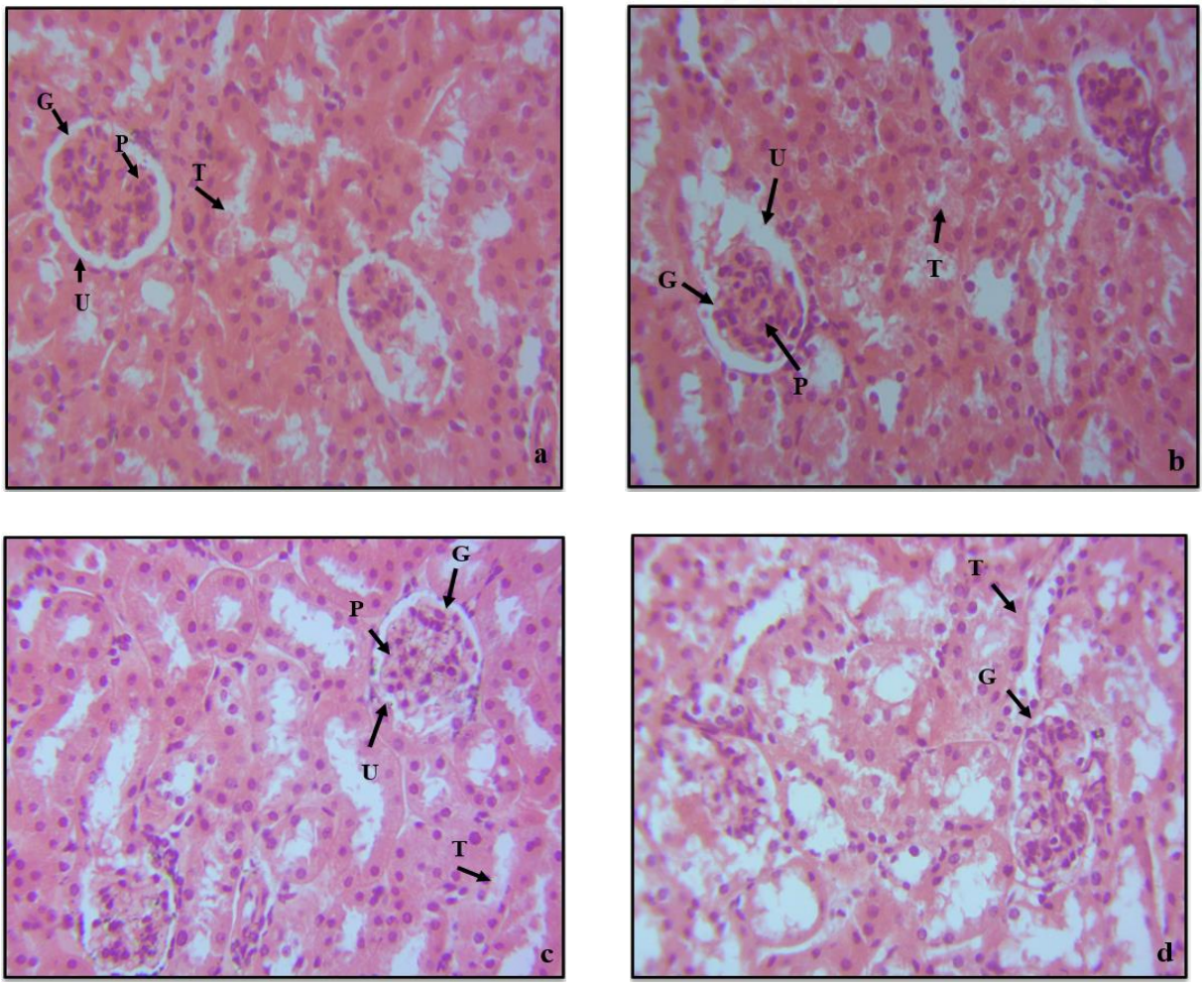


Figure 4.15 *Figure Photomicrographs of mice kidney. (a) Represents the kidney section of normal mouse in normal physiological state. (b) and (c) represents kidney section of mice given 500 and 2500 mg/kg of plant extract respectively with no sign of any structural and physiological abnormality. (d) represents kidney section for mouse given 5000 mg/kg of plant extract. This slide shows Section shows infiltration of glomerulus by acute and chronic inflammatory cells surrounding tissues show increase tubules and infarction and few viable tubules are infiltrated by chronic inflammatory cells showing signs of toxicity.*

4.8 Oral Glucose Tolerance Test

Oral glucose tolerance test was conducted to investigate the glucose homeostasis in normal mice. Blood glucose level was checked after time intervals. These results showed that there is significant potential of glucose reduction in plant extract as compared to the normal group which was not given any treatment.

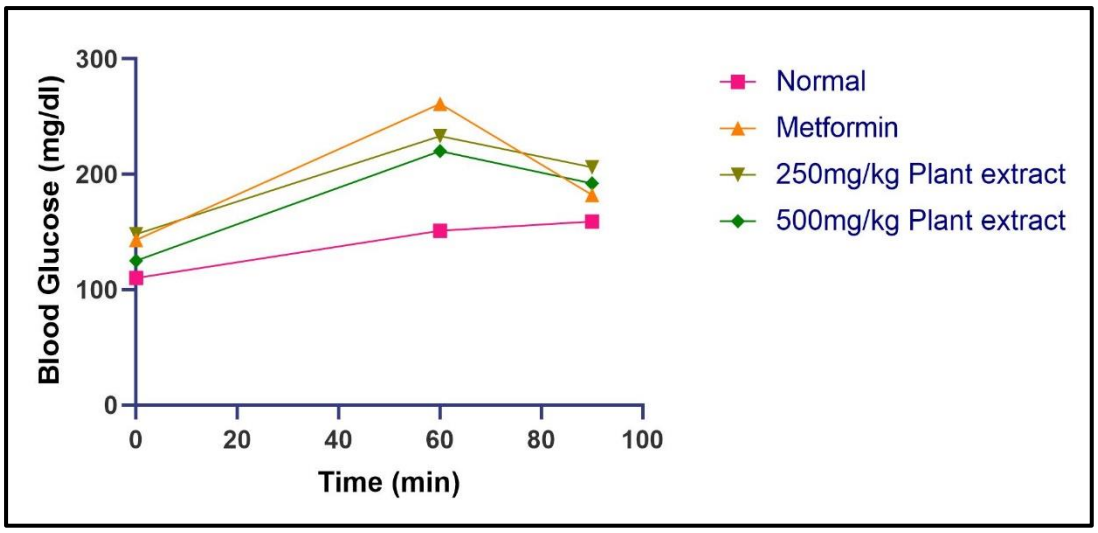


Figure 4.16 *Evaluation of glucose tolerance potential in normal glucose loaded mice. The metformin and extract treated group show reduction in glucose level.*

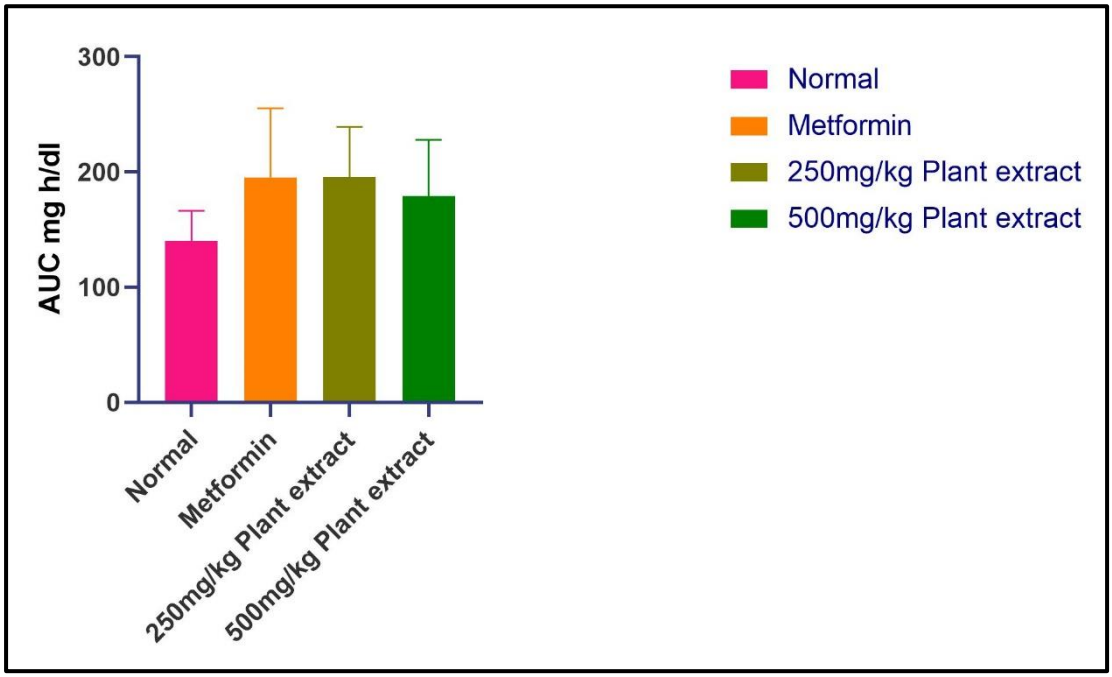


Figure 4.17 *Area under the curve estimated for oral glucose tolerance. The metformin and extract treated groups showed the significant area under the curve as compared to normal group.*

4.9 Effect of Plant Extract on Body Weight of Normal and Diabetic Mice

Effect on body weight on diabetic mice groups treated differently was evaluated by measuring weight after 4 days and it was recorded in the form of graph shown below. Mice treated with STZ and given no treatment show continuous decrease in body

eight due to pancreatic inflammation on the other hand, mice groups treated with metformin and plant extract show increase in body weight. Among these groups, mice group given 250mg/kg of plant extract showed significantly higher affectivity on body weight. After 14 days of treatment, the body weight loss due to STZ was significantly reversed.

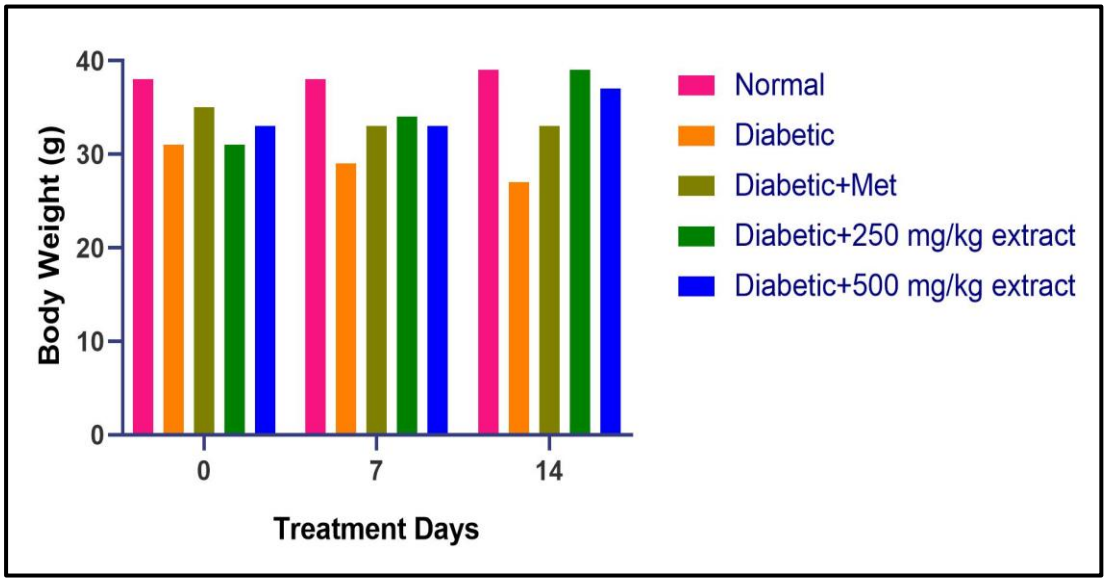


Figure 4.18 *Effect of Cestrum diurnum leaves extract on body weight*

4.10 Effect of Plant Extract on Glucose Reduction

Glucose reduction potential of plant extract was investigated for the groups treated differently by measuring fasting blood glucose level every week. Diabetic group treated with plant extract showed significantly decrease in blood glucose level as compared to the group treated with metformin for first seven days of treatment. During second week, reduction in fasting blood glucose level was observed similar to metformin. It was also observed that group treated with 250mg/kg of extract showed significantly higher activity than other groups.

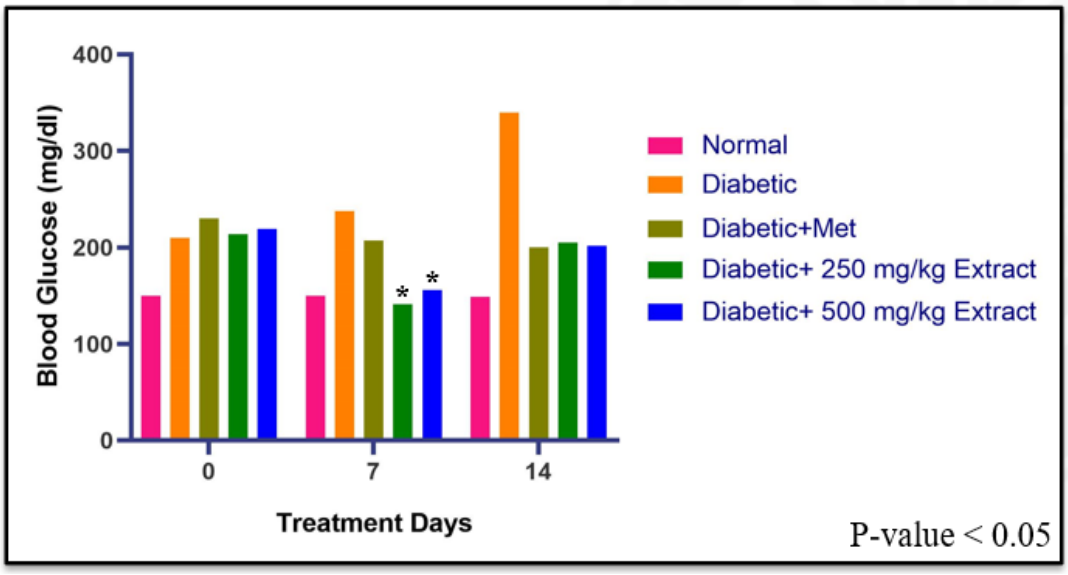


Figure 4.19 *Effect of Cestrum diurnum leaves extract on fasting blood glucose level*

4.11 Histopathological Analysis of Liver

For the histopathological analysis of liver, slides were observed under 10X. Normal physiology was observed in normal group. Diabetic group shows distorted morphology around the central vein while groups treated with metformin, 250 and 500 mg/kg of plant extract shows restoration of normal physiology around central vein.

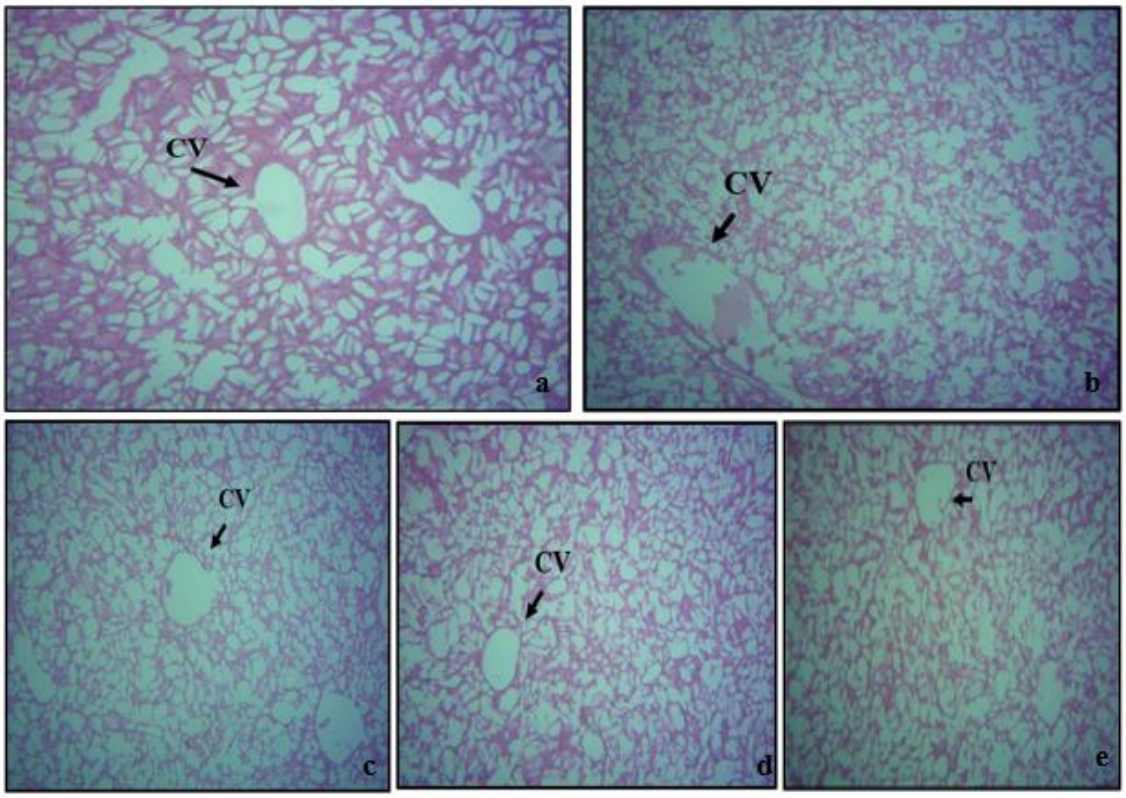


Figure 4.20 *Histopathological analysis of Liver. A= shows normal physiology of Liver, B= shows distorted physiology of liver, C= Shows Liver section from mice treated with metformin, D=shows liver section from mice treated with 250mg/kg of extract E= shows section from 500mg/kg of plant extract treated group*

4.12 Histopathological Analysis of Kidney

In the histopathological analysis of kidney, the normal group shows normal physiological state of cell and tissue around glomerulus. Diabetic group shows distorted morphology of kidney while the groups treated with metformin and plant extract shows restoration of cells around glomerulus.

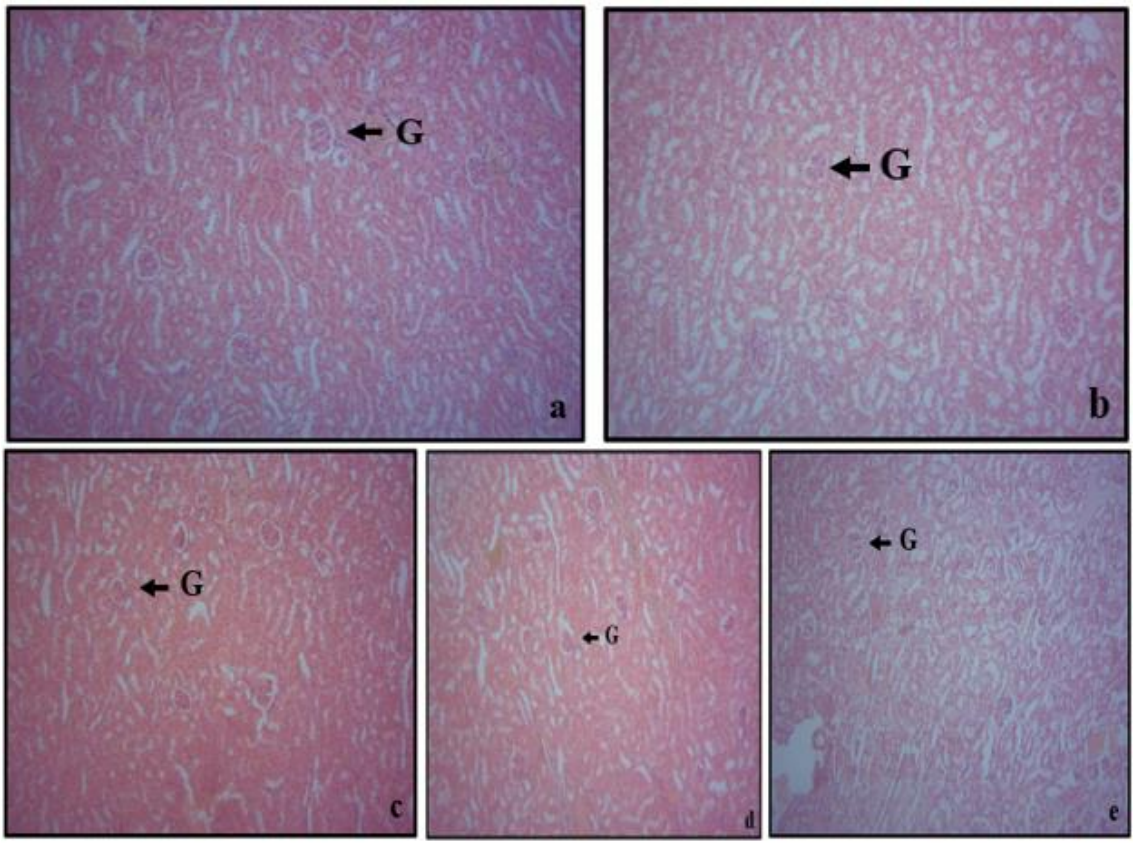


Figure 4.21 *Histopathological analysis of Kidney. A= shows normal physiology of Kidney, B= shows distorted physiology of Kidney, C= Shows Kidney section from mice treated with metformin, D=shows Kidney section from mice treated with 250mg/kg of extract E= shows section from 500mg/kg of plant extract treated group*

5 Discussion

Diabetes Mellitus (DM) is one of the oldest known diseases to mankind. DM is a metabolic disease, divided into 3 types, characterized by high blood glucose levels which need to be monitored periodically. It is primarily characterized by (i) the inability of pancreatic β-cells to produce insulin and (ii) the inability of body tissues to respond to insulin effectively. Insulin insensitivity and insufficiency are the leading causes of insulin resistance leading to cell damage and abnormalities. The main therapeutic target is to restore the ability of beta cells to produce insulin and target tissues to respond effectively towards insulin (Wu et al., 2014b).

Medicinal plant has great therapeutic potential, and they are being explored and utilized since ages. *Cestrum diurnum*, member of family Solanaceae, has been explored due to presence of wide range of bioactive compounds in it. Leaves of cestrum diurnum have been used locally to treat skin diseases, burns, and swellings. Volatile oils made from cestrum diurnum have been in use against mosquitoes due to its sweet and strong smell .(Khatun et al., 2014)This study was carried out to investigate the anti-diabetic potential of ethanolic extract of *Cestrum diurnum* leaves.

Preliminary qualitative phytochemical screening has confirmed the presence of alkaloids, phenols, flavonoids, glycosides, tannins, cardiac glycosides, carbohydrates, amino acids and deoxy sugars in it which shows that this plant is source of a wide range of phytochemicals that can prove its therapeutic efficiency (Prasad et al., 2013). To evaluate the phytochemical contents, quantitative phytochemical screening was conducted. Total phenolic test has revealed that the plant extract possesses 1105 mg of phenolic content per gram while total flavonoid test shows that 181.62 mg of

flavonoid content is present per gram of plant extract. Phenolics and flavonoids are the strong antioxidants found in plants.

Antioxidant activity of plant extract is also evaluated by using DPPH radical scavenging assay which shows that the plant extract has significantly higher antioxidant as compared to ascorbic acid taken as standard. The antioxidant properties of medicinal plants may complement the disease defense they provide. Free radicals are known to harm cells by covalent binding and lipid peroxidation, which results in tissue injury. Because of their capacity to scavenge free radicals, natural antioxidant agents have garnered particular attention (Rao, 2017).

Anti-inflammatory activity of ethanolic extract of *C. diurnum* was evaluated by using albumin denaturation assay. Protein denaturation is a well-known contributor to inflammation. The potential of plant extract to suppress protein denaturation was investigated as aspect of the process of the anti-inflammation effect. The test shows that at maximum concentration of 1000ug/mL, extract shows significantly higher activity than standard but below this concentration the inhibitory activity of extract is lower than standard.

Alpha amylase inhibition assay was carried to explore the anti-diabetic efficacy of ethanolic extract of cestrum diurnum. Since a-amylase is one of the key enzymes that break down starch into simple sugars, a-amylase inhibitors can slow down the digestion of carbohydrates and reduce rates of glucose uptake. As a result, they can increase glucose tolerance in diabetic individuals and lower attenuated postprandial plasma glucose levels (Leelaprakash & Dass, n.d.). Therefore, alpha amylase test was focused on this study for estimation of anti-diabetic effect of extract. This test shows

that plant extract has significantly higher inhibitory activity against alpha amylase than standard and proves anti-diabetic potential of *Cestrum diurnum.*

Hemolytic toxicity assay was carried out to predict the toxic hemolysis cause by drug. Drugs induce toxicity is rare, but it has serious effects on human body. test was carried out on human blood taken on same day (Fazel Nabavi et al., 2012). The effect of extract on human RBCs was evaluated and the extract was proved to be harmless to RBCs as the maximum hemolysis percentage of 30.3 was observed at 1000 ug/mL.

Antimicrobial testing was carried out at two different concentrations against two different, gram positive and gram negative, bacteria. Disk diffusion method was used for this purpose which shows zero percent activity of extract against both bacteria in comparison with ampicillin standard. It shows that up to concentration of 2mg/mL, the extract shows no anti-bacterial activity, but it should be studied further at higher concentration. The results were in accordance with the study conducted by Panghal et al., 2011 which shows that antimicrobial activity of cestrum diurnum ethanolic extract against several bacteria was zero up to concentration 4mg/ml.

Oral acute toxicity assay was carried out before conducted experiment on mice models to calculate the safest dose. The tendency of a chemical to induce adverse effects "quite fast" following one oral dosage or a four-hour air exposure is known as acute toxicity (also known as lethal toxicity). It is also known as the "Median Lethal Dose" at times. The lethal dose (LD50) of a chemical is primarily the concentration at which half (i.e., 50%) of a group of a specific animal species, typically rats or mice, would die when the substance enters their bodies through a specific method. It is often represented as the quantity of chemical delivered milligram per kilogram (for larger subjects) or per 100 g (for smaller animals) of the test animal's body weight (C & A,

2014). Median lethal dose of plant extract was calculated to be 3535.53 mg/kg bwt which means that it is safe to use up to this dose.

The effect of plant extract on body weight was also assessed. Streptozotocin has negative impact on pancreatic cells leading to inflammation and necrosis. This results in the negative impact on body weight (Gebremeskel et al., 2020). In this study, it was observed that the body weight of normal control increases within normal range while for diabetic control, it keeps decreasing below normal range. Groups treated with standard and plant extract shows that the body weight loss was reversed which confirms that the plant extract has healthy effect on body weight.

The in-vivo glucose reduction potential of plant extract was evaluated through diabetic mice model. This study reveals that the group treated with 250mg/kg of plant extract show higher glucose reduction potential than standard and 500mg/kg dose. Fasting blood glucose level was recorded after every week. Treatment was given for 14 days. During first week, higher reduction potential was observed than standard by both 250 and 500mg/kg dose group. On the other hand, during second week, the reduction in blood glucose level was similar to the standard.

Conclusion

The study reveals that the ethanolic extract of *Cestrum diurnum* leaves extract possess significant antioxidant, anti-inflammatory, anti-diabetic potential. It also reveals the healthy effect on body weight and remarkable reduction in blood glucose level which shows that the extract can be a potential source of antioxidants and alpha amylase inhibitors for future considerations. This study also reveals the acute toxicity and hemolytic toxicity potential of *Cestrum diurnum*.

References

Aamir, A. H., Ul-Haq, Z., Mahar, S. A., Qureshi, F. M., Ahmad, I., Jawa, A., Sheikh, A., Raza, A., Fazid, S., Jadoon, Z., Ishtiaq, O., Safdar, N., Afridi, H., & Heald, A. H. (2019). Diabetes Prevalence Survey of Pakistan (DPS-PAK): Prevalence of type 2 diabetes mellitus and prediabetes using HbA1c: A population-based survey from Pakistan. In *BMJ Open* (Vol. 9, Issue 2). BMJ Publishing Group. https://doi.org/10.1136/bmjopen-2018-025300

Acharya, E., & Pokhrel, B. (2006). *Siwakoti) and B. Pokhrel / Our Nature* (Vol. 4).

Aronoff, S. L., Berkowitz, K., & Shreiner, B. (2004). Glucose Metabolism and Regulation: Beyond Insulin and Glucagon. In *Diabetes Spectrum* (Vol. 17, Issue 3). http://diabetesjournals.org/spectrum/article-pdf/17/3/183/558189/0183.pdf

Beseni, B. K., Bagla, V. P., Njanje, I., Matsebatlela, T. M., Mampuru, L., & Mokgotho, M. P. (2017). Antioxidant, Antiglycation, and Hypoglycaemic Effect of Seriphium plumosum Crude Plant Extracts. *Evidence-Based Complementary and Alternative Medicine*, *2017*. https://doi.org/10.1155/2017/6453567

Bhattacharjee, I., Kumar Chatterjee, S., Ghosh, A., Chandra, G., & Professor, Ds. (2011). Antibacterial activities of some plant extracts used in Indian traditional folk medicine Asian Pacific Journal of Tropical Biomedicine. In *Asian Pacific Journal of Tropical Biomedicine*. www.elsevier.com/locate/apjtb

C, O. J., & A, U. A. (2014). Oral acute toxicity (LD 50) study of methanol extract of Annona senegalensis leaf in albino mice. *Sky Journal of Biochemistry Research*, *3*(5), 46–048. http://www.skyjournals.org/SJBR

Chemistry, P. (n.d.). *Phytochemical and Biological Evaluation of Cestrum diurnum (Solanaceae) MASTER OF PHILOSOPHY*.

Cole, J. B., & Florez, J. C. (2020). Genetics of diabetes mellitus and diabetes complications. In *Nature Reviews Nephrology* (Vol. 16, Issue 7, pp. 377–390). Nature Research. https://doi.org/10.1038/s41581-020-0278-5

Dabholkar, N., Rapalli, V. K., & Singhvi, G. (2021). Potential herbal constituents for psoriasis treatment as protective and effective therapy. In *Phytotherapy Research* (Vol. 35, Issue 5, pp. 2429–2444). John Wiley and Sons Ltd. https://doi.org/10.1002/ptr.6973

Edward J Boyko, S. K. D. J. M. P. S. H. S. (2021). *diabetesatlas*. https://diabetesatlas.org/

El-Demerdash, M. M., El-Sayed, A. S., Georg, N. M., Abou-Elnour, A., & Nosier, H. (2021). Biosystematic studies of some Egyptian species of Cestrum (Solanaceae). *Molecular Biology Reports*, *48*(5), 4497–4515. https://doi.org/10.1007/s11033-021-06471-1

El-Kebbi, I. M., Bidikian, N. H., Hneiny, L., & Nasrallah, M. P. (2021). Epidemiology of type 2 diabetes in the Middle East and North Africa: Challenges and call for action. *World Journal of Diabetes*, *12*(9), 1401–1425. https://doi.org/10.4239/wjd.v12.i9.1401

Fatima, S., Akhtar, M. F., Ashraf, K. M., Sharif, A., Saleem, A., Akhtar, B., Peerzada, S., Shabbir, M., Ali, S., & Ashraf, W. (2019). Antioxidant and alpha amylase inhibitory activities of Fumaria ofcinalis and its antidiabetic potential against alloxan induced diabetes. *Cellular and Molecular Biology*, *65*(2), 50–57. https://doi.org/10.14715/cmb/2019.65.2.8

Fazel Nabavi, S., Nabavi, S. M., Hellio, C., Alinezhad, H., Zare, M., Azimi, R., & Baharfar, R. (2012). Antioxidant and antihemolytic activities of methanol extract of Hyssopus angustifolius. In *Journal of Applied Botany and Food Quality* (Vol. 85).

Fouad, M. A., Mohamed, K. M., Kamel, M. S., Matsunami, K., & Otsuka, H. (2008). Cesdiurins I-III, steroidal saponins from Cestrum diurnum L. *Journal of Natural Medicines*, *62*(2), 168–173. https://doi.org/10.1007/s11418-007-0211-2

Galicia-Garcia, U., Benito-Vicente, A., Jebari, S., Larrea-Sebal, A., Siddiqi, H., Uribe, K. B., Ostolaza, H., & Martín, C. (2020). Pathophysiology of type 2 diabetes mellitus. In *International Journal of Molecular Sciences* (Vol. 21, Issue 17, pp. 1–34). MDPI AG. https://doi.org/10.3390/ijms21176275

Gebremeskel, L., Tuem, K. B., & Teklu, T. (2020). Evaluation of antidiabetic effect of ethanolic leaves extract of becium grandiflorum lam. (lamiaceae) in streptozotocin-induced diabetic mice. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, *13*, 1481–1489. https://doi.org/10.2147/DMSO.S246996

Ghosh, A., & Chandra, G. (2006). Biocontrol efficacy of Cestrum diurnum L. (Solanaceae: Solanales) against the larval forms of Anopheles stephensi. *Natural Product Research*, *20*(4), 371–379. https://doi.org/10.1080/14786410600661575

Ghosh, A., Rawani, A., Mondal, R. P., & Chandra, G. (2021). Mosquito larvicidal and antimicrobial activities of synthesized silver nanoparticles (AgNP) using mature fruit extract of Cestrum diurnum L. In *Indian Journal of Natural Products* (Vol. 12, Issue 4).

Giugliano, D., Ceriello, A., & Esposito, K. (2008). *Glucose metabolism and hyperglycemia 1-5*. https://academic.oup.com/ajcn/article/87/1/217S/4633392

Goldstein, B. J. (n.d.). *Insulin Resistance as the Core Defect in Type 2 Diabetes Mellitus*.

Halban, P. A., Polonsky, K. S., Bowden, D. W., Hawkins, M. A., Ling, C., Mather, K. J., Powers, A. C., Rhodes, C. J., Sussel, L., & Weir, G. C. (2014). β-Cell failure in type 2 diabetes: Postulated mechanisms and prospects for prevention and treatment. *Journal of Clinical Endocrinology and Metabolism*, *99*(6), 1983–1992. https://doi.org/10.1210/jc.2014-1425

Hills, A. P., Arena, R., Khunti, K., Yajnik, C. S., Jayawardena, R., Henry, C. J., Street, S. J., Soares, M. J., & Misra, A. (2018). Epidemiology and determinants of type 2 diabetes in south Asia. In *The Lancet Diabetes and Endocrinology* (Vol. 6, Issue 12, pp. 966–978). Lancet Publishing Group. https://doi.org/10.1016/S2213-8587(18)30204-3

Hooda, M. S., Pal, R., Bhandari, A., & Singh, J. (2014). Antihyperglycemic and antihyperlipidemic effects of Salvadora persica in streptozotocin-induced diabetic rats. *Pharmaceutical Biology*, *52*(6), 745–749. https://doi.org/10.3109/13880209.2013.869607

Jawale, C. (2014a). *LARVICIDAL ACTIVITY OF SOME SAPONIN CONTAINING PLANTS AGAINST THE DENGUE VECTOR AEDES AEGYPTI* (Vol. 3, Issue 1). www.sciencejournal.in

Jawale, C. (2014b). *LARVICIDAL ACTIVITY OF SOME SAPONIN CONTAINING PLANTS AGAINST THE DENGUE VECTOR AEDES AEGYPTI* (Vol. 3, Issue 1). www.sciencejournal.in

Kalita, D., Holm, D. G., LaBarbera, D. v., Petrash, J. M., & Jayanty, S. S. (2018). Inhibition of α-glucosidase, α-amylase, and aldose reductase by potato polyphenolic compounds. *PLOS ONE*, *13*(1), e0191025. https://doi.org/10.1371/journal.pone.0191025

Khatun, A., Chowdhury, U. K., Jahan, A., & Rahman, M. (2014). Cytotoxic and thrombolytic activity of the aerial part of Cestrumdiurnum L. (Solanaceae). *Pharmacologyonline*, *1*.

Khatun, A., Rahman, M., Nesa, Mst. L., Looi, C. Y., Wong, W. F., Hazni, H., bin Mahdzir, M. A., Uddin, S. J., Awang, K., & Shilpi, J. A. (2022). Analgesic, anti-inflammatory and NF-κB inhibitory activity of aerial parts of Cestrum diurnum. *Clinical Phytoscience*, *8*(1). https://doi.org/10.1186/s40816-022-00340-5

Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. (2009). www.atcc.org

Leelaprakash, G., & Dass, S. M. (n.d.). INVITRO ANTI-INFLAMMATORY ACTIVITY OF METHANOL EXTRACT OF ENICOSTEMMA AXILLARE. In *Int. J. Drug Dev. & Res* (Vol. 3, Issue 3). http://www.ijddr.in

Maddaloni, E., Cavallari, I., Napoli, N., & Conte, C. (2018). Vitamin D and Diabetes Mellitus. *Frontiers of Hormone Research*, *50*, 161–176. https://doi.org/10.1159/000486083

Marchetti, P., Suleiman, M., de Luca, C., Baronti, W., Bosi, E., Tesi, M., & Marselli, L. (2020). A direct look at the dysfunction and pathology of the β cells in human type 2 diabetes. In *Seminars in Cell and Developmental Biology* (Vol. 103, pp. 83–93). Elsevier Ltd. https://doi.org/10.1016/j.semcdb.2020.04.005

Mohamed, K. M., Fouad, M. A., Matsunami, K., Kamel, M. S., & Otsuka, H. (2007a). A new norlignan glycoside from Cestrum diurnum L. *Arkivoc*, *2007*(13). https://doi.org/10.3998/ark.5550190.0008.d09

Mohamed, K. M., Fouad, M. A., Matsunami, K., Kamel, M. S., & Otsuka, H. (2007b). A new norlignan glycoside from Cestrum diurnum L. *General Papers ARKIVOC*, 63–70.

Narayan, K. M. V., Kondal, D., Daya, N., Gujral, U. P., Mohan, D., Patel, S. A., Shivashankar, R., Anjana, R. M., Staimez, L. R., Ali, M. K., Chang, H. H., Kadir, M., Prabhakaran, D., Selvin, E., Mohan, V., & Tandon, N. (2021). Incidence and pathophysiology of diabetes in South Asian adults living in India and Pakistan compared with US blacks and whites. *BMJ Open Diabetes Research and Care*, *9*(1). https://doi.org/10.1136/bmjdrc-2020-001927

Naz, R., & Bano, A. (2013). Phytochemical screening, antioxidants and antimicrobial potential of Lantana camara in different solvents. *Asian Pacific Journal of Tropical Disease*, *3*(6), 480–486. https://doi.org/10.1016/S2222-1808(13)60104-8

Panghal, M., Kaushal, V., & Yadav, J. P. (2011). *In vitro antimicrobial activity of ten medicinal plants against clinical isolates of oral cancer cases*. http://www.ann-clinmicrob.com/content/10/1/21

Parveen, S., Hussain, M. M., & Khan, M. (2018). COMBINED EFFECT OF PIOGLITAZONE AND LEVO-CARNITINE ON SERUM LIPIDS AND ADIPONECTIN IN STREPTOZOTOCIN INDUCED TYPE 2 DIABETIC MICE. In *Pak J Physiol* (Vol. 14, Issue 3). http://www.pps.org.pk/PJP/14-3/Shahida.pdf7

Prasad, M. P., Prabhu, A., Thakur, M. S., & Ruparel, Y. M. (2013). Phytochemical screening, anti-oxidant potential and antimicrobial activities in three species of cestrum plants. *International Journal of Pharma and Bio Sciences*, *4*(2).

Prasanna Bandi, L., Acharya, S., Hegde, V., & Scholar, P. (2018). Pages 101-108 Ayurpharm-International Journal of Ayurveda and Allied Sciences 101 Research Article Cite This Article Lakshmi Prasanna Bandi, Shripathi Acharya, Veeraj Hegde MD. Clinical efficacy of Dinamallika (Cestrum diurnum) in Kitibha kustha (Psoriasis). *Ayurpharm Int J Ayur Alli Sci*, *7*(7), 101–108. www.ayurpharm.comwww.ayurpharm.com

Prema, T. P., & Raghuramulu, N. (1994). *FREE VITAMIN D, METABOLITES IN CESTRUM DZURNUM LEAVES* (Vol. 37, Issue 3).

Punthakee, Z., Goldenberg, R., & Katz, P. (2018). Definition, Classification and Diagnosis of Diabetes, Prediabetes and Metabolic Syndrome. *Canadian Journal of Diabetes*, *42*, S10–S15. https://doi.org/10.1016/j.jcjd.2017.10.003

Ramkissoon, J. S., Mahomoodally, M. F., Ahmed, N., & Subratty, A. H. (2013). Antioxidant and anti-glycation activities correlates with phenolic composition of tropical medicinal herbs Asian Pacific Journal of Tropical Medicine. In *Asian Pacific Journal of Tropical Medicine*. www.elsevier.com/locate/apjtm

Rao, A. (2017). PHYTOCHEMICAL ANALYSIS AND ANATOMICAL STUDY OF TWO SPECIES OF CESTRUM FROM CHANDIGARH. *International Journal of Pharmaceutical Sciences and Research*, *8*(12), 5234–5240. https://doi.org/10.13040/IJPSR.0975-8232.8(12).5234-40

Renu, K., Tripathi, R. D., & Dixit, S. N. (1980). Fungitoxic properties of Cestrum diurnum. *Naturwissenschaften*, *67*(3). https://doi.org/10.1007/BF01073626

SADIQ, S. (2020). *Phytochemical and Biological Evaluation of Cestrum diurnum (Solanaceae) (Doctoral dissertation)*. the islamia university of bahawalpur, pakistan.

Shadab, H., Khan, M. N., Ul Haq, F., Ali, H., El-Seedi, H. R., & Musharraf, S. G. (2020). Cross-mixing study of a poisonous Cestrum species, Cestrum diurnum in herbal raw material by chemical fingerprinting using LC-ESI-QTOF-MS/MS. *Arabian Journal of Chemistry*, *13*(11), 7851–7859. https://doi.org/10.1016/j.arabjc.2020.09.016

Singh Brar, R., & Angad, G. (2011). *Cholecalciferol (Vitamin D3) toxicity in animals*. https://www.researchgate.net/publication/252063370

Taneera, J., Lang, S., Sharma, A., Fadista, J., Zhou, Y., Ahlqvist, E., Jonsson, A., Lyssenko, V., Vikman, P., Hansson, O., Parikh, H., Korsgren, O., Soni, A., Krus, U., Zhang, E., Jing, X. J., Esguerra, J. L. S., Wollheim, C. B., Salehi, A., … Groop, L. (2012). A systems genetics approach identifies genes and pathways for type 2 diabetes in human islets. *Cell Metabolism*, *16*(1), 122–134. https://doi.org/10.1016/j.cmet.2012.06.006

Unuofin, J. O., & Lebelo, S. L. (2020). Antioxidant Effects and Mechanisms of Medicinal Plants and Their Bioactive Compounds for the Prevention and Treatment of Type 2 Diabetes: An Updated Review. In *Oxidative Medicine and Cellular Longevity* (Vol. 2020). Hindawi Limited. https://doi.org/10.1155/2020/1356893

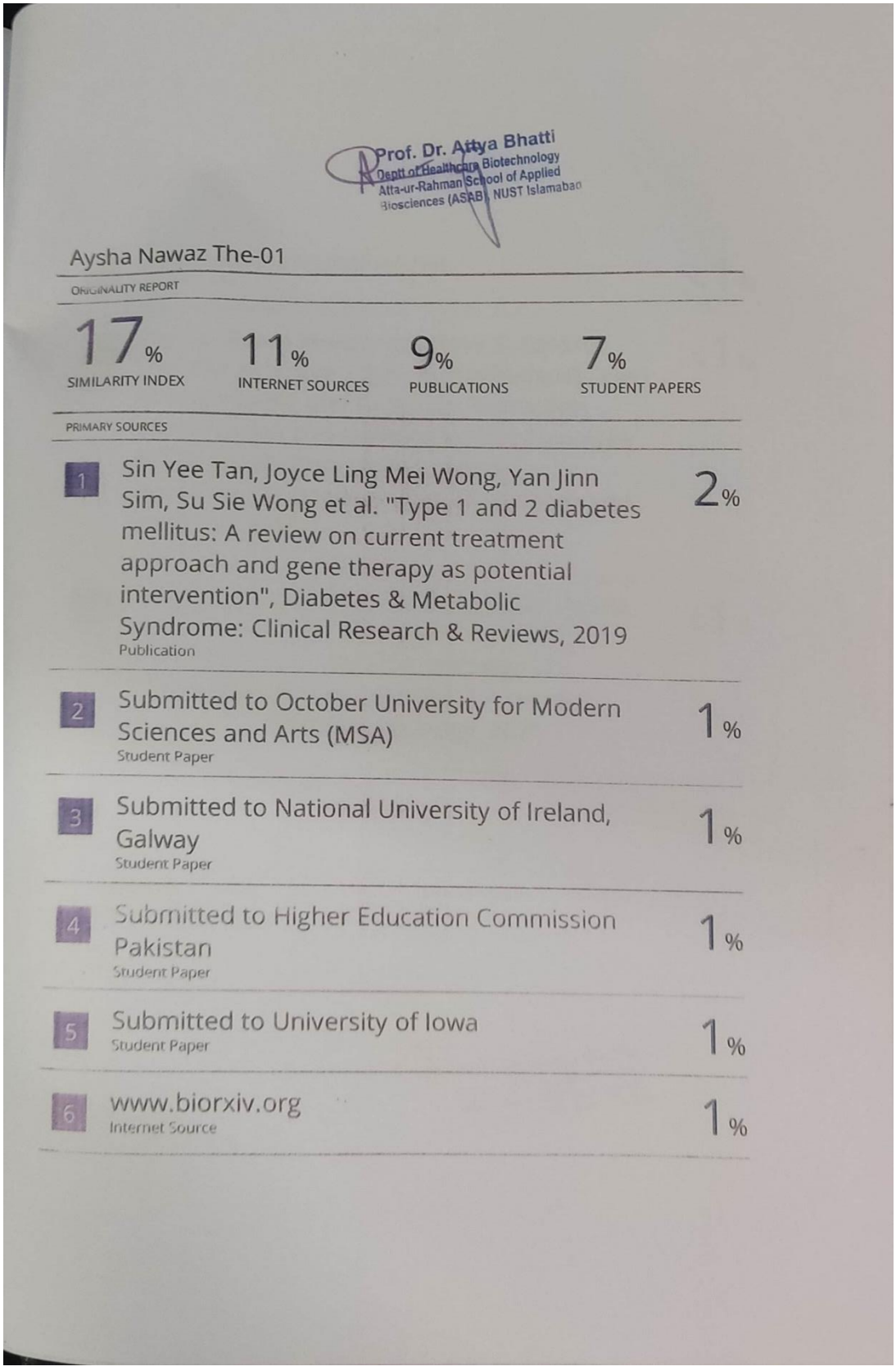
Wasserman, R. H., Corradino, R. A., & Krook ~-~, L. P. (1975). *BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS CESTRUM*

DiURNUM: A DOMESTIC PIANT WITH i, 25-D iHYDROXYCHOLECALC !FEROL-LIKE ACTIVITY I (Vol. 62, Issue 1).

Wu, Y., Ding, Y., Tanaka, Y., & Zhang, W. (2014a). Risk factors contributing to type 2 diabetes and recent advances in the treatment and prevention. In *International journal of medical sciences* (Vol. 11, Issue 11, pp. 1185–1200). https://doi.org/10.7150/ijms.10001

Wu, Y., Ding, Y., Tanaka, Y., & Zhang, W. (2014b). Risk factors contributing to type 2 diabetes and recent advances in the treatment and prevention. In *International journal of medical sciences* (Vol. 11, Issue 11, pp. 1185–1200). https://doi.org/10.7150/ijms.10001

Yamamoto, W. R., Bone, R. N., Sohn, P., Syed, F., Reissaus, C. A., Mosley, A. L., Wijeratne, A. B., True, J. D., Tong, X., Kono, T., & Evans-Molina, C. (2019). Endoplasmic reticulum stress alters ryanodine receptor function in the murine pancreatic cell. *Journal of Biological Chemistry*, *294*(1), 168–181. https://doi.org/10.1074/jbc.RA118.005683



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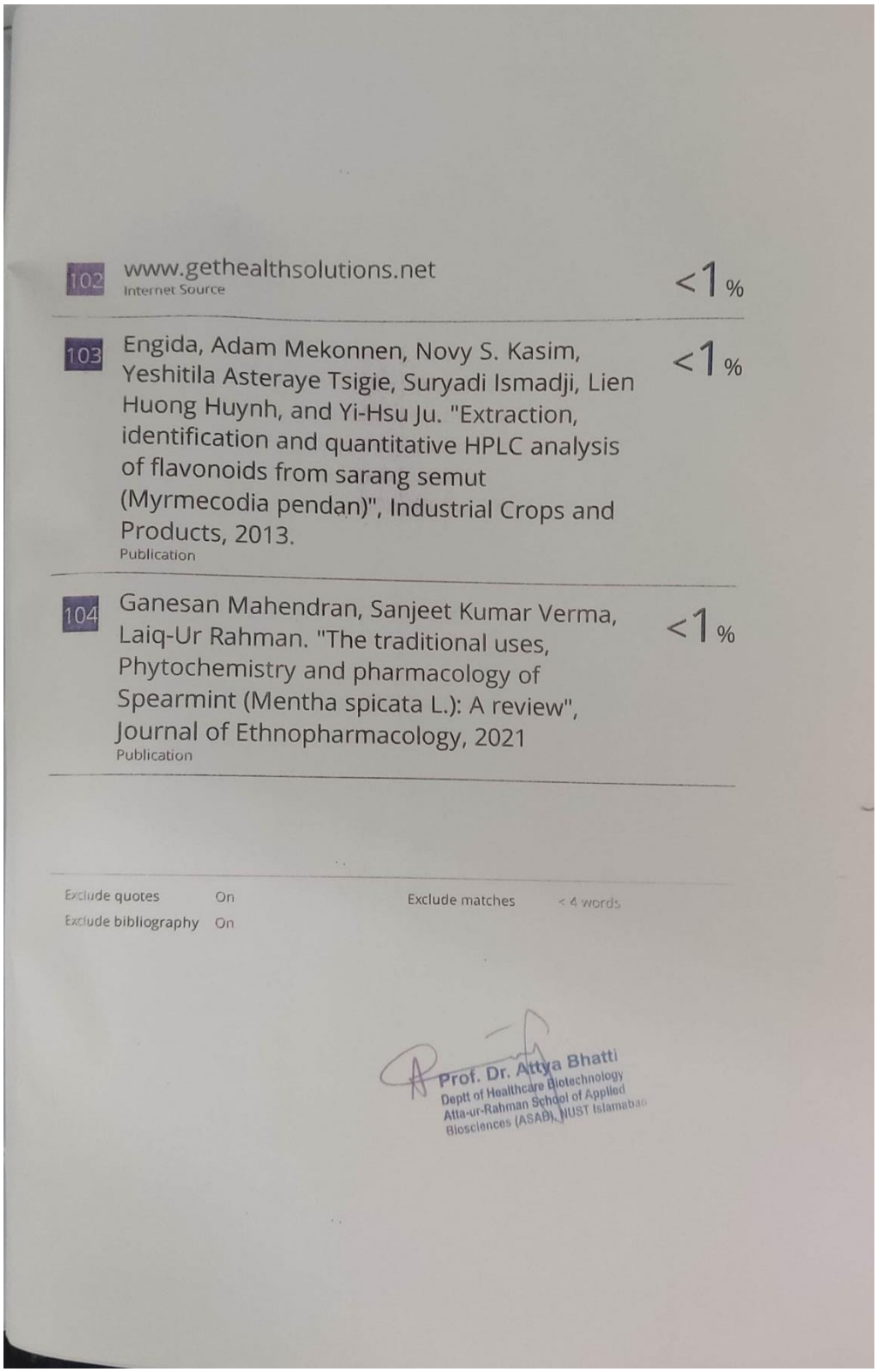

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