Evaluation of nephroprotective potential of *Gymnema sylvestre* and *Pterocarpus marsupium* extracts in diabetic Wistar rats



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MASTER OF VETERINARY SCIENCE

IN

VETERINARY PHARMACOLOGY AND TOXICOLOGY

BY

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Enrolment No. V-1716/17

COLLEGE OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY

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(2025)

CERTIFICATE

This is to certify that the thesis entitled **"Evaluation of nephroprotective potential of *Gymnema sylvestre* and *Pterocarpus marsupium* extracts in diabetic Wistar rats"** submitted by **Dr. Shikha Verma,** Enrollment No. **V-1716/17** in partial fulfilment of the requirements for the award of the **Master of Veterinary Science** in **Veterinary Pharmacology and Toxicology** of the **U. P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan, Mathura (U.P.)** India is a bonafide research work carried out by her under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Dated ..13/01/2025

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%	:	Percent (age)
<	:	Less than
>	:	Greater than
B cells	:	Beta cells
°C	:	Degree Celsius
µ	:	Micro
µM	:	Micromolar
b.wt.	:	Body weight
BSA	:	Bovine Serum Albumin
CAT	:	Catalase
CDNB	:	1-Chloro-2,4-dinitrobenzene
DF	:	Dilution Factor
DMSO	:	Dimethyl Sulfoxide
DTNB	:	5,5-dithiobis-2-nitrobenzoic acid
EC	:	Molar Extinction Coefficient
et al.	:	et alibi
g	:	Gram (s)
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
GPx	:	Glutathione peroxidase
GRase	:	Glutathione reductase
GSH	:	Reduced Glutathione
GS	:	*Gymnema sylvestre*
GST	:	Glutathione-S-Transferase
H_2O_2	:	Hydrogen Peroxide
HRP	:	Horseradish peroxidase
i.e.	:	That is
INF¥	:	Interferon Gamma
IL6	:	Interleukin 6
JAK2	:	Janus Kinase2
LPO	:	Lipid Peroxidation
M	:	Molar

MDA	:	Malondialdehyde
Met	:	Metformin
mg	:	Milligram
min	:	Minute (s)
mM	:	Milimoles
MT	:	Massion's Trichome
MTT	:	Diphenyltetrazolium Bromide
n	:	Number of observation
NADP	:	Nicotinamide adenine dinucleotide
NADPH	:	Nicotinamide adenine dinucleotide phosphate
NSS	:	Normal Saline Solution
PBS	:	Phosphate Buffer Saline
PM	:	*Pterocarpus marsupium* extract
ROS	:	Reactive Oxygen Species
S.E.	:	Standard Error
SDS	:	Sodium dodecyl sulphate
sec	:	Second
SOD	:	Superoxide Dismutase
STAT3	:	Signal transducer and activator of transcription 3
STZ	:	Streptozotocin
TBA	:	Thiobarbituric acid
TCA	:	Trichloroacetic acid
UV	:	Ultraviolet

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Shikha Verma

(Shikha Verma)

Place: Mathura (U.P.)

Date: 13/01/2025

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ABSTRACT

The present study was designed to assess the antidiabetic and hypoglycemic potential of *Pterocarpus marsupium* and *Gymnema sylvestre*, where metformin was used as a standard drug central group to compare the efficacy of the herbs. Thirty-six adult male Wistar rats weight 150-200 were randomly divided into six groups I, group II, group III, group IV, group V, and group VI, with six animals in each group & fed on a high-fat diet (except control) for four weeks after generation of obese model rats of different groups except healthy control & obese groups were administered with streptozotocin @ 35 mg/kg but injection intraperitoneally further, metformin, extracts of PM & GS were administered by oral gavage for the perusal of 60 days continuously at the due rate of 50, 300 and 400 mg/kg body weight, respectively. Results demonstrate significant ($p < 0.05$) restoration of glycemic control, improved lipid profiles, and mitigation of diabetes-induced complications. PM and GS treatment markedly reduced fasting blood glucose (FBG) and glycated haemoglobin (HbA1c) levels. Additionally, these agents ameliorated oxidative stress, evident by improved antioxidant enzyme activities (SOD, CAT) and reduced lipid peroxidation in renal tissues. Histopathological analysis revealed that PM and GS significantly restored pancreatic β-cell architecture and renal integrity, reducing inflammation and fibrosis. These findings align with their antioxidant and anti-inflammatory properties. Moreover, PM exhibited pronounced nephroprotective effects, while GS showed efficacy in lipid metabolism regulation, highlighting their complementary therapeutic roles. In conclusion, the study establishes the potential of PM and GS as effective, antidiabetic agents for T2DM therapies. Their bioactive compounds, which mitigate hyperglycemia, oxidative stress, and associated complications, make them promising candidates for future clinical applications.

Keywords: *Pterocarpus marsupium*, *Gymnema sylvestre*, Fasting blood glucose, Streptozotocin



Introduction

CHAPTER-1

INTRODUCTION

Diabetes mellitus, commonly known as diabetes, is not merely one disease; it is defined as a complex group of endocrine metabolic disorders characterized by sustained hyperglycemia, or elevated blood sugar levels. This condition arises from one or both of two primary issues: either the pancreas fails to produce adequate amounts of insulin, which is known as a defect in insulin secretion, or the body's cells become resistant to insulin's effects, a scenario referred to as a defect in insulin action. The resulting insulin deficiency leads to chronic hyperglycemia and disturbances in the metabolism of carbohydrates, fats, and proteins, ultimately affecting multiple bodily systems (Chen et al., 2020; Ahmed et al., 2024).

Diabetes mellitus is primarily classified into three categories: insulin-dependent diabetes mellitus (IDDM, identified as Type 1), non-insulin-dependent diabetes mellitus (NIDDM, recognized as Type 2), and gestational diabetes mellitus (GDM). Type 1 DM is an autoimmune condition characterized by an inflammatory response that targets the pancreatic islets, ultimately destroying insulin-secreting beta cells. In contrast, Type 2 diabetes primarily involves peripheral insulin resistance, where the body's cells do not respond adequately to insulin signals, compounded by an impaired capacity of the pancreas to secrete insulin in sufficient quantities (Deshmukh and Jain, 2015; Antar et al., 2023). Gestational diabetes represents another significant clinical challenge, as it can lead to major health issues for both mothers and infants if not effectively managed during pregnancy (Modelzeski et al., 2022). The role of hormones such as estrogen and progesterone is particularly noteworthy; these hormones can diminish the sensitivity of target tissues to insulin, making females more prone to developing Type 2 diabetes compared to males. Moreover, symptoms of diabetes in females are often more prevalent during specific reproductive phases, such as the estrus and diestrus periods (Davison et al., 2005).

In recent decades, the global incidence of diabetes has increased dramatically. The International Diabetes Federation (IDF) projects that the number of people diagnosed with diabetes will reach approximately 643 million worldwide by 2030, with this number expected to escalate to 783.2 million by 2045. In India, about 8.9%

of the population is currently affected, with future projections estimating that the number of individuals with diabetes could rise to around 100.95 million by 2030 and possibly 134.23 million by 2045 (Sun et al., 2022; Nanditha et al., 2024).

Diabetes mellitus carries serious health risks and can lead to multiple complications categorized into macrovascular and microvascular issues. Macrovascular complications include serious conditions such as coronary heart disease and strokes, while microvascular complications frequently manifest as peripheral neuropathy, retinopathy, diabetic nephropathy, and peripheral vascular disease. Additionally, emerging research has identified a range of other health concerns associated with diabetes, including increased risks of certain cancers, infections, liver disease, functional disabilities, and cognitive impairments (Tomic et al., 2022).

Diabetic nephropathy is of particular concern and is characterized by persistent albuminuria, thickening of the glomerular basement membrane, glomerular sclerosis, and inflammation, which collectively contribute to mesangial cell expansion and hypertrophy, ultimately leading to progressive kidney dysfunction (Varghese and Jialal, 2023). Studies indicate that diabetic nephropathy can develop in 20% to 50% of individuals living with diabetes and is regarded as a leading cause of end-stage kidney disease (ESKD) (Selby and Taal, 2020). The underlying mechanisms that drive diabetic nephropathy are primarily related to chronic hyperglycemia, which initiates the production of reactive oxygen species (ROS) and activates several harmful pathways, including protein kinase C, the polyol pathway, the hexosamine pathway, the renin-angiotensin-aldosterone system (RAAS), and the formation of advanced glycation end products (AGE).

In the realm of veterinary medicine, diabetes mellitus is recognized in small and wild animals, mirroring the classifications found in humans. Studies have shown that cats predominantly exhibit Type 2 diabetes, while dogs are typically diagnosed with Type 1 diabetes mellitus (Niaz et al., 2018; Rand et al., 2020). The prevalence of diabetes among cats is estimated to be approximately 7.4 per 1,000, whereas about 1 in every 300 dogs is affected by the condition.

To manage diabetes and mitigate its life-threatening complications, a variety of strategies have been developed. These include dietary adjustments, various forms

of medication, and regular exercise. The effectiveness of these interventions is influenced by a multitude of factors, including the rate of nutrient uptake by the body, the actions of digestive enzymes on food, insulin's activity, and how tissues respond to insulin (Boucher et al., 2014). A broad array of pharmacological agents is available for diabetes treatment, including insulin itself, sulfonylureas, meglitinides, thiazolidinediones (TZD), dipeptidyl peptidase 4 (DPP-4) inhibitors, sodium-glucose co-transporter 2 (SGLT2) inhibitors, α-glucosidase inhibitors, GLP-1 receptor agonists, and amylin analogues (Mohazan and Mohazan, 2024; Chaudhary et al., 2017). However, many conventional antihyperglycemic medications can have significant adverse effects, including hypoglycemia, lactic acidosis, weight gain or loss, and increased risks of cancers and cardiovascular diseases. As a result, the search for novel anti-diabetic agents is ongoing, with a strong emphasis on enhancing the proliferation and functional capacity of pancreatic β cells (Osadebe et al., 2014; Sodikov et al., 2024). Notably, certain plant species have demonstrated capabilities for β cell regeneration, suggesting a promising avenue for diabetes treatment.

Public interest in herbal medicine has grown significantly, particularly various phytochemicals contained within plant extracts. Research indicates that over 1,200 plant species are recognised for possessing antidiabetic properties (Lankatillake et al., 2019) and serve as promising sources of bioactive compounds that could contribute to diabetes management. The World Health Organization (WHO) supports the integration of medicinal plants within dietary practices as a viable strategy for controlling diabetes. Alarmingly, approximately four billion people in developing counries, utilize these plants in various forms of traditional medicine to manage metabolic diseases such as diabetes (Chaudhary et al., 2018).

The importance of medicinal plants, along with vitamins and essential minerals with anti-hypoglycemic properties, cannot be overstated in the context of diabetes management. These natural products not only play a crucial role in the prevention of the disease but are also recognized as vital sources for pharmaceutical product development and therapeutic applications. In many developing countries, reports suggest that up to 90% of the population depends on plants and their derivatives for traditional medicine for primary health care (WHO, 2002). The WHO has catalogued 21,000 plants used for medicinal purposes around the globe, with approximately 2,500 species found in India. Many of these plants exhibit properties

that promote the regeneration of β cells, making them invaluable candidates for the development of effective anti-diabetic treatments (Modak et al., 2007; Rahman et al., 2022).

Various plant species have been recognized for their antidiabetic potential, including *Pterocarpus marsupium, Gymnema sylvestre, Catharanthus roseus, Carthamus tinctorius, Momordica charantia, Gynostemma pentaphyllum, Glycyrrhiza glabra, Smilax glabra, Psidium guajava, Rehmannia glutinosa, Santalum spicatum, Ocimum tenuiflorum, Rhizoma fagopyri, Rosa rugosa, Caulis polygoni, Fructus amomi, Rhizoma alpiniae officinarum, Folium ginkgo, Cortex cinnamomi, Marrubium radiatum,* and *Aloe vera*, which have all been documented as effective hypoglycemic agents (Salehi et al., 2019). Additionally, plants such as *Allium sativum, Azadirachta indica, Berberine, Pterocarpus marsupium, Gymnema sylvestre, Curcumin,* and *Vinca rosea* have been identified for their potential to regenerate β cells (Shivakumar and Deepa, 2023).

Pterocarpus marsupium (PM) and *Gymnema sylvestre* (GS) have been integral to diabetes treatment for thousands of years, underscoring their significance in traditional medicine (Kashif et al., 2023). The heartwood and bark of Pterocarpus marsupium have garnered attention for their hypoglycemic effects and ability to stimulate the regeneration of pancreatic β cells (Nzute et al., 2023). The beneficial effects of PM include stimulating insulin release, exhibiting actions similar to insulin, providing antioxidant protection, reducing dyslipidemia, and demonstrating antiglycation effects, particularly through its latex (Perera, 2016; Raina et al., 2020).

Similarly, *Gymnema sylvestre*, which is also known by names such as *Madhunashini, Gurmar*, and *Meshashrunga*, is a member of the *Asclepiadaceae* family and is indigenous to southwestern India, Australia, and tropical Asia. This plant is widely recognized for its potent antidiabetic properties and has been utilized extensively in folk medicine, Ayurveda, and homeopathy. The active components in *Gymnema sylvestre*, including gymnemic acid, gurmarin, and gymnemagerinin, are known to possess antidiabetic, anti-sweetener, and anti-inflammatory properties. Research has demonstrated that extracts from Gymnema leaves can stimulate the pancreas to enhance insulin secretion, thus playing a significant role in managing blood glucose levels (Rahman and Husen, 2023).

This study aims to explore the potential protective impacts of these plant extracts on kidney function and structure in the context of diabetes, addressing an important aspect of diabetic complications and contributing to the understanding of alternative therapeutic strategies in diabetes management.

2. Objective

- To evaluate the nephroprotective effect of *Gymnema sylvestre* and *Pterocarpus marsupium* extracts in diabetic Wistar rats.



Review of Literature

CHAPTER-2

REVIEW OF LITERATURE

Diabetes, or chronic hyperglycemia, is a metabolic disorder caused by either a lack of insulin secretion, impaired insulin action, or both. Insulin plays an important role as an anabolic hormone, significantly affecting the metabolism of carbohydrates, lipids, and proteins (Ruze et al., 2023). The word "diabetes" originates from Greek, meaning “to pass through,” whereas "mellitus" is derived from Latin, referring to honey or sweetness (Kaul et al., 2013).

In cases of chronic hyperglycemia, the defect can either stem from inadequate insulin secretion or from cells failing to respond effectively to insulin produced by the β cells of the pancreas. This dysfunction results in classical symptoms such as polyuria (increased urination), polydipsia (increased thirst), and polyphagia (increased hunger). Diabetes is recognized as the fifth leading cause of morbidity and mortality worldwide. Diabetes mellitus encompasses several categories, including Type 1 diabetes mellitus (T1DM), Type 2 diabetes mellitus (T2DM), maturity-onset diabetes of the young (MODY), gestational diabetes, and neonatal diabetes. Type 1 diabetes occurs primarily due to the autoimmune destruction of beta cells in the pancreas, leading to an absolute lack of insulin. This destruction culminates in a significant imbalance between insulin levels and their sensitivity, thereby causing a functional deficit of insulin. Insulin resistance, a major concern in diabetes, has multifactorial origins but is commonly associated with obesity and ageing (Chandrashekhran and Weiskirchen, 2024).

Type 2 diabetes is particularly prevalent, accounting for at least 90% of all diagnosed cases of diabetes (WHO, 2016). It is closely related to obesity; however, visceral obesity poses a greater risk for the development of T2DM compared to a high body mass index, liver fat, or ectopic fat deposits (Padhi et al., 2020). Individuals with Type 2 diabetes often exhibit a combination of insulin resistance and diminished beta cell function, which may occur with or without an absolute deficiency of insulin (Lu X et al., 2024). This condition is frequently referred to as "adult-onset diabetes."

Both type 1 and type 2 diabetes significantly affect populations in developed and developing nations, leading to various complications. Diabetic individuals are prone to macrovascular conditions such as coronary heart disease, stroke, and peripheral arterial disease. Additionally, they may experience microvascular complications, including diabetic kidney disease, retinopathy, and peripheral neuropathy, all of which are closely linked to diabetes (Demir et al., 2021).

Diabetic nephropathy (DN) is a primary microvascular complication and is considered the leading cause of end-stage renal disease (ESRD) and chronic kidney disease (CKD) globally, contributing significantly to morbidity and mortality among affected individuals (Pugliese et al., 2019). Elevated glucose levels in the blood induce specific cellular effects in the kidneys, impacting various cell types, including endothelial cells, smooth muscle cells, mesangial cells, podocytes, and cells in the tubular and collecting duct systems, as well as inflammatory cells. Glomerular hyperfiltration, a hemodynamic feature associated with early diabetes, has been correlated with the development of diabetic nephropathy (Selby and Taal, 2020).

Well-known risk factors for diabetes mellitus include hypertension, hyperlipidemia, obesity, race/ethnicity, and increasing age, particularly individuals aged 40 years or older. A history of impaired fasting glucose or glucose tolerance, as well as previous gestational diabetes, also heightens these risks (Bereda and Bereda, 2021).

Certain medications have been identified as contributors to the development of diabetes mellitus. Thiazide diuretics can induce hypokalemia due to potassium depletion, which subsequently impairs insulin secretion. Moreover, thiazides may inhibit the conversion of proinsulin to insulin and increase the mobilization of free fatty acids, promoting insulin resistance.

Verapamil, a non-dihydropyridine calcium channel blocker, prevents β cells from absorbing calcium into the cytosol, thereby inhibiting insulin release stimulated by glucose, glucagon, and sulfonylureas. Additionally, beta-adrenoceptor blockers can partially counteract insulin resistance induced by weight gain while also inhibiting insulin release from pancreatic beta cells (Arefanian et al., 2024).

HIV protease inhibitors can lead to peripheral lipodystrophy, hyperlipidemia, and insulin resistance in muscle, while also obstructing the conversion of proinsulin to insulin. These protease inhibitors may attach to proteins involved in lipid metabolism, causing an increase in circulating fatty acids that can disrupt insulin signalling. Furthermore, beta-adrenoceptor agonists may induce insulin secretion through an increase in hepatic glucose production, leading to hyperglycemia.

Fluoroquinolones limit insulin release by blocking ATP-sensitive potassium channels, whereas niacin through acceleration of free fatty acid mobilization can enhance insulin resistance in skeletal muscle. Some estrogen replacement therapies and oral contraceptive pills have been shown to decrease insulin sensitivity in non-diabetic women, with certain implantable hormonal contraceptives possibly contributing to changes in carbohydrate metabolism, including diminished glucose tolerance and increased insulin resistance.

Glucocorticoids induce gluconeogenesis through post-receptor pathways, thereby reducing insulin sensitivity in both the liver and peripheral tissues. Additionally, atypical antipsychotics, specifically clozapine and olanzapine, have been found to alter receptor binding properties, consequently increasing insulin resistance. Conventional antipsychotics can sometimes result in hyperglycemia, while phenothiazines inhibit insulin secretion from pancreatic beta cells (Bereda, 2022).

3.2 Epidemiological Status of Diabetes Mellitus

3.2.1 International Status

Diabetes is one of the top ten global causes of death, ranking prominently alongside respiratory, cancerous, and cardiovascular diseases. It accounts for more than 80% of premature deaths resulting from non-communicable diseases (NCDs). The presence of diabetes significantly increases the likelihood of mortality from all causes, with estimates suggesting that it raises the risk of death by two to three times. Infections, heart disease, stroke, chronic kidney disease, chronic liver disease, and various forms of cancer are all linked to increased mortality in individuals with diabetes. Despite improvements in overall population health and life expectancy, diabetes remains the second largest contributing factor to the global decline in health-adjusted life expectancy (Lin et al., 2020).

Globally, diabetes predominantly affects "middle-aged" individuals, specifically those between the ages of 40 and 59. This demographic trend has grave social and economic ramifications. Notably, 77% of all diabetics worldwide reside in low- and middle-income countries, highlighting the disease's prevalence in these regions. The World Health Organization has documented a steady increase in the number of people with diabetes, from 151 million in the year 2000, when the IDF Diabetes Atlas was first introduced, to 285 million in 2009, reaching 382 million in 2013, and 425 million by 2017. Alarmingly, about 50% of individuals living with diabetes remain undiagnosed, particularly in developing nations. Projections indicate that by 2045, the prevalence of diabetes will rise to 10.9%. In 2019, the global number of individuals with diabetes was approximately 463 million, and it is expected to surge by 51%, reaching 700 million by 2045. Based on data from the IDF, it is estimated that a person dies from diabetes or its complications every seven seconds. In 2019, total fatalities reached 4.2 million, with over 50% of these deaths occurring in individuals under 60 years of age (IDF, 2019).

3.2.2 National Status

In India, persistent hyperglycemia is a hallmark of diabetes, and the number of individuals diagnosed with this condition is projected to rise sharply—from 74.2 million in 2021 to an alarming 124.9 million by 2045. This trend underscores the growing health emergency posed by diabetes in the country. Factors contributing to this rise include genetic predisposition, increasing rates of abdominal obesity, and a higher prevalence of metabolic risk factors such as dyslipidemia and hypertension. According to the International Diabetes Federation's (IDF) 2021 report, the total prevalence of diabetes among adults aged 20 to 79 in India is 8.3% (ranging from 7.3% to 9.3%), which is somewhat lower than the global prevalence of 10.5%. The age-adjusted prevalence of diabetes in India is reported to be 9.6% (8.5% to 10.6%).

The fifth wave of the National Family Health Survey (NFHS-5), conducted between 2019 and 2021, revealed that men were more likely than women to exhibit elevated blood sugar levels (greater than 140 mg/dl) (15.6% vs. 13.5%) (Sahadevan et al., 2023).

A recent report, which is considered "one of the first comprehensive studies covering all states of India" to assess the burden of non-communicable diseases (NCDs), highlighted that approximately 31 million Indians developed diabetes

between 2019 and 2021. While the incidence of diabetes is comparatively lower in rural areas, projections indicate that these figures are expected to soar in the coming years. Goa has reported the highest prevalence of the disease, at 26.4%, followed closely by Puducherry and Kerala, both nearing 25%. In India, the total prevalence of diabetes stands at 11.4%, while 35.5% of the population has hypertension. Additionally, the nation faces a concerning overall population rate of 40% for abdominal obesity, with rates reaching 50% among women.

In recent years, India has witnessed a significant increase in the number of individuals living with diabetes, pre-diabetes, hypertension, and both generalized and abdominal obesity. These individuals are more susceptible to non-communicable diseases and face increased risk of life-threatening conditions, including strokes. The prevalence of generalized obesity and abdominal obesity in India was found to be 28.6% and 39.5%, respectively. Furthermore, 15.3% of the population has been identified as having pre-diabetes, and 24% of individuals exhibit hypercholesterolemia, a condition characterized by fat accumulation in the arteries, which elevates the risk of heart attacks and strokes (Anjana et al., 2023).

3.3 Diabetes in Animals

Diabetes mellitus in small and wild animals encompasses all types of diabetes that are recognized in humans (Niaz et al., 2018). This condition is a common endocrine disorder notably observed in cats and dogs, where it is frequently diagnosed due to its similarities with human diabetes (Rees and Alcolado, 2004; Rand, 2020). Most feline cases are characterized by Type 2 diabetes or non-insulin-dependent diabetes mellitus, while among dogs, Type 1 diabetes mellitus is more prevalent. Breeds particularly predisposed to diabetes mellitus include cocker spaniels, Labradors, German shepherds, dachshunds, and beagles (Hussain et al., 2024).

Statistically, the prevalence of diabetes in cats is approximately 7.4 per 1,000, while around 1 in 300 dogs are diagnosed with the condition Generally, canine diabetes mellitus tends to be diagnosed clinically in dogs aged between 4 to 18 years (Guptill et al., 2003; Catchpole et al., 2005; Fall et al., 2007). Notably, the highest incidence of diabetes in cats is found around the age of 8 years, with certain breeds such as Siamese, Burmese, and Maine Coon showing a higher predisposition to the condition (Hoening, 2002; Reusch, 2014).

3.4 Insulin Signalling Mechanism and Regulation of Blood Glucose

Insulin signaling is a critical pathway for energy metabolism within the human body. The binding of insulin to its receptor triggers a cascade of mechanisms that facilitate the translocation of glucose, the body's primary source of energy (Lubenia et al., 2024).

When blood glucose levels rise, the β cells in the islets of the pancreas are stimulated to release insulin. Insulin itself is a peptide hormone consisting of a 51-amino acid polypeptide formed from two chains linked by disulfide bonds (Weiss and Philipson, 2015). The intricate insulin signal transduction pathways in pancreatic β cells are mediated through insulin receptors (IR), including the insulin-like growth factor 1 (IGF-1) receptor and other insulin-related receptors. The insulin receptor activates various substrates, specifically insulin receptor substrates (IRS), through a series of signaling molecules that include IR tyrosine kinase, phosphoinositide 3-kinase (PI3K), and its downstream effectors protein kinase B (PKB, also referred to as AKT), mitogen-activated protein kinase (MAPK), MAPK kinase (MAPKK/MEK), raf proteins, and rat sarcoma (Ras) proteins (small G proteins) (Lee et al., 2022).

The regulation of blood glucose relies on a negative feedback loop involving the release of insulin and glucagon. Glucose serves as the primary energy source, entering the bloodstream through processes such as intestinal absorption, gluconeogenesis, and glycogenolysis (Piero, 2006).

Insulin action begins with its binding to and activation of the cell-surface receptor, which comprises two α subunits and two β subunits linked by disulfide bonds. Upon insulin binding to the extracellular α subunits of the receptor, a signal is transmitted across the plasma membrane, activating the intracellular tyrosine kinase domain of the β subunit. This process undergoes a series of intramolecular transphosphorylation reactions, initiating a signaling cascade that phosphorylates the insulin substrate receptor (IRS). The activated IRS subsequently binds to SH2 domain proteins, such as phosphatidylinositol 3-kinase and phosphotyrosine phosphatases (Chandrasekaran and Weiskirchen, 2024). PI3-kinase operates via serine and threonine kinases, including Akt/protein kinase B (PKB), protein kinase C (PKC), and PI-dependent protein kinases 1 and 2.

Furthermore, insulin sends signals to the liver to convert excess glucose into glycogen for storage. It also stimulates other cells throughout the body, such as

skeletal muscle cells, cardiac muscle cells, and adipose tissue cells, to utilize glucose from the bloodstream by translocating glucose transporter-4 (GLUT4) to the cell surface. GLUT4 is insulin-dependent, meaning that its expression on the cell surface relies on insulin's presence. In a basal state without insulin, GLUT4 is sequestered within the intracellular compartments, where it facilitates the inward transport of glucose from the blood.

In cases of diabetes, there is a dysfunction either in the synthesis or secretion of insulin, as seen in Type 1 diabetes mellitus, or the development of resistance to insulin or its inadequate production, as observed in Type 2 diabetes (Deshmukh et al., 2015).

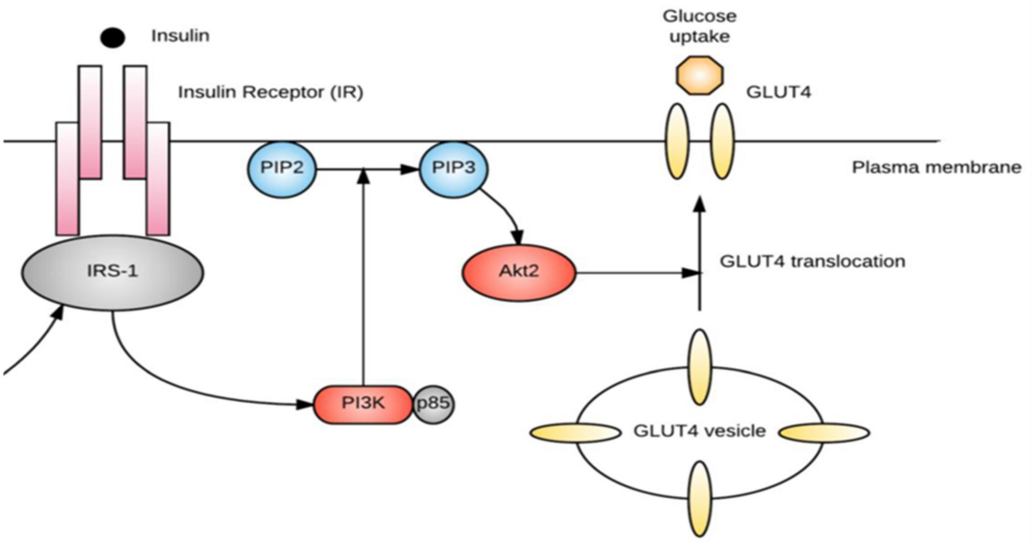


Fig. 2.1: Insulin Signalling Pathway (Jasmine et al., 2018)

3.4.1 Insulin Resistance

Throughout the life cycle, fluctuations in insulin sensitivity are common, with insulin resistance notably evident during puberty, pregnancy, and the aging process. Type 2 diabetes mellitus (T2DM) is closely linked to insulin resistance, and both conditions are significantly associated with obesity. In obese individuals, insulin resistance primarily arises from dysfunctional adipose tissue (Ahmed et al., 2021). The relationship between obesity and T2DM is well established, as these two conditions often coexist and exacerbate each other. Research indicates that variations in adipokines such as resistin, adiponectin, and leptin play critical roles in the development of insulin resistance and the onset of diabetes (Lankatillake et al., 2019).

Adiponectin, for instance, is known for its anti-inflammatory and anti-atherogenic properties, in addition to its function as an insulin sensitizer. Conversely, leptin, a hormone with orexigenic activity, helps regulate energy balance by suppressing appetite. The dysregulation of these metabolic pathways contributes to the development of insulin resistance (Artasensi et al., 2020). In obese individuals, adipose tissue also releases pro-inflammatory cytokines, glycerol, non-esterified fatty acids, and other substances that have been reported to contribute to the development of insulin resistance.

Inflammatory cytokines can diminish the insulin signaling activity in both adipocytes and hepatocytes by inhibiting key proteins like IRS1 and PPARϒ or by increasing plasma-free fatty acids through the stimulation of lipolysis while blocking triglyceride synthesis (Ye, 2013). The inflammatory process in adipocytes accelerates the overflow of fatty acids from adipose tissue to skeletal muscle and the liver. This leads to ectopic fat deposition, which ultimately contributes to the development of insulin resistance and T2DM (Wu and Ballantyne, 2020).

3.5 Complications of Diabetes

Prolonged hyperglycemia can precipitate severe complications, including retinopathy, neuropathy, kidney disorders, and cardiovascular diseases. The complications of diabetes mellitus often arise from long-term exposure to elevated blood sugar levels, resulting from malfunctions in insulin metabolism and the dysfunction of biological macromolecules such as proteins, lipids, carbohydrates, and nucleic acids. The complications of diabetes mellitus have rapidly become the predominant causes of morbidity and mortality globally (Liu et al., 2020). These complications can be broadly classified into macrovascular conditions, such as cardiovascular and cerebrovascular diseases, and microvascular complications, which include neuropathy, retinopathy, and nephropathy. In both types of diabetes mellitus, higher urine albumin excretion (proteinuria) or a decreased glomerular filtration rate in the kidneys are indicative of diabetic nephropathy, a significant microvascular complication (Zheng et al., 2020).

3.5.1 Diabetic Nephropathy

Diabetic kidney disease (DKD) is a major microvascular complication that impacts approximately 40% of people with diabetes. It is currently the leading cause of end-stage kidney disease (ESKD) worldwide, affecting about 700 million

individuals. The proportion of ESKD patients with incidents attributed to diabetes has risen from 22.1% to 31.3%, and the global prevalence of ESKD patients associated with diabetes grew from 19.0% in 2000 to 29.7% in 2015. By 2017, diabetes was recognised as the principal etiological factor in half of all ESKD cases in the United States. With the current count of individuals affected by diabetes at 564 million worldwide, projections estimate that this figure will rise to 600 million by 2035 and reach 783 million by 2045 (Naaman and Bakris, 2023).

Diabetic nephropathy not only requires treatments such as dialysis or renal transplantation but also affects up to 50% of individuals with diabetes and is associated with a markedly higher risk of cardiovascular morbidity and mortality. Although chronic albuminuria and progressive renal function deterioration are hallmarks of diabetic kidney disease (DN), it is increasingly recognized that the clinical course and presentation of kidney disease in diabetes can be quite variable. Today, a wide range of patients with diabetes exhibiting albuminuria or reduced renal function are classified under diabetic kidney disease (DKD) (Selby and Taal, 2020).

The primary pathological changes linked to diabetic nephropathy include extracellular matrix (ECM) deposition, thickening of the glomerular basement membrane (GBM), mesangial expansion, and renal tubular atrophy. These changes ultimately lead to glomerulosclerosis and renal interstitial fibrosis. The mechanisms driving the progression of diabetic kidney disease are multifaceted, involving aberrant hemodynamics, autophagy, apoptosis, inflammation, fibrosis, oxidative stress, and alterations in intestinal flora (Cai et al., 2024).

3.5.2 Pathophysiology of Diabetic Nephropathy

The pathophysiology of diabetic kidney disease (DKD) can be understood through the interaction of three major processes, each influencing the condition based on an individual's genetic makeup. This genetic interplay accounts for the observed variability in the hemodynamic, metabolic, and inflammatory components of DKD.

Key players in hemodynamic regulation include renin, angiotensin, and aldosterone, all of which are vital for maintaining nephron homeostasis. Renin is an essential enzyme in the activation of the renin-angiotensin-aldosterone system (RAAS). The RAAS triggers the production of angiotensin II, which binds strongly to two distinct receptors, AT1 and AT2. Activation of the AT1 receptor increases efferent arteriolar resistance in the nephron, raising intraglomerular pressure to sustain

the renal filtration rate. Conversely, the activation of the AT2 receptor opposes the function of AT1 by modulating renal vasodilating prostaglandin production and exerting a protective counter regulatory influence on blood pressure regulation.

Moreover, high levels of angiotensin II lead to increased adrenal aldosterone secretion and the induction of fibrogenic chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor-β (TGF-β), alongside macrophage activation, which fosters an inflammatory environment factors that can contribute to renal injury (Carey et al., 2000; Naaman and Bakris, 2023).

Among the early and well-characterized observations in patients with Type 1 and Type 2 diabetes, increases in intraglomerular pressure, facilitated by RAAS activation, have been observed in 75% and 40% of cases, respectively (Premaratne et al., 2015). The initial alterations in glomeruli exposed to hyperglycemia, leading to mesangial enlargement, are driven by angiotensin II and endothelin. Over time, persistent inflammation contributes to the development of glomerulosclerosis (Mauer et al., 2009).

Podocytes play a crucial role in maintaining the selective nature of the glomerular filtration barrier (Barutta et al., 2022). The noted increase in albuminuria among diabetic patients is often attributed to podocyte loss and foot process effacement resulting from injury to the podocytes themselves. Impairments in tubule-glomerular feedback can further exacerbate glomerular hemodynamic alterations, stemming from tubular dysfunction (Poursharif et al., 2022). In diabetic conditions, elevated glucose levels stimulate the upregulation of glucose and sodium transporters, such as SGLT1 and SGLT2, to enhance reabsorption (Tuttle, 2017; Heerspink et al., 2016). A diminished supply of salt to the distal nephron leads to negative tubule-glomerular feedback, which can contribute to these hemodynamic changes.

Studies focusing on Type 1 diabetes indicate an increase in local angiotensin production to improve glomerular perfusion, resulting in afferent arteriole dilation and efferent arteriole constriction (Tuttle, 2017; Grabias et al., 2014). Restoration of tubulo-glomerular equilibrium is achieved by preventing glucose reabsorption at the proximal tubule and promoting its excretion, which lowers intraglomerular pressure and reduces hyperfiltration (Tsimihodimos et al., 2017; Ferrannini et al., 2014). However, such processes become ineffective if autoregulation is impaired and

estimated glomerular filtration rate (eGFR) falls below 45 mL/min/1.73 m² (Picken et al., 2016).

The co-occurrence of hyperglycemia, insulin resistance, and dyslipidemia initiates multiple dysregulated metabolic pathways that are intrinsically linked to oxidative stress and inflammatory processes, creating a vicious cycle where one process exacerbates another (Pichler et al., 2017; Hojs et al., 2016). Notably, the polyol and protein kinase C (PKC) pathways are particularly significant as they elevate oxidative stress levels and reduce endothelial nitric oxide synthase activity, resulting in increased levels of endothelin-1 and vascular endothelial growth factor.

Inflammation is prominently involved in this cycle, being facilitated by endothelial instability and the action of nuclear factor-kappa B (NF-κB)-mediated cytokines, including TNF-α and interleukin-6 (IL-6). Advanced glycation end products (AGEs) are also more likely to accumulate in a hyperglycemic environment. These compounds, characterized by a diverse array of nonenzymatically glycated structures, exert disruptive effects on cellular functions when interacting with their receptors (RAGEs), which are widely dispersed throughout the kidney. This interaction leads to the activation of NF-κB and subsequent secretion of pro-inflammatory cytokines (TNF-α and IL-6). AGEs are known to be associated with impaired vasodilation in diabetes, as they enhance the production of reactive oxygen species and diminish the bioavailability of nitric oxide originating from the endothelium (Khalid et al., 2022).

Fibrosis and inflammation are interrelated factors critically involved in the pathogenesis of DKD. Emerging data underscore a complex interplay involving the RAS-related C3 botulinum toxin substrate 1 (Rac1) and the mineralocorticoid receptor (MR) signaling pathways, which drive the inflammatory processes culminating in renal fibrosis. Animal studies have long established the adverse effects of MR activation on inflammation and fibrosis, providing a therapeutic rationale for using MR antagonists. Comparative benefits of MR blocking on both the kidneys and vasculature have been supported by various animal investigations (Tostes et al., 2002; Rocha et al., 2002; Naaman and Bakris, 2023).

The aldosterone-responsive distal nephron experiences MR activation, which contributes to potassium excretion and sodium reabsorption. When plasma volume is reduced or potassium levels notably increase, the activation of the renin-angiotensin

system triggers aldosterone release. Chronic high sodium intake, as evident in many Western and Asian diets, can be pathologic, even though sodium is essential for survival in low-sodium environments (Nakamura et al., 2022).

The consequences of high sodium consumption coupled with inappropriate aldosterone signaling manifest as hypertension, a primary driver of glomerular damage and fibrosis. Similar to aldosterone, the mineralocorticoid receptor exhibits a comparable binding affinity for cortisol and corticosterone. Typically, the enzyme 11B-dehydrogenase isoenzyme 2 (11B-HSD2) is co-expressed in these cells, counteracting cortisol and mitigating MR over-activation. While MRs are expressed on numerous cell types beyond the distal nephron including podocytes, fibroblasts, vascular cells, and macrophages these cells often lack the co-expression of 11B-HSD2, leading to unchecked MR activation (Barrera et al., 2019).

In instances of obesity, dyslipidemia, insulin resistance, and hyperglycemia, the expression of mineralocorticoid receptors is markedly increased. This rise in receptor activity leads to increased gene transcription of profibrotic factors such as TGF-β1, connective tissue growth factor, plasminogen activator inhibitor-1 (PAI-1), and various extracellular matrix proteins, all of which contribute to the progression of diabetic kidney disease (Tesch et al., 2017).

3.5.3 Stages of Diabetic Nephropathy

Approximately 20–30% of individuals with diabetes progress to develop diabetic nephropathy (DN), which represents a continuum beginning with microalbuminuria and advancing to overt nephropathy or macroalbuminuria, ultimately resulting in end-stage renal failure (ESRF). This progression can be divided into several distinct stages:

Stage 1: Early hypertrophy and hyperfunction of the kidneys.

Stage 2: Presence of glomerular lesions without clinical symptoms of disease.

Stage 3: Incipient diabetic nephropathy, marked by microalbuminuria, where urine albumin excretion ranges from 30 to 300 mg/day.

Stage 4: Overt diabetic nephropathy or macroalbuminuria, characterized by urine albumin excretion exceeding 300 mg/day.

Stage 5: End-stage renal disease (Parchwani and Upadhyah, 2012).

3.5.4 Diagnosis of Diabetic Kidney Disease

Diabetic nephropathy is traditionally diagnosed by the onset of proteinuria accompanied by a progressive decline in renal function. Conversely, non-proteinuric diabetic kidney disease refers to patients with diabetes who show reduced renal function and vascular complications in the absence of proteinuria. It is recommended that individuals with diabetes regularly monitor their urine albumin and renal function levels, with the preferred screening test being the urine albumin-to-creatinine ratio obtained from a first-morning void spot collection (Person and Rossing, 2018).

Several important urinary biomarkers indicative of tubular injury associated with early diabetic nephropathy are utilized to assess renal involvement in diabetes management. These biomarkers include components intrinsic to nephron function, such as nephrin and podocalyxin from epithelial cells (podocytes), collagen and laminin from the glomerular basement membrane, and vascular endothelial growth factor (VEGF). Additionally, degeneration markers like neutrophil gelatinase-associated lipocalin (NGAL), N-acetyl-β-D-glucosaminidase (NAG), and Kidney Injury Molecule-1 (KIM-1), as well as serum cystatin C, are also important for assessment (Gluhovschi et al., 2016; Jung and Yoo 2022).

Neutrophil gelatinase-associated lipocalin (NGAL) is a 25 kDa protein secreted by neutrophils and various epithelial cell types, including renal tubular cells. This protein is a member of the lipocalin family and is produced in response to tubular injury, serving as an indicator of tubular function. Kidney Injury Molecule-1 (KIM-1) is a transmembrane protein whose expression significantly increases upon cellular injury or dedifferentiation and is typically undetectable in normal proximal tubule cells. Its presence in urine is considered highly specific for kidney damage, making it a valuable biomarker for detecting renal proximal tubule injury and aiding in early diagnosis (Uwaezuoke, 2017).

The enzyme N-acetyl-beta-D-glucosaminidase (NAG), primarily located in the proximal tubule, has demonstrated efficacy as an indicator of renal tubular dysfunction (Fiseha and Tamir, 2016). An updated review of urine biomarkers utilized in the early diagnosis of diabetic nephropathy highlights their significant role in ensuring timely intervention and treatment (Gluhovschi et al., 2016; Liu et al., 2022).

3.6 Diabetes Mellitus and Pancreatic β-cell Dysfunction

Insulin resistance is often the initial step in the pathophysiology of Type 2 diabetes mellitus (T2DM). To compensate for insulin resistance, there is an increase in β-cell insulin production, which helps maintain normal blood glucose levels. However, this compensatory mechanism is insufficient due to the ongoing loss of both β-cell mass and function, ultimately leading to overt diabetes as the β-cells fail to adequately counteract the insulin resistance (Wysham and Shubrook, 2020).

The primary detrimental factors that contribute to β-cell dysfunction include glucotoxicity, lipotoxicity, and glucolipotoxicity (Weir, 2020). Chronic exposure to elevated glucose levels suppresses β-cell activity, diminishing glucose-stimulated insulin secretion (GSIS) and promoting β-cell apoptosis. In contrast, short-term exposure to elevated glucose can stimulate β-cell growth (Donath et al., 1999).

Glucotoxicity refers to the irreversible structural and functional damage sustained by β-cells due to prolonged exposure to high glucose concentrations (Prentki et al., 2020). Under conditions of moderate to high hyperglycemia (30-60 mmol/L), increased levels of reactive oxygen species (ROS) can lead to β-cell apoptosis (Cai et al., 2024).

Despite long-standing interest, the fundamental mechanisms by which hyperglycemia induces β-cell dysfunction remain poorly understood. One potential strategy to mitigate β-cell damage in diabetic conditions is to limit excessive glucose metabolism. Numerous studies have demonstrated that increased oxidative stress, inflammation, and mitochondrial dysfunction triggered by high hyperglycemia and free fatty acids—are key contributors to β-cell failure and death. The interplay between insulin resistance and β-cell dysfunction serves to elevate blood glucose levels and accelerate the progression of Type 2 diabetes. These β-cell functional abnormalities commonly begin during the prediabetic phase and progressively worsen as the disease advances (Zhao et al., 2023).

Oxidative stress is frequently associated with β-cell destruction in the progression of T2D (Giacco and Brownlee, 2010; Asmat et al., 2016). Typically, excessive oxidative stress results from an overload of free radicals, particularly reactive oxygen species (ROS), which hinder the capacity of intracellular antioxidants to neutralize these harmful substances. This oxidative stress can inflict damage on proteins, lipids, and DNA, leading to a variety of detrimental effects. Increased

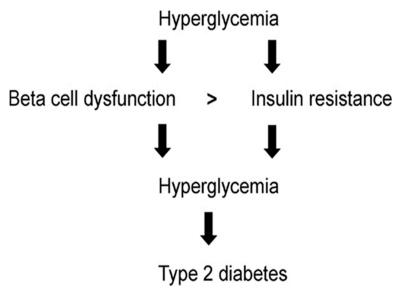
oxidative stress is often linked to a complex of lipid and lipoprotein abnormalities that may further exacerbate diabetic complications, as many T2D patients exhibit dyslipidemia (Krauss, 2004). Furthermore, pancreatic islets lack sufficient antioxidant enzyme expression, making them particularly susceptible to oxidative stress throughout the development of diabetes (Lenzen et al., 1996; Drews et al., 2010; Zhao et al., 2023).

Obesity or excessive fat accumulation within the pancreas is one of the primary drivers of oxidative stress, insulin resistance, and β-cell dysfunction in T2D (Rohm et al., 2022; Singh et al., 2017). Enhancing β-cell function and reducing oxidative stress can be achieved by increasing intracellular antioxidant levels, which might provide a viable approach to combat diabetes-related challenges (Lei and Vatamaniuk, 2011; Wang and Wang, 2017). The detrimental link between oxidative stress and inflammation often leads to adverse effects that accelerate the pathogenesis of Type 2 diabetes. Specifically, these complications are closely associated with the progression of diabetes-related issues, such as retinopathy, neuropathy, nephropathy, and tissue damage (Halim and Halim, 2019).

3.6.1 Hyperglycemia and Pancreatic β-cell Dysfunction

β-cell dysfunction is a primary driver of Type 2 diabetes mellitus (T2DM), a condition that is further complicated by insulin resistance (Ashcroft and Rorsman, 2012). The intricate relationship between insulin resistance and β-cell dysfunction underscores the pathogenesis of diabetes. Hyperglycemia plays a pivotal role in this dynamic, simultaneously inducing both β-cell dysfunction and insulin resistance, which ultimately accelerates the progression of T2DM (Cerf, 2013).

Elevated glucose levels lead to oxidative stress and endoplasmic reticulum (ER) stress, alongside cytokine-induced inflammation, all of which contribute to β-cell stress. This ongoing stress results in a progressive loss of β-cell function, culminating in β-cell exhaustion and eventual cellular death (Ma et al., 2018).



3.6.2 Hyperglycemia-Induced Oxidative Stress

Excessive levels of reactive oxygen species (ROS) produced during hyperglycemia lead to oxidative stress, which promotes the formation of advanced glycation end-products (AGEs). Additionally, it activates protein kinase C (PKC) and results in the heightened activity of the hexosamine and sorbitol pathways. Collectively, these biochemical events contribute to the emergence of insulin resistance, impaired insulin secretion, and endothelial dysfunction (Papachristoforou et al., 2020).

The dysregulation of several cellular pathways affected by oxidative stress, triggered by hyperglycemia, either promotes cellular dysfunction or results in the apoptosis of damaged cells. As a consequence, oxidative stress has been recognised as a crucial factor in the development of diabetic retinopathy. Hyperglycemia stimulates the synthesis of AGEs, induces oxidative damage, and elevates levels of the protein hypoxia-inducible factor (HIF)-1 alpha. This elevation activates vascular endothelial growth factor (VEGF) and enhances transactivation functions, leading to microvascular damage (Chen et al., 2020).

Oxidative stress and metabolic disorders are considered the two main pathogenic factors responsible for insulin secretion disorders (Rani et al., 2017). Numerous cellular alterations associated with oxidative stress and compromised redox signaling often resulting from lipotoxicity and glucotoxicity due to chronic overfeeding contribute to the development of T2DM (Tan et al., 2022; Eguchi et al., 2022). Additionally, oxidative stress exacerbates β-cell dedifferentiation by being linked to inflammation, ER stress, hypoxia, and mitochondrial damage. This oxidative stress hampers the function and maintenance of pancreatic β-cells, which is widely understood to be connected to the onset of diabetes and its subsequent complications.

In the islets of individuals with T2D, oxidative stress-induced β-cell dysfunction leads to the inactivation and cytoplasmic translocation of key transcription factors such as MafA, NKX6.1, and Pdx1 (Gua et al., 2013). Under oxidative stress, these transcription factors become dephosphorylated and inactive due to the promotion of phosphatase activity, notably SH2-containing tyrosine-protein phosphatase and protein tyrosine phosphatase 1B (PTP1B), effectively blocking the insulin response (Nandi and Saxena, 2020). Moreover, oxidative stress results in the suppression of the mammalian target of rapamycin complex 1 (mTORC1) signalling

pathway, suggesting a potential role for mTOR in β-cell dedifferentiation. This suppression of mTOR signaling is linked to a decrease in β-cell mass and further reductions in insulin secretion (Wang et al., 2016).

3.7 β-cell Regeneration and Workload Hypothesis

The workload hypothesis emphasizes the necessity of reducing stress on β-cells in individuals with Type 2 diabetes mellitus (T2DM) to interrupt the cycle of β-cell damage and enhance glycemic control. Research has indicated that the persistence of glycemic control in patients treated with non-insulin secretagogues, such as metformin and thiazolidinediones, is often superior to that seen with insulin secretagogues like sulfonylureas (Kahn et al., 2006). Insulin therapy itself is known to enhance β-cell activity and induce periods of β-cell rest, thereby allowing recovery (Weng et al., 2008; Gerstein et al., 2012).

Furthermore, glucagon-like peptide-1 (GLP-1) receptor agonists, which are a form of incretin-based therapy, facilitate weight reduction, which further alleviates stress on the β-cells. Clinical evidence suggests improved glycemic persistence with GLP-1 receptor agonists compared to DPP-4 inhibitors (Nauck et al., 2014; Farngren and Ahren 2019). Similarly, sodium-glucose cotransporter 2 (SGLT2) inhibitors, which increase glucose excretion in urine and promote weight loss, have been shown to enhance β-cell function, at least in part by lowering the workload of β-cells (Ferrannini et al., 2014). These medications also demonstrate better long-term glycemic durability compared to sulfonylureas (Delprato et al., 2015).

3.7.1 Pancreatic Cell Mass/β-cell Regeneration

Diabetes, whether Type 1 or Type 2, is characterized by a relative or absolute deficiency of β-cells. Therefore, strategies aimed at replenishing lost functional or absolute β-cell mass are critical for alleviating the burden of the disease. Broadly, there are two primary approaches to restore β-cell function: (1) replacement therapy using islets harvested from cadavers or β-cells derived from induced pluripotent stem cells (iPSCs) or human embryonic stem cells (hESCs), and (2) inducing endogenous regeneration (Balakrishnan et al., 2020).

However, β-cell replacement therapy is unlikely to become widely accessible to the hundreds of millions affected by diabetes globally. Since most diabetic patients retain some β-cell function, developing pharmacological strategies that encourage the

proliferation and regeneration of these remaining cells presents a promising alternative for reversing diabetes (Wang et al., 2021).

Regenerative therapy is increasingly viewed as a crucial aspect of diabetes treatment, encompassing the development of feasible therapeutic strategies that promote β-cell expansion (Tiwari et al., 2015). Research into phytochemical compounds that possess the ability to regenerate β-cells may offer effective treatment options for patients experiencing significant loss of functional islet cells (Hosseini et al., 2015).

3.7.2 Mechanisms of β-cell Regeneration

Type 1 and Type 2 diabetes mellitus are characterized by decreased β-cell mass and impaired function of existing β-cells. Various cellular signalling pathways linked to β-cell survival and proliferation have been identified, though many of the pathways and molecular interactions associated with β-cells remain poorly understood (Jiang et al., 2018).

3.7.2.1 PI3K-AKT/PKB Signaling Pathway

The regulation of β-cell function primarily hinges on the phosphatidylinositol 3-kinase (PI3K) signalling pathway, which is intricately coordinated by a network of mediators. AKT/PKB, a downstream target of PI3K, modulates the expression of several crucial genes involved in β-cell proliferation, such as the mammalian target of rapamycin (mTOR), glycogen synthase kinase-3 (GSK3), and Forkhead box protein O1 (FOXO1) (Wang et al., 2015)

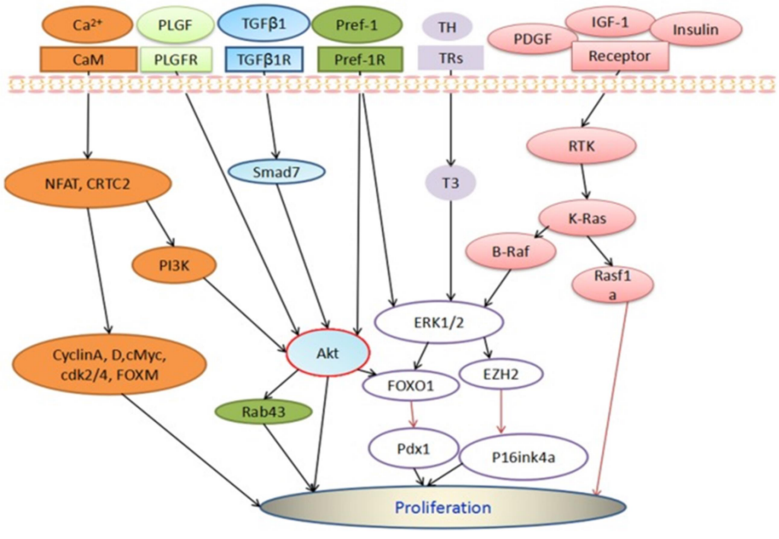


Fig. 2.2: PI3K-AKT/PKB Signaling Pathway

3.7.2.2 Wnt/β-catenin Signaling Pathway

The Wnt/β-catenin signalling pathway plays a vital role in cellular proliferation (Macdonald et al., 2009). According to Yu et al. (2018), the compound dioscin has been shown to stimulate β-cell proliferation via the Wnt/β-catenin signalling cascade. Additionally, studies in cultured mouse islets demonstrated that the Wnt signalling pathway promotes β-cell proliferation while inhibiting apoptosis, thereby maintaining β-cell functionality.

3.7.2.3 Growth Factor Signaling Pathways

Several growth factors, including insulin-like growth factor 1 (IGF-1), nerve growth factor (NGF), members of the transforming growth factor (TGF)-β superfamily, epidermal growth factor (EGF) family members, and placental growth factor (PLGF), have been linked to the regulation of β-cell proliferation through their respective receptors (PLGF, TGFβR, NGFR, EGFR, and IGF-1R). These mitogenic signals are associated with multiple downstream pathways that influence β-cell proliferation and viability, including the PI3K-AKT-mTOR pathway, ERK pathway, and Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathways (Wang et al., 2010).

3.7.2.4 Calcium-Mediated Signaling Pathways

Calcium ions serve as crucial second messengers that regulate the synthesis of hormones and enzymes such as insulin and play a role in apoptosis. Maintaining calcium homeostasis i.e., sustaining normal intracellular Ca^{2+} concentrations is vital for cell survival. Previous research suggests that disruptions in calcium homeostasis can accelerate β-cell death and may lead to the onset of Type 1 diabetes mellitus (Ahn et al., 2015). PI3K signaling promotes the autocrine function of pancreatic β-cells by inhibiting endoplasmic reticulum Ca^{2+} ATPase, thereby increasing intracellular Ca^{2+} levels and activating calcium-mediated signaling pathways. Elevated intracellular calcium concentrations further stimulate calmodulin (CaM), which subsequently activates various transcription factors (Roper et al., 2002; Lawrence et al., 2011).

3.7.2.5 ERK/MAPK Signaling Pathway

In humans, three distinct types of Ras proteins exist: H-Ras, K-Ras, and N-Ras. K-Ras, as a key downstream target of several cellular signaling pathways that govern growth, proliferation, and differentiation, holds considerable importance

(Nussinov et al., 2015). K-Ras has been shown to induce the anti-proliferative X-associated protein (Rasf1a) pathway, thereby inhibiting cell proliferation, as demonstrated by Chamberlain et al. Consequently, K-Ras may exert a negative regulatory effect on β-cell proliferation, suggesting that downregulation of the K-Ras gene could promote proliferation by reducing Rassf1a activity (Chamberlin et al., 2014).

3.7.2.6 Nerve Growth Factor (NGF) Signaling

Nerve growth factor (NGF) plays a crucial role in neuronal cell development and can also influence the function of non-neuronal cells (Zhang et al., 2017; Rosenbaum et al., 1998). It is notable that NGF is stored within secretory vesicles in β-cells of the islets. Upon appropriate stimulation of β-cell receptors, NGF can elicit both autocrine and paracrine effects (Rosenbaum et al., 1998). The primary receptors through which NGF acts are the tropomyosin receptor kinase A (trkA) and the p75 neurotrophin receptor (P75 NTR). When phosphorylated, trkA mainly regulates signaling associated with cell survival, differentiation, and proliferation (Freund and Frossard, 2015). In contrast, P75 NTR can recruit specific intracellular effectors depending on whether it is bound to NGF or in its unbound form (Roux and Barker, 2002).

3.7.2.7 Hormonal Signaling Pathways

Hormones such as growth hormone (GH), prolactin (PRL), glucagon-like peptide 1 (GLP-1), and thyroid hormone (TH), along with their respective receptors (TR, GHR, GLP-1R, IGF-1R, and PRLR), are linked to several signalling pathways, including ERK, JAK-STAT, and PI3K-AKT, which influence β-cell survival, growth, proliferation, and differentiation (Huang and Chang, 2014). Specifically, thyroid hormone receptors (TR) are expressed in various cell lines, and T3 has demonstrated a stimulatory effect on cellular growth. The presence of TR in the pancreas suggests that T3 may promote the proliferation of pancreatic β-cells, with T3-induced β-cell proliferation being mediated through the ERK signalling pathway (Kim et al., 2014). Additionally, GLP-1 can activate multiple signalling pathways in β-cells, including cAMP-PKA, PI3K-AKT, ERK1/2, and Wnt pathways (Campbell and Drucker, 2013).

3.7.2.8 JAK-STAT Signalling Pathway

The JAK-STAT pathway, a relatively newer signalling cascade, has been associated with various cytokines, including interleukins (IL-1, IL-2, and IL-6), as well as hormones like GH, leptin, placental lactogens, erythropoietin (EPO), and PRL (Brooks et al., 2014; Kiu and Nicholson, 2012). This pathway plays a role in regulating critical biological processes, including cell division, proliferation, and apoptosis. Activated STAT proteins can stimulate the expression of suppressors of cytokine signaling (SOCS), which act as protein repressors of activated STAT through their transcription (Linossi et al., 2013; Shuai and Liu, 2005). SOCS proteins serve as negative modulators of the JAK-STAT pathway by terminating upstream signaling processes, with SOCS1, SOCS2, SOCS3, and CISH identified as key contributors (Trengove and Ward, 2013; Liongue et al., 2016).

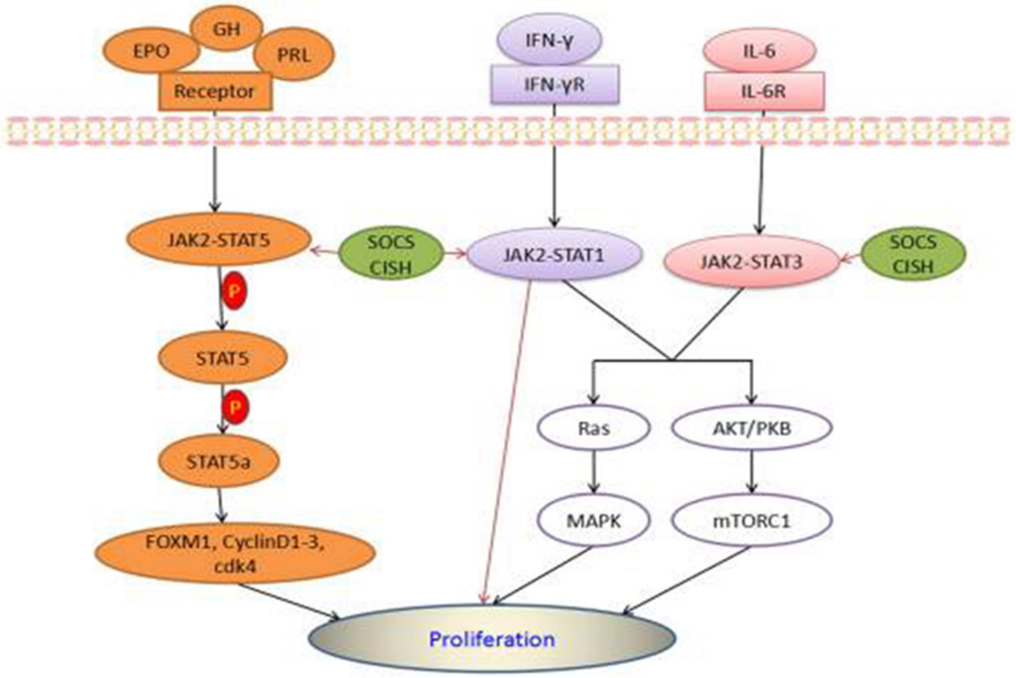


Fig. 2.3: JAK-STAT Signaling Pathway

3.7.2.9 TLR4 Signalling Pathway

Toll-like receptor 4 (TLR4), which is primarily expressed in the innate immune system, has been linked to diabetes mellitus (Shao et al., 2014). In Type 2 diabetes mellitus, the nuclear factor-κB (NF-κB) signalling pathway may become activated, leading to increased TLR4 expression. While NF-κB is not directly implicated in the inflammatory response associated with diabetes, it likely plays a

significant role in mediating insulin response and regulating glucose metabolism. The binding of NF-κB to the promoters of innate immune genes is believed to positively regulate TLR4 expression, especially for pro-inflammatory cytokines like IL-1β and tumour necrosis factor-α (TNF-α) (Sepehri et al., 2015).

3.8 Therapeutic Management of Diabetes Mellitus

The primary objective of contemporary diabetes management is to regulate and lower blood glucose levels effectively. Both conventional and novel medications function by stimulating the pancreatic islet's β-cells to release increased amounts of insulin, blocking the action of hormones that raise blood glucose, improving the sensitivity of insulin receptor sites, preventing the hydrolysis of glycogen in the liver, and enhancing the body's ability to utilise glucose (Tran et al., 2020).

Oral hypoglycemics are commonly prescribed for Type 2 diabetes, while insulin replacement therapy remains the standard treatment for Type 1 diabetes. Various classes of medications, including insulin secretagogues, biguanides, insulin sensitisers, alpha-glucosidase inhibitors, incretin mimetics, amylin antagonists, and sodium-glucose co-transporter-2 (SGLT2) inhibitors, play roles in managing diabetes (Gallwitz, 2016).

These pharmacological agents can be used alone or in combination with other hypoglycemics; however, relying solely on oral hypoglycemic medications is often insufficient to meet a patient's therapeutic goals (Tan et al., 2019). The traditional drugs come with disadvantages, such as the risk of severe hypoglycemia, weight gain, diminished therapeutic effectiveness due to inappropriate or ineffective dosing regimens, low potency, altered side effects attributed to drug metabolism, and lack of target specificity, not to mention issues with drug solubility and permeability (Padhi et al., 2020).

A novel and potentially effective treatment avenue for diabetes mellitus involves cell-based therapies, such as those utilizing stem cells—unspecialized cells that can differentiate into specialized cells capable of multilineage regeneration, depending on the tissue type (Hamad et al., 2021; Sena et al., 2010). A multifaceted approach is required to identify various therapeutic options that can address the management and treatment challenges of diabetes mellitus. Current research prominently focuses on enhancing both the quantity and functionality of β-cells. Many plants are known to possess properties that facilitate the replenishment of β-

cells. Consequently, medicinal plants may offer a cost-effective alternative to anti-diabetic medications, with fewer adverse effects (Rahman et al., 2022). Ethnopharmacological Approaches in the Treatment and Management of Diabetes

Medicinal plants have long been a source for discovering new therapeutic agents, playing a crucial role in the management of various ailments, including diabetes mellitus (Suhitha et al., 2015). In many traditional medical systems, particularly in less affluent nations, these plants have been utilized for centuries to treat and manage diabetes. The general population tends to favor the use of medicinal plants for basic healthcare due to their cultural acceptance, compatibility with human physiology, and low toxicity profiles (Gang et al., 2023).

The World Health Organization (WHO) has endorsed the use of medicinal plants as therapeutic agents for diabetes mellitus and has emphasized the need for further research into the hypoglycemic properties of various species. Notably, the WHO has identified 21,000 plants globally used for medicinal purposes, with approximately 2,500 of those found in India (Modak et al., 2007). Ethnopharmacological studies are invaluable for uncovering potential pharmaceutical candidates within these plants (Howes et al., 2020).

Medicinal plant-derived therapies are believed to be naturally effective and safe, with fewer side effects compared to synthetic alternatives. Their accessibility, lower costs, and reduced complexities make them suitable for managing diabetes effectively. Herbal remedies exert various mechanisms to lower blood glucose levels, including enhancing insulin production, reducing insulin resistance, and protecting pancreatic β-cells from damage (Alam et al., 2022).

A selection of medicinal plants known for their hypoglycemic effects includes *Acacia arabica, Aegle marmelos, Allium cepa, Allium sativum, Aloe vera, Annona squamosa, Azadirachta indica, Berberis vulgaris, Camellia sinensis, Capsicum frutescens, Cassia alata, Cinnamomum zeylanicum, Eucalyptus globulus, Eugenia jambolana, Momordica charantia, Panax ginseng, Punica granatum, Swertia chirayita, Trigonella foenum-graecum, Pterocarpus marsupium,* and *Gymnema sylvestre*. The antidiabetic properties of these plants are believed to be mediated through multiple pathways, including stimulating insulin secretion from pancreatic β-cells, enhancing insulin binding to its receptors, reducing insulin resistance, and improving glucose tolerance. Other mechanisms of action include boosting glucose

metabolism, improving β-cell mass and function, and increasing plasma insulin levels, thereby reducing overall blood glucose concentrations (Ansari et al., 2022).

3.8.1 *Momordica charantia*

Momordica charantia, commonly known as bitter melon, is one of the most popular vegetables in tropical regions, particularly in Vietnam, India, China, and East Africa. This plant's anti-hyperglycemic effects are attributed to its primary secondary metabolites, including charantin, momordicin, p-insulin, and vicine (Lankatillake et al., 2019).

3.8.2 *Allium cepa*

Onions, or *Allium cepa*, contain sulfur-containing compounds such as allyl propyl disulfide (APDS) and diallyl disulfide (allicin), which are their principal active ingredients. The positive effects of onions on blood glucose and cholesterol levels are generally associated with significant antioxidant activity, notably demonstrated by increased superoxide dismutase activity (Drobiova et al., 2011).

3.8.3 *Azadirachta indica*

Azadirachta indica, a member of the Meliaceae family, has been utilized in traditional medicine for treating various conditions, including diabetes, for centuries. Its seeds, stem bark, and leaves possess hypoglycemic properties that enhance insulin secretion from pancreatic β-cells (Singab et al., 2014).

3.8.4 *Ocimum sanctum*

Ocimum sanctum, or holy basil, belongs to the Lamiaceae family. Almost all parts of this plant are used in the treatment of various ailments. Eugenol is identified as the active constituent of *Ocimum sanctum*, contributing to its medicinal effects (Pattanayak et al., 2014).

3.8.5 *Allium sativum*

Allium sativum, commonly known as garlic, belongs to the Amaryllidaceae family. Its leaves and bulbs are frequently utilized for their medicinal benefits, particularly in significantly lowering fasting blood sugar levels (Kumar et al., 2011).

3.8.6 *Solanum nigrum*

Solanum nigrum, part of the Solanaceae family, has leaves known for their antihyperglycemic and hypolipidemic effects. Extracts from *S. nigrum* have been

shown to markedly decrease blood glucose levels and other lipid parameters (Patel et al., 2012).

3.8.7 *Aloe vera*

Aloe vera, belonging to the Asphodiloideae family, has been recognized for its medicinal properties for centuries. This plant is used to treat hyperlipidemia and is effective in managing Type 2 diabetes mellitus (Etxeberria et al., 2012).

3.8.8 *Eugenia jambolana*

Eugenia jambolana, also known as Jamun or black plum, is part of the Myrtaceae family. A traditional preparation involves boiling its seeds in water, which has been used for medicinal purposes (Baliga et al., 2013).

3.8.9 *Pterocarpus marsupium*

Pterocarpus marsupium, native to India, Nepal, and Sri Lanka, is commonly referred to as the Indian Kino Tree or Malabar Tree. This plant, known in Hindi as Vijayasar or Bija and in Sanskrit as Asana, belongs to the Fabaceae family. It serves as a key remedy in various traditional medical systems, including homeopathy and Ayurveda. Pterocarpus marsupium contains numerous phytoconstituents, such as pterosupin, pterostilbene, liquiritigenin, isoliquiritigenin, epicatechin, kinoin, kinotannic acid, kino-red beta-eudesmol, and marsupol, which are concentrated within its structure (Katiyar et al., 2016). For centuries, this plant has been used to treat diabetes mellitus (Jung et al., 2006; Danao et al., 2023).

The compound (-)-epicatechin, sourced from the bark of Pterocarpus marsupium, exhibits properties such as antioxidative activity, antihyperglycemic effects, anti-inflammatory characteristics, hyperinsulinemic action, and hepatoprotection (Gairola et al., 2010). Consumption of water stored overnight in tumblers made from the heartwood of this tree has been reported to lower blood glucose concentrations in diabetic individuals. Additionally, (-)-epicatechin has been found to enhance insulin release. In studies involving STZ-induced diabetic rats, the ethanol extract of Pterocarpus marsupium heartwood administered over ten days increased serum insulin levels (Mishra et al., 2013). This extract may support antiglycation activity by reducing ROS formation or scavenging ROS generated through the auto-oxidation of sugars and oxidative degradation of Amadori products. Furthermore, AGE formation was significantly inhibited by the alcohol extract (Gupta

et al., 2017). In STZ-induced rats, administration of the methanolic extract over six days resulted in observed enzymatic activity for catalase and superoxide dismutase (Joshi et al., 2013). The flavonoids and phenolic compounds present in *Pterocarpus marsupium*, such as liquiritigenin, pterosupin, and marsupin, possess antihyperglycemic and antihyperlipidemic properties. Extracts from this plant inhibit AGE formation (Gupta et al., 2017) and demonstrate significant inhibitory effects on α-amylase and α-glucosidase activity (Poongunran et al., 2015).

Moreover, the extract can lower inflammatory cytokines and reduce protein expression of apoptotic markers, such as the enzyme caspase-3, alongside pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). Histopathological examinations have shown improvements in both liver and pancreatic tissues. Notably, the flavonoid fraction of Pterocarpus marsupium has been shown to induce regranulation of pancreatic β-cells, which is believed to contribute to the plant's hypoglycemic effects. The pure flavonoid (-)-epicatechin has demonstrated antihyperglycemic properties in diabetic rats, raising the cAMP content in pancreatic islets, which has been associated with increased insulin release and conversion of proinsulin to insulin (Pari et al., 2018).

3.8.10 *Gymnema sylvestre* (GS)

Gymnema sylvestre, a valued herb belonging to the Asclepiadaceae family, is prevalent across various regions, including tropical Africa, China, Australia, Indonesia, Japan, Vietnam, and India. The term "Gymnema" derives from the Hindu word "Gurmar," meaning "destroyer of sugar," reflecting its purported ability to balance excessive sugar levels in the body (Saneja et al., 2010).

The active component in *Gymnema sylvestre*, known as gymnemic acid, has been isolated and shown to contribute to weight loss, possess antidiabetic and anti-obesity effects, and inhibit glucose absorption. Extracts from the plant effectively reduce triglyceride accumulation in both the liver and muscles, as well as the buildup of fatty acids in the bloodstream (Pothuraju et al., 2014). Furthermore, the constituents of *Gymnema sylvestre* inhibit glucose absorption within the small intestine. Reports indicate that the herb enhances hepatic and muscle glucose uptake, promotes glycogen synthesis, enhances glycolysis and gluconeogenesis, and mitigates glycosylation of haemoglobin and plasma proteins in rabbits. Additionally, *Gymnema sylvestre* may bolster glycemic control by promoting insulin release from the

pancreatic islets of Langerhans. It addresses numerous etiological factors related to diabetes, including chronic inflammation, obesity, enzymatic deficiencies, and pancreatic cell functionality (Ahmed et al., 2010).

In studies involving diabetic Wistar rats over a duration of 18 to 20 months, administration of *Gymnema sylvestre* extract alongside standard oral medications resulted in a significant decrease in blood glucose levels, glycosylated hemoglobin, and glycosylated plasma proteins. Moreover, the doses of conventional medications could be reduced (Aralelimath and Bhise, 2012). The extract of *Gymnema sylvestre* demonstrated potent antioxidant activity by neutralizing DPPH free radicals, underscoring its potential as a rich natural source of antioxidants (Bahera, 2019).

3.9 Experimental Animal Models of Diabetes

Rats are highly valuable animal models in diabetes research, offering several advantages over mice and other species. These advantages include their simpler physiological response and the extensive body of existing research, which helps in understanding the metabolic profiles and pathologies associated with various stages of Type 2 diabetes. However, despite the benefits of rat models, mouse models remain widely utilized in diabetes research, particularly for studies related to obesity and Type 2 diabetes. The insights gained from mouse models provide a crucial foundation for preclinical investigations, facilitating a deeper understanding of processes such as inflammation, insulin resistance, and the exploration of potential treatment options.

A significant advancement in rat models of diabetes was introduced by Reed et al. (2000) with their novel HFD/STZ rat model, also known as the high fat diet / streptozotocin (STZ) rat model. This model was specifically developed to simulate the natural progression of diabetes pathology, capturing the complex interactions that characterize the disease. In this model, 7-week-old Sprague-Dawley rats are subjected to a diet consisting of 40% kcal from fat for a duration of two weeks. This dietary intervention leads to the onset of insulin resistance, as evidenced by parallel glucose clearance profiles observed in both fat and lean rats.

Following the two-week dietary phase, the rats are administered a single intravenous dose of STZ (50 mg/kg) after undergoing an overnight fasting period. This administration of STZ is critical for inducing a hyperglycemic state, as it selectively targets the pancreatic β-cells. Three days post-STZ treatment, the rats exhibiting elevated blood glucose levels are identified and subsequently subjected to

testing for their responsiveness to metformin, an established antidiabetic medication. The observed reduction in blood glucose levels in response to metformin further substantiates the relevance of the HFD/STZ rat model for studying Type 2 diabetes, as it closely mimics the corresponding conditions found in human diabetics (Skovsø, 2014).

Streptozotocin (STZ) itself is a widely recognized compound utilized to induce diabetes mellitus in various experimental animal models, including rats, mice, rabbits, and guinea pigs. As a nitrosourea compound, STZ has distinct properties that contribute to its effectiveness; it acts as a toxic glucose analogue and a N-acetyl glucosamine analogue, allowing it to accumulate specifically in pancreatic β-cells through uptake via the GLUT-2 transporter.

The mechanism by which STZ induces diabetes can vary based on the dosage administered. At high doses, STZ selectively targets pancreatic β-cells due to its alkylating properties, which closely resemble glucose, thus facilitating its uptake via the GLUT-2 carrier protein (Furman, 2015). This targeted action leads to significant destruction of β-cells while having minimal effects on the α-cells of the pancreas and other extra-pancreatic cells. Conversely, at low doses, STZ induces an immune and inflammatory response that releases glutamic acid decarboxylase, a crucial autoantigen associated with autoimmune diabetes. This response triggers the destruction of β-cells and the subsequent development of a hyperglycemic state characterized by inflammatory infiltrates, particularly lymphocytes within the pancreatic tissue.

High-dose STZ administration typically involves a single delivery method, either intravenously or intraperitoneally, which leads to extensive β-cell destruction and a rapid onset of diabetic symptoms (Kottaisamy et al., 2021).

Materials and Methods

CHAPTER-3

MATERIALS AND METHODS

The present study evaluated the nephroprotective effect of *Pterocarpus marsupium* and *Gymnema sylvestre* against experimentally high-fat diet fed-streptozotocin-induced type 2 diabetes in male Wistar rats. General parameters like feed and water intake, body weight, per cent weight gain, organ weight (absolute and relative), haemato-biochemical indices, oxidative stress biomarkers in the kidney and histoarchitecture of the pancreas and kidney, western blot study of sodium -glucose transporter-2 (SGLT2) expression in kidney tubule and genes related to pancreatic regeneration were studied

Place of investigation

The present research work was conducted within the facilities of the Department of Veterinary Pharmacology and Toxicology and Veterinary Pathology College of Veterinary Science and Animal Husbandry, U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan (DUVASU), Mathura, (U.P.).

The study was taken up after the approval of Institutional Animal Ethical Committee (IAEC), U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan (DUVASU) (Project No. IAEC/24/1/36 letter No. 145 / IAEC/ 24 Dated 14-5-2024) as per guidelines of CPCSEA, Govt. of India.

The present study includes:

- Preparation of hot ethanolic extract of *Pterocarpus marsupium* heartwood and bark
- Preparation of hot methanolic extract of *Gymnema sylvestre* leaves
- Development of high-fat diet fed obese rats
- Development of high-fat diet fed-streptozotocin-induced type 2 diabetic Wistar rat model.
- Evaluation of efficacy of ethanolic extract of *Pterocarpus marsupium, and methanolic extract* of *Gymnema sylvestre* fed for sixty days of the experimental period on type 2 diabetes-induced male Wistar rats.

3.1 Materials used

Table 3.1 (a) Chemicals, reagents and drugs used in the present study

Chemicals	Make/Company
3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT)	HiMedia
5,5'dithiobis (2- nitrobenzoic acid) (DTNB)	HiMedia
Citric acid monohydrate	Merck
Concentrated Hydrochloric acid	Qualikems
Concentrated sulphuric acid	Qualikems
Cupric sulphate	HiMedia
D-Glucose	HiMedia
Diethyl ether	Research Lab
Disodium hydrogen phosphate	CDH
Eosine staining solution 2% w/v	LobaChemie
Ethanol	Research Lab
Ethylene diamine tetra acetic acid disodium salt	Qualigens
Ferric chloride	Qualigens
Folin-Ciocalteau reagent	HiMedia
Formaldehyde solution 37-41% GR	Rankem
Glutathione Reductase	Sigma-Aldrich
Hematoxylin staining solution	LobaChemie
Iodine	Fischer Scientific
Liquid ammonia	SRL
Metformin (Glyciphage)	Franco-Indian Pharmaceuticals
Paraffin wax	CDH
Potassium chloride	BDH
Potassium dihydrogen phosphate	BDH
Potassium iodide	Rankem
Pyrogallol	HiMedia
Reduced glutathione	HiMedia
Sodium Azide	HiMedia
Sodium bicarbonate	Merck
Sodium carbonate	HiMedia
Sodium chloride	Merck
Sodium hydroxide	Rankem
Sodium potassium tartrate	Qualigens
Streptozotocin	M P Biomedicals
Sucrose	SRL
Thiobarbturic acid	HiMedia
Trichloro acetic acid	HiMedia
Trisodium citrate	Merck

Table 3.1 (b) Diagnostic kits used in the present study

Kits	Make
LDL- Cholesterol	Arkray Healthcare
HDL- Cholesterol	
Triglycerides	
Total Cholesterol	
Glucose	
Glycated haemoglobin (HbA1c)	Truechemi
Insulin Rat ELISA Kit	Thermo Fisher Scientific
RNA isolation kit	Thermofisher
Revert Aid First Strand cDNA Synthesis Kit	

Table 3.1(c) Equipment/Instruments used in the present study

Equipment	Make/Company
Accu Chek Performa Glucometer	Accucheck Performa
Autoclave	Sonar, India
BOD incubator	Sonar
Electronic balance	Shimadzu
ELISA reader	Span Diagnostic/auto-reader 4011
Hematology autoanalyser	Mindray
Hot air oven	Instron
Microtome	Leica
pH meter	Systronics
Refrigerated Centrifuge	REMI, C-30BL
Semi-autoanalyser	Erba, Manheim
Shaker	Genei
Soxhlet apparatus	Perfit India
Ultra-low deep freezer,	New Brunswick Scientific
Water bath	Macflow

Table 3.1 (d) Chemicals and antibodies used for Western Blot

Chemicals and antibodies	Make/Company
2- Mercaptoethanol	Amresco
Acrylamide	Amresco
Ammonium persulphate	Amresco
Bis- acrylamide	Amresco
Bovine serum albumin	HiMedia
Bradford 1x dye reagent	Bio-Rad
Commasie Brilliant Blue	SRL
Diamino benzene (DAB)	Sigma
Glacial acetic acid	Qualigens
Glycerol	HiMedia
Glycine	Amresco
HRPO- conjugated anti- mouse polyclonal secondary antibody	Santa Cruz
HRPO- conjugated anti- rabbit secondary antibody	Abcam
Hydrogen peroxide	HiMedia
Methanol	Rankem
Native membrane protein extraction kit	Calbiochem-Proteo extract
Nickel chloride	Empura
Polyvinylidene fluoride (PVDF) Membrane	Millipore
PRECISION PLUS PROTEIN- Kaleidioscope prestained protein standard (10-250 kD)	Bio-Rad
Protease inhibitor cocktail, mammalian	Amresco
SGLT-2 monoclonal primary antibody	Santa Cruz
Skimmed milk powder	Santa Cruz
Sodium Dodecyl sulphate (SDS)	Amresco
Tetra methyl ethylene diamine (TEMED)	Amresco
Tris base	Amresco
Tris HCl	Amresco
Tween 20	Amresco
β- Actin primary antibody	Santa Cruz

3.2 Procurement and maintenance of experimental animals

Healthy adult male Wistar rats weighing 150-200 g were procured from Disease Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar. Rats were kept in polyacrylic cages and housed in laboratory conditions. Before the start of the experiment, animals were kept in the laboratory conditions for seven days for acclimatization and maintained under standard management conditions with daily 12 hours of light-dark cycle along with *ad libitum* balanced pelleted feed (Ashirwad Feed Industries, Chandigarh, India)

3.3 Plant materials

The dried stem and bark of *Pterocarpus marsupium* were brought from Ayurvedic Medical College, Ron, Karnataka.

Dried leaves of Gymnema sylvestre were procured from Panchvati Herbals, Rangnathpura, Bengaluru (Karnataka).

3.3.1 Preparation of hot methanolic extract of *Gymnema sylvestre* leaves

Hot methanolic extract of shade-dried and coarsely powdered leaves of *Gymnema sylvestre* was prepared using a soxhlet apparatus by hot percolation method. About 40-45 g of coarsely grounded powder of leaves were placed in a porous cellulose thimble in a soxhlet apparatus (capacity 500 ml). The thimble was placed in an extraction chamber above the flask containing 500 ml of 80% methanol. The flask was heated and the solvent was allowed to evaporate. The temperature (40° C) was adjusted according to the boiling temperature of the solvent in the flask. The extraction process was allowed to continue for several hours (approximately 48 hr.) involving 20-22 cycles till the solvent in the siphon tube of the extractor became completely colourless. Thereafter, the extract obtained was concentrated in a rotator vacuum evaporator under reduced pressure at 40° C and 70 RPM to obtain semisolid mass which was later kept in a BOD incubator at 37° C for further evaporation of the solvent and the percent yield was calculated. The harvested extract was then transferred into air tight containers and stored in refrigerator (4° C) till used.

3.3.2 Preparation of ethanolic extract of *Pterocarpus marsupium* (PM) heartwood and bark

Ethanolic extract of *Pterocarpus marsupium* prepared using a soxhlet apparatus by hot percolation method. The dried stem and bark of Pterocarpus were

ground in an electrical grinder to a coarse powder and sieved. About 35-45 g of coarsely ground powder of *Pterocarpus marsupium* was placed in a porous cellulose thimble in a soxhlet apparatus (capacity 500 ml). The thimble was placed in the extraction chamber above the flask, which contained 500 ml of 100% ethanol. The flask was heated and the solvent was allowed to evaporate. The temperature (40 ^{0}C) was adjusted according to the solvent's boiling temperature in the flask. The extraction process was allowed to continue for 48 hours involving 20-22 cycles till the solvent in the siphon tube of the extractor became completely colourless. After that, the extract obtained was concentrated in an incubator at 37 ^{0}C to obtain semisolid mass, and the per cent yield was calculated. The harvested extract was transferred into air-tight containers and stored in the refrigerator (4 ^{0}C) till used.

3.4 Experimental Design

Thirty-six adult healthy male Wistar rats weighing 150-200 g were randomly divided into six groups of six animals in each and subjected to different treatments as detailed below and housed in polypropylene cages.

Groups	Treatment	Mode of administration	Exposure period	Number of animals
I.	Healthy Control	Oral gavage (NSS)	60 days	6
II.	Obese	Oral gavage (NSS)	60 days	6
III.	Obese Diabetic	Oral gavage (NSS)	60 days	6
IV.	Obese Diabetic + Metformin	Oral gavage (NSS)	60 days	6
V.	Obese Diabetic + *Pterocarpus marsupium* ethanolic extract (PMEE)	Oral gavage	60 days	6
VI.	Obese Diabetic + *Gymnema sylvestre* methanolic extract (GSME)	Oral gavage	60 days	6

Note: Metformin @ 50 mg/kg body weight, *Pterocarpus marsupium* @ 300 mg/kg body weight, and *Gymnema sylvestre* @ 400 mg/kg body weight.

After dividing normal healthy rats into six groups (as mentioned above) their blood samples were collected into clot-activated tubes (for serum) after overnight fasting for estimation of fasting blood glucose, triglycerides, and total cholesterol.

3.5 Preparation of high-fat diet (HFD) and development of obese Wistar rat model

HFD was prepared in our laboratory in accordance to Suman et al. (2016) with minor modifications. HFD was prepared by using normal pelleted feed (365 g), lard (310 g), mixture of vanaspati ghee and coconut oil in ratio of 2:1 (50 g), soybean chunks and/or milk powder (250 g), yeast powder (1 g), sodium chloride (1 g), dl-methionine (3 g) and vitamin and mineral supplement (20 ml as syrup). All ingredients were mixed. The mixture was then kept in a freezer to solidify and after brought at room temperature for further use. The rats of each group except the healthy control group were fed a high-fat diet prepared, ad libitum for a period of four weeks to develop an obese rat model.

3.6 Induction of type 2 diabetes (metabolic syndrome) in HFD-fed-Obese male Wistar rats

After four (04) weeks of dietary manipulation with HFD, rats were injected with a single dose of Streptozotocin @ 35 mg/kg body weight dissolved in 0.1 M citrate buffer (pH 4.5) intraperitoneally as per previous studies carried out in our laboratory. Streptozotocin-injected rats were closely monitored for their behaviour and any other clinical signs and were given 10% sucrose water to avoid hypoglycemic shock on the day of injection. Blood samples were collected after 72 hrs of STZ administration to measure fasting blood glucose (>200mg/dl), serum cholesterol (>80 mg/dl), and serum triglyceride (>50 mg/dl) and change in body weight as 8% of initial weight to ensure true diabetic rat model. Rats that were under the above criteria were included in the present study.

3.6.1 Composition of citrate buffer (0.1 M, pH 4.5)

1) 0.1M Trisodium citrate dihydrate: 1.47 g of sodium citrate tribasic dihydrate was dissolved in 50 ml distilled water and stored at room temperature.
2) 0.1M Citric acid monohydrate: 1.05 g of citric acid monohydrate was dissolved in 50 ml distilled water and stored at room temperature.

Fresh citrate buffer was prepared every time by adding 2 parts of 0.1 M trisodium citrate with 3 parts of 0.1 M citric acid monohydrate to make 0.1 M citrate buffer (pH 4.5) and kept it on ice.

Note: Streptozotocin is stable for 3 to 5 minutes after reconstitution, so it was made sure to administer streptozotocin within 3 minutes after reconstitution in citrate buffer. Metformin was administered orally at 50 mg/kg body weight, an ethanolic extract of *Pterocarpus marsupium* at 300 mg/kg body weight, and *Gymnema sylvestre* at 400 mg/kg body weight (the dose used was decided from previous studies carried out in our laboratory).

3.7 Parameters studied

3.7.1 General Parameters

During the entire study period animals were closely observed for any apparent clinical signs or symptoms. Daily feed and water intake (measured and calculated on weekly basis), weekly body weight, and body weight gain. At the end of the experiment, animals were sacrificed by cervical dislocation and organ weight (relative and absolute) were assessed.

3.7.2 Hematobiochemical parameters

3.7.2.1 Collection of blood samples

Blood samples of experimental rats were collected after overnight fasting on days 1, 3, 15, 30, 45, and 60^{th} days after induction of diabetes from retro-orbital plexus in EDTA and clot-activated tubes for glucose estimation using Accu-Chek Performa, Roche Glucometer, and other biochemical parameters were determined at the end of the experiment in serum and whole blood samples. Clotted blood was centrifuged at 4500 rpm for 10 min at 4 °C for serum collection. Serum samples were stored at -40 °C for further analysis while parameters performed on whole blood samples performed on the same day of sample collection

3.7.2.2 Haematological parameters

Haemoglobin (Hb), total leukocyte count (TLC), total erythrocyte count (TEC), and platelet count were estimated in whole blood using a haematology auto-analyser (Mindray, The Netherlands).

3.7.2.3 Glycated haemoglobin (HbA1c) assay

On the 60th day, glycated haemoglobin (HbA1c) assay was performed on whole blood samples using commercially available Truechemi Diagnostics kits and a multimode microplate reader spectra-max i3x.

3.7.2.4 Serum biochemical parameter

Total cholesterol, triglycerides, low-density lipoproteins (LDL), and high-density lipoproteins (HDL) were estimated in serum samples using commercially available kits of Arkray Healthcare Pvt. Ltd. with the help of a semi-auto analyser (Erba-Manheim, Germany).

3.8 Oxidative stress markers in kidney tissue

Oxidative stress-related parameters in the kidney were determined by employing different assay procedures and using a multimode microplate reader spectra-max i3x.

3.8.1 Preparation of tissue homogenate

In 2 ml of ice-cold saline, 200 mg of tissue sample was weighed and taken for GSH estimation. 200 mg sample was weighed separately and taken in 2 ml of 0.02 M EDTA. By using a tissue homogenizer, homogenates were prepared under cold conditions and were centrifuged for 10 min at 3000 rpm. The supernatant was used for the estimation of different oxidative stress-related parameters.

(a) Lipid peroxidation

The extent of lipid peroxidation was evaluated in terms of malonaldehyde (MDA) production, determined by the method as described by Shafiq-ur-Rehman (1984).

Reagents

1) TCA (10 %): 10 g of TCA was dissolved in distilled water and final volume was made up to 100 ml.
2) 0.67 % TBA: 0.67 g of TBA was dissolved in 100 ml of distilled water and warmed up for complete dissolution.

Procedure

1 ml of homogenate supernatant was incubated at 37 ^{0}C for 2 h. To each sample, 1 ml of 10 % TCA was added. After thoroughly mixing, it was centrifuged at 2000 rpm for 10 min. To 1ml of supernatant liquid, an equal amount of 0.67 % TBA was added and kept in a boiling water bath for 10 min, cooled and diluted with 1ml distilled water. The absorbance was taken at 535 nm.

Calculation

The LPO concentration was calculated by using the molar extinction coefficient (EC) of the MDA-TBA complex at 535 nm, i.e. 1.56 x 10^8 /M/cm and results were expressed in nM MDA formed /g of wet tissue.

LPO nM MDA formed /g tissue

$$= \frac{\text{OD} \times \text{Total volume of reaction mixture}}{\text{EC} \times \text{Amount of sample}} \times (10^9 \times \text{DF} \times 2)$$

(b) Reduced glutathione (GSH)

For determination of GSH free –SH group was estimated using DTNB method of Sedlak and Lindsay (1968). For this, 10 % tissue homogenate was prepared in 0.02 M EDTA.

Reagents

1) DTNB (0.01M): 99 mg of DTNB in 25 ml of methanol.
2) 1M Tris-buffer (pH 8.9): 121g of Tris base was dissolved in 950 ml of distilled water. pH was adjusted to 8.9 and final volume was made up to 1000 ml by addition of distilled water.
3) TCA (50 %): 50 g of TCA was dissolved in distilled water and final volume was made up to 100ml.

Procedure

To 1 ml of homogenate supernatant, 0.2 ml of 50% TCA solution and 0.8 ml of distilled water were added and kept at room temperature for 15 min. This solution was centrifuged at 3000 rpm for 15 min. 0.4 ml of supernatant was taken and to this 0.8 ml of Tris buffer was added, followed by 0.2 ml DTNB (0.01M) and after mixing, the absorbance was read at 412 nm within 5 min. Reagent blank had no sample and sample blank was without DTNB.

Calculation

The concentration of GSH was calculated by using the extinction coefficient (EC) 1300 M^{-1} cm^{-1} and results were expressed in mM GSH/g of wet tissue.

$$\text{GSH (Mm/g of tissue)} = \frac{\text{OD} \times \text{Total volume of reaction mixture}}{\text{EC} \times \text{Amount of sample taken}} \times (1000 \times \text{DF})$$

DF = Dilution factor

(c) Superoxide Dismutase

Superoxide dismutase was estimated as per the method described by Madesh and Balasubramanian (1998).

Reagents

1) 100 µM pyrogallol: 6.3 mg of pyrogallol was dissolved in 5 ml of distilled water; 1ml of this solution was added to 100 ml of distilled water.
2) 1.25 mM MTT: 2.58 mg MTT was dissolved in 5ml distilled water
3) Phosphate buffer saline: PBS was prepared by dissolving NaCl (8 g), KCl (0.2 g), KH_2PO_4 (0.2 g), Na_2HPO_4 (0.94 g) in about 800 ml of distilled water and the volume was then made to one litre with distilled water.

Procedure

Different reagents were added in sample, control and blank as shown below. The absorbance was read at 570 nm against distilled water.

Reagent	Test /sample	Blank	Control
PBS	650 µl	650 µl	650 µl
MTT	30 µl	30 µl	30 µl
Homogenate	10 µl	-	-
Pyrogallol	75 µl	75 µl	75 µl
Incubate at RT for 5 min			
DMSO	750 µl	750 µl	750 µl
Homogenate	-	-	-
DW	-	-	10 µl
Take OD at 570 nm			

Calculation

Superoxide Dismutase was expressed as SOD units / mg of protein

$$Y\ \% = \frac{\text{OD of test}}{\text{OD of blank}}$$

SOD (U /mg protein) = (mg protein in 10 µl homogenate /Y %) x 50 x DF

Where,

DF = dilution factor

Y % = % inhibition of MTT by SOD

d) Catalase

Catalase in tissue homogenate was estimated and calculated by the method of Bergmeyer (1983).

Reagents

1) Phosphate buffer (50 mM pH 7.0)
   A) KH_2PO_4 - 1.37 g/ 200 ml DW (50 mM)
   B) Na_2HPO_4 - 1.42 g/ 200 ml DW (50 mM)

Solution A and B were mixed in a ratio of 1:1.5 (v/v) and pH was adjusted to pH 7.0.

2) H_2O_2 (10 mM) 0.1 ml H_2O_2 of 30% was diluted to 100 ml DW. The solution was checked at 230 nm, and the concentration was adjusted by using molar extinction coefficient of H_2O_2.

Procedure

In a test tube, 2 ml phosphate buffer and 10 µl homogenate were added and the contents were transferred to cuvette. By adding 1 ml of H_2O_2 directly into the cuvette, the reaction was started. OD was recorded at every 30 second for 3 min at 240 nm against blank (DW).

Calculation

The activity of catalase was expressed as mM H_2O_2 utilized /min/ mg protein and calculated using the formula:

Catalase mM H_2O_2 utilized /min/ mg protein =

$$\frac{\Delta OD/time}{0.067} \times \frac{\text{total volume of reaction}}{\text{amount of sample taken}} \times 1/\text{ mg protein in 10 µl}$$

e) Total Protein

Total protein was estimated by method of Lowry et al. (1951).

Reagents

1) Reagent A: 2 % Sodium Carbonate in 0.1N NaOH (2 g Na_2Co_3 in 100 ml of 0.1N NaOH)
2) Reagent B: 1 % copper sulphate (100 mg of $CuSo_4$ was dissolved in10 ml of distilled water and stored in amber coloured bottle)
3) Reagent C: 2 % sodium potassium tartarate (200 mg of sodium potassium tartarate was dissolved in10 ml of distilled water)
4) Reagent D (Alkaline copper sulphate): It was prepared by mixing 49 ml of reagent A with 0.5 ml of each of reagent B and C just before estimation.

5) Folin-Ciocalteau reagent (1N): 2 ml Folin-Ciocalteau reagent was diluted with 2 ml of distilled water. Fresh solution was prepared.
6) Bovine Serum Albumin: 50 mg of BSA was dissolved in 50 ml of distilled water and used as standard.

Procedure

To 20 µl of sample (1:100) in a test tube 980 µl of distill water and 2.5 ml alkaline copper sulphate solution were added. The reaction mixture was allowed to stand for 10 minutes at room temperature. Thereafter, Folin-Ciocalteau reagent (0.25ml) was added and tubes were shaken immediately. The whole mixture was allowed to stand for 30 minutes at room temperature and absorbance was read at 750 nm. For the BSA standard, 100 µl was added to 900 µl, for blank to 1000 µl of distilled water, and other solutions were added as per the sample.

Calculation

Protein (µg/ml) = OD of test/OD of standard x 100 x1/0.02

3.9 Estimation of Diabetic Nephropathic marker

Estimation of cystatin C in serum by ELISA kit.

3.10 Histopathological Examination of Tissues

3.10.1 Haematoxylin and Eosin Staining of Pancreas and Kidney

Tissues isolated from rats from all the groups were fixed in 10% buffered formal-saline for 48 h, dehydrated in ascending alcohol grades and embedded in paraffin wax. Approximately 5-6 µm thick sections were prepared and stained with hematoxylin and eosin (H & E) for assessment of the general morphology under light microscopy (Luna, 1968).

a) Dehydration of tissue and preparation of blocks and slides:

Tissues from 10% neutral buffered formalin were taken out to remove formalin from the tissue and kept under running tap water for overnight

↓

Remove water as much as possible with the help of tissue paper

↓

Dehydrate the tissue in ascending grades of ethanol for 30 min each

↓

50% ethanol

↓

70% ethanol

↓

90% ethanol

↓

Absolute ethanol - I

↓

Absolute ethanol - II

↓

Kept in acetone for 20 minutes

↓

Kept in xylene and benzene mixture (1:1) for 20 minutes

↓

The tissue was kept in molten paraffin for 3 hours

↓

Blocks were prepared by adding molten paraffin to the tissue and kept at room temp. for 0.5 hr for solidification, then kept in refrigerator at 4˚C for 1 h

↓

Sections (5-6µm) were cut using a microtome (Leica, ICC50) and tissue sections were transferred on glass slides

↓

Slides were kept in the oven for 1 h to melt the paraffin wax

b) Hematoxylin and Eosin staining of Kidney and Pancreas (H & E staining)

Xylene I (2 min)

↓

Xylene II (2 min)

↓

Xylene + Alcohol (1:1; 2 min)

↓

Absolute ethanol (2 min)

↓

90, 80, 70 and 50 % ethanol (for 2 min each)

↓

Distilled water (2 min)

↓

Hematoxylin (3 min)

↓

Tap water (1-2 min)

↓

Acid water (few dips)

↓

Ammonia water (few dips)

↓

Eosin (1 min)
↓
80 and 90 % ethanol (few dips each)
↓
Absolute ethanol I (few dips)
↓
Absolute ethanol II (few dips)
↓
Xylene + Alcohol (1:1; few dips)
↓
Xylene I (3 min)
↓
Xylene II (3 min)
↓
Slides were dried and mounted in DPX and kept for 3 h to dry the DPX solution.
↓
After fixing, the slides were examined using a computerized microscope (Leica, ICC50) to determine the histological details of different organs.

3.10.2 Chrome alum Haematoxylin-Phloxine staining

a) Chemical Solutions

i) Bouin's fixative

Saturated picric acid (aqueous) – 750 ml

Concentrated formaldehyde (37-40%) – 250 ml

Glacial acetic acid – 50 ml

ii) Potassium Permanganate solution

Potassium permanganate – 1.5gm

Distilled water – 500 ml

iii) 5% sodium bisulfite solution

Sodium bisulfite – 5 g

Distilled – 100 ml

iv) Chromium-Alum Hematoxylin solution

Solution A (Stock)

Hematoxylin – 1g

Distilled water – 100ml

Solution B (Stock)

Chromium potassium sulfate – 3g

Distilled water – 100ml

Working Solution

Solution A (Stock) – 100m

Solution B (Stock) – 100ml

Potassium iodate – 0.2g

(The working solution was boiled until a deep blue color appeared and filtered before each use.)

v) 1% acid alcohol solution

Conc. HCl – 1 ml

95% Alcohol – 99 ml

vi) 0.5% phloxine B solution

Phloxine B – 0.5 g

Distilled water – 100ml.

vii) 5% Phosphotungstic acid solution

Phosphotungstic acid – 5g

Distilled water to make – 100 ml

b) Fixation of tissue

Pancreas was fixed into 10% neutral buffered formalin solution for 72 h. The paraffin sections were mordanted in Bouin's fixative for 16 h before staining.

c) Staining procedure

Deparaffinized and hydrated section in distilled water

↓

Mordanted in Bouin's solution for 16 h

↓

Washed in tap water to remove picric acid for 15 min

↓

Kept in potassium permanganate solution for 1 min

↓

Differentiated in sodium bisulfite solution for 1min (till slides become transparent)

↓

Washed well under tap water

↓

Kept in chromium hematoxylin solution for 10 min

↓

Differentiated in an acid alcohol solution for 45 seconds

↓

Washed in tap water until the section became clear blue

↓

Kept in phloxine B solution for 7 min.

↓

Rinsed in distilled water

↓

Kept in the phosphotungstic acid solution for 5 min

↓

Washed in tap water for 5 min. The section regained its red colour

↓

Dehydrated in 95% alcohol (10 dips)

↓

Absolute alcohol

↓

Xylene (two changes)

↓

Slides were dried and mounted in DPX and kept for 3 h to dry the DPX solution.

↓

After fixing, the slides were examined using a computerized microscope (Leica, ICC50) to determine the histological details of the pancreas

3.10.3 Masson Trichrome staining procedure for cholegen

1) Deparaffinize and hydrate to distilled water.
2) Incubate in Bouin's fixative solution at 56º C to 60º C for 60 minutes in a hot air oven.
3) Wash in running tap water until the yellow colour disappears and rinse in two changes of distilled water.
4) Stain nuclei with Weigert's Iron Hematoxylin working solution for 10 minutes.
5) Wash in running tap water and rinse in two changes of distilled water.
6) Stain in Biebrich Scarlet Acid Fuchsin solution for 2 minutes.
7) Rinse in three changes of distilled water.
8) Place slides in Phosphomolybdic and Phosphotungstic Acid Solution for 15 minutes.

9) Drain slides and transfer to Aniline Blue solution for 5 minutes.

10) Rinse in three changes of distilled water.

11) Differentiate in 1% Glacial Acetic Acid Solution for 1-2 minutes.

12) Rinse in two changes of distilled water.

13) Dehydrate, clear and do cover slip with DPX mountant.

3.11 mRNA expression studies in rat pancreas

3.11.1 RNA isolation

Pancreas tissue samples from rats of all groups were collected in liquid nitrogen and stored immediately at -80 ºC. Total RNA was isolated with PureLink™ RNA mini kit (Invitrogen, USA) as per the manufacturer's instructions and stored at -80 ºC. RNA's purity was checked by a Biophotometer (Eppendorff, Germany) and quantitated as follows:

$$1\ OD_{260} = 40\mu g$$

RNA samples having an A260/280 ratio of 1.8-2.0 and an A260/230 ratio >1.6 were used for cDNA synthesis and further gene analysis.

3.11.2 cDNA synthesis

By using Revertaid® First strand cDNA synthesis kit (Thermo Scientific, USA) cDNA synthesis was performed from the mRNA present in the total RNA by following the manufacturer's instructions. In autoclaved PCR tubes 1000 ng RNA was mixed thoroughly with 1µl of oligo dT primer and the final volume was made 12 µl by adding nuclease-free water. The solution was incubated at 65ºC for 5 minutes in a Gradient thermocycler (T-100, Biorad). To this, 4 µl MMLV reverse transcriptase buffer (5X), 2 µl dNTPs Mix (10mM), 1 µl RiboLock RNase Inhibitor (20 U/µL) and 1 µl RevertAid M-MuLV reverse transcriptase (200 U/µL) were added and made the total volume 20 µL. Then incubated at 42 ºC for 60 minutes and 70 ºC for 5 minutes in a thermocycler.

3.11.3 Primer validation

Primer sequences were obtained from the previous publications and checked from the NCBI nucleotide database. Reverse transcription real-time polymerase chain reaction (RT-qPCR) analysis was performed for cDNA samples by using respective primers and annealing temperatures.

3.11.4 Description of primers

Gene	Primer sequences	Amplicon size (bp)	References
STAT3	F: 5′- CACCCATAGTGGGAGCCCTTGGA-3′ R: 5-TGAGTGCAGTGACCAGGACAGA-3′	127	Zhang et al. (2024)
JAK2	F:5′-TTTGAAGACAGGGACCCTACACAG -3′ R:5′-TCATAGCGGCACATCTCCACA -3′	101	Zhang et al. (2024)
IL6	F: 5′-TGTTCTCAGGGAGATCTTGG -3′ R:5'-TCCAGGTAGAAACGGAACTC -3′	204	Zhang et al. (2023a)
IFN¥	F: 5′-AGGAAAGAGCCTCCTCTTGG -3′ R:5'-TCTACCCCAGAATCAGCACC -3′	346	Xie et al. (2001)
GAPDH	F: 5′-CAGCAATGCATCCTGCAC-3′ R:5'-GAGTTGCTGTTGAAGTCACAGG-3′	417	Nakahara et al. (2004)

3.11.5 Reverse transcriptase polymerase chain reaction (RT-PCR)

The cDNA was checked for quality by performing PCR with standard GAPDH primers. The polymerase chain reaction was standardized for each gene using cDNA from rat pancreas. The reaction was carried out at different annealing temperatures and the optimum annealing temperatures for different genes were determined. The protocol performed according to Sambrook and Russel, 2001 as follows

S. No.	Steps	Temperature and duration
1.	Initial denaturation	95 ºC for 5 min
2.	Denaturation	95 ºC for 1 min
3.	Annealing	56 ºC to 62 ºC for 1 min
4.	Extension	72 ºC for 1 min
5.	Step 2-4 repetition	40 cycles
6.	Final extension	72 ºC for 5 min
7.	Held at	4 ºC

3.11.6 Agarose gel electrophoresis

DNA amplified by PCR was subjected to agarose gel electrophoresis as described by Sambrook and Russel (2001). DNA amplified by PCR was subjected to agarose gel electrophoresis as described by Sambrook and Russel (2001). Four fifty and/or sixty-milligram agarose (1.5% or 2%, respectively) were dissolved in 30 ml IX TAE buffer by heating and then allowed to cool to 50 °C. In this, 1.0 µl ethidium bromide (final concentration 0.5 µg/ml) was added and mixed thoroughly. The mixture was poured onto a gel-casting tray fitted with the acrylic comb and allowed to solidify. Once the gel had formed, a few ml of TAE was added near the comb, which was later removed carefully, and the gel was immersed in an electrophoresis tank containing IX TAE buffer. 1 µl of 6X DNA loading dye was mixed with 5 µl of DNA samples and loaded into the wells. Electrophoresis was carried out at 85 volts until the tracking dye reached the end. The DNA bands were visualized as bands under UV illumination and documented. Based on the standard 1 kb plus DNA ladder (10488085, Invitrogen, USA), the size of the PCR products produced was estimated.

3.11.7 Reverse transcription real-real-time quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR reactions were run on a real-time PCR system (QuantStudio3, Applied Biosystem) and analysed by using Quantstudio™ Design and Analysis Software v1.2.x.

RT-qPCR was performed using the SYBER Green master mix (PowerUp™ SYBER™ Green master mix [2X]; Thermofischer Scientific, USA). Each reaction consisted of 5µl SYBER Green master mix, 0.5/0.25µl from 10pmol/µl stock solution forward and reverse primers of the respected gene, 1µl of cDNA and final volumed made 10µl by adding nuclease-free water (NFW). The real-time PCR reaction was started with UDG activation at 50ºC for 2 minutes, Taq polymerase activation at 95ºC for 2 minutes followed by 40 cycles of amplification with denaturation at 95ºC for 15 sec, annealing (temperature as mentioned for gene-specific primer pairs) for 30sec and extension at 72ºC for 1 minute each.

To assess the specificity of the amplified product, a dissociation curve was generated at temperatures of 60ºC through 95ºC. The results were expressed as threshold cycle values (C_T). This value is the cycle number when the fluorescence of the reporter dye is automatically adjusted by the instrument, that was used for the generation of C_T values. As previously stated by Livak and Schmittgen (2001), the 2-Ct technique was employed to examine the relative change in gene expression. Using the equation "fold change =$2^{-\Delta\Delta Ct}$" [where $\Delta\Delta C_T = (C_{T,\ \text{target gene}} - C_{T,\ GAPDH})$ treatment - $(C_{T,\ \text{target gene}} - C_{T,\ GAPDH})$ control] "one may determine the fold change in gene expression. The housekeeping gene GAPDH was used to adjust the gene-specific amplification for any differences in RNA input. Evaluation of $2^{-\Delta\Delta Ct}$ for each experimental group (aside from Control) reveals the fold change in gene expression compared to Control (fold change in Control = 1). The outcomes were examined about the CT (minimum threshold of amplification) values of the reference gene (GAPDH) and the target gene.

Note: Despite multiple attempts and repeated trials, the intended procedure using RT-PCR could not be successfully executed. As a result, an alternative approach was adopted for the analysis. The quantification was carried out using an ELISA kit by BT LAB (Rat interleukin-6 Cat. No. E0135Ra), (Rat interferon

gamma Cat. No. E0103Ra), (Rat tyrosin protein kinase JAK-2 Cat. No. E2490Ra) which provided reliable and reproducible results. This methodological adjustment was necessary to ensure the accuracy and feasibility of the study while maintaining the integrity of the experimental objectives.

3.12 Preparation of kidney tissue samples for Western Blot analysis of SGLT-2 protein

The kidney tissue samples was homogenised using tissue homogeniser and the membrane and soluble fractions were isolated from tissue by Native Membrane Protein Extraction Kit (Calbiochem-Proteo Extract).

For western blot analysis of SGLT-2 following solutions were used:

3.12.1 Recipe of Solutions

(a) 30% acrylamide mix for stacking gel and resolving gel: 29.2 g acrylamide and 0.8 g bis acrylamide was dissolved in 60 ml of distilled water. The solution was filtered and the volume was made up to 100 ml. It was stored in dark bottle at 4°C.

(b) Stock buffer for resolving gel: 1.5M Tris-base buffer (pH 8.8): 18.15 g Tris base was dissolved in 90 ml distilled water and pH was adjusted to 8.8 and the final volume made up to 100 ml

(c) Stock buffer for stacking gel: 0.5 M Tris-base buffer (pH 6.8): Dissolve 6 g of Tris base in 90 ml distilled water and adjust the pH up to 6.8 and final volume was made up to 100 ml.

(d) 10% SDS: 10 g SDS was dissolved in 90 ml distilled water and mixed well and then final volume was made up to 100 ml.

(e) Ammonium per sulphate: (10%) (prepared fresh every time): 0.1 g of APS was dissolved in 1 ml distilled water.

(f) Electrode buffer:

25 mM Tris	:	3.025 g
Glycine	:	18.75 g
Methanol	:	10 ml
SDS	:	1g

Distilled Water to make 100 ml

(g) Staining solution:

Coomassie Brilliant Blue	:	0.15 g
Methanol	:	50 ml
Glacial acetic acid	:	10 ml

Distilled Water to make 100 ml

First 20 ml of MGD (methanol, glacial acetic acid, distilled water) solution was taken and added 0.15 gm of coomassie brilliant blue dye was added and dissolved and filtered. Then after filtrate was added to rest of the MGD solution.

(h) De-staining solution:

Glacial acetic acid	:	50 ml
Methanol	:	200 ml
Distilled Water	:	500 ml

(i) Transfer Buffer (pH 8.3):

25 M Tris	:	3.0285 g
19.2 mM Glycine	:	14.41 g
10% Methanol	:	100 ml

Distilled Water to make 1000 ml

(j) TBST (Tris Buffer Saline Tween 20):

20 mM Tris Base (pH 7.4):	:	1.12 g
150 mM NaCl	:	4.38 g
0.05% Tween 20	:	25 µl

Distilled water to make 500 ml

(k) TBS (Tris Buffer Saline) pH 7.5:

20 mM Tris Base	:	2.42 g
150 mM NaCl	:	8.75 g

Distilled water to make 1000ml

(l) DAB solution:

2.5 M Tris HCl (pH 7.4)	:	1.25 ml
DAB	:	0.2g
8% $NiCl_2$	:	150 µl

The volume was made upto 25 ml with distilled water and the solution was filtered, and then added 7.5 µl H_2O_2.

(m) Blocking solution: Two gram of skimmed milk powder was dissolved in 50 ml of TBST solution.

3.12.2 Western Blot Procedure

i) Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

(a) Gel casting

The gel casting apparatus was arranged and 10% resolving gel was prepared using the recommended compositions. The reaction mixture was gently put in between the sandwich of glass plates. One layer of water was poured over the gel reaction mixture and the gel was allowed to polymerize at room temperature. After polymerization of the resolving/separating gel, isobutanol/water was drained off. The reaction mixture for 5% stacking gel was prepared and it was poured gently over the resolving gel in between the sandwich of glass plates. The teflon comb was carefully placed into the top of the stacking gel and allowed to polymerise the gel at room temperature. After polymerization of stacking gel, the comb was removed carefully and the wells were washed thoroughly with electrode buffer to remove any unpolymerized gel material.

(b) Gel loading and running of gel

The isolated protein samples with 2X gel loading or sample buffer in a proportion of 4:1 was mixed and boiled at 100°C for 5 minutes in a boiling water bath and was allowed to cool down. The samples in the wells were loaded with the help of gel-loading tips. The electrophoresis tank was filled with electrode buffer and the vertical gel electrophoresis apparatus was run at 45 Volt till the tracking dye crossed the stacking gel. Thereafter, the gel was run at 90 volt till the tracking dye reached the bottom of the gel.

(c) Staining of Gel

SDS-PAGE gel was stained with Coomassie brilliant blue R-250 stain to visualize the separated protein band. The gel was immersed in staining solution and placed on a slowly rotating platform for 5 hours at room temperature. The staining solution was removed by briefly rinsing with deionized water. The gel was immersed in de-staining solution and rotated slowly on rocking platform for 24 hours, changing the de-staining solution three or four times. The gel was 7% stored in acetic acid in a plastic container.

(ii) Transfer of proteins and reaction with specific antibodies

This technique is popularly used to detect protein of interest from the protein mixture. Proper separation of the protein samples was ensured in the gel and the protein bands from SDS-PAGE gel were transferred to a polyvinylidene fluoride (PVDF) membrane by semi- dry transfer method. Thereafter, the desired protein band was detected using specific antibodies.

(a) Transfer method

The proteins were separated using a vertical gel electrophoresis, dipping the gel into distilled water and thereafter in the transfer buffer. PVDF membrane and filter pads were dipped in transfer buffer. While using PVDF membrane, the membrane was first activated by dipping in methanol. Over the platform of semi-dry apparatus, the wet filter paper pads were placed first and then the activated membrane. Then the gel was placed over the membrane. It was again kept with one or two layer of wet filter paper pads over the gel and a little amount of transfer buffer was put at the top of the sandwich. Proper care was taken to remove any entrapped air bubbles in between the sandwich. The apparatus was run at 10 Volt for 30 minutes.

(b) Blocking and developing membrane using specific antibodies

After transferring the protein bands into the membrane, the membrane was kept in blocking solution for 2 hours at 4°C. Then the membrane was washed with TBST 3-4 times and the membrane was treated with primary antibody (SGLT-2 mouse monoclonal antibody to SGLT-2 in dilution of 1:500) for overnight at 4°C. The membrane was again washed with TBST 3-4 times and it was treated with secondary antibody (SGLT-2 HRP- conjugated anti mouse IgG polyclonal antibody in dilution of 1: 500) for 2 hours at room temperature. Then the membrane was washed with

TBST 3-4 times. The diaminobenzidine (DAB) solution was prepared and put over the membrane and waited till brown colour band developed. After colour development, the membrane was washed with distilled water and scanned for further use.

Note: Initially, the detection of SGLT2 was intended to be performed using Western blot analysis. However, despite multiple attempts and optimizations, the procedure did not yield successful results due to some technical limitations. Consequently, an alternative approach was adopted, and the quantification of SGLT2 was carried out using the BT LAB Rat SGLT2 ELISA Kit (Cat. No. E1382Ra) this method provided reliable and reproducible results, ensuring the accuracy of the study while maintaining the integrity of the experimental objectives.

3.13 Immunohistochemical Examination of Pancreas

In addition to the previously mentioned biochemical and histopathological assessments, immunohistochemistry (IHC) was performed on pancreatic tissue to evaluate insulin. This additional parameter was included to further elucidate the histological and molecular changes in pancreatic β-cells across different experimental groups."

In pancreas immunohistochemical examination is done. Paraffin blocks were sectioned at 5 μm and glass slides coated with poly-L-lysine. The slides were dried overnight at 37°C to optimize adhesion. Sections were deparaffinized through xylene, and tissues were rehydrated in sequentially graduated ethyl alcohol. Slides were incubated in hydrogen peroxide in methanol for 10 min to reduce nonspecific background staining due to endogenous peroxidase. The sections were washed twice, in phosphate buffer solution (PBS). Then, tissues were boiled in 1: 100 citrate buffer solution for 10 min and cooled for 20 min. The cooled tissues were washed four times in PBS before the application of blocking serum for 5 min. Then, a primary antibody was applied; tissues were incubated for 30 min at room temperature. They were rinsed 4 times in PBS, given an application of biotinylated anti-polyvalent antibody, and incubated for 10 min at room temperature. After being washed three times in PBS, streptavidin peroxidase was applied and the samples were incubated for 10 min at room temperature, and then rinsed 4 times in PBS. Tissues were further incubated for

20 min at room temperature in a solution of DAB chromogen. After being washed in PBS, tissues were counter-stained with Mayer's hematoxylin, washed in water, and coverslips were applied with mounting media. For negative control, the primary antibody was not added to the sections. fields of each slide were examined under the 20x objective of the microscope.

3.14 Statistical analysis

Results are expressed as Mean ± SEM. Statistical analysis of data was performed using GraphPad Prism version 8 and SPSS version 16 by One-way ANOVA followed by Tukey 's-b Multiple Comparisons Post-Hoc Tests. A value of $p<0.05$ was considered as statistically significant.



Results

CHAPTER-4

RESULTS

The present study was designed to evaluate the nephroprotective potential of extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* in HFD-fed obese male Wistar rats where in parameters like body weight, organ weight, (absolute and relative), feed & water intake, hemato-biochemical indices, lipid profile, oxidative stress parameters, histopathological changes in the kidney & pancreas, nephropathy and pancreatic regenerative markers were studied.

The male Wistar rats were randomly divided into six groups namely control (group I), obese (group II), obese diabetic (group III), obese diabetic + metformin (group IV), obese diabetic + *Pterocarpus marsupium* (V), obese diabetic + *Gymnema sylvestre* (VI), consisting six animals in each group. Rats of all groups except control group rats were offered a high-fat diet (HFD) for four weeks to develop obese rats. After four weeks a single dose of streptozotocin (STZ) @ 35mg/body weight was administered intraperitoneally to develop HFD-fed obese diabetic rats. The rise in body weight by 8%, cholesterol (>80 mg/dl), and triglyceride (> 50mg/dl) after HFD and a significant rise in fasting blood glucose (>200mg/dl) after 72 hours of administration of STZ confirm the development of the metabolic syndrome.

4.1 Effect of high-fat diet (HFD) on body weight, serum triglyceride, total cholesterol and fasting blood glucose (FBG) for development of obese Wistar rat model.

Table 4.1 summarises changes in body weight, serum triglyceride, total cholesterol, and fasting blood glucose. After 4 weeks of high-fat diet feeding, rats showed significant ($p < 0.05$) increases in body weight, triglycerides, total cholesterol, and fasting blood glucose. These changes indicate the development of an obese rat model.

After the development of an obese model, STZ was administered intraperitoneally to obese rats. The significant rise in fasting blood glucose level after 72 hours of STZ administration indicates the development of a diabetic rat model.

Table 4.1 Effect of high-fat diet (HFD) on body weight, serum triglyceride, total cholesterol and fasting blood glucose (after STZ @35mg/body weight on rats of different groups.

S. No.	Group	Body Weight (g)		%Weight gain	Triglyceride (mg/dl)		Cholesterol (mg/dl)		Fasting Blood Glucose(mg/dl)	
		Before HFD	After HFD	After completion Of HFD	Before HFD	After HFD	Before HFD	After HFD	1ST Day	3RD Day After STZ
I	Control	181.0 ±3.1[a]	220.7 ±4.4[a]	21.93	63.4 ±2.6[a]	64.5 ±1.8[a]	120.0 ±2.3[a]	125.7 ±51.3[a]	93.5 ±6.1[a]	92.5 ±4.3[a]
II	Obese	172.2 ±9.4[a]	263.5 ±4.4[b]	53.01	64.3 ±1.6[a]	86.3 ±2.3[b]	120.0 ±0.8[a]	172.9 ±70.6[b]	102.8 ±4.0[a]	93.0 ±4.7[a]
III	Obese Diabetic	181.3 ±4.4[a]	281.3 ±10.0[b]	55.16	63.9 ±1.4[a]	91.0 ±1.5[b]	120.4 ±1.9[a]	171.8 ±70.1[b]	90.3 ±3.9[a]	351.8 ±39.3[b]
IV	Obese Diabetic + Metformin	174.2 ±3.5[a]	261.5 ±6.5[b]	50.11	64.0 ±1.7[a]	88.0 ±2.1[b]	120.3 ±1.9[a]	171.3 ±69.9[b]	93.7 ±3.9[a]	391.0 ±16.9[b]
V	Obese Diabetic + PM (EE)	169.2 ±8.4[a]	266.2 ±6.4[b]	57.33	64.2 ±1.6[a]	89.6 ±2.4[b]	120.6 ±0.9[a]	172.4 ±70.4[b]	94.8 ±5.5[a]	388.3 ±25.3[b]
VI	Obese Diabetic + GS	171.8 ±8.8[a]	266.8 ±6.0[b]	55.30	63.6 ±1.2[a]	88.7 ±2.3[b]	118.9 ±2.2[a]	165.9 ±67.7[b]	92.0 ±4.9[a]	346.2 ±42.5[b]

Values (Mean ± SEM; n=6) bearing different superscripts (a and b) in the same column differ significantly ($p<0.05$) using one way ANOVA following Tukey's B multiple comparison Post Hoc test.

4.2 Effect of oral administration of metfoemin, extracts of *Pterocarpus marsupium*, and *Gymnema sylvestre* on feed (g), and water intake (ml) of HFD-fed obese diabetic rats.

The weekly changes in feed intake (g) and water intake (ml) in different groups of rats are presented in Tables 4.2 & 4.3 and Fig. (4.2) & (4.3), respectively compared to the healthy control, the obese and obese diabetic groups showed consistently and significantly ($p < 0.05$) increased feed and water intake during the 2nd to 8th week of the experimental period. However, treatment groups (IV, V and VI) showed a markedly significant reduction in feed and water intake compared to obese and obese diabetic rats and almost comparable with healthy control group rats.

4.3 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* on body weight of HFD-fed obese diabetic rats.

The change in weekly body weight and per cent weight gain (g) were recorded in different treatment groups are summarized in Table (4.4) and Fig. (4.4)

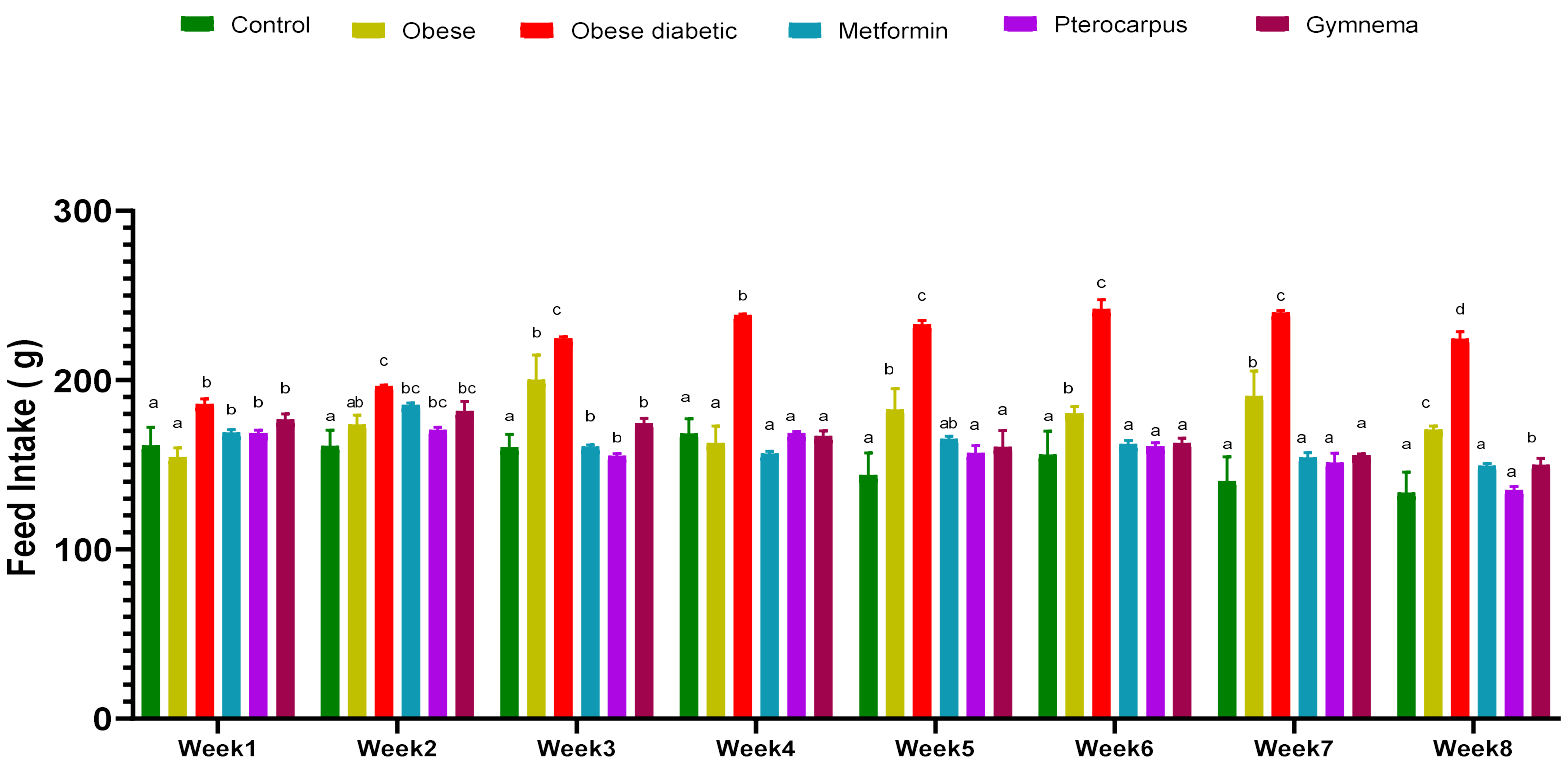
At the end of the experimental period in the 8th week a progressive and significant ($P < 0.05$) decrease in body weight in rats of the obese diabetic group with a negative weight gain of 30.75% and an increase in obsess group of rats (307.3 ± 6.6) as compared to healthy control (282.2 ± 5.6). However, an appreciable and significant ($p < 0.05$) increase was recorded in metformin, PM and GS-treated groups compared to obsess diabetic groups and were almost comparable to the healthy control group.

Table 4.2 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on weekly feed intake (g) of HFD-fed obese diabetic rats.

S. No.	Group (s)	Feed intake (g)							
		1st Week	2nd Week	3rd Week	4th Week	5th Week	6th Week	7th Week	8th Week
I.	**Healthy Control**	161±6.4^{a}	160.3±5.7^{a}	159.6±4.6^{a}	168±5.3^{a}	156.5±7.8^{a}	155.3±8.2^{a}	150±8.4^{a}	143±7.3^{a}
II.	**Obese**	154±3.5^{a}	171.4±3.4^{ab}	174.5±8.7^{b}	175.5±5.9^{a}	182±7.2^{b}	185.5±2.5^{b}	190.2±8.8^{b}	192.3±1.4^{b}
III.	**Obese Diabetic**	185.5±1.9^{b}	196±0.6^{c}	224.2±0.8^{c}	238±0.6^{b}	232.5±1.6^{c}	241.7±3.4^{c}	239.6±0.9^{c}	224±2.6^{d}
IV	**Obese Diabetic + Met**	182±1.5^{b}	184.8±2.3^{bc}	190±5.5^{b}	168.8±1.5^{a}	164.8±1.1^{ab}	161.8±1.4^{a}	153.8±1.9^{a}	149±0.9^{a}
V	**Obese Diabetic + PM (EE)**	180±2.6^{b}	184.8±5.4^{bc}	194.3±3.8^{b}	170.5±1.4^{a}	156.5±2.8^{a}	160.4±1.5^{a}	150.8±3.4^{a}	144.6±1.5^{a}
VI	**Obese Diabetic + GS (ME)**	176.4±2.1^{b}	181.3±3.5^{bc}	180.3±3.3^{b}	166.5±2.1^{a}	160.4±5.8^{a}	162.4±1.8^{a}	155.3±0.6^{a}	149.5±2.5^{b}

Values (Mean ± SEM; n=6) bearing different superscripts (a, b, c and d) in the same column and values bearing different superscripts (a, b, c, d and e) in the same row differ significantly ($p<0.05$) using one way ANOVA following Tukey's B multiple comparison Post Hoc test.

Fig 4.2 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on weekly feed intake (g) of HFD-fed obese diabetic rat



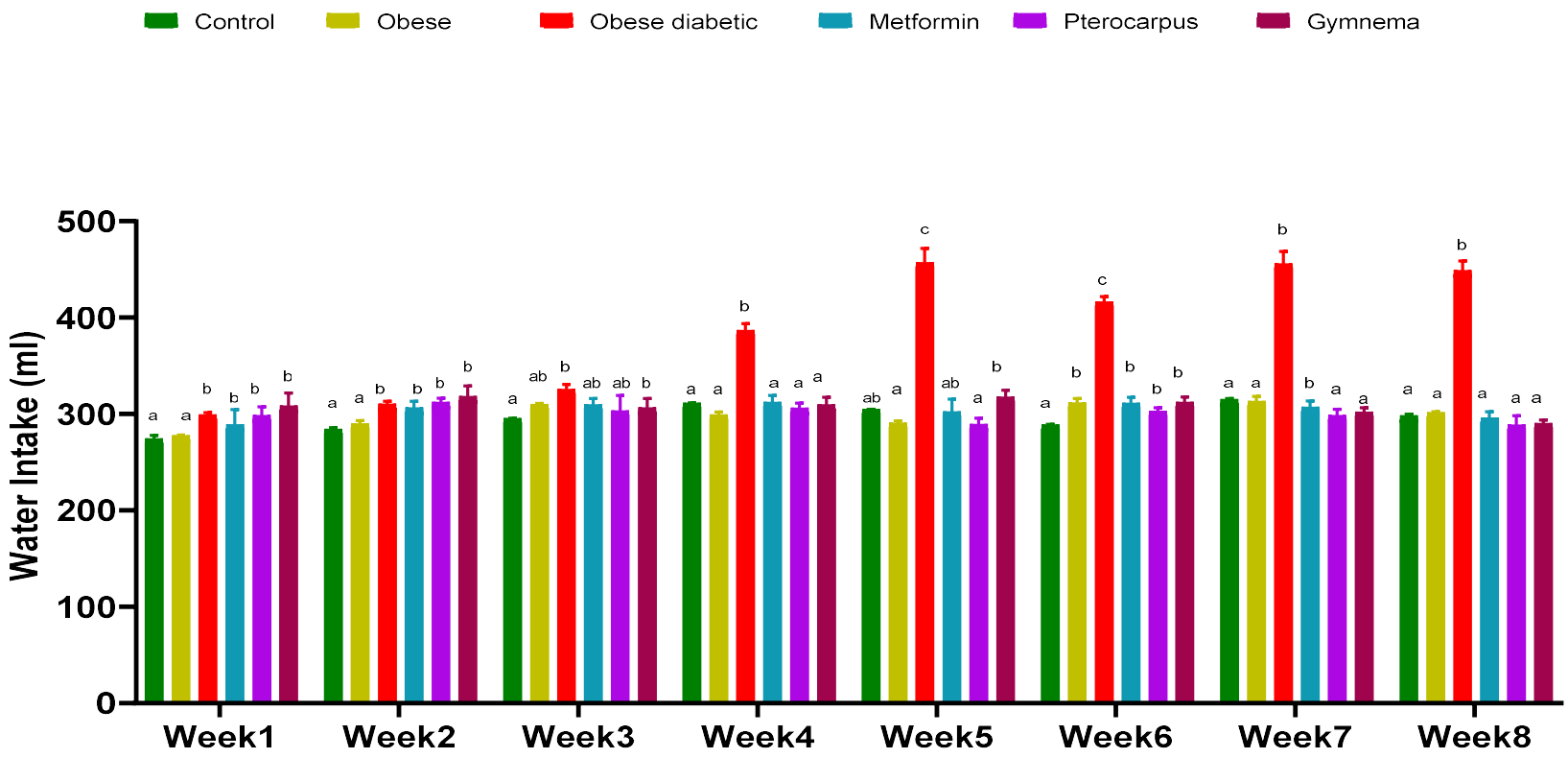
Value (mean ± SEM; n=6) bearing different superscripts in the histogram differ significantly ($p<0.05$)

Table 4.3 Effect of oral administration of metformin and extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on weekly water intake (ml) of HFD-fed obese diabetic rats.

S. No.	Group (s)	Water intake (ml)							
		1st Week	2nd Week	3rd Week	4th Week	5th Week	6th Week	7th Week	8th Week
I.	Healthy Control	273.6±2.3^{a}	283.1±1.3^{a}	294.6±0.4^{a}	290.5±0.5^{a}	289.0±0.3^{ab}	287.9±0.7^{a}	292.3±0.9^{a}	293.4±1.3^{a}
II.	Obese	276.5±0.9^{a}	289.2±2.2^{a}	299.2±1.0^{ab}	298.5±1.9^{a}	289.8±1.6^{a}	306.8±3.0^{b}	312.4±3.4^{a}	300±1.0^{a}
III.	Obese Diabetic	298.2±1.7^{b}	318.4±2.1^{b}	324.8±3.3^{b}	385.8±4.4^{b}	456.4±8.8^{c}	415.7±3.5^{c}	455.2±7.8^{b}	447.9±6.3^{b}
IV.	Obese Diabetic + Metformin	288.2±9.4^{ab}	312.6±4.4^{b}	308.7±4.2^{ab}	311.3±4.4^{a}	301.6±7.9^{ab}	310.3±3.9^{b}	306.4±4.1^{a}	294.9±4.2^{a}
V.	Obese Diabetic + PM(EE)	297.5±5.7^{ab}	311.4±2.7^{b}	305.6±9.5^{ab}	305.3±3.3^{a}	288.3±4.1^{a}	302.1±2.4^{b}	297.6±4.0^{a}	296.6±6.1^{a}
VI.	Obese Diabetic + GS (ME)	307.3±8.4^{b}	317.5±6.5^{b}	312.7±6.0^{b}	308.6±5.1^{a}	316.8±4.5^{b}	311.5±3.5^{b}	301.2±3.0^{a}	299.4±2.5^{a}

Values (Mean ± SEM; n=6) bearing different superscripts (a, b and c) in the same column differ significantly ($p<0.05$) using one way ANOVA following Tukey's B multiple comparison Post Hoc test.

Fig 4.3 Effect of oral administration of metformin and extracts of *Pterocarpus marsupium, Gymnema sylvestre* continuously for 60 days on weekly water intake (ml) in HFD-fed obese diabetic rats.



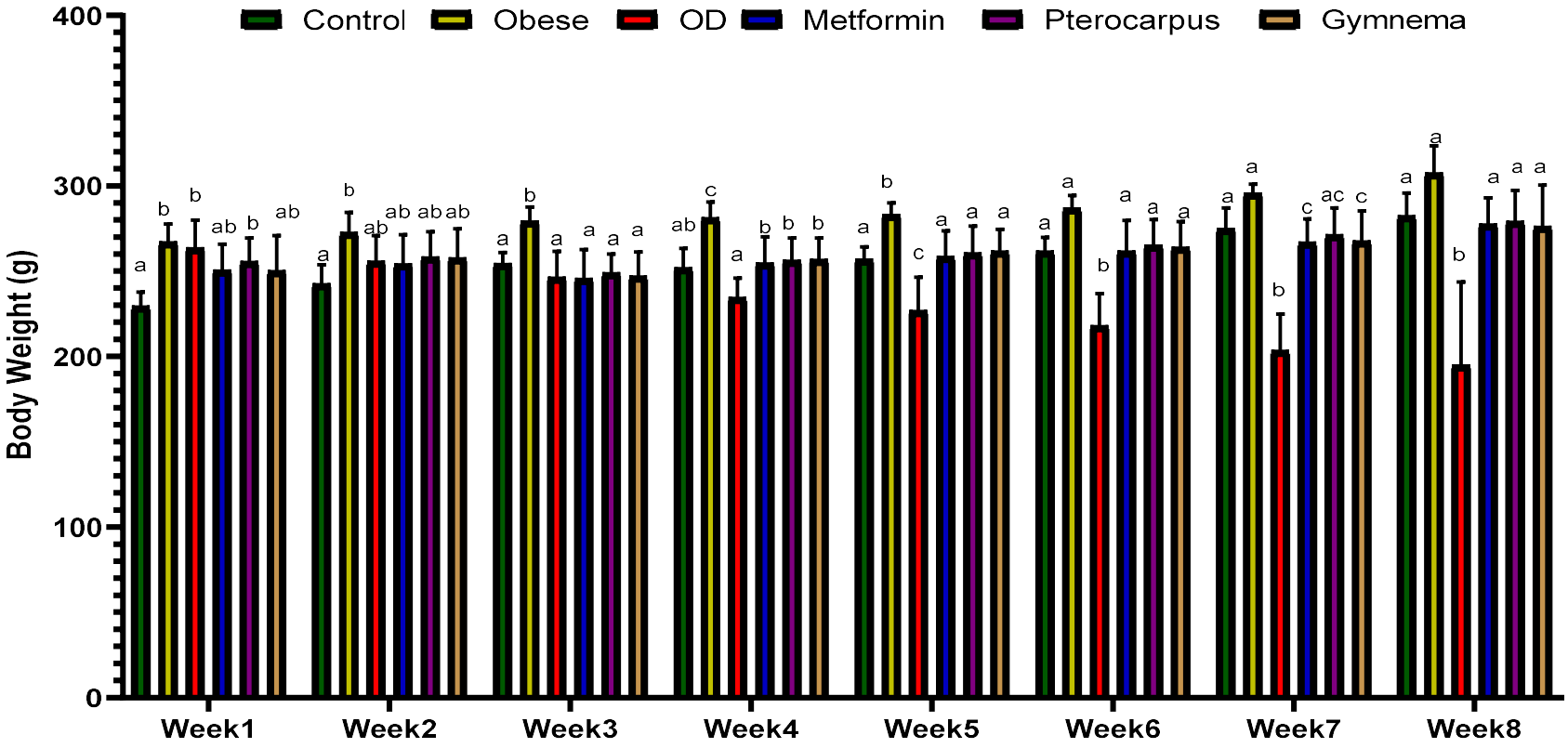
Value (mean ± SEM; n=6) bearing different superscripts in the histogram differ significantly ($p<0.05$)

Table 4.4 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium*, and *Gymnema sylvestr* continuously for 60 days on weekly body weight (g) in HFD-fed obese diabetic rat

S. No.	Group (s)		Body weight gain(g)								
		Day 0	1st Week	2nd Week	3rd Week	4th Week	5th Week	6th Week	7th Week	8th Week	% Weight gain
I.	Healthy Control	220.7 ±3.1^{a}	229.2 ±3.6^{a}	242.3 ±4.7^{ab}	254.2 ±2.8^{a}	251.7 ±4.8^{ab}	256.7 ±3.1^{a}	261.5 ±3.4^{a}	274.7 ±5.1^{a}	282.2 ±5.6^{a}	27.86
II.	Obese	263.5 ±9.4^{b}	266.8 ±4.4^{b}	272.5 ±4.9^{b}	279.0 ±3.5^{b}	281.0 ±3.9^{c}	283.0 ±2.9^{b}	286.7 ±3.2^{a}	295.5 ±2.2^{a}	307.3 ±6.6^{a}	16.62
III.	Obese Diabetic	281.3 ±4.4^{b}	263.3 ±6.8^{b}	255.5 ±6.3^{ab}	246.0 ±6.4^{a}	234.2 ±4.8^{a}	226.7 ±8.1^{c}	217.7 ±7.9^{b}	203.3 ±8.8^{b}	194.8 ±20.0^{b}	-30.75
IV.	Obese Diabetic + Metformin	261.5 ±3.5^{b}	250.3 ±6.3^{ab}	254.0 ±7.1^{ab}	245.5 ±7.0^{a}	254.5 ±6.4^{b}	258.3 ±6.3^{a}	261.5 ±7.5^{a}	266.7 ±5.7^{c}	277.3 ±6.4^{a}	6.042
V.	Obese Diabetic + PM (EE)	266.2 ±8.4^{b}	255.3 ±5.8^{ab}	258.0 ±6.2^{ab}	248.8 ±4.7^{a}	256.0 ±5.5^{b}	260.5 ±6.6^{a}	264.8 ±6.3^{a}	271.2 ±6.5^{ac}	278.8 ±7.6^{a}	4.73
VI.	Obese Diabetic + GS(ME)	266.8 ±8.8^{b}	250.0 ±8.6^{ab}	257.5 ±7.1^{ab}	246.8 ±5.9^{a}	256.5 ±5.3^{b}	261.3 ±5.4^{a}	263.7 ±6.3^{a}	267.5 ±7.3^{c}	276.0 ±10.0^{a}	3.44

Values (Mean ± SEM; n=6) bearing different superscripts (a, b and c) in the same column differ significantly ($p<0.05$) using one way ANOVA following Tukey's B multiple comparison Post Hoc test.

Fig 4.4: Graph showing the effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on weekly body weight (g) in HFD-fed obese diabetic rats.



Value (mean ± SEM; n=6) bearing different superscripts in the histogram differ significantly ($p<0.05$)

4.4 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium*, and *Gymnema sylvesre* treatment on absolute (g) and relative organ weight (g/100 g body weight) on t HFD-fed obese diabetic rats.

The result of absolute organ weights (g) and relative organ weight of liver, kidneys, heart, and pancreas in rats after 60 days of the experimental period are represented in Table 4.5 & 4.6 and Fig. 4.5 & Fig. 4.6, respectively. The present data revealed a mild to moderate but non-significant decrease in the absolute weight of the liver, kidney, heart and pancreas, in an obese diabetic group of rats. Whereas a non-significant increase in the treatment group (IV, V and VI) when compared among different groups.

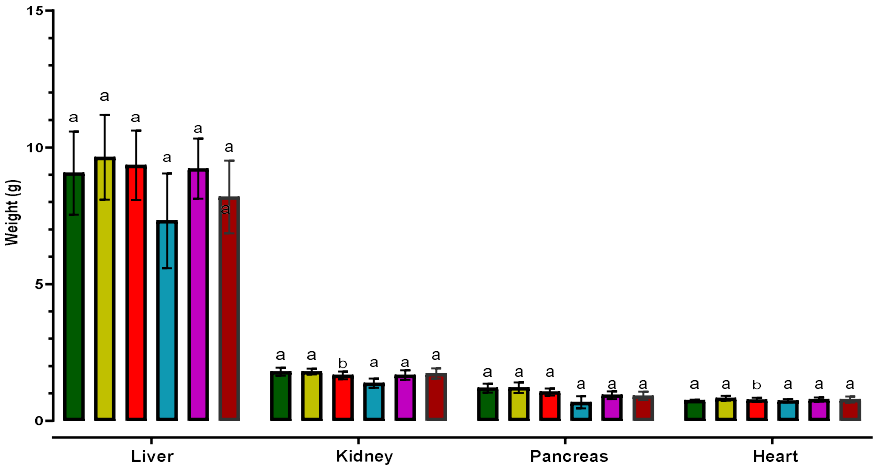
On the other hand, no significant increase in relative organ weight of liver and kidney was recorded in the obese diabetic group of rats and other groups did not show any significant change when compared among other groups.

Table 4.5 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on absolute organ weights (g) in HFD-fed obese diabetic rats.

S. No.	Group (s)	Absolute Organ Weights (g)			
		Liver	Kidneys	Heart	Pancreas
I.	Healthy Control	9.06 ± 0.62^{a}	1.80 ±0.06^{a}	0.80 ±0.04^{a}	1.00 ±0.07^{a}
II.	Obese	9.64 ± 0.63^{a}	1.80 ±0.04^{a}	0.86 ±0.03^{a}	1.21 ±0.10^{a}
III.	Obese Diabetic	7.31 ± 0.71^{b}	1.38 ±0.04^{b}	0.75 ±0.07^{b}	0.58 ±0.09^{b}
IV	Obese Diabetic + Metformin	9.34 ± 0.52^{a}	1.66 ±0.06^{a}	0.88 ±0.05^{a}	1.06 ±0.05^{ac}
V	Obese Diabetic + PM (EE)	9.22 ± 0.45^{a}	1.67 ±0.07^{a}	0.86 ±0.05^{a}	0.94 ±0.06^{abc}
VI.	Obese Diabetic + GS (ME)	8.81 ± 0.54^{a}	1.73 ±0.08^{a}	0.77 ±0.06^{a}	0.91 ±0.06^{bc}

Values (Mean ± SEM; n=6) bearing different superscripts (a, b and c) in the same column differ significantly ($p<0.05$) using one way ANOVA following Tukey's B multiple comparison Post Hoc test

Fig 4.5 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on absolute organ weights (g) of liver, kidneys, heart and pancreas in HFD-fed obese-diabetic rats.



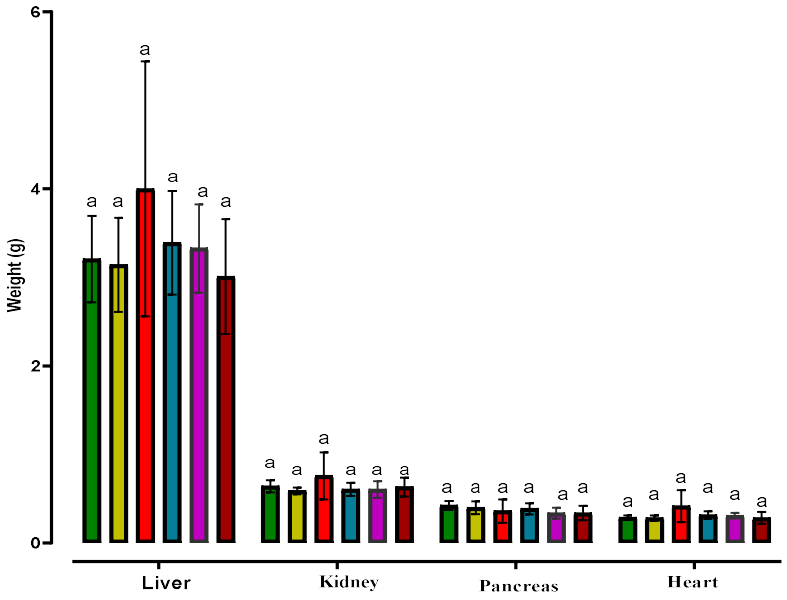
Value (mean ± SEM; n=6) bearing different superscripts in the histogram differ significantly ($p<0.05$)

Table 4.6 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium*, *Gymnema sylvestre* continuously for 60 days on relative organ weights (g) in HFD-fed obese diabetic rats.

S. No.	Group (s)	Relative organ weights (g/100 g body wt.)			
		Liver	Kidneys	Heart	Pancreas
I.	Healthy Control	3.21 ± 0.20^a	0.64 ±0.03^a	0.28 ±0.01^a	0.42 ±0.02^a
II.	Obese	3.14 ± 0.22^a	0.59 ±0.02^a	0.35 ±0.13^a	0.39 ±0.03^a
III.	Obese Diabetic	4.00 ± 0.59^a	0.76 ±0.11^a	0.41 ±0.73^a	0.36 ±0.05^a
IV	Obese Diabetic + Metformin	3.39 ± 0.24^a	0.60 ±0.03^a	0.32 ±0.18^a	0.38 ±0.03^a
V	Obese Diabetic + PM (EE)	3.33 ± 0.20^a	0.60 ±0.04^a	0.31 ±0.11^a	0.34 ±0.03^a
VI	Obese Diabetic + GS (ME)	3.01 ± 0.26^a	0.63 ±0.04^a	0.28 ±0.27^a	0.34 ±0.03^a

Values (Mean ± SEM; n=6) bearing different superscripts in the same column differ significantly ($p<0.05$) using one way ANOVA following Tukey's B multiple comparison Post Hoc test

Fig 4.6 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium*, *Gymnema sylvestre* continuously for 60 days on relative organ weights (g) of liver, kidneys, heart and pancreas on HFD-fed obese diabetic rats.



(Histograms bearing different superscripts differ significantly, p<0.05)

4.5 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium and Gymnema sylvestre* continuously for 6o days on fasting blood glucose (mg/dl) level of HFD-fed obese diabetic rats.

Data of fasting blood glucose level revealed a significant ($p<0.05$) increase in obese diabetics (309.3 ± 26.8) compared to healthy control (98.2 ± 2.5) on the 60th day of the experimental period after a single injection of STZ, whereas mild to moderate but non-significant increase in obese group of rats (107.3 ± 4.6). While groups treated with metformin, PM and GS the level of fasting blood glucose were recorded to be significant ($p < 0.05$) reduced by 108.7 ± 11.7, 116.5 ± 11.76, and 129.3 ± 17.8, respectively compared to obese diabetics and non-significantly higher than control group of rats. The level of glucose in the obese diabetic group remains constantly higher from day 1 to day 60 (table 4.7 and fig. 4.7).

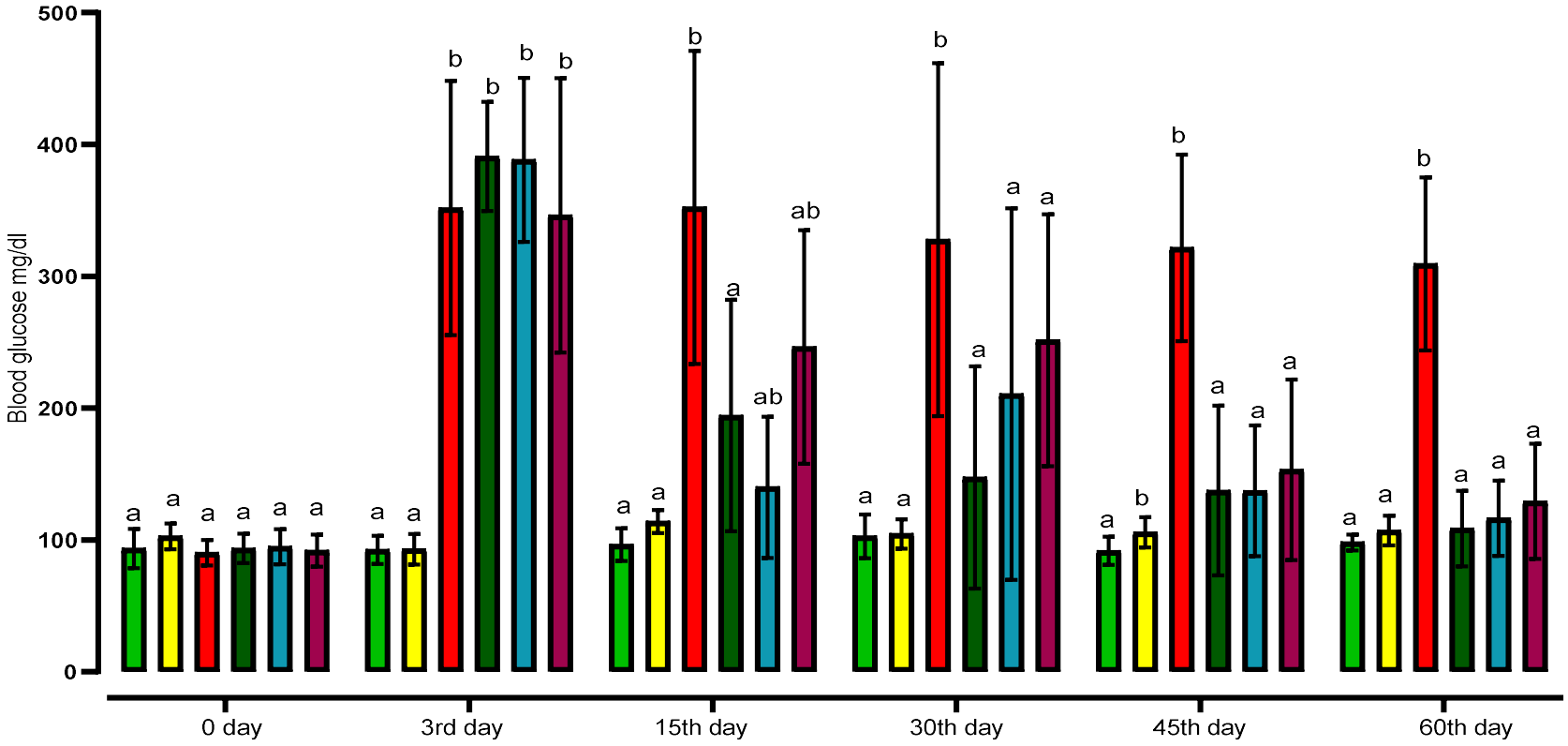
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Table 4.7 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on fasting blood glucose (mg/dl) in HFD-fed obese diabetic rats.

S. No	Group (s)	Fasting blood glucose (mg/dl)					
		1st day	3rd day	15th day	30th day	45th day	60thday
I.	**Healthy Control**	93.5±6.1[a]	92.5±4.3[a]	96.5±5.1[a]	102.8±6.8a	91.8±4.4[a]	98.2±2.5[a]
II.	**Obese**	102.8±4.0[a]	93.0±4.7[a]	114.0±3.6[a]	104.7±4.6	105.8±4.7[a]	107.3±4.6[a]
III.	**Obese Diabetic**	90.3±3.9[a]	351.8±39.3[b]	352.3±48.5[c]	327.8±54.7[c]	321.7±28.8[b]	309.3±26.8[b]
IV.	**Obese Diabetic + Metformin**	93.7±4.7[a]	391±16.9[b]	194.3±35.8[a]	157.5±34.4[a]	137.5±26.3[a]	108.7±11.7[a]
V.	**Obese Diabetic + PM (EE)**	94.8±5.5[a]	388.3±16.9[b]	190.0±21.9[a]	210.7±57.5[bc]	147.3±20.3[a]	116.5±11.6[a]
VI.	**Obese Diabetic + GS (ME)**	92.0±4.9[a]	346.2±42.5[b]	246.5±36.2[bc]	251.5±39.0[bc]	153.3±28.0[a]	129.3±17.8[a]

Values (Mean ± SEM; n=6) bearing different superscripts (a, b and c) in the same column differ significantly ($p<0.05$) using One way ANOVA following Tukey's B Multiple Comparison Post Hoc Test

Fig 4.7: Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on fasting blood glucose (mg/dl) level in HFD-fed obese diabetic rats.



(Histogram bearing different superscripts differ significantly, $p<0.05$)

4.6 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* on per cent HbA1c of HFD-fed obese diabetic rats

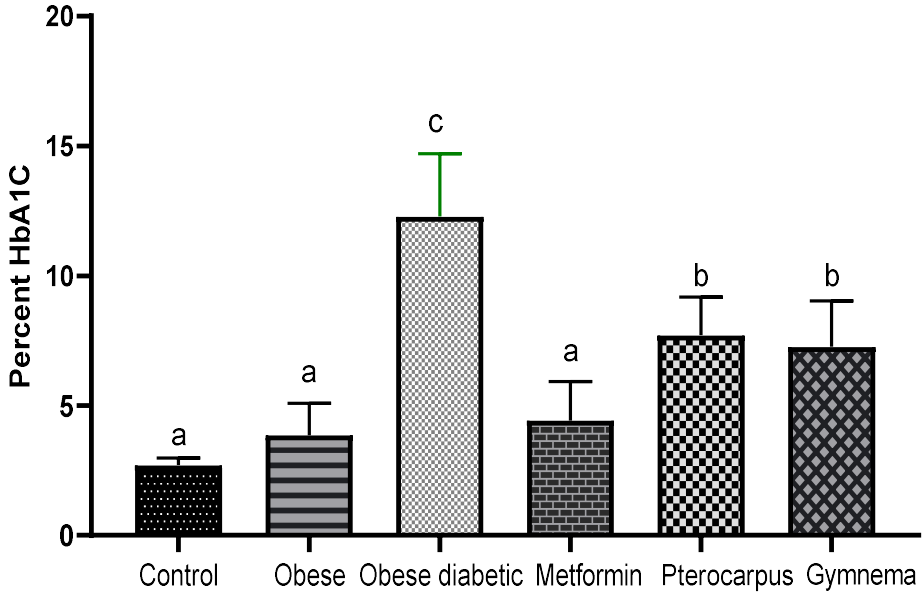
Values presented in Table 4.8 and illustrated in Fig. 4.8 show changes in glycated haemoglobin (HbA1c) recorded on the 60th day wherein a significant ($p<0.05$) increase in per cent HbA1c in obsess diabetic group (12.28 ± 0.99) as compared to the healthy control group (2.71 ± 0.11). whereas, in metformin-treated group (4.44 ± 0.61) has been found to be more effective in reducing % HbA1c by 4.44 ±0.61 and values are near to the value of the healthy control than PM (7.72 ± 0.59) and (7.27 ± 0 .72).

Table 4.8 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on HbA1c (HbA1c%) of HFD-fed obese diabetic rats.

S. No.	Group (s)	Per cent HbA1c
		60th day
I.	Healthy Control	2.71 ± 0.11^{a}
II.	Obese	3.86 ± 0.50^{a}
III.	Obese Diabetic	12.28 ± 0.99^{c}
IV.	Obese Diabetic + Metformin	4.44 ± 0.61^{a}
V.	Obese Diabetic+ PM (EE)	7.7 ± 0.59^{b}
VI.	Obese Diabetic + GS (ME)	7.27 ± 0.72^{b}

Values (Mean ± SEM; n=6) bearing different superscripts (a, b and c) in the same column differ significantly ($p<0.05$) using one way ANOVA following Tukey's B Multiple Comparison Post Hoc Test

Fig 4.8 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* and metformin continuously for 60 days on per cent HbA1c of HFD-fed obese diabetic rats.



(Histograms bearing different superscripts differ significantly, $p<0.05$)

4.7 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* on total leucocyte count (TLC), erythrocytes (RBCs), haemoglobin (Hb) and platelets count of HFD-fed obese diabetic rats.

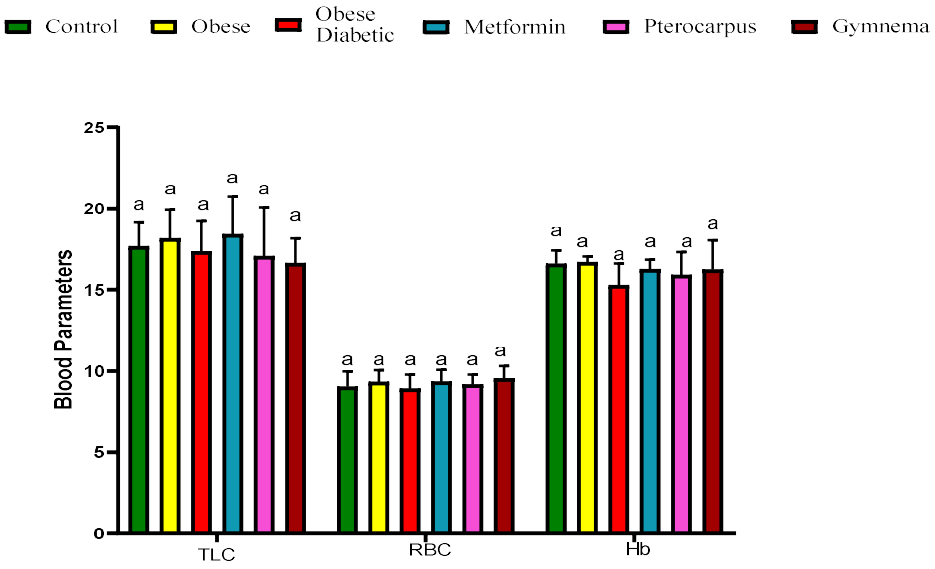
No significant change in TLC, RBC and haemoglobin has been observed when compared among different groups. At the end of the experimental period of 60 days, mild to moderate reduction in platelets count was recorded in obsess diabetic group of rats, though it was non-significant when compared with other groups (Table- 4.9 & Fig. 4.9).

Table 4.9 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on total leucocyte count (TLC), erythrocytes (RBCs), per cent haemoglobin (Hb%) and platelets count of HFD-fed obese diabetic rats.

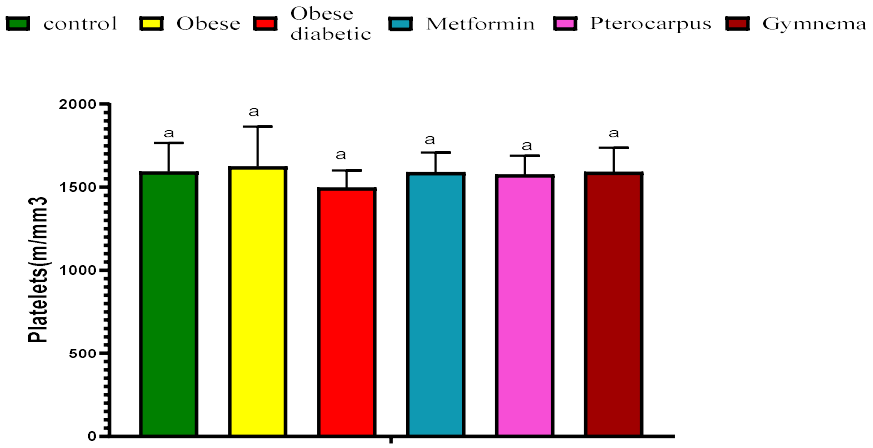
S. No.	Groups	TLC ($m/mm^{3)}$)	RBC ($m/mm^{3)}$)	Hb (g/dl)	Platelets ($m/mm^{3)}$)
I.	Healthy Control	17.7 ±0.6[a]	9.0 ±0.4[a]	16.6 ±0.3[a]	1592.5 ±71[a]
II.	Obese	16.2 ±0.7[a]	9.3 ±0.3[a]	17.2 ±0.1[a]	1623.2 ±98[a]
III.	Obese Diabetic	17.4 ±0.8[a]	8.9 ±0.4[a]	15.3 ±0.6[a]	1495.5 ±42[a]
IV.	Obese Diabetic + Metformin	18.4 ±0.9[a]	9.3 ±0.3[a]	16.3 ±0.2[a]	1589.2 ±48[a]
V.	Obese Diabetic + PM (EE)	17.1 ±1.2[a]	9.1 ±0.2[a]	15.9 ±0.6[a]	1574.2 ±46[a]
VI.	Obese Diabetic + GS (ME)	16.6 ±0.6[a]	9.5 ±0.3[a]	16.2 ±0.7[a]	1589.8 ±60[a]

Values (Mean ± SEM; n=6) bearing different superscripts in the same column differ significantly ($p<0.05$) using one way ANOVA following Tukey's B Multiple Comparison Post Hoc Test

Fig 4.9 (A & B) Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* on total leucocyte count (TLC), erythrocytes (RBCs), haemoglobin (Hb) and platelets count of HFD-fed obese diabetic rats. (Histograms bearing different superscripts differ significantly, $p<0.05$)



(A)



Platelets (m/mm3)

(B)

4.8 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium*, and *Gymnema sylvesre* on lipid profile [triglyceride (TRI), total cholesterol (TC), high-density lipoproteins (HDL) and low-density lipoproteins (LDL)] of HFD-fed obese diabetic rats.

Distributions in lipid profile are a common phenomenon in hyperglycemic/diabetic patients, the results obtained are summarized in Table 4.10 and illustrated in Fig. 4.8 (A-D). Serum triglyceride, total cholesterol and low-density lipoprotein levels have been found to be significantly ($P<0.05$) higher in the obese diabetic group, whereas moderate reduction in high-density lipoproteins levels in the obese diabetic group of animals compared to healthy control and an obese group of rats. To control dyslipidemia, the treatment group that is metformin, PM and GS have been observed to be almost equally effective for all four parameters recorded, however, PM treatment group has been observed to be more effective in maintaining serum triglyceride levels.

Table 4.10 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on lipid profile Triglyceride (TRI), Total Cholesterol, High-Density Lipoproteins (HDL) and Low-Density Lipoproteins (LDL) of HFD-fed obese diabetic rats.

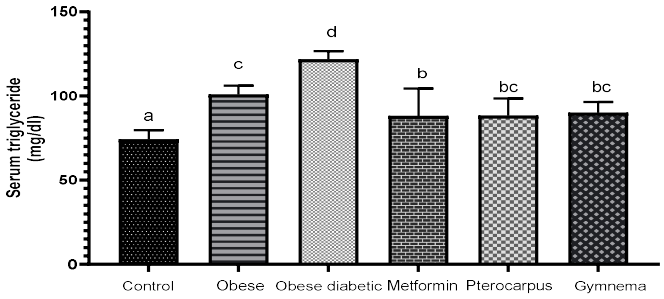
S. No.	Group(s)	TRI (mg/dl)	Total cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
I.	Healthy Control	80.7 ±2.27^{a}	124.9 ±4.08^{a}	33.3 ±0.76^{a}	37.1 ±2.0^{a}
II.	Obese	96.9 ±2.21^{c}	179.1 ±5.43^{c}	21.2 ±1.22^{a}	44.0 ±2.0^{ab}
III.	Obese Diabetic	115.7 ±2.00^{d}	210.8 ± 3.98^{d}	18.2 ±1.46^{a}	75.5 ±1.7^{c}
IV.	Obese Diabetic + Metformin	91.5 ±4.50^{b}	160.4 ±3.38^{b}	28.2 ±1.62^{a}	47.5 ±2.9^{b}
V.	Obese Diabetic + PM (EE)	78.9 ±4.10^{bc}	163.4 ±5.53^{bc}	29.2 ±1.38^{a}	48.2 ±1.3^{b}
VI.	Obese Diabetic + GS (ME)	93.3 ±2.67^{bc}	163.7 ±3.72^{bc}	29.0 ±0.92^{a}	48.4 ±1.6^{b}

Values (Mean ± SEM; n=6) bearing different superscripts (a, b and c) in the same column differ significantly ($p<0.05$) using one way ANOVA following Tukey's B multiple comparison Post Hoc test

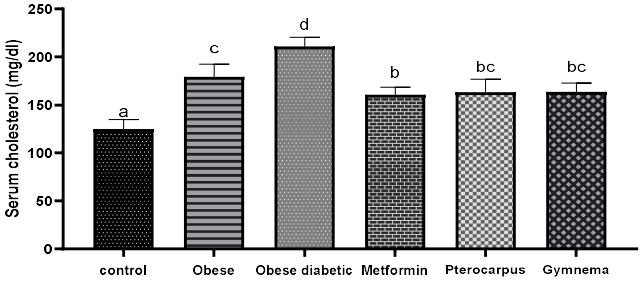
Fig 4.10 (A-D) effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on triglycerides (TRI), total cholesterol (TC), high-density lipoproteins (HDL) and low-density lipoproteins (LDL) of HFD-fed obese diabetic rats.

(Histograms bearing different superscripts differ significantly, $p<0.05$)

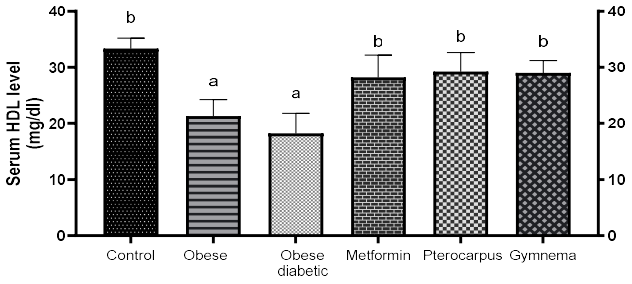
(A)



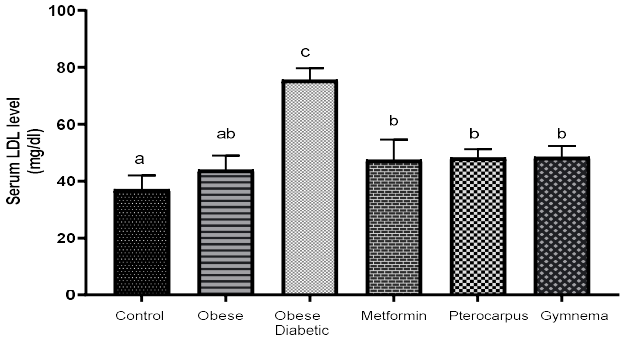
(B)



(C)



(D)



4.9 Effect oral administration of metformin, extracts of *Pterocarpus marsupium*, and *Gymnema sylvestre* and metformin on oxidative stress indices in kidney tissue of HFD-fed obese diabetic rats.

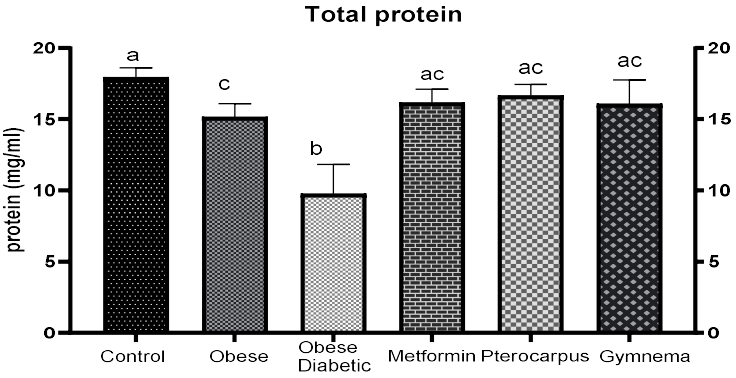
Table 4.12 and Fig. 4.12 (A, B, C, D, E) revealed the extent of lipid peroxidation in terms of malonaldehyde (MDA) levels and antioxidant biomarkers i.e. reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) in kidney tissue of rats of different groups are summarised in Table 4.12 and illustrated in Fig 4.12 (A-E). A perusal of data revealed significantly ($p<0.05$) higher content of lipid peroxidation and a decrease in the levels of GSH, protein and activities of SOD, CAT in obese and obese diabetic groups of rats when compared to healthy control group. Treatment with PM, GS and metformin has been observed to have antioxidation potential and mild to moderate and significantly ($p<0.05$) decreased the levels of LPO and improved protein level, GSH content and activities of antioxidant enzymes effectively and made to reverse oxidative stress produced in kidney tissues due to glucotoxicity.

Table 4.11 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on Kidney tissue protein, lipid peroxidation (LPO), reduced glutathione (GSH) levels, superoxide dismutase (SOD) and catalase (CAT) activity of HFD-fed obese diabetic rats.

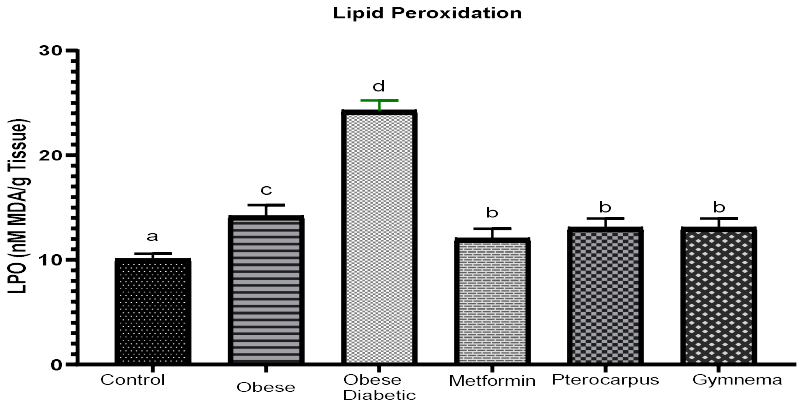
S. No.	Groups	LPO (nM MDA /g of tissue)	GSH (mM/g of tissue)	SOD (U /mg protein)	Catalase (mM H_2O_2 utilized min/ mg protein)	Protein (mg/ml)
I.	Healthy Control	10.0 ± 0.20^{a}	$4.0\pm.08^{a}$	17.4 ± 0.45^{a}	156.3 ± 0.52^{a}	17.9 ± 0.27^{a}
II.	Obese	15.8 ± 1.01^{c}	$2.9\pm.09^{c}$	13.5 ± 0.37^{c}	151.8 ± 1.19^{c}	15.1 ± 0.38^{c}
III.	Obese Diabetic	24.2 ± 0.41^{d}	$1.6\pm.06^{b}$	8.3 ± 0.29^{b}	132.2 ± 1.24^{b}	9.7 ± 0.84^{b}
IV.	Obese Diabetic+ Metformin	13.0 ± 0.37^{b}	$3.3\pm.05^{d}$	15.1 ± 1.46^{ac}	152.5 ± 0.86^{c}	16.2 ± 0.39^{ac}
V.	Obese Diabetic+ PM (EE)	12.0 ± 0.40^{b}	$3.6\pm.058^{e}$	16.0 ± 0.62^{ac}	155.2 ± 0.94^{ac}	17.0 ± 0.24^{ac}
VI.	Obese Diabetic+ GS (ME)	12.5 ± 0.31^{b}	$3.5\pm.07^{ed}$	16.4 ± 0.41^{ac}	154.0 ± 0.65^{ac}	16.5 ± 0.31^{ac}

Values (Mean ± SEM; n=6) bearing different superscripts (a, b and c) in the same column differ significantly ($p<0.05$) using one way ANOVA following Tukey's B Multiple Comparison Post Hoc Test

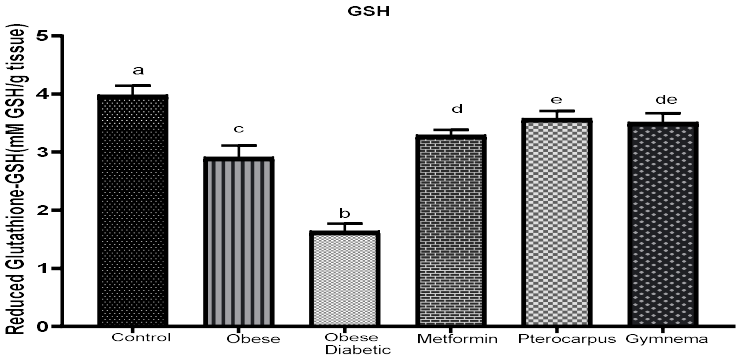
Fig 4.11 (A-E) Effect of oral administration of metformin, extracts of Pterocarpus marsupium and *Gymnema sylvestre* continuously for 60 days on kidney tissue protein, lipid peroxidation (LPO), reduced glutathione (GSH) levels, superoxide dismutase (SOD) and catalase (CAT) activity of HFD-fed obese diabetic rat. (Histograms bearing different superscripts differ significantly, $p<0.05$)



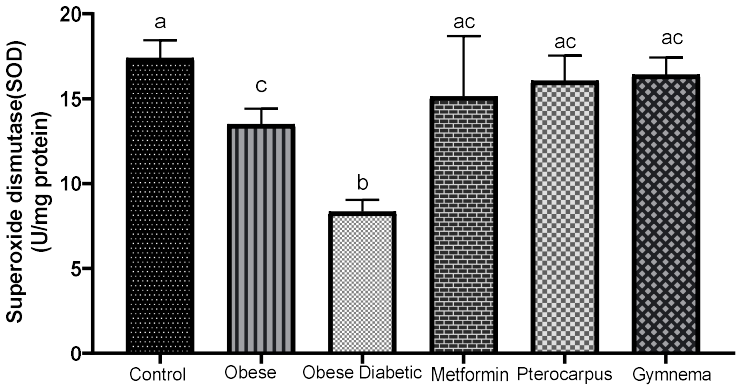
A)



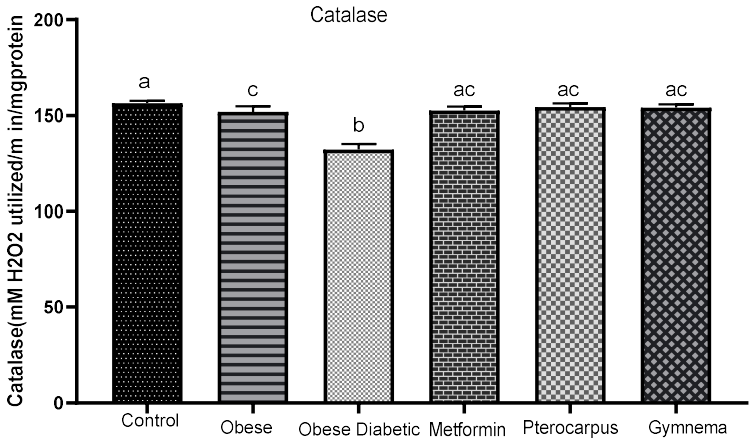
(B)



(C)



(D)



(E)

4.10 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on serum cystatin-C levels of HFD-fed obese diabetic rats.

Cystatin-c is a novel biomarker of nephrotoxicity and has been reported to be at all higher levels during renal dysfunction during diabetic nephropathy. The results obtained for cystatin-C of the present study are summarized in Table 4.11 and depicted in Fig. 4.9, the result revealed a significant ($p< 0.05$) increase in cystatin- C levels in obsess diabetic groups of rats (35.6±1.7) when compared to control (4.5±0.6), however, obese group of rats also showed a rise in cystatin-C level by (13.9±1.2) and was significant. The treatment group (metformin, PM, GS) was observed to reduce the

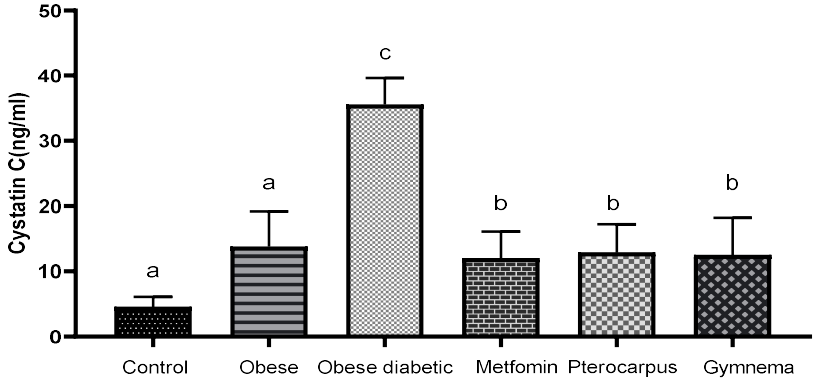
levels of cystatin -C appreciably but were statistically non-significant as compared to obese diabetics.

Table 4.12 Effect of oral administration of ethanolic extract of *Pterocarpus marsupium*, methanolic extract of *Gymnema sylvestre* and metformin continuously for 60 days on serum cystatin C levels of high-fat diet-fed obese-streptozotocin-induced type 2 diabetic rats.

S. No.	Group (s)	Cystatin - C(ng/ml)
		60th day
I.	Healthy Control	4.5 ± 0.6^{a}
II.	Obese	13.9 ± 1.2^{b}
III.	Obese Diabetic	35.6 ± 1.7^{c}
IV.	Obese Diabetic + Metformin	12.0 ± 1.7^{b}
V.	Obese Diabetic + PM (EE)	12.9 ± 1.8^{b}
VI.	Obese Diabetic + GS (ME)	12.6 ± 2.0^{b}

Values (Mean ± SEM; n=6) bearing different superscripts (a, b and c) in the same column differ significantly ($p<0.05$) using one way ANOVA following Tukey's B Multiple Comparison Post Hoc Test

Fig 4.12 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on serum cystatin C of HFD-fed obese-diabetic rats.



(Histograms bearing different superscripts differ significantly, $p<0.05$)

4.11 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on gross morphology of kidney and Pancreas in HFD-fed obese diabetic rats.

Following the necropsy, no significant gross changes were observed in the kidneys and pancreas. Fig. 4.13 (A -F) and Fig. 4.13 (A- F) depicted kidney & pancreas gross morphology.



Image 4.13 A: Control



Image 4.13 B: Obese



Image 4.13 C: Obese Diabetic

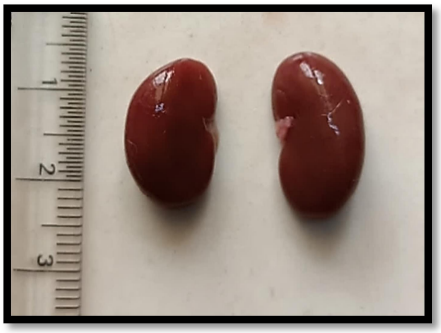


Image 4.13 D: Metformin



Image 4.13 E*: Pterocarpus marsupium*



Image 4.13 F*: Gymnema sylvestre*

Fig. 4.13 (A-F) Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on gross morphology of kidney of HFD-fed obese-diabetic rats



Image 4.14 A: Control

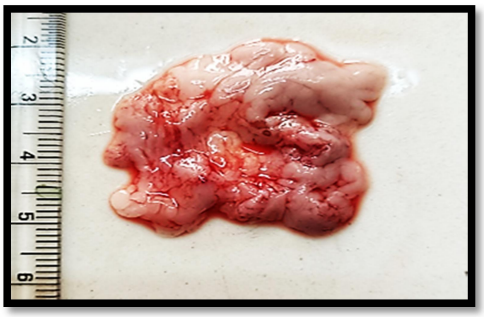


Image 4.14 B: Obese

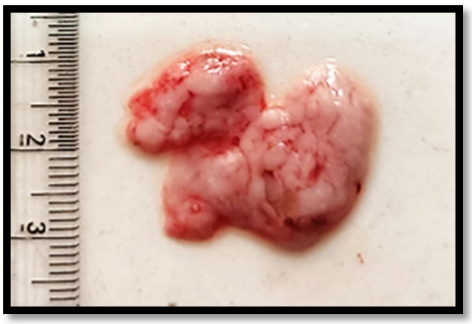


Image 4.14 C: Obese Diabetic



Image 4.14 D: Metformin



Image 4.14 E: *Pterocarpus marsupium*

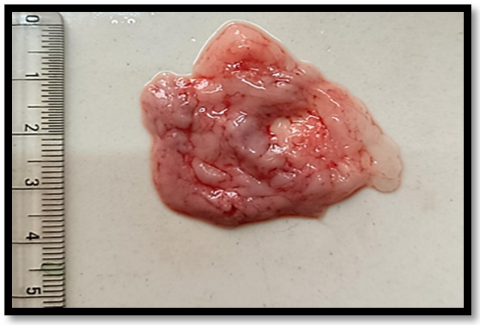


Image 4.14 F: *Gymnema sylvestre*

Fig. 4.14 (A-F) Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on gross morphology of Pancreas of HFD-fed obese-diabetic rats

4.12 Effect of oral administration of metformin, extracts of *Pterocarpus* and *Gymnema sylvestre* on histopathological changes of kidney on HFD-fed obese-diabetic rats.

Histopathological changes in the kidneys of different groups are illustrated in Figure 4.15- 4.16. Fig 4.17 (A-E) depicted the histopathological changes observed in the H & E staining of the kidneys of rats of different experimental groups.

The kidneys of the healthy control group (Fig. 4.15 A) showed normal glomerular and tubular histoarchitecture. Moderate Congestion was seen in the obese group (Fig. 4.15 B), whereas degeneration and necrosis of glomeruli and congestion of blood vessels were seen in the obese diabetic group (Fig. 4.15 C). In the treatment group, GS (Fig. 4.15 E) showed mild congestion, while metformin (Fig. 4.15 D) and PM (Fig. 4.15 E) recovered the normal histoarchitecture of glomeruli and tubules.

Histopathological changes using a special staining procedure (Masson's Trichrome staining) observed in the kidneys of rats of different treatment groups are depicted in Fig 4.16 (A-E) which revealed normal renal histoarchitecture in the healthy control and obese group rats, Fig 4.16 (A) except certain fatty changes in the obese group Fig. 4.16 (B).

Stained sections of the obese diabetic group Fig. 4.16 (C) showed deposition of excess collagen fibres causing necrosis and replacing tubules and glomeruli. It also showed severe deposition of collagen fibres around the blood vessels thus, causing constriction of blood vessels. Among treatment groups, the metformin (Fig. 4.16 D), PM (Fig.4.16 E) and GS (Fig 4.16 F) treated group presented mild changes in kidney parenchyma tissue. GS-treated group (Fig 4.16 F) showed mild deposition of collagen and degeneration of tubules whereas the Pterocarpus *marsupium-treated* group (Fig 4.16 E) revealed mild deposition of collagen fibres around glomeruli along with mild swelling of renal tubules but nearly normal architecture as compared to the control group, the metformin-treated group also showed deposition of collagen fibre in glomeruli and mild changes in both glomeruli and tubules.

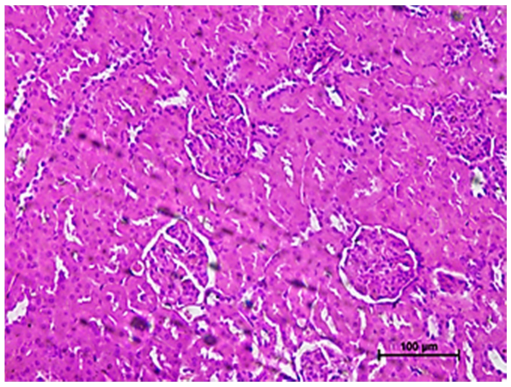


Fig 4.15 A Healthy Control: Kidney tissue section showing normal histoarchitecture of glomeruli and tubules. H&Ex200x

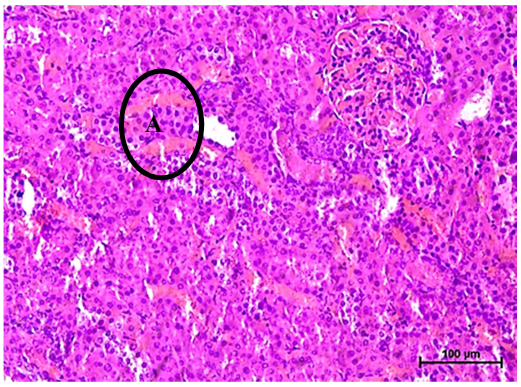


Fig 4.15 B Obese: Kidney tissue section showing **A.** Moderate Congestion. H&Ex200x

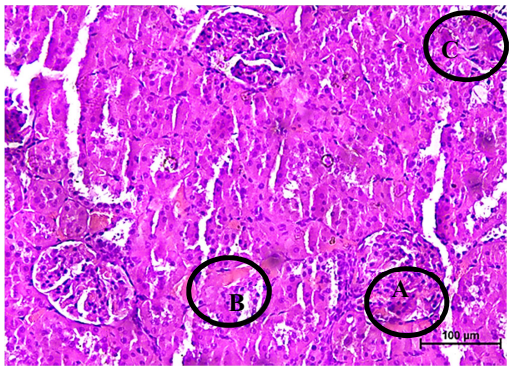


Fig 4.15 C Obese Diabetic: Kidney tissue section showing **A.** glomeruli with haemorrhages, **B.** Congestion of blood vessels and **C.** glomerular degeneration H&Ex200x

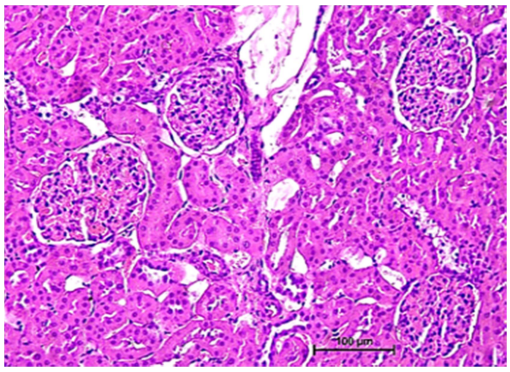


Fig 4.15 D Obese Diabetic + Meformint: Kidney tissue section showing normal histoarchitecture of glomeruli and tubules. H&Ex200x

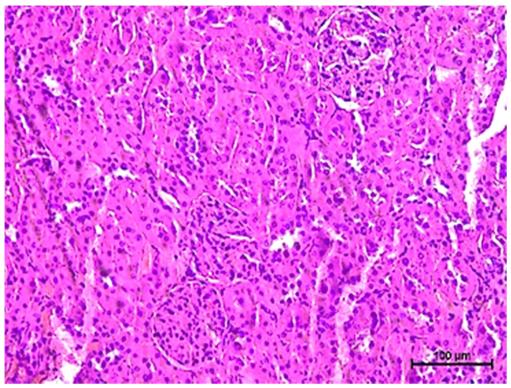


Fig 4.15 E Obese Diabetic + PM: Kidney tissue section showing normal glomeruli with mild congestion in tubule vascular changes H&Ex200x

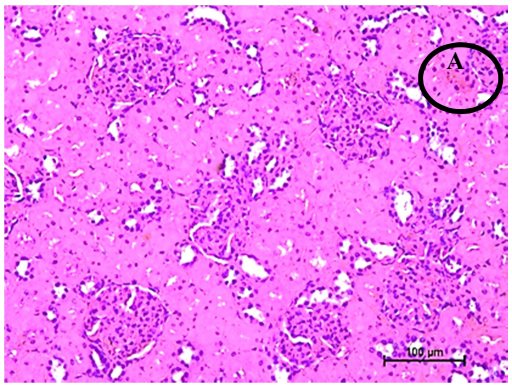


Fig 4.15 F Obese Diabetic + GS: Kidney tissue section showing **A.** Mild Changes in glomeruli and congestion. H&Ex200x

Fig. 4.15 (A-F) shows the effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* on hematoxylin and eosin staining (H&E) of kidney of HFD-fed obese-diabetic rats

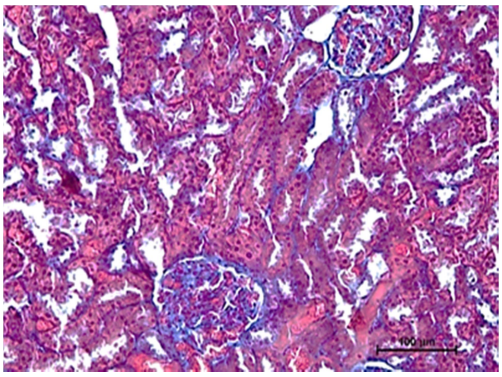


Fig 4.16 A control: Kidney tissue section showing less blue colour deposition showing normal histoarchitecture MTx200x

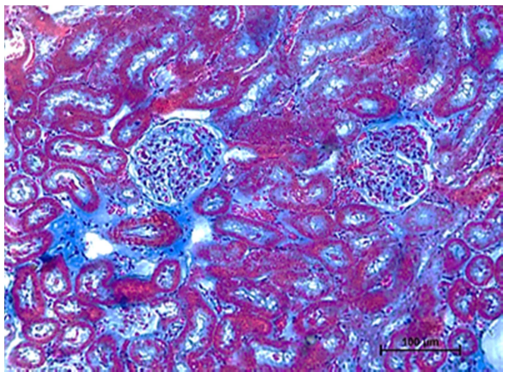


Fig 4.16 B Obese: Kidney tissue section showing more-blue colour diposition MTx200x

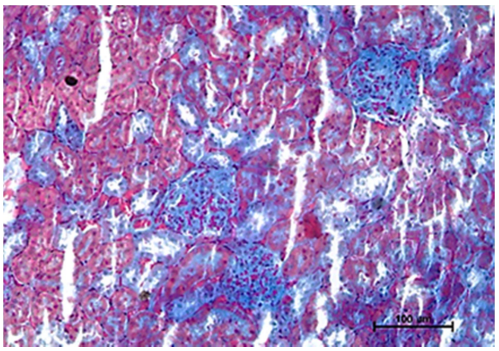


Fig 4.16 C Obese Diabetic: Kidney tissue section showing deposition of more blue colour collagen fibre in glomeruli causing necrosis of glomerular tissue and tubule MTx200x

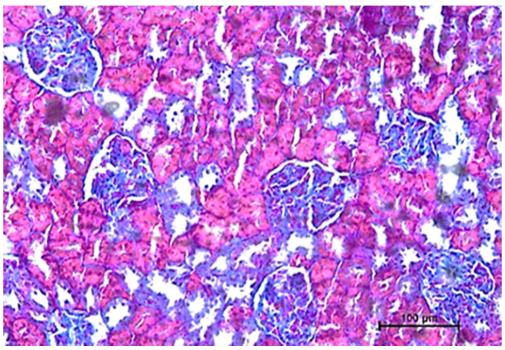


Fig 4.16 D Obese Diabetic + Metformin: Kidney tissue section showing normal histoarchitecture of glomeruli and tubules MTx200x

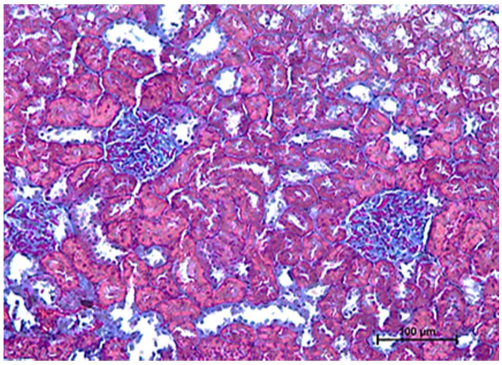


Fig 4.16 E Obese Diabetic + PM: Kidney tissue section showing mild deposition of blue colour collagen fibre in glomeruli MTx200x

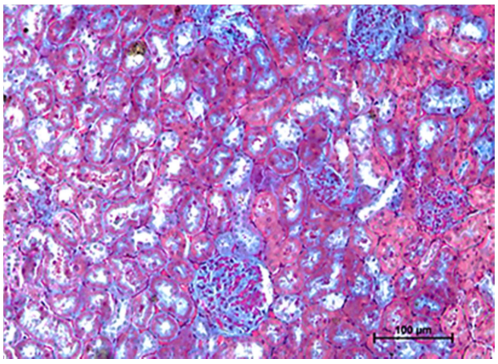


Fig 4.16 F Obese Diabetic + GS: Kidney tissue section showing A. Mild changes in tubule and in glomeruli and congestion. MTx200x

Fig. 4.16 (A-F) effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on massion' s trichome staining of kidney of HFD-fed obese diabetic rats

4.13 Effect of oral administration of metformin, extracts of *Pterocarpus* and *Gymnema sylvestre* on histopathological changes of the pancreas on high-fat diet-fed obese-diabetic rats

The H & E staining of pancreatic tissue of the healthy control group (Fig.4.17 A) showed the normal structure of pancreatic islets and compact cells. Obese group rats also (Fig. 4.17 B) show normal structures of pancreatic islets but slightly less compact cells. The obese diabetic group showed shrunken, irregular outlines and distorted islets (Fig. 4.17 C). In treatment groups (metformin, PM and GS) (Fig 4.17 D, Fig. 4.17 E, 4.17F), pancreatic islets were restored with increased size of ilets and regular borders.

The chrome alum hematoxylin-phloxine staining of the pancreas of the healthy control (Fig 4.18A) and obese group (Fig 4.18B) showed healthy islets with alpha and beta cells, in the obese diabetic group (Fig 4.18C), shrunken islet with degeneration of beta cells was observed. Metformin (Fig 4.18 D), PM (Fig 4.18 E) and *Gymnema* (Fig 4.18 F) treated groups showed compact and regular margins with improved beta cell mass compared to the obese diabetic group.

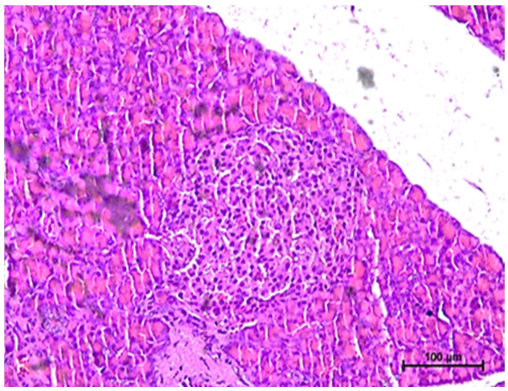


Fig 4.17 A. Healthy Control: Pancreas showing healthy granular darkly stained nuclei and compact cells. H&E 200x

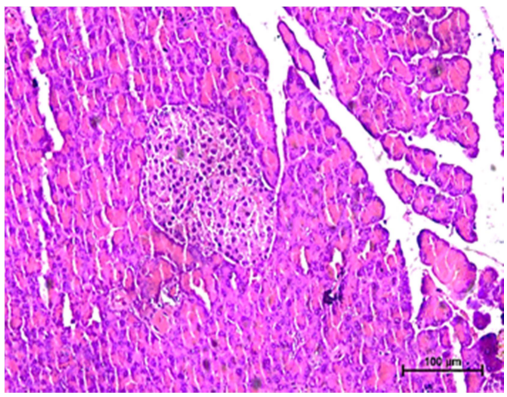


Fig 4.17 B Obese: Pancreas showing islets having islet cells with granular cytoplasm and dark nuclei. H&E 200x

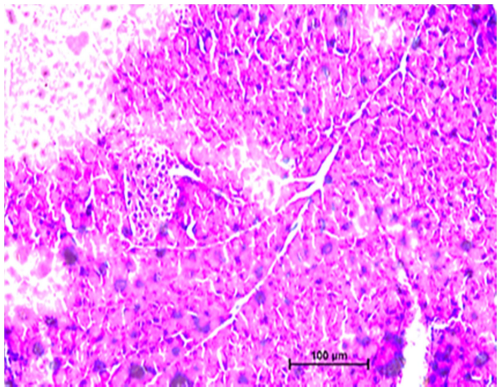


Fig 4.17 C Obese Diabetic: Pancreas showing A. Shrunken, irregular outline and distorted islet of Langerhans. H&E 200x

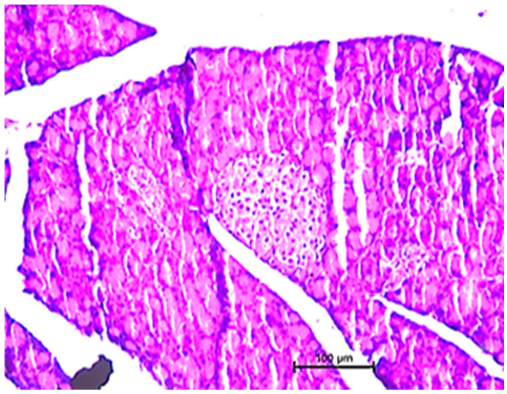


Fig 4.17 D Obese Diabetic + Metformin: Pancreas showing almost near to normal structure of islets of the pancreas. H&E 200x

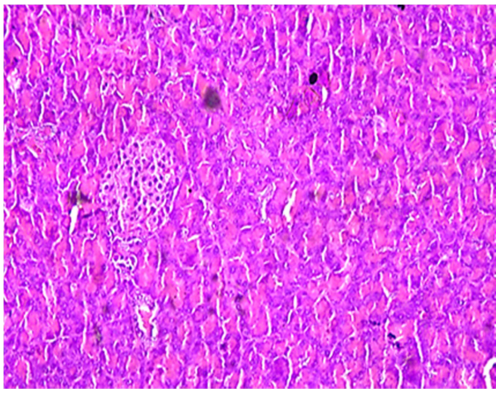


Fig 4.17 E Obese Diabetic + PM: Pancreas showing an increased number of islet cells, and darker nuclei compared to the diabetic group. H&E 200x

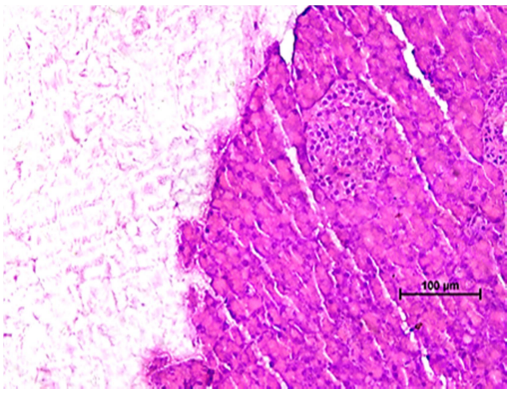


Fig 4.17 F Obese Diabetic + GS: Pancreas showing increased number of islet cells, darker nuclei compared to diabetic group. H&E 200x

Fig. 4.17 (A-F) Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on hematoxylin and eosin staining (H&E) of Pancreas of HFD-fed obese-diabetic rats.

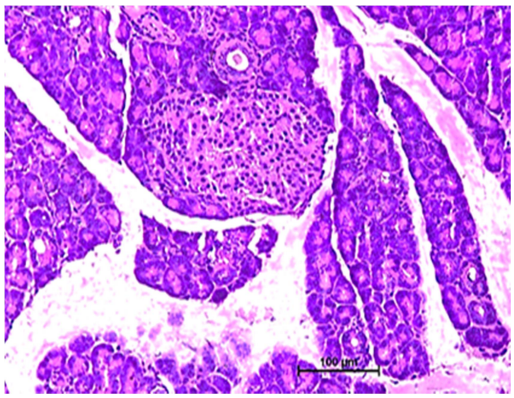


Fig 4.18 A Healthy Control: Pancreas showing healthy islet with A. Alpha cells B. beta cells. 200x.

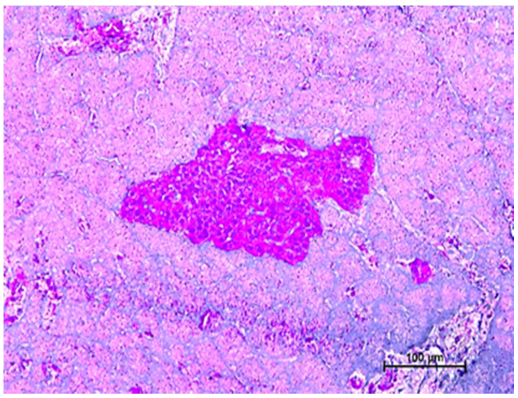


Fig 4.18 B Obese: Pancreas showing islets with beta cells.200x.

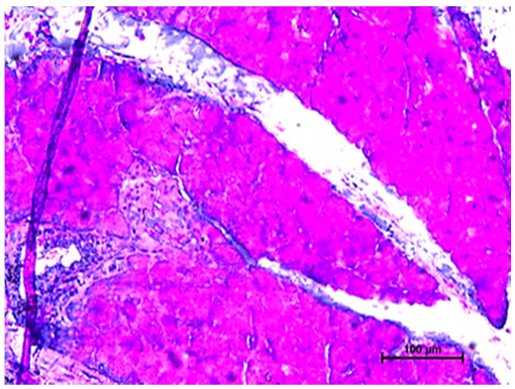


Fig 4.18 C Obese Diabetic: Pancreas tissue showing A. shrunken Islet with degeneration of beta cells.200x

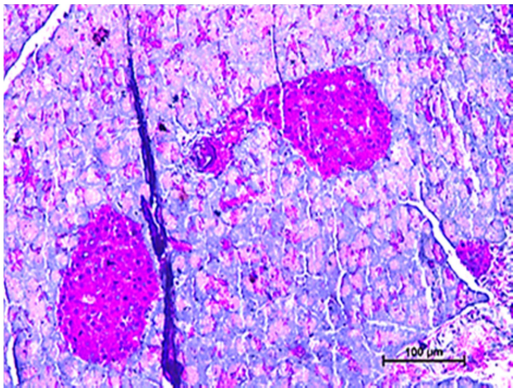


Fig 4.18 D Obese Diabetic + Met: Pancreas showing A. Compact and regular margins B. Improved beta cell mass compared to obese diabetic group.200x

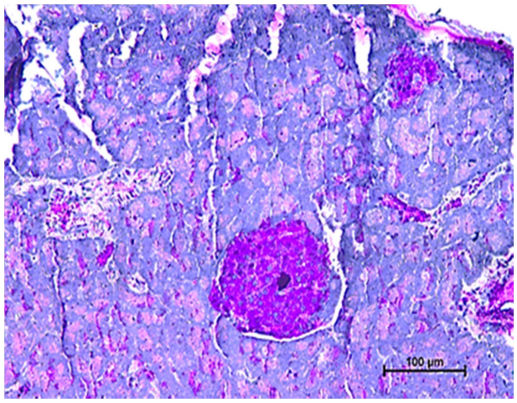


Fig 4.18 E Obese Diabetic + PM: Pancreas showing A. Compact and regular margins B. Increased beta cells compared to obese diabetic group.20x.

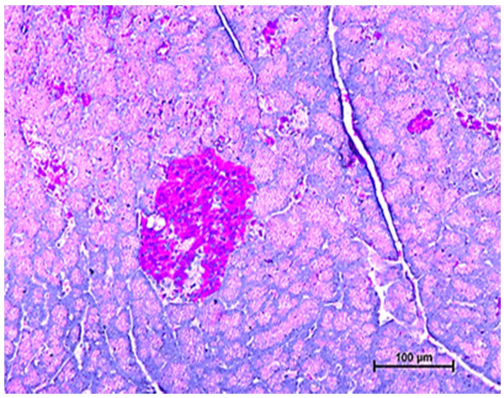


Fig 4.18 F Obese Diabetic + PM: Pancreas showing A. Compact and regular margins B. Increased beta cells compared to obese diabetic group.200x.

Fig. 4.18 (A-F) Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on chrome alum hematoxyline-phloxine staining of Pancreas of HFD-fed obese diabetic rats

4.14 Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on Iummunohistochemistry of Pancreatic beta cells on HFD-fed obese-diabetic rats

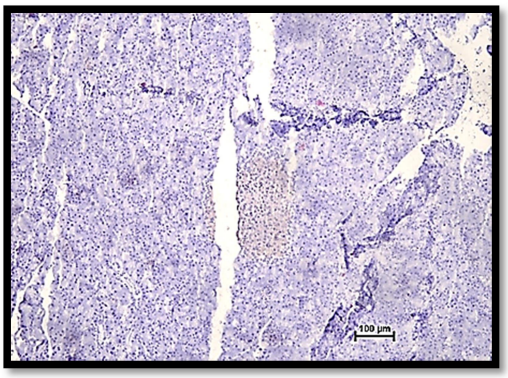


Fig. 4.19 A Negative Control: Pancreas showing no immunolabeling against beta cells. 100x

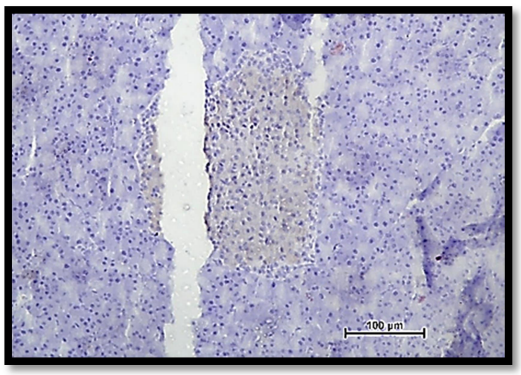


Fig. A (200x)

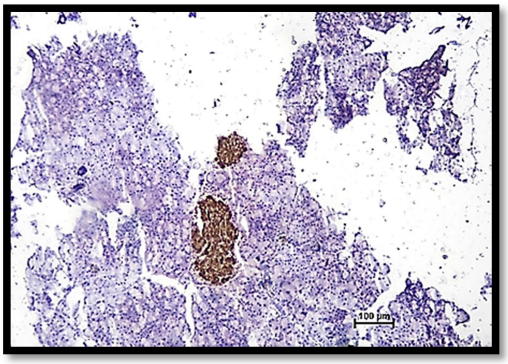


Fig. 4.19 B Healthy Control: Pancreas showing strong immunoreactivity of insulin in beta cells. 100x

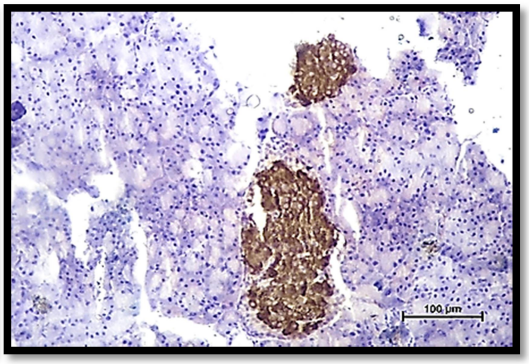


Fig. B (200x)

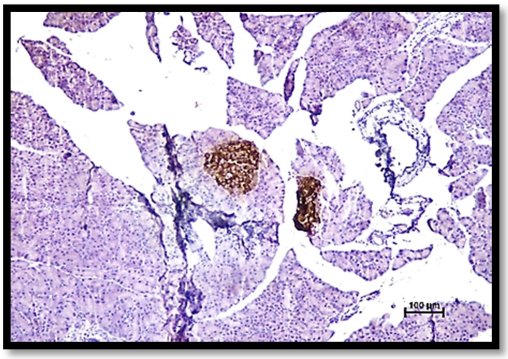


Fig. 4.19 C Obese : Pancreas showing strong immunoreactivity of insulin in beta cells. 100x

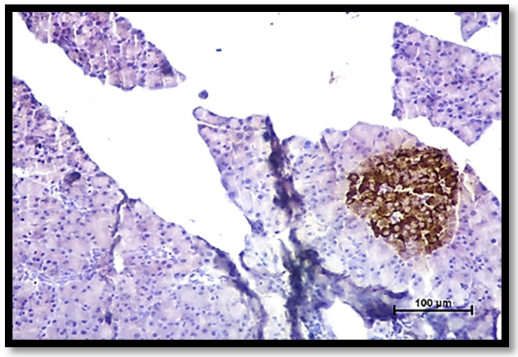


Fig. C (200x)

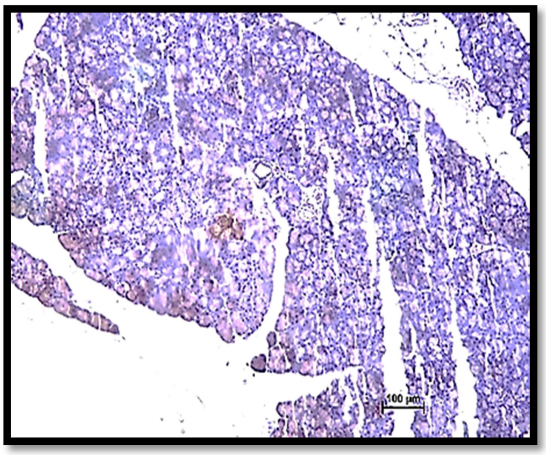


Fig. 4.19 D Obese Diabetic Marked reduction in immunochemical reaction of insulin in beta cells. 100x

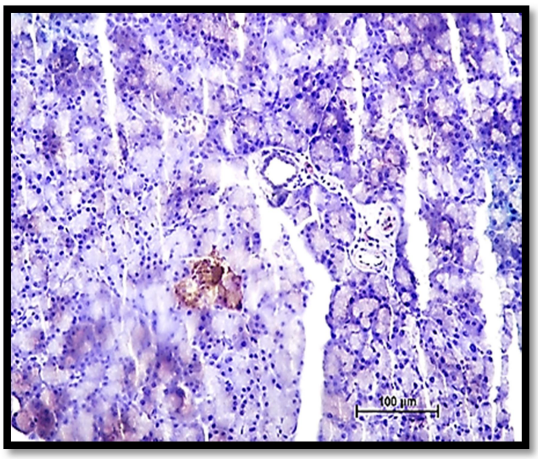


Fig. D (200x)

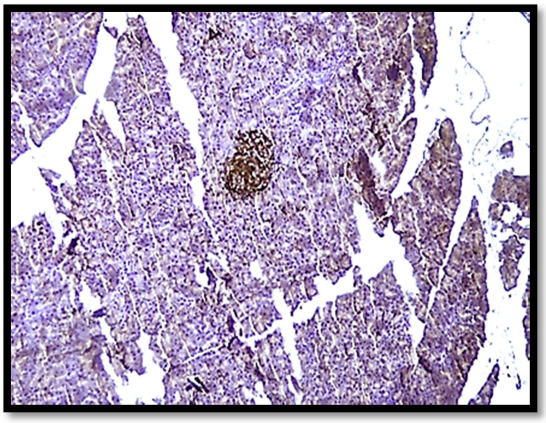


Fig. 4.19 E Pancreas showing strong immunoreactivity and increase in no. of beta cells. 100x

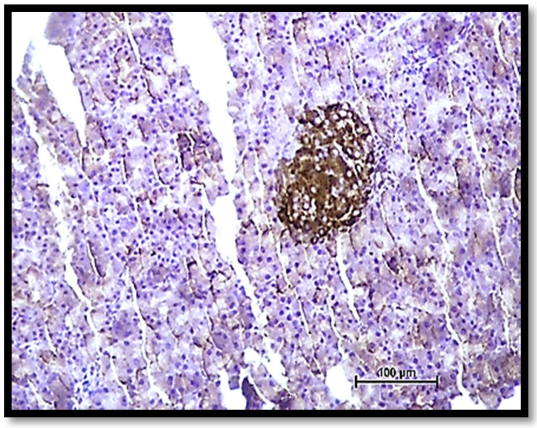


Fig. E (200x)

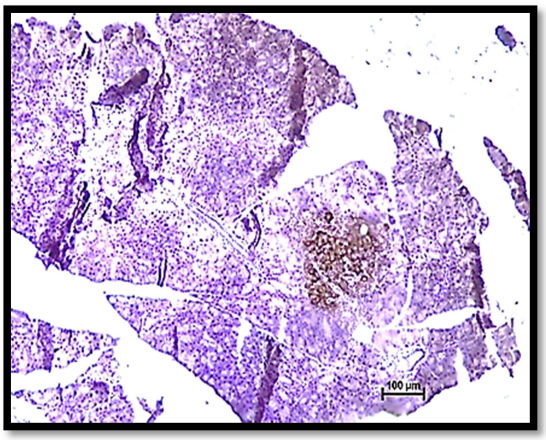


Fig. 4.19 F Pancreas showing increase no. and area of beta cells having immunoreactivity. 100x

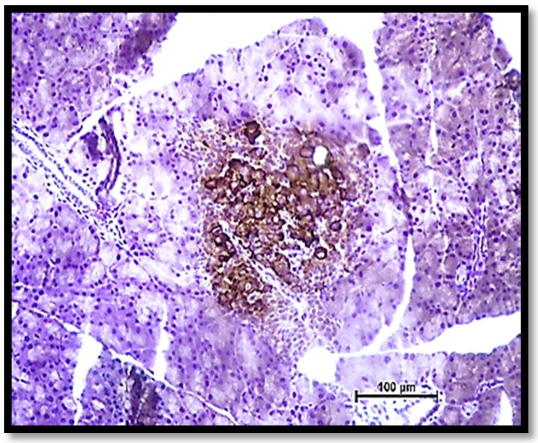


Fig. F (200x)

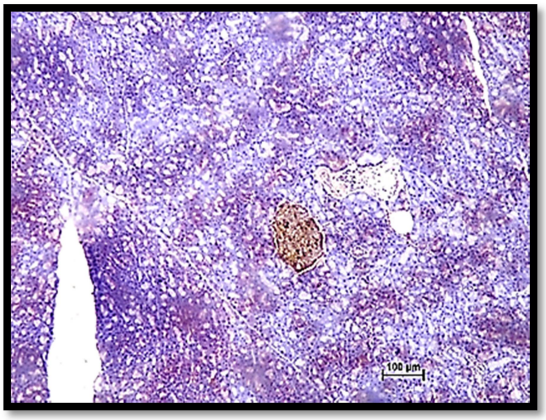


Fig. 4.19 G Pancreas showing comparativly increase no. of beta cells and area of immunoreativity than obese diabetic. 100x

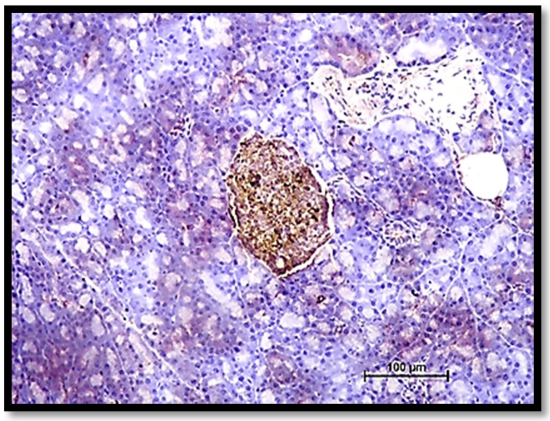


Fig. G (200x)

4.15 Effect of oral administration of metformin and the extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on pancreatic interleukin -6 (IL-6), JAK-2, and IFN levels in pancreatic tissue of HFD -fed obese diabetic rats.

Persual of the data presented in Table (4.13) and Fig (4.20 A, B, C) observed in pancreatic tissue revealed a significant ($p<0.05$) increase in the levels of IL-6, JAK-2, and IFN gamma in an obese and obese diabetic group of rats compared to a healthy control group of rats, whereas the groups treated with metformin , PM and GS showed marked and significant decrease of the above parameters compared to obese and obese diabetic rats however observations are comparable to healthy control groups of rats.

Effect of oral administration of metformin, and the entrance of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on sodium-glucose transporter -2 levels in kidney tissue of HFD -fed-obese – transporter-2 levels in kidney tissue of HFD-fed obese- diabetic rats.

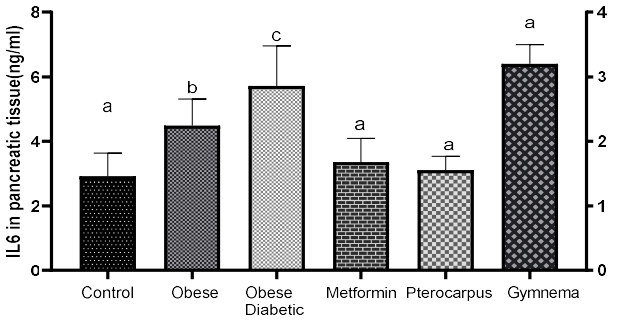
Table 4.13: Effect of oral administration of metformin and extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on pancreatic IL6, JAK-2, IFN gamma levels of HFD-fed obese-diabetic rats

S. No.	Group (s)	IL6 (ng/ml)	JAK-2 (ng/ml)	IFN gamma (ng/ml)
I.	Healthy Control	2.9 ± 0.29^{a}	1.4 ± 0.11^{a}	45.2 ± 2.0^{a}
II.	Obese	4.5 ± 0.33^{b}	1.8 ± 0.05^{ab}	56.6 ± 3.5^{bc}
III.	Obese Diabetic	5.7 ± 0.50^{c}	2.2 ± 0.23^{b}	62.2 ± 3.5^{c}
IV.	Obese Diabetic + Metformin	3.3 ± 0.30^{a}	1.6 ± 0.04^{a}	47.7 ± 1.5^{ab}
V.	Obese Diabetic + PM (EE)	3.1 ± 0.18^{a}	1.5 ± 0.12^{a}	47.5 ± 2.0^{ab}
VI.	Obese Diabetic + GS (ME)	3.2 ± 0.12^{a}	1.6 ± 0.06^{a}	51.4 ± 2.2^{ab}

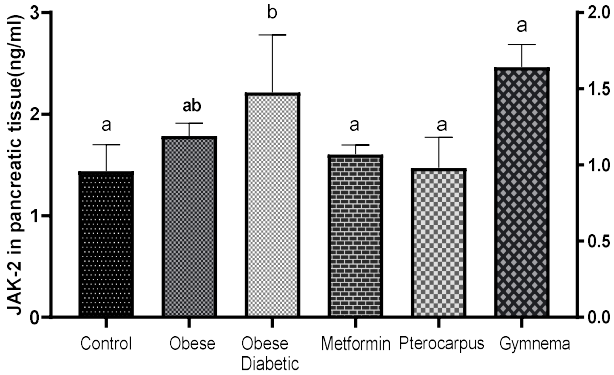
Values (Mean ± SEM; n=6) bearing different superscripts (a, b and c) in the same column significantly ($p<0.05$) using one way ANOVA following Tukey's B multiple comparison Post Hoc test

Fig. 4.20: Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on pancreatic IL6 level of HFD-fed obese-diabetic rats.

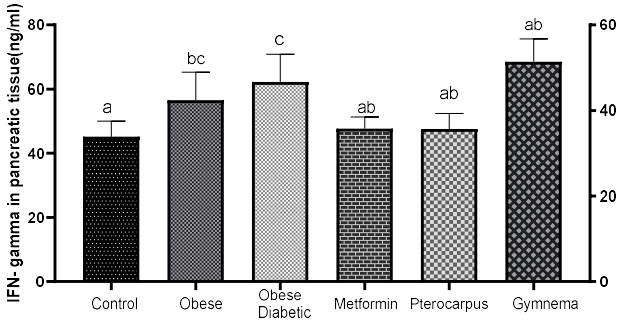
(Histograms bearing different superscripts differ significantly, $p<0.05$)



(A)



(B)



(C)

4.16 Effect of oral administration of metformin, and the entrance of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on sodium-glucose transporter -2 levels in kidney tissue of HFD -fed-obese – transporter-2 levels in kidney tissue of HFD-fed obese- diabetic rats.

The SGLT2 levels in kidney tissue (Table 4.14 & Fig. 4.21) revealed the following observations: SGLT2 levels in obese diabetic rats were elevated to 5.7 ± 0.50 ng/ml, demonstrating a significant increase compared to healthy control, obese and treatment groups ($p < 0.05$). Treatment with metformin significantly reduced SGLT2 levels to 3.3 ± 0.30 ng/ml, restoring values towards the healthy control group ($p < 0.05$).

Administration of extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* reduced SGLT2 levels showing a significant decrease compared to the obese diabetic group and comparable to the healthy control group ($p < 0.05$).

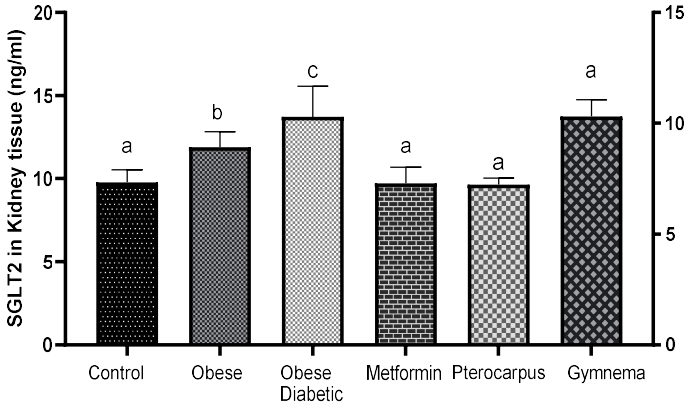
Table 4.14: Effect of oral administration of metformin, and the entrance of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on sodium-glucose transporter -2 levels in kidney tissue of HFD -fed-obese – transporter-2 levels in kidney tissue of HFD-fed obese- diabetic rats.

S. No.	Group (s)	SGLT2 (ng/ml)
I.	Healthy Control	9.78 ± 0.31^{a}
II.	Obese	11.89 ± 0.38^{b}
III.	Obese Diabetic	13.72 ± 0.75^{c}
IV.	Obese Diabetic + Met	9.72 ± 0.40^{a}
V.	Obese Diabetic + PM	9.65 ± 0.16^{a}
VI.	Obese Diabetic + GS	10.31 ± 0.31^{a}

Values (Mean ± SEM; n=6) bearing different superscripts (a, b, and c) in the same column significantly ($p<0.05$) using one-way ANOVA following Tukey's B multiple comparison Post Hoc test

Fig. 4.21: Effect of oral administration of metformin, extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* continuously for 60 days on SGLT2 level in the kidney of HFD-fed obese-diabetic rats.

(Histograms bearing different superscripts differ significantly, $p<0.05$)





Discussion

CHAPTER- 5

DISCUSSION

Diabetes mellitus, a group of metabolic disorders characterised by hyperglycemia, arises from insufficient insulin production or insulin resistance (Chen et al., 2020a). Type 1, Type 2, and gestational diabetes are distinguished by their underlying mechanisms. In India, about 8.9% of the population is currently affected, with future projections estimating that the number of individuals with diabetes could rise to around 100.95 million by 2030 and possibly 134.23 million by 2045 (Sun et al., 2022). The global prevalence is alarmingly high, with significant consequences including macrovascular and microvascular complications such as cardiovascular diseases, strokes and diabetic nephropathy (Selby & Taal, 2020). Obesity, physical inactivity, hypertension, hyperlipidemia, gestational diabetes, increasing age, differences in ethnic groups and hereditary factors are risk factors that can contribute to the development of Diabetes Mellitus (Marinho et al., 2013). To manage diabetes and mitigate its life-threatening complications, a variety of strategies have been developed. Nowadays, the ethnopharmacological approach is gaining popularity in the treatment of diabetes and its complications due to its efficacy, low incidence of side effects and low cost. Various plant species have been recognized for their antidiabetic potential. Medicinal plants such as *Pterocarpus marsupium* and *Gymnema sylvestre*, offer potential therapeutic benefits due to their hypoglycemic, antilipidemic and β-cell regenerative properties (Perera et al., 2015; Rahman & Husen, 2023). Research into these plants could lead to novel treatments, addressing the urgent need for effective diabetes management, especially concerning diabetic nephropathy.

5.1. Development of high-fat diet-fed (obese) streptozotocin-induced type-II diabetic rodent model

A rodent model of type 2 diabetes mellitus (T2DM) was established by employing a two-stage protocol. Initially, insulin resistance was induced by administering a high-fat diet (HFD) for four weeks. This dietary intervention resulted in characteristic metabolic abnormalities, including abdominal adiposity, dyslipidemia, hyperglycemia, and impaired insulin sensitivity. further development of

the obese model was confirmed by estimation of serum total cholesterol (TC) and triglycerides levels after 4 weeks of high-fat diet feeding and comparing them with initial values before high-fat diet feeding. Increased levels of TC, and triglycerides confirm the development of obesity. This model is similar to Kaydash et al., 2020 and Suman et al., 2016). Subsequently, a low-dose streptozotocin (STZ) injection of 35mg/kg body weight was administered intraperitoneally to induce a state of mild β-cell dysfunction (Wickramasinghe et al. 2022). Animals showing fasting blood glucose (>200mg/dl) after hours of STZ injection confirm the diabetic rats model. This combination of HFD and low-dose STZ successfully established a model closely mirroring the early stages of human type 2 diabetes, characterised by a clinically relevant metabolic syndrome phenotype (Furman 2021). This approach is consistent with established protocols in the literature, reflecting the efficacy of this model in recapitulating key features of human disease pathogenesis (Srinivasan et al., 2005; Mansor et al., 2013). The mechanism of STZ-induced β-cell damage involves the selective destruction of pancreatic islet cells by alkylation of DNA, resulting in reduced DNA synthesis and release of insulin (Elisa et al., 2009; Seedevi et al.,2020). The resulting hyperglycemia underscores the importance of glycemic control in mitigating the chronic complications associated with T2DM. This model provides a robust and translatable platform for investigating the efficacy of potential therapeutic interventions targeting the multifaceted pathophysiological aspects of T2DM.

5.2 General health parameters (Weekly feed and water intake, body weight, absolute organ weight and relative organ weight)

Type 2 diabetes is a disease with a high global prevalence, characterized by chronic hyperglycemia, insulin resistance, polyphagia, polydipsia, polyuria, and changes in body weight (Barragan et al., 2019). These parameters were demonstrated in this study, with increased feed and water intake in obese diabetic and obese group with marked weight loss in obese diabetic compared to control. The findings of the present study are parallel to the findings of (Miaffo et al., 2021; Silva-Reis et al., 2023) In the present study, the rats showed remarkably increased food and water intake after the development of metabolic syndrome and tended to consume more of the diet. Insulin is crucial for glucose uptake into cells, particularly in muscle and fat tissues. In diabetic states, insulin deficiency or resistance prevents cells from utilizing glucose for energy. The energy deficit at the cellular level sends signals to the

hypothalamus to increase food intake, even though blood glucose levels are high. Diabetes alters levels of hormones like leptin and ghrelin, which regulate appetite. Reduced leptin sensitivity or increased ghrelin levels can further drive hyperphagia (Yang et al., 2021).

At the same time, poor glycaemic control and unavailability of carbohydrates as an energy source results in excessive catabolism of proteins to provide amino acids for gluconeogenesis leading to muscle wasting and weight loss during insulin deficiency in diabetic rats (Panigrahi et al., 2016). In diabetes, elevated blood glucose levels exceed the renal threshold for glucose reabsorption. Glucose spills into the urine, leading to osmotic diuresis, which increases water loss and causes dehydration. This dehydration stimulates thirst centres in the hypothalamus, resulting in excessive drinking (Hahm et al., 2011; Barragan et al., 2019). The tested extracts of *Gymnema sylvestre*, *Pterocarpus marsupium* and metformin reduced the magnitude of food and water intake compared to the diabetic group this could be the result of better control of hyperglycemia in diabetic rats (Miaffo et al., 2021). The findings of the present study showed an appreciable decrease in body weight and per cent weight gain in diabetic groups of rats and treatment with extracts of *Pterocarpus marsupium*, *Gymnema sylvestre,* improved the body weight and reduced weight loss compared to the diabetic group (Miaffo et al., 2021). The results obtained from the previous studies also showed a significant decrease in body weight in animals fed an HFD and streptozotocin-induced diabetes, compared to the normal control group. The weight loss observed in obese diabetic rats after STZ administration results from a combination of insulin deficiency, enhanced fat and protein breakdown, severe metabolic dysregulation, dehydration and elevated TNF-α in diabetic rats which inhibits the uptake of free fatty acids from circulation and accelerates the lipolysis in adipose tissue, leading to weight loss in type 2 diabetes. These mechanisms are consistent with findings reported by Srinivasan et al. (2005), Skovsø et al. (2014), Goyal et al. (2016) and Zhang et al. (2019). Lu et al., (2021) observed that the weekly body weights were significantly reduced with a significant increase in food intake in type 2 diabetic mice compared to control mice indicating utilization of fatty acids and proteins as energy sources due to their inability to use carbohydrates as an energy source. *Pterocarpus marsupium* contains bioactive compounds like (-)-epicatechin, pterostilbene, and marsupin, which have been shown to reduce blood glucose levels

by enhancing insulin sensitivity and secretion. The antioxidant properties of *Pterocarpus marsupium* protect pancreatic β-cells from oxidative damage, preserving insulin function and reducing the catabolic effects of diabetes. Modulation of cytokine TNF-α by the aqueous extract of *Pterocarpus marsupium* is possibly due to flavonoids present in the extract where flavonoids have been shown to have an inhibitory effect on TNF-α (Saraf et al., 2007). *Gymnema sylvestre* extract also improved body weight in diabetic rats since it promotes β-cell regeneration and enhances insulin secretion, improving glucose uptake and utilization. This will further reduce muscle and fat breakdown and facilitate weight gain. Ahmed et al. (2010) reported that *Gymnema sylvestre* enhanced pancreatic β-cell function in diabetic rats, leading to better glycemic control and improved body weight. Improved lipid metabolism by reducing triglyceride and cholesterol levels, mitigating lipotoxicity, and aiding weight restoration would be the possible reasons. By lowering blood glucose levels, the extract reduces osmotic diuresis and dehydration and thus conservation of body fluids helps prevent weight loss associated with diabetes (Baskaran et al., 2015). extract improved lipid profiles in diabetic rats, indirectly supporting weight gain by reducing metabolic stress. By lowering blood glucose levels, the extract reduces osmotic diuresis and dehydration. This conservation of body fluids helps prevent weight loss associated with diabetes. Prakash et al. (1986) demonstrated that *Gymnema sylvestre* reduced hyperglycemia and its associated complications, including polyuria, in diabetic rats Metformin, known for its weight-modulating effects, appears to prevent drastic weight loss while stabilizing metabolism in diabetic conditions (Kim et al., 2018).

Mild to moderate and significant reduction in organ weight of liver, kidney, heart and pancreas in diabetic group rats as compared to healthy control group rats. in the present study are parallel to the observations of (Sun et al., 2023; Hajam et al., 2020). The reduction in the organ weight may be due to deleterious effect of constant hyperglycemia leads to oxidative damage of liver, kidney, heart and pancreatic tissue, which might have caused the depletion of cellular mass of the liver, glomerular hypertrophy and damage to tubular cells of kidney, potential cardiotoxic effect leads to cardiomyopathy and reduced myocardial mass of heart and beta cell loss pancreatic atrophy respectively. The restoration of organ weight in treatment group towards the value of healthy control group could be due to that metformin reduces hepatic glucose

production through the inhibition of gluconeogenesis and activation of AMP-activated protein kinase (AMPK), which improves energy metabolism and reduces oxidative stress in the liver (Bailey et al., 2024). PM contains bioactive compounds like pterostilbene and flavonoids, which exhibit hepatoprotective properties by scavenging free radicals and reducing oxidative stress (Mukhtar et al., 2005; Sathyaraj et al., 2011) whereas, GS extract inhibits glucose absorption and improves glycemic control, reducing the burden on the liver to regulate blood glucose levels (Shanmugasundaram et al., 1990; Kashif et al., 2017). The restored kidney weight in treatment groups is likely due to the nephroprotective effects mediated by antioxidant properties and improved glycemic control of the kidney (Mukhtar et al., 2005; Oyedemi et al., 2011). Restoration of the heart in the treatment groups demonstrates the protective role by mitigating oxidative stress and improving cardiac metabolism. Treatment with metformin, PM and GS restored pancreatic weight and reversal of pancreatic damage was observed with metformin, PM and GS indicating their role in preserving pancreatic integrity and β-cell function. This is consistent with evidence showing the regenerative effects of PM and GS on pancreatic β-cells (Mukhtar et al., 2005; Sathyaraj et al., 2011).

5.3 Hemato-Biochemical Parameters

A significant increase in fasting blood glucose levels, in the diabetic group of rats in correlation with the findings (Sun et al., 2023; Hajam et al., 2020). Streptozotocin leads to causes pancreatic β-cell death (apoptosis and necrosis) via different mechanisms, including DNA alkylation, depletion of cellular NAD+ levels and thus energy deprivation, increasing oxidative stress, and increasing nitric oxide production (Srinivasan et al., 2005; Ghasemi and Jeddi, 2023). The pancreatic β-cells have the important function of producing and secreting insulin, insulin is required for the absorption of glucose from the bloodstream into different cells, and decreased insulin level leads to the development of hyperglycemia (Wilcox et al., 2005; Patel et al., 2006; Dludla et al., 2023; Huang et al., 2024)) which is the main characteristic of diabetes mellitus type 2 (Caturano et al., 2023). Administration of PM, GS extract and metformin significantly lowered blood glucose levels. *Pterocarpus marsupium* contains bioactive compounds such as pterostilbene, marsupium, and flavonoids, which exhibit anti-diabetic properties, regenerating pancreatic beta cells, Enhancing insulin secretion, and inhibiting glucose absorption in the gut (Mukhtar et al., 2005;

Sathyaraj et al., 2011; Manne et al., 2020). *Gymnema sylvestre* also has been reported to lower blood glucose by Inhibiting glucose absorption in the intestine, stimulating insulin secretion and protecting pancreatic beta cell damage, significantly reducing fasting blood glucose levels and improving glycemic control in diabetic patients. Metformin acts primarily by Reducing hepatic glucose production, improving insulin sensitivity in peripheral tissues and enhancing glucose uptake in skeletal muscles (Bailey et al., 2023; Inzucchi et al., 2014; Hunter et al., 2018).

The haematopoietic system is very sensitive and serves as an important index of physiological and pathological status in both animals and humans. The present study shows Non-significant but appreciable increase in total leucocyte count (TLC) in the Obese Diabetic group of animals indicating a mild inflammatory response due to diabetes induction. Inflammatory processes are commonly observed in diabetic conditions due to increased oxidative stress and immune response. The Obese Diabetic group (Group III) had the lowest RBC count (8.9±0.4 million/mm^3), which may be indicative of anaemia or impaired erythropoiesis, a common complication in diabetes due to kidney damage or altered erythropoietin levels. The Obese Diabetic group showed reduced haemoglobin levels is consistent with the observed RBC count reduction. A decrease in Hb in diabetic rats is often due to oxidative stress and erythropoietic disturbances. Platelet dysfunction and low counts are common in diabetic conditions due to vascular complications and increased inflammation. These findings are similar to (Singh et al., 2012; Mahjabeen et al., 2022; Patel et al., 2023). The findings suggest that Metformin, *Pterocarpus marsupium*, and *Gymnema sylvestre* have beneficial effects on hematological parameters in obese-streptozotocin-induced diabetic rats. These treatments may help alleviate blood-related complications commonly associated with diabetes, possibly through anti-inflammatory, antioxidant, and metabolic regulatory mechanisms. A study reported that metformin treatment in diabetic rats improved red blood cell production and restored haematological abnormalities associated with diabetes. Treatment with *Pterocarpus marsupium* extract resulted in restored RBC count and improved haemoglobin levels in diabetic rats (Patel et al., 2023). Whereas, *Gymnema sylvestre* treatment restored platelet count and hemoglobin percentage in diabetic rats (Sharma et al., 2023).

The present study reflects poor glycemic control and is consistent with the diabetic condition induced by streptozotocin, a significant increase in per cent

glycated haemoglobin in the obese diabetic group was present and the findings are similar to the observation of Natarajan et al. (2023). Streptozotocin induces pancreatic β-cell destruction, leading to hyperglycemia and elevated HbA1c levels. Elevated HbA1c levels are a hallmark of diabetes due to the chronic exposure of haemoglobin to high glucose levels, resulting in non-enzymatic glycation (Choudhary et al., 2017). Treatment groups of Pterocarpus marsupium, Gymnema sylvestre and metformin reflect moderate reduction in per cent HbA1c, whereas, metformin is a first-line drug for the management of type-2 diabetes and present findings are in line with the previous studies reported by Kumar et al. (2023), Patel et al. (2023) and Sharma et al. (2023). In Pterocarpus group rats HbA1c is significantly lower than the diabetic group but higher than the metformin-treated group. *Pterocarpus marsupium* and *Gymnema sylvestre* is known for their antidiabetic properties, which are attributed to their ability to improve insulin sensitivity, regenerate pancreatic β-cells, ability to enhance insulin secretion and reduce oxidative stress in diabetic conditions (Kumar et al., 2023).

5.4 Lipid Profile (Triglyceride (TRI), Total Cholesterol, Low-Density Lipoproteins (LDL) and High-Density Lipoproteins (HDL)

Insulin resistance impairs the normal inhibitory effect of insulin on hormone-sensitive lipase in adipose tissue, leading to enhanced lipolysis and elevated free fatty acid (FFA) levels in the bloodstream. These excess FFAs are taken up by the liver, promoting increased hepatic triglyceride synthesis and secretion of very low-density lipoprotein (VLDL), which subsequently elevates serum TG and LDL-C levels. Additionally, insulin resistance hampers the activity of lipoprotein lipase, an enzyme crucial for the hydrolysis of TG-rich lipoproteins, further contributing to hypertriglyceridemia (Chen et al., 2020).

Dyslipidemia is usually manifested by high levels of TC, TG, LDL and low levels of HDL (Ghosh et al., 2015; Safhi et al., 2019).

The levels of serum lipid profiles, triglycerides, total cholesterol, and low-density lipoprotein were found to be significantly increased while high-density lipoproteins were found to be substantially decreased in obese and obese diabetic group rats when compared with healthy control, this finding shows similarity with previous investigations performed by (Zhang et al., 2017; Sun et al., 2023; Abduh et al., 2023). After administration of PM, GS extract and metformin for 60 days, lipid

profile was improved in treatment groups showed promising decrease in the leveld of TRI, TC, LDL and increase in HDL values towards healthy control groups, indicating that PM, GS extract and metformin can effectively reverse the disorder of lipid metabolism in T2DM rats.

The present findings showing similarty with the studies carried out by (Modak et al., 2007; Maruthupandian and Mohan, 2011; Mishra et al., 2013; Pari et al., 2018). In normal metabolism insulin activates the enzyme lipoprotein lipase and hydrolyses triglycerides and the deficiency in insulin results in inactivation of these enzymes thereby causing hypertriglyceridemia. The significant reduction of serum lipid levels in diabetic rats after treatment with *Gymnema sylvestre* and P. marsupium treatment may be directly attributed to improvements in insulin levels, which finally leads to decrease in the synthesis of fatty acid and cholesterol (Maruthupandian and Mohan, 2011; Mishra et al., 2013). Both PM and GS possess antioxidant activities that reduce oxidative stress, a contributor to dyslipidemia in diabetes Insulin promotes lipid storage by increasing lipogenesis and suppressing lipolysis by dephosphorylating hormone-sensitive lipase (Carpentier et al., 2021).

5.5 Oxidative stress indices in kidney

In the present study, the effects of ethanolic extract of *Pterocarpus marsupium* (PM), methanolic extract of *Gymnema sylvestre* and metformin were evaluated in high-fat diet-fed obese-streptozotocin-induced type 2 diabetic rats. Significant alterations were observed in kidney tissue protein, lipid peroxidation (LPO), reduced glutathione (GSH) levels, superoxide dismutase (SOD), and catalase (CAT) activity when compared to healthy control rats. The diabetic rats exhibited significantly elevated LPO suggesting increased oxidative stress and lipid membrane damage in the kidneys. This result aligns with previous studies demonstrating an increase in oxidative stress and lipid peroxidation in diabetic rats (Mahmoud et al., 2012; Abduh et al., 2023). In addition, the diabetic rats showed markedly reduced GSH levels a critical antioxidant that plays an essential role in scavenging free radicals and protecting tissues from oxidative damage (Elsayed et al., 2020). These results are consistent with reports that oxidative stress in diabetes leads to a depletion of GSH, which further exacerbates tissue damage (Mahmoud et al., 2017). Furthermore, the activities of antioxidant enzymes such as SOD and CAT were significantly decreased in diabetic rats, indicating a compromised antioxidant defense system. The decrease in

SOD and CAT activities is a common feature of oxidative stress in diabetic conditions (Ghosh et al., 2015; Rosen et al., 2001).

Treatment with metformin and PM extract led to a significant reduction in LPO and MDA respectively while GSH levels were restored respectively. These treatments also significantly improved the activities of SOD and CAT indicating an improvement in antioxidant capacity (Pari et al., 2018; Mishra et al., 2013). Additionally, GS treatment also showed significant improvements in all parameters: LPO, MDA, GSH, SOD and CAT suggesting its potential as an antioxidant in ameliorating oxidative damage in diabetic kidneys.

present findings are consistent with previous studies, where *Pterocarpus marsupium* and *Gymnema sylvestre* have shown significant antioxidant activity and protective effects against oxidative stress in diabetic animals (Rizvi and Zaid, 2001; Satheesh and Pari, 2006). The improvement in antioxidant status, as observed with PM and GS treatments, may help in protecting kidney tissues from oxidative damage associated with diabetes and obesity. The improved kidney function and reduced oxidative stress observed in this study support the potential therapeutic effects of PM, GS, and metformin in alleviating diabetes-induced kidney damage suggest natural antioxidants can mitigate kidney damage and improve oxidative stress markers in diabetic conditions (Mahmoud et al., 2012; Abduh et al., 2023).

5.6 Reanal Injury Biomarker (Cystatin C)

Elevated serum cystatin C is a reliable marker for detecting early renal dysfunction in diabetes (Rivellese et al., 2004; Macisaac et al., 2006). The present study demonstrates that serum cystatin C levels are significantly elevated in obese and obese diabetic rats compared to healthy controls, with the highest levels observed in the obese diabetic group. Increased levels of cystatin C in obese diabetic rats can be attributed to several factors (Crinigan et al., 2015). Diabetes and obesity are known to induce hyperglycemia, oxidative stress, and inflammation, leading to damage to renal tissues and impaired filtration function. Hyperglycemia results in glycation end products and hemodynamic changes, contributing to glomerular injury and subsequent increases in serum cystatin C (Abduh et al., 2023). The reduction in cystatin C levels following treatment with metformin, *Pterocarpus marsupium*, and *Gymnema sylvestre* reflects their potential therapeutic role in managing renal dysfunction in diabetic

conditions. Metformin reduces renal injury by lowering blood glucose levels and preventing oxidative damage, thereby normalizing cystatin C levels (Sun et al., 2023). Both PM and GS have been shown to possess nephroprotective properties. PM has demonstrated antioxidant activity, reducing oxidative stress-induced renal damage, while GS improves glycemic and lipid profiles, indirectly benefiting renal function (Abduh et al., 2023).

5.7 Histopathology of Kidney and Pancreas

Histopathological examination is a crucial method for assessing the structural alterations in tissues, particularly in conditions like diabetes, where various organs undergo pathological changes due to hyperglycemia and oxidative stress. In the current study, histopathological slides of kidney tissues from different experimental groups were evaluated to assess the effects of high-fat diet-induced obesity and subsequent diabetes on tissue morphology and to observe the potential protective effects of *Pterocarpus marsupium* (PM) extract, *Gymnema sylvestre* (GS) and metformin.

After heamatoxilin – eosin staining of different groups, the kidney tissue from the healthy control group showed normal histological architecture with well-preserved glomeruli, tubules and interstitial spaces, consistent with previous studies that report the structural integrity of tissues in healthy rats. In contrast, the kidney tissue of obese diabetic rats exhibited significant pathological changes, including glomerular hypertrophy, tubular degeneration, and infiltration of inflammatory cells and the findings are consistent with previous reports that high-fat diet-induced obesity and streptozotocin (STZ)-induced diabetes lead to kidney damage due to oxidative stress, and inflammation produced by glucotoxicity (Elsayed et al., 2020; Mahmoud et al., 2012). The increased lipid accumulation in the renal tissue could exacerbate inflammation and fibrosis, contributing to diabetic nephropathy (Kumar et al., 2022).

Interestingly, the histopathological damage observed in the kidney tissue of diabetic rats was significantly attenuated upon treatment with metformin, PM, GS and metformin-treated group showed improved glomerular and tubular integrity, as well as reduced inflammatory cell infiltration, suggesting their potential renoprotective effect (Pari et al., 2018; Maruthupandian and Mohan, 2011). By improving insulin

sensitivity, reducing oxidative stress, and preventing inflammation, thus preserving kidney function.

PM and GS possess various bioactive compounds that may help in reducing oxidative stress, thereby preserving the integrity of kidney tissues and preventing the progression of diabetic nephropathy potentially through their antioxidant, anti-inflammatory, and insulin-sensitizing properties. These findings suggest that natural compounds, such as those from PM and GS, may serve as effective adjuncts to conventional therapies for managing diabetes-induced organ damage (Germoush et al., 2019; Mahmoud et al., 2017).

The histopathological examination of kidney tissues stained using Masson's Trichrome stain revealed significant variations across the experimental groups. The normal control and obese control groups displayed normal renal histoarchitecture with minor fatty changes in the obese control group, aligning with the findings of previous studies indicating the impact of obesity on renal morphology (Patel et al., 2022). The obese diabetic group exhibited severe pathological changes, including excessive collagen deposition leading to necrosis, replacement of renal tubules and glomeruli, and pronounced collagen deposition around blood vessels, resulting in vascular constriction. These findings are consistent with the well-documented fibrotic changes and vascular remodeling associated with diabetes-induced nephropathy (Li et al., 2023). The progression of renal fibrosis in diabetic conditions is often linked to chronic hyperglycemia and oxidative stress, promoting extracellular matrix accumulation and subsequent structural damage (Gong et al., 2022).

Among the treatment groups, the metformin-treated group showed moderate improvement in renal histoarchitecture with mild collagen deposition in glomeruli and limited changes in tubules and glomeruli. This observation aligns with metformin's known role in reducing renal fibrosis and oxidative stress through activation of AMPK pathways (Zhang et al., 2023b). Similarly, the *Gymnema sylvestre* (GS)-treated group demonstrated mild collagen deposition and tubular degeneration, indicative of partial protection against fibrotic damage, possibly due to its anti-inflammatory and antioxidant properties (Kumar et al., 2023).

The *Pterocarpus marsupium* (PM)-treated group presented the most favorable outcome, showing minimal collagen deposition around glomeruli, mild swelling of

renal tubules, and an overall near-normal renal architecture. These results are consistent with PM's potential to mitigate renal fibrosis through modulation of TGF-β signaling pathways and inhibition of oxidative stress (Rathore et al., 2022). The findings collectively suggest that while all treatments showed varying degrees of protection against diabetes-induced renal damage, PM extract exhibited the most promising nephroprotective effects, followed by GS and metformin. The observed improvements highlight the therapeutic potential of these treatments in mitigating diabetic nephropathy, supporting their use as complementary or alternative therapeutic options.

Findings from Chrome Alum Hematoxylin-Phloxine staining corroborated the H&E results. Healthy and obese control groups exhibited well-structured islets with intact alpha and beta cells. In contrast, the obese diabetic group displayed shrunken islets and degeneration of beta cells, which are hallmarks of diabetes-induced beta-cell dysfunction (Wang et al., 2023). Treatment with metformin, PM and GS significantly improved the beta cell mass and restored compact islet margins. Among these, PM treatment showed the most notable improvement, consistent with its known role in promoting beta-cell regeneration and antiapoptotic effect (Rathore et al., 2022).

The restoration of pancreatic islet structure and function in the treatment groups highlights the potential of PM, metformin, and *Gymnema sylvestre* as therapeutic agents for diabetes management. These findings are consistent with previous studies demonstrating their ability to modulate oxidative stress, reduce inflammation, and enhance beta-cell survival and function (Mishra et al., 2021; Kumar et al., 2022). The results emphasize the significant protective effects of PM, GS and metformin in preserving pancreatic histoarchitecture and beta-cell integrity, offering promising strategies for mitigating diabetes-related pancreatic damage.

5.8 Estimation of IL6, JAK2 and IFN gamma in pancreatic tissue

Type 2 diabetes mellitus, characterized by chronic inflammation often involves elevated levels of pro-inflammatory cytokines such as interleukin-6 (IL-6). IL-6 plays a dual role in metabolic regulation and inflammation, with excessive levels contributing to β-cell dysfunction and insulin resistance in diabetes (Donath et al., 2019).

In present study, significantly elevated pancreatic IL-6 levels were observed in the obese and obese diabetic groups compared to the healthy control group. The increase in IL-6 levels in the obese diabetic group aligns with findings by Pickup et al. (2004), which indicate that chronic hyperglycemia and insulin resistance exacerbate inflammation, leading to elevated cytokine production. The obese group also displayed higher IL-6 levels, reflecting the role of adipose tissue in secreting inflammatory cytokines during obesity-induced metabolic stress.

The elevated IL-6 levels in the obese and obese diabetic groups reflect the role of systemic inflammation in the progression of metabolic disorders. Prolonged exposure to elevated IL-6 disrupts pancreatic β-cell function, exacerbating hyperglycemia and insulin resistance. By reducing IL-6 levels, the tested interventions mitigate inflammatory damage and potentially restore metabolic balance.

The administration of metformin and extracts of *Pterocarpus marsupium,* and *Gymnema sylvestre* significantly ($p < 0.05$) reduced IL-6 levels in in experimental and obese diabetic rats. These values are comparable to the healthy control group, indicating their effectiveness in mitigating inflammation. Metformin's anti-inflammatory effects are well-documented, attributed to its ability to activate AMP-activated protein kinase (AMPK), which suppresses inflammatory pathways (Bai et al., 2021).

Pterocarpus marsupium contains bioactive compounds like (-)-epicatechin and pterostilbene, which possess anti-inflammatory properties and modulate cytokine levels, including IL-6 (Saraf et al., 2007). Similarly, anti-inflammatory effects of *Gymnema sylvestre* likely to be mediated by its saponins and flavonoids, reduced oxidative stress and suppress of pro-inflammatory cytokines, as reported by Baskaran et al. (2015).

The observations in the present study of this study contrast with the findings by Rodrigues et al. (2020), who reported limited effects of natural plant extracts on cytokine modulation in severely diabetic rodents, potentially due to differences in dose, duration or animal model used.

Type 2 diabetes mellitus is characterized by chronic metabolic disturbances and inflammation, involved the dysregulation of key signaling pathways such as the

Janus kinase 2 (JAK2) pathway. JAK2 is a critical mediator in cytokine signalling and contributes to insulin resistance and β-cell dysfunction in diabetes (Shi et al., 2017).

In the present study, pancreatic JAK2 levels were significantly elevated in the obese and obese diabetic groups compared to the healthy control group. The increased JAK2 levels in the obese diabetic group align with findings by Klover et al. (2005), who demonstrated that upregulation of the JAK/STAT pathway contributes to inflammation and insulin resistance in diabetic conditions. The elevation observed in the obese group is likely due to the pro-inflammatory environment created by adipose tissue, which activates JAK2 signalling even in the absence of hyperglycemia.

Pterocarpus marsupium contains anti-inflammatory bioactive compounds which may suppress JAK2 signalling through their ability to reduce oxidative stress and modulate cytokines like TNF-α (Saraf et al., 2007). Similarly, *Gymnema sylvestre*, known for its insulin-sensitizing, hypoglycemic and anti-inflammatory properties, may downregulate JAK2 signalling by reducing oxidative and inflammatory stress, as reported by Baskaran et al. (2015). Elevated JAK2 levels in obese and obese diabetic groups highlights the role of this signalling pathway in mediating inflammation and insulin resistance. Prolonged activation of JAK2 disrupts pancreatic function, exacerbating β-cell dysfunction and hyperglycemia. By reducing JAK2 levels, the tested treatments mitigate the inflammatory response and help preserve pancreatic function (Baskaran et al., 2015).

The obese diabetic group continuously fed for sixty days with metformin, *Pterocarpus marsupium*, and *Gymnema sylvestre* significantly reduced JAK2 levels. These reductions brought JAK2 levels closer to those of the healthy control group, suggesting effective modulation of this pro-inflammatory pathway. Metformin's ability to suppress JAK2 activation has been linked to its role in reducing cytokine-induced inflammation via AMPK activation and inhibition of STAT3 signalling (Bai et al., 2021).

IFN-γ, a cytokine secreted by activated T cells and natural killer cells, plays a significant role in perpetuating inflammation, β-cell dysfunction, and insulin resistance in diabetes (Donath et al., 2019).

In the present study, pancreatic IFN-γ levels were significantly elevated in the obese and obese diabetic groups compared to the healthy control group. The increase

in IFN-γ levels in the obese diabetic group aligns with findings by Maedler et al. (2002), who demonstrated that elevated levels of IFN-γ exacerbate β-cell apoptosis and contribute to the pathophysiology of type 2 diabetes. Elevated levels in the obese group are likely attributed to chronic low-grade inflammation induced by excess adipose tissue, which stimulates cytokine production even in prediabetic states.Metformin's ability to suppress IFN-γ production is supported by its known anti-inflammatory effects via AMPK activation, which downregulates pro-inflammatory cytokines (Bai et al., 2021).

Present study reported that treatment with *Pterocarpus marsupium* and *Gymnema sylvestre* significantly reduced pancreatic IFN-γ levels in obese diabetic rats compared to the untreated obese diabetic group. These reductions suggest that treatments effectively mitigated inflammation in the pancreatic tissue.

Pterocarpus marsupium contains bioactive compounds which reduce oxidative stress and inflammation, leading to the downregulation of cytokines such as IFN-γ (Saraf et al., 2007). Similarly, *Gymnema sylvestre* anti-inflammatory and antioxidant properties have been shown to modulate cytokine production, reducing IFN-γ levels and promoting pancreatic β-cell survival (Baskaran et al., 2015).

Metformin and *Pterocarpus marsupium* demonstrated significant reductions in IFN-γ levels, the effect of *Gymnema sylvestre* was less pronounced, as it's IFN-γ levels remained slightly higher at 51.4 ± 2.2 ng/ml. This observation aligns with Rodrigues et al. (2017), who noted that while *Gymnema sylvestre* reduces inflammation, its efficacy may vary depending on the dosage and treatment duration.

5.9 Estimation of SGLT2 in kidney tissue homogenate

Sodium-glucose cotransporter-2 (SGLT2), expressed in the renal proximal tubules, plays a significant role in glucose reabsorption and is often upregulated in diabetic states, contributing to hyperglycemia. In this study, SGLT2 levels were significantly increased in the obese and obese diabetic groups compared to the healthy control group, indicating renal adaptations to hyperglycemic conditions. Elevated SGLT2 levels in the obese diabetic group (5.7 ± 0.50 ng/ml) are consistent with findings by Mather and Pollock (2011), who reported increased renal glucose reabsorption in diabetes due to SGLT2 overexpression.

The administration of metformin, and extracts of *Pterocarpus marsupium*, and *Gymnema sylvestre* significantly reduced SGLT2 levels in obese diabetic rats, bringing them closer to those of the healthy control group. The reductions were indicating their efficacy in mitigating SGLT2 upregulation. Metformin, known to decrease renal glucose reabsorption by inhibiting SGLT2 indirectly, has been widely documented for its renoprotective effects (Bajpai and Kumar, 2020). Similarly, the bioactive compounds in *Pterocarpus marsupium*, such as (-)-epicatechin and pterostilbene, may suppress SGLT2 expression by improving insulin sensitivity and glycemic control (Saraf et al., 2007). *Gymnema sylvestre,* known for it's role in regenerating pancreatic β-cells and improving insulin secretion, may act to lower glucose levels, reducing the stimulus for SGLT2 overexpression (Baskaran et al., 2015).

The obese group in the present study also exhibited elevated SGLT2 levels (4.5 ± 0.33 ng/ml). This increase is likely due to insulin resistance and mild hyperglycemia, which drive compensatory mechanisms in the kidney. However, these levels were lower than those observed in the obese diabetic group, aligning with the understanding that diabetes exacerbates renal SGLT2 expression due to prolonged hyperglycemic stimuli (Mather and Pollock, 2011).

In conclusion, the study demonstrates that metformin and the extracts of *Pterocarpus marsupium* and *Gymnema sylvestre* effectively reduce renal SGLT2 levels in obese diabetic rats. These findings suggest a potential therapeutic role in managing hyperglycemia by modulating renal glucose reabsorption. Further studies focusing on the molecular mechanisms of SGLT2 downregulation by these treatments could provide deeper insights.



Summary and Conclusions

CHAPTER-6

SUMMARY AND CONCLUSIONS

The present study was performed to study the nephroprotective potential of *Pterocarpus marsupium* and *Gymnema sylvestre*, in experimental obese diabetic male Wistar rats. Diabetes is a metabolic disorder characterised by chronic hyperglycemia, dyslipidemia and insulin resistance (Miaffo et al., 2021). In the present study, type 2 diabetes was induced in rats by fed on a high-fat diet for 4 weeks followed by the injection of a low dose of streptozotocin (STZ), antidiabetic and pancreatic regeneration activity of *Pterocarpus marsupium and Gmnema slvestre* was evaluated.

After feeding HFD for a period of four weeks, a single intraperitoneal injection of streptozotocin @ 35 mg/ kg body weight was administered for induction of type II diabetes obese rat model with fasting blood glucose > 200 mg/dl was used for further studies. Ethanolic extract of *Pterocarpus marsupium* @ 300 mg/kg body weight *Gymnema sylvestre* @ 400 mg/kg and metformin @ 50 mg/kg body weight were orally administered continuously for 60 days in experimental obese diabetic male Wistar rats.

Parameters viz feed and water intake, body weight, body weight gain, absolute and relative organ weight, fasting blood glucose, HbA1c, haematological parameters, lipid profile, oxidative stress indices, renal injury biomarker, the histopathological architecture of pancreas & kidney tissue and genes of pancreatic regeneration and activity of sodium-glucose transporter -2 in kidney were recorded at the end of the experimental period of 60 days as mentioned here under.

Feed and water intake was increased significantly in rats of the obese diabetic group compared to the healthy control group, whereas PM, GS extract and metformin-treated groups showed values at par with the healthy control group. The obese group showed a significant increase in weekly body weight whereas, the obese diabetic group showed a significant decrease when compared to the healthy control group. PM, GS and metformin-treated groups showed a significant rise in body weight compared to the obese diabetic group and improved body weight gain towards the healthy control group. A marked increase in absolute organ weights of the liver, kidney, heart and pancreas in the obese group whereas a significant decrease in

absolute organ weights of liver, kidney, heart and pancreas in the obese diabetic group when compared to the healthy control group was recorded. a marked increase in the pancreas of an obese diabetic was recorded, however relative organ weights non-significantly increased in obese rats. In obese diabetic groups, the relative organ weight of the liver, kidney and pancreas decreased non significantly while mild to moderate increase in the relative weight of the kidney. PM, GS extracts and metformin-treated groups restored the absolute and relative organ indices towards the healthy control group and were comparable.

Significant increases in fasting blood glucose at different time intervals and HbA1c at the end of the experimental period of 60 days and haematological indices in different groups of rats, were observed in obese diabetic groups of rats, where no significant but marked increase in TLC, decrease in RBC, Hb and platelets count was recorded in the obese diabetic group, however, fasting blood glucose, and HbA1c were recorded on higher side in obese diabetic group of rats whereas, treatment with PM, GS extracts and metformin have been found effective in restoring the values of these parameters towards the values of healthy control group.

Increase in triglycerides, total cholesterol and LDL levels significantly whereas significant decreases in HDL levels were recorded in obese and obese diabetic groups. *Pterocarpus marsupium*, *Gymnema sylvestre* and metformin-treated groups corrected dyslipidemia induced after STZ injection when compared to the obese and obese diabetic group.

Oxidative stress indices were assessed in kidney tissues. Oxidative stress evaluation of kidney tissue revealed a significant increase in the LPO and a decrease in the level of GSH, Catalase, and SOD in obese and obese diabetic groups, while treatment groups showed antioxidant properties with improved indices in kidney tissue.

Renal injury marker, Serum Cystatin c level was significantly increased in the obese diabetic group compared to the healthy control group however, PM, GS extract and metformin-treated rats showed a significant decrease compared to obese diabetic rats.

Histopathological examination of the kidney and pancreas using hematoxylin and eosin staining, Masson's trichrome in kidney tissue and chrome alum

hematoxylin-phloxine staining in pancreas was performed. H& E staining revealed that obese and obese diabetic groups showed degenerative changes in kidney. In the Diabetic group kidney degeneration, necrosis of glomeruli, and Congestion of blood vessels were seen. Whereas, in the pancreas only the obese diabetic group showed degenerative changes and showed shrunken, irregular outlines & distorted islets. In PM, GS and metformin treatment restored the histoarchitecture of the organs. The masson's trichome staining is also done in kidney tissue which shows necrosis of glomeruli and tubules due to more collegen fibre diposition in glomeruli of kidney tissue in obese and obese diabetic groupWhereas treatment with PM, GS and metformin restored the renal normal histoarchitecture. The chrome alum hematoxylin-phloxine staining of the pancreas showed shrunken Islets with degeneration of beta cells in the obese diabetic group. PM, GS and metformin displayed improved histoarchitecture of islets of the pancreas.

Based on the results of the present study, it may be concluded that:

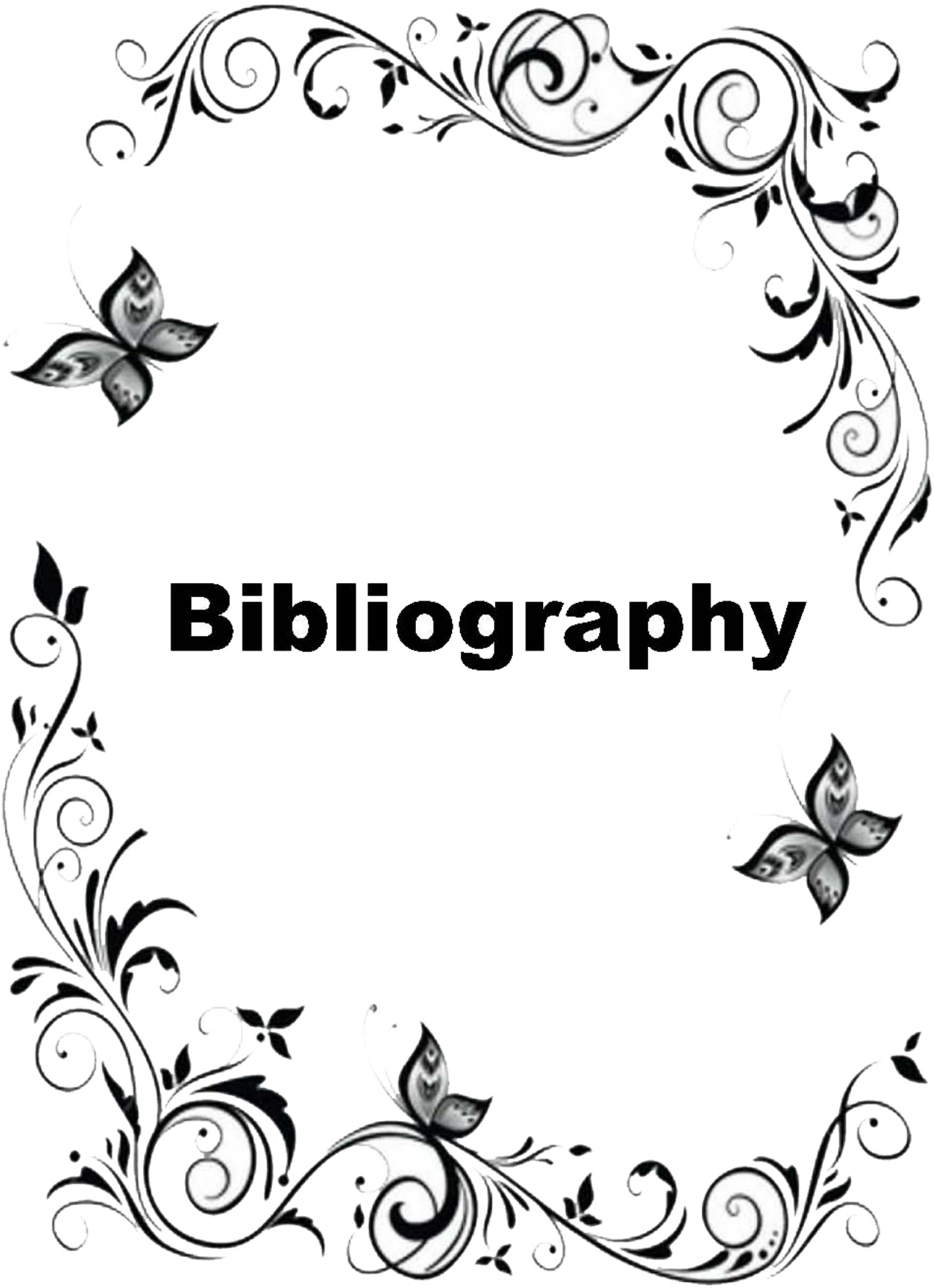
1. Feeding high-fat diet (HFD) continuously for four weeks and a single intraperitoneal injection of streptozotocin @ 35 mg/kg body weight. developed a consistent obese type II diabetic rat model.
2. *Pterocarpus marsupium* and *Gymnema sylvestre* showed antidiabetic activity by decreasing fasting blood glucose levels significantly in diabetic rats, which is the main characteristics of diabetes mellitus. Other clinical signs like polyphagia and polydipsia decline in body weight which are observed in diabetic conditions were also reduced by *Pterocarpus marsupium* and *Gymnema sylvestre* extracts.
3. Significant reduction in percent glycated haemoglobin (HbA1c %) level which is an important biomarker of diabetes, PM and GS extracts showed potent hypoglycemic activity and reduction in HbA1c %, which was comparable to metformin.
4. The significant rise in triglycerides, total cholesterol, LDL levels and decrease in HDL levels showed the development of dyslipidemia in obese and obese diabetic groups. PM extract and metformin significantly improved these lipid profile parameters towards healthy control group.
5. Diabetic rats developed oxidative stress characterized by increased MDA levels and decreased activities of antioxidant enzymes (SOD and CAT) and

GSH content in the liver and pancreas. Administration of the extract of PM, GS and metformin for 60 days resulted in a decrease in MDA levels and an increase in CAT and SOD activities, which showed potent antioxidant properties of *Pterocarpus marsupium* and *Gymnema sylvestre.*

6. reduction in serum cystatin C level which is early kidney marker shows that PM and GS extracts has renoprotective potential by mitigating the effect of chronic hyperglycemia and oxidative stress.
7. Histopathological examination of kidney and pancreatic tissue showed degenerative changes produced due to hyperglycemia-induced oxidative stress and dyslipidemia. PM, GS extract and metformin treatment restored the histoarchitecture towards a healthy control group. Restoration of pancreatic islets with improved beta cell mass was observed in PM and GS extract-treated rats.
8. Administration of PM, GS extracts, and metformin significantly reduced IL-6, JAK2, IFN gamma levels in pancreatic tissue, suggesting their anti-inflammatory properties in mitigating diabetes-induced inflammation.
9. Treatment with PM, GS extracts, and metformin for 60 days significantly reduced SGLT2 expression, indicating their potential role in modulating renal glucose handling and improving glycemic control.
10. Based on the analysis of all the results and observations mentioned above, it can be concluded that the ethanolic extract of *Pterocarpus marsupium* and the methanolic extract of *Gymnema sylvestre* exhibit significant hypoglycemic potential by improving blood glucose levels, reducing glycated hemoglobin, and normalizing the altered lipid profile while protecting cells against oxidative stress. Histopathological and immunohistochemical analyses showed the restoration of pancreatic islets with improved β-cell mass in PM- and GS-treated rats. Furthermore, gene expression analysis in pancreatic tissue revealed a significant reduction in IL-6, JAK2, and IFN-γ expression following treatment, indicating the anti-inflammatory potential of these extracts in mitigating diabetes-associated pancreatic inflammation.
11. Based on the analysis of all the results and observations mentioned above, it can be concluded that the ethanolic extract of *Pterocarpus marsupium* and the methanolic extract of *Gymnema sylvestre* exhibit significant renoprotective effects in diabetic rats. The treatment effectively reduced SGLT2 expression in

the kidney, thereby modulating renal glucose reabsorption and improving glycemic control. Additionally, a reduction in serum cystatin C levels was observed, suggesting the potential of these extracts in protecting against early kidney dysfunction associated with diabetes. Furthermore, treatment with PM and GS extracts mitigated oxidative stress in renal tissue by decreasing MDA levels and enhancing antioxidant enzyme activities (SOD, CAT, and GSH), thereby reducing hyperglycemia-induced renal damage. Histopathological examination confirmed the restoration of kidney architecture, indicating the protective role of these plant extracts against diabetes-induced nephropathy.

The present study indicates that both *Pterocarpus marsupium* and *Gymnema sylvestre* possess significant antidiabetic and nephroprotective potential, comparable to metformin, by ameliorating hyperglycemia, dyslipidemia, oxidative stress, and promoting the restoration of pancreatic β-cell function and renal tissue architecture. Further research is warranted to fully elucidate the mechanisms underlying these effects and explore their therapeutic potential in the management of diabetic nephropathy.



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I also undertake that patent, if any, arising out of research work conducted during the programme shall be filed by me only with due permission of the competent authority of U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan (DUVASU), Mathura (UP).

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