ABBREVIATIONS

4-OH-Ile	:	4-hydroxyisoleucine
A1C	:	Glycated haemoglobin
ADP	:	Adenosine diphosphate
AMPK	:	AMP-activated protein kinase
ATP	:	Adenosine triphosphate
BW	:	Body weight
CA	:	Carcinoma
CBN	:	Combination group
Cyp7a1	:	Cholesterol-7α-hydroxylase
DC	:	Diabetic Control group
DM	:	Diabetes Mellitus
DPP-IV	:	Dipeptidyl peptidase – IV
DRPM	:	Diabetes related premature death
FA	:	Fatty acid
FBG	:	Fasting blood glucose
FPG	:	Fasting plasma glucose
FXR	:	Farnesoid X receptor
G6pc	:	Glucose-6-phosphatase
GK	:	Goto–Kakizaki
GLP-1	:	Glucagon-like peptide 1
GLUT2	:	Glucose Transporter – 2
HFD	:	High-fat diet
HLA	:	Human leukocyte antigen
HNF4	:	Hepatocyte nuclear factor 4

IDDM	:	Insulin dependent diabetes mellitus
IUGR	:	Intrauterine growth retardation
L-PK	:	Pyruvate kinase – liver type
MET	:	Metformin group
NAD	:	Nicotinamide adenine dinucleotide
NC	:	Normal Control group
NFBG	:	Neonatal fasting blood glucose
NIDDM	:	Non-insulin dependent diabetes mellitus
OGTT	:	Oral glucose tolerance test
OLETF	:	Otsuka Long-Evans Tokushima Fat rat
PK	:	Pyruvate kinase
SD	:	Sprague Dawley
SGLT-2	:	Sodium Glucose Transport protein – 2
SHR	:	Spontaneous hypertensive rats
STZ	:	Streptozotocin
T1D	:	Type 1 diabetes
T1DM	:	Type 1 Diabetes Mellitus
T2D	:	Type 2 diabetes
T2DM	:	Type 2 Diabetes Mellitus
TCI	:	Traditional, Complementary and Integrative Medicine
TFG	:	*Trigonella foenum-graecum*
USD	:	US dollars
WHO	:	World Health Organization
ZDF	:	Zucker Diabetic Fatty
ZF	:	Zucker Fatty

ABSTRACT

Introduction & Objectives

This study was designed to evaluate the effect of *Trigonella foenum-graecum* (TFG) on the expression of L-PK, G6pc and Cyp7a1 genes in the liver tissue of experimentally induced diabetic rats. The study also intended to evaluate the interaction of TFG and metformin at the genetic level involving the same respective genes.

Methodology

The study in the beginning included 38 rats divided into five groups with eight rats in all groups except the normal control group which had six rats. Groups: normal control (NC), diabetic control (DC), metformin (MET), TFG and combination of metformin and TFG (CBN). Diabetes was induced in 32 rats by a combination of HFD given for two weeks & single low-dose intraperitoneal injection of Streptozotocin (STZ) of 30 mg/kg. Rats with random blood sugar (RBS) levels > 200 mg/dl after 72 hours of STZ injection were included in the study. Respective diets were continued throughout the study. Test compounds or vehicle were administered for 30 days by oral route. RBS and body weight were tested on day 1 and day 30 of the experiment. At the end of the study, all rats were euthanized, and liver tissue was harvested for gene expression analysis. The Real-time PCR technique was used to analyse gene the expression.

Results

The MET, TFG, and CBN groups significantly lowered the RBS levels of diabetic rats. However, there was no significant difference in body weight before and

after treatment in any of the groups. Metformin reduced L-PK gene expression by 3.5-fold, whereas TFG and CBN increased it by 37.9-fold and 1.3-fold, respectively. MET, TFG, and CBN all upregulated G6pc gene expression by 9.6-fold, 16.6-fold, and 17.9-fold, respectively. All groups upregulated Cyp7a1 expression, however metformin only upregulated it by 4.8-fold, whereas the TFG and CBN groups upregulated it by a substantial amount with 50.2-fold and 50.4-fold respectively.

Conclusion

This research has helped us better grasp TFG's anti-diabetic action, and the probable mechanisms involved. Furthermore, co-administration of metformin and TFG revealed an undesirable pharmacological interaction on glucose metabolism and bile acid synthesis at the genetic level. This is critical for people who wish to take metformin with TFG for ostensibly better blood glucose management and it is advisable to avoid combining metformin and TFG. Additional research would be required to substantiate the data obtained by the current study. Also, the current investigation has proven Cyp7a1 to be an essential therapeutic target for both metformin and TFG due to its involvement in both, bile acid synthesis and anti-diabetic effect.

Keywords:

Type 2 diabetes mellitus, Fenugreek, Metformin, Gene expression, Pyruvate kinase, Glucose-6-Phosphatase, Cyp7a1

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Effect of *Trigonella foenum-graecum* on gene expression in liver tissue of experimentally induced diabetic rats

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INTRODUCTION

The global prevalence of diabetes mellitus has risen dramatically in recent years, with about 422 million individuals affected. In 2014, it afflicted 8.5 % of people aged 18 and upwards. In 2019, it took the lives of 1.5 million people. To get a more accurate picture of diabetes-related mortality, deaths caused by higher-than-optimal blood glucose through cardiovascular disease, chronic renal illness, and tuberculosis should be included. In 2012, another 2.2 million people died as a result of excessive blood glucose levels (the most recent year for which data is available).[1]

Between 2000 and 2016, the DRPM rose by 5%. From 2000 to 2010, Diabetes related premature death (DRPM) fell in high-income nations, but subsequently grew from 2010 to 2016. DRPM, on the other hand, surged in lower-middle-income countries across both time periods.[1]

Diabetes is estimated to cost the world health system USD 760 billion each year, according to the International Diabetes Federation (IDF). By 2030, these direct costs are estimated to reach USD 825 billion, and by 2045, they will reach USD 845 billion.[2]

Diabetes has progressively increased in India and around the world in the last quarter-century, with India accounting for a significant portion of the worldwide burden. It has increased dramatically in India, from 26 million in 1990 to 65 million in 2016, with Tamil Nadu, Kerala, and New Delhi having the greatest prevalence.[3] India presently has roughly 69.1 million diabetics and is predicted to have the second greatest number of diabetes cases after China, with the figure expected to reach 79.4 million by 2030. [4,5] Despite tremendous scientific discoveries that have resulted in the

creation and availability of a wide range of safe and effective oral and injectable pharmaceutical medications for diabetes, its prevalence and morbidity remain serious medical issues worldwide. Based on the most recent Diabetes Country Profile from the WHO – Diabetes Country Profiles, 2016, DM affects 7.9 % of males and 7.5 % of females in India. DM was also found to be connected with a 2% death rate. [6]

According to a 2007 study, around 2.2 billion USD would be required to adequately treat all cases of T2D in India. Diabetes and its consequences are likely to have a greater impact on total health-care spending in the coming years, which will have substantial implications for health-care systems and financial sustainability.[7] As a result, the need to identify more cost-effective and safer treatment alternatives for diabetes and its complications is becoming increasingly pressing.

As per ADA-2021, the current pharmacotherapy for Type 2 DM includes metformin as the first line agent along with lifestyle modifications. This is then followed by adding additional classes of drugs depending upon the presence or absence of individual risk factors for atherosclerotic cardiovascular disease, chronic kidney disease or heart failure. In either case, any individual whose HbA1c is not adequately controlled or is persistently high, will be prescribed two different drug classes, in some cases even three.

This leads to polypharmacy and may result, in drug-drug interactions and additional adverse effects. Common adverse effects seen with commonly prescribed antidiabetic drugs include hypoglycaemia, weight changes, anorexia, flatulence, etc. Apart from this there are certain peculiar adverse effects restricted to certain classes of drugs like dipeptidyl peptidase-IV (DPP-IV) inhibitors causing pancreatitis. Sodium glucose transport protein – 2 (SGLT-2) inhibitors causing genitourinary

infections and Fournier's gangrene, GLP-1 receptor agonists and amylin agonists increasing the risk of thyroid cancer as well as thiazolidinediones increasing the risk of congestive cardiac failure and bladder cancer and causing macular oedema. Parenteral medications have the disadvantage of being injectable, which leads to poor compliance. [8,9]

Although synthetic oral hypoglycaemic medicines used in conjunction with insulin are the most common method of managing diabetes, they do not completely reverse the disease's problems and, as previously mentioned, have significant adverse effects. This is the driving factor behind the search for new anti-diabetic medicines[10]. Despite tremendous advances in the treatment of diabetes with oral antidiabetic drugs during the last three decades, diabetic patient treatment outcomes are still far from ideal.[11]

Natural products, particularly those of plant origin, are the primary target for identifying viable lead candidates and will play a critical role in future dru g development efforts.[12–14] Plant-based preparations are among the main essential players of all current medicines, especially in rural regions, due to their ease of availability, low cost, and fewer adverse effects.[15] Many plants have long been thought to be a major source of effective anti-diabetic medicines. Medicinal herbs are utilised to treat diabetes in impoverished nations, particularly to alleviate the financial load of conventional drugs on the people.[16] Nowadays, medicinal plants are being used to treat a variety of ailments, including diabetes[17], since they include phytoconstituents such as flavonoids, terpenoids, saponins, carotenoids, alkaloids, and glycosides, which may have anti-diabetic properties.[18]

The WHO Traditional Medicine Strategy 2014–2023 was designed and launched in 2013, emphasising the need for integrating traditional and complementary medicine known as Traditional, Complementary and Integrative Medicine (TCI) to promote universal healthcare and ensure the quality, safety, and efficacy of such medicines. According to WHO data, 88 % of Member States have acknowledged their usage of TCI, which equates to 170 countries.[19]

Traditional medicines, largely plant pharmaceuticals, are used by more than half of the population in underdeveloped nations for their basic health care requirements because they are natural, safe, non-narcotic, and have fewer side effects. Furthermore, because these therapies are cost-effective, preventive, and curative, WHO has advised that traditional knowledge systems be combined with other interventions in order to reach the objective of "Health for All" in a cost-effective manner.[20]

Fenugreek, also known as *Trigonella foenum-graecum*, is a powerful herb that is one of the few recognized to have anti-diabetic properties.[21,22] It has also been discovered to have a wide range of therapeutic qualities. Fenugreek has been shown to have antidiabetic, gastric stimulant, and galactogogue (lactation-inducing) properties, as well as antilipidemic, hypocholesterolemic, antioxidant, anti-inflammatory, hepatoprotective, antibacterial, antifungal, antiulcer, antilithigenic, anticarcinogenic, and other medicinal properties.[23]

Even with the widespread use of herbal medicines in India, few, if any, have been examined for their genetic effects. As a result, unravelling the mechanisms of these medicines and knowing how they produce therapeutic benefits is critical.

Therefore, the purpose of this study was to evaluate the effect of *Trigonella foenum-graecum* on gene expression of selected genes in the liver tissue of rats affected by T2DM induced by a combination of HFD and STZ. Hence, the given study was conceived and conducted.

OBJECTIVES

The objectives of the study were as follows:

- **Primary Objective**

To determine the effect of administration of *Trigonella foenum-graecum* on gene expression of pyruvate kinase – liver type (L-PK) gene, glucose-6-phosphatase (G6pc) gene, and Cyp7a1 gene in liver tissue of Streptozotocin (STZ) + High Fat Diet (HFD) induced diabetic male Wistar rats.

- **Secondary Objective**

To study the interaction of *Trigonella foenum-graecum* with Metformin on gene expression of L-PK gene, G6pc gene, and Cyp7a1 gene in liver tissue of STZ + HFD induced diabetic male Wistar rats.

REVIEW OF LITERATURE

3.1 Diabetes Mellitus

3.1.1 Definition

Diabetes, according to the WHO, is a chronic condition that arises when either the pancreas does not make enough insulin or the body is unable to adequately use the insulin that is produced.[1] It is defined as a chronic metabolic disorder that affects various organ systems and can cause secondary pathophysiologic changes because of metabolic dysregulation in the body. It is frequently referred to as an endocrine condition, and it places a significant burden on both the individual with diabetes and the health-care system.[8]

3.1.2 Diagnostic Criteria[24]

Diabetes can be diagnosed using plasma glucose criteria, such as fasting plasma glucose (FPG) or 2-hour plasma glucose (2-h PG) values obtained via a 75-gram oral glucose tolerance test (OGTT), or A1C criteria.

Criteria for diagnosis of diabetes
FPG ≥ 126 mg/dL (7.0 mmol/L). Fasting is defined as no calorie intake for at least 8 hours. *
OR
2-h PG ≥ 200 mg/dL (11.1 mmol/L) during OGTT. The test should be performed as described by WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.*
OR
A1C ≥ 6.5% (48 mmol/mol). The test should be performed in a laboratory using a method that is NGSP certified & standardized to the DCCT assay. *
OR
In a patient with classic symptoms of hyperglycaemia or hyperglycaemic crisis, a random plasma glucose ≥ 200 mg/dL (11.1 mmol/L).
DCCT – Diabetes Control and Complications Trial *In the absence of unequivocal hyperglycaemia, diagnosis requires two abnormal test results from the same sample or in two separate test samples.

3.1.3 Classification

As per ADA, Diabetes is divided into the following broad categories:[24]

1. Type 1 diabetes, also known as autoimmune diabetes or non-insulin dependent diabetes mellitus (NIDDM), accounts for about 5% to 10% of all diabetic cases. In these patients, autoimmune T-cell-mediated inflammation and beta-cell death are the primary causes of diabetes. In children and adolescents, it accounts for 80-90 percent of diabetes cases. Type 1 diabetes has also been linked to distinct HLA types, with DR or DQ types being the most common.[25]
2. Type 2 diabetes, more than 90-95 percent of diabetic patients suffer from this form of condition. Insulin resistance is the most common cause, which causes the body's organs to demand more insulin. These patients do not require

insulin, which distinguishes them from people with T1DM. Insulin resistance is also linked to obesity, essential hypertension, nephropathy, dyslipidaemia, low HDL, and high LDL.[26]

3. Specific types of diabetes caused by other factors, such as monogenic diabetes syndromes (such as neonatal diabetes and maturity-onset diabetes of the young), drug- or chemical-induced diabetes (such as with glucocorticoid use, or after organ transplantation), and exocrine pancreas disorders (such as cystic fibrosis and pancreatitis).
4. Gestational diabetes mellitus (diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation)

Type 1 and type 2 diabetes are the most widespread types of diabetes out of these.

3.2 Glucose Metabolism[27]

One of the body's primary functions is to maintain energy balance. The regulation and return of energy molecules such as glucose and FAs in the body is a complex process involving all cells. The main metabolic organs in the body are adipose tissue, skeletal muscle, and the liver. Normoglycemic and normolipidemic levels in the circulation represent the normal activity of these metabolic organs. One of the first stages towards understanding the pathophysiology of metabolic illnesses like metabolic syndrome, diabetes, and dyslipidemia is to understand the biochemistry of carbohydrates and lipids.

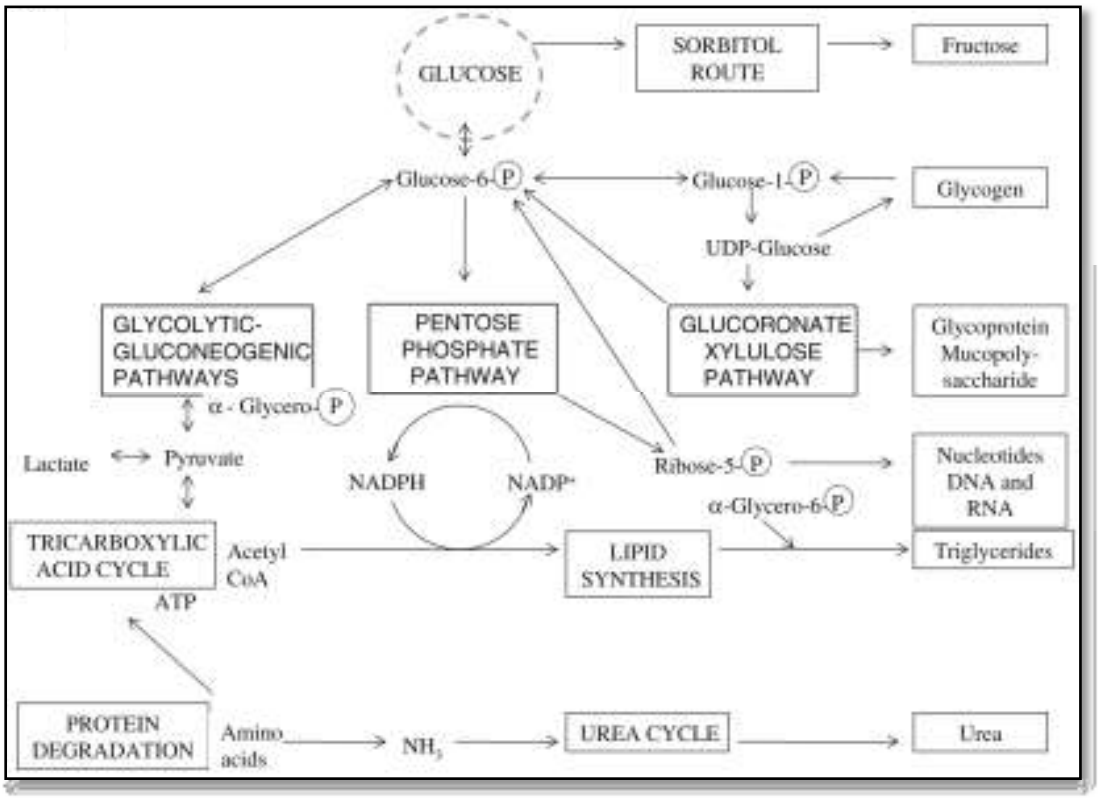


Figure 1: Central role of glucose in carbohydrate, fat and protein metabolism[28]

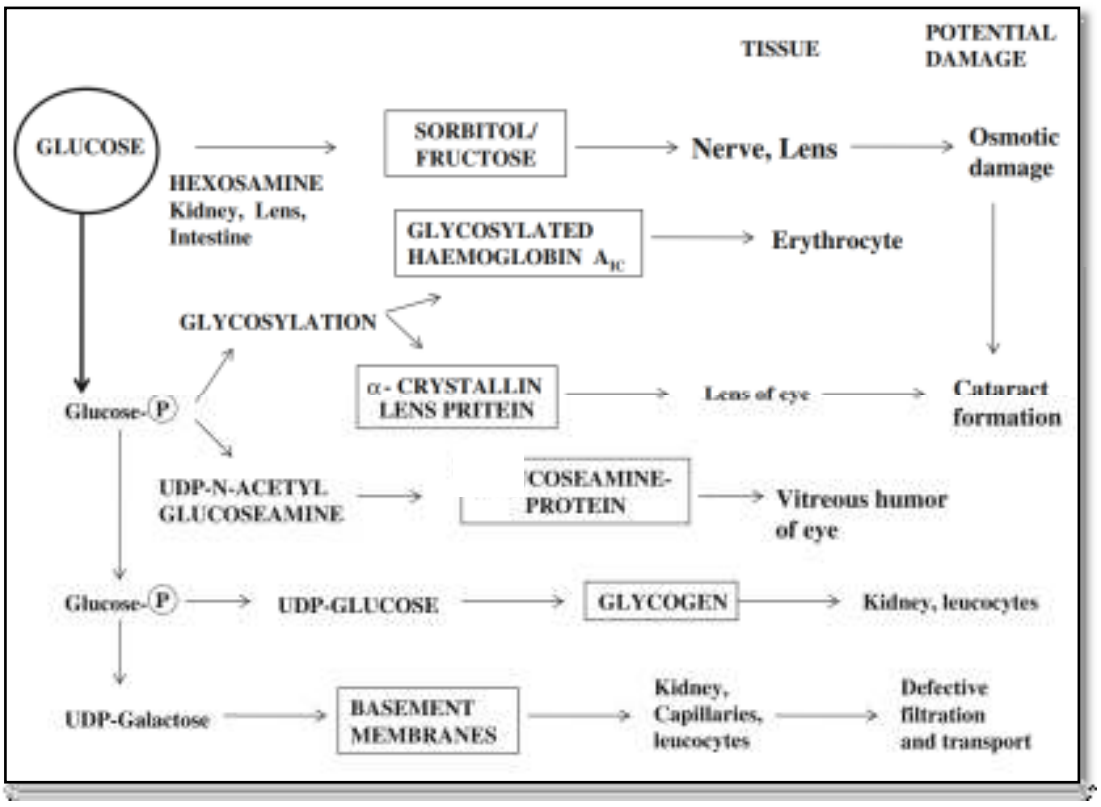


Figure 2: Glucose over-utilization and induced pathological changes in tissues resulting from non-insulin-requiring pathways. Inter-relationships among alternative routes of glucose metabolism [28]

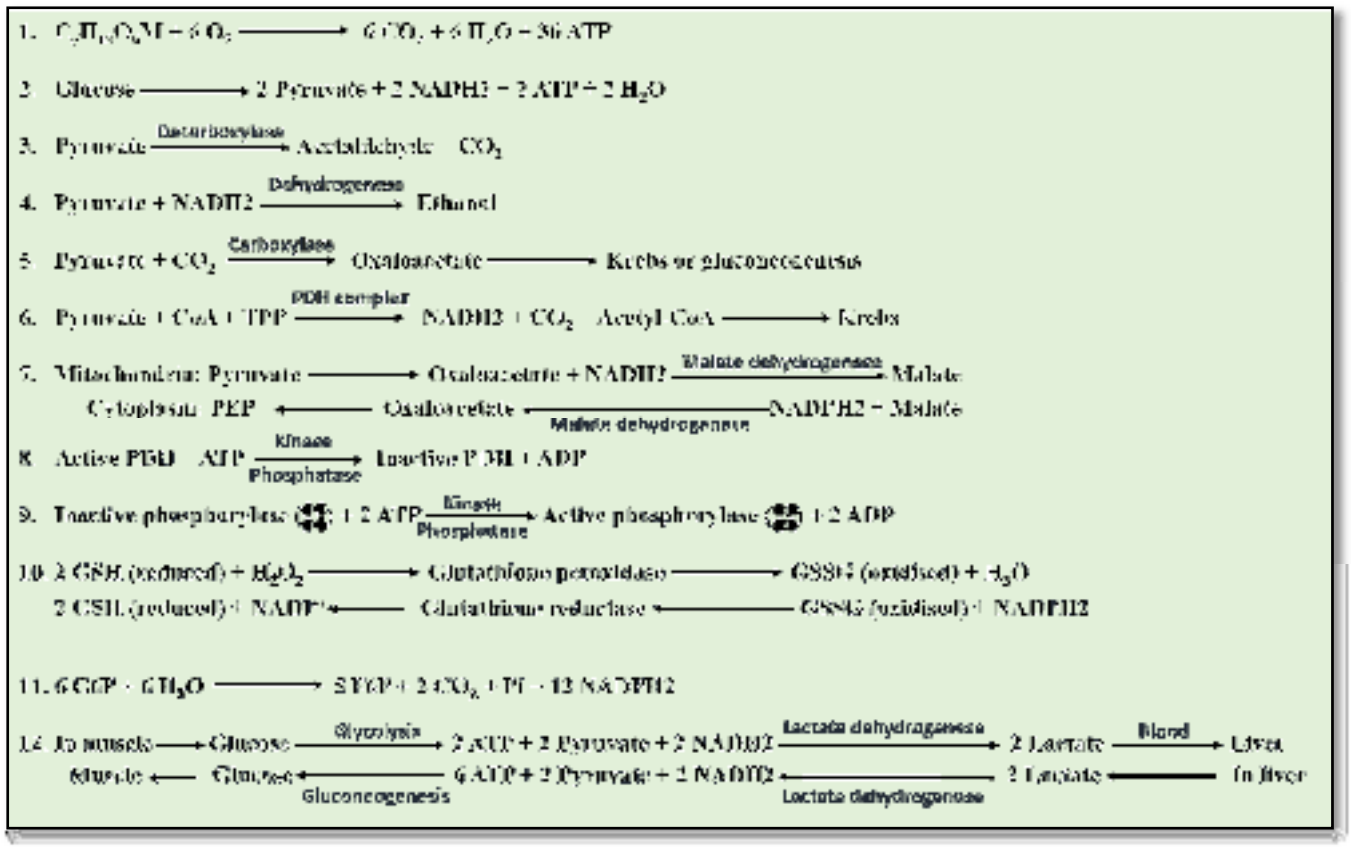


Figure 3: Summary of Biochemical Reactions in Carbohydrate Metabolism[27]

3.3 Brief pathophysiology of T2DM[29]

T2DM is an example of a complex, multifaceted disease. Environmental variables like sedentary lifestyle and food choices are undeniably important. Disease concordance is 35 percent to 60 percent in monozygotic twins, compared to nearly half in dizygotic twins, showing that genetic factors play a role in its etiology. This level of agreement is much higher than in type 1 diabetes, indicating that type 2 diabetes has a greater genetic component. More than a dozen susceptibility loci known as "diabetogenic" genes have been found in recent large-scale genome wide association studies, adding to the evidence for a genetic basis. Unlike T1D, however, there is no evidence of an autoimmune component, and the condition is not linked to immunological tolerance and regulatory genes (e.g., HLA, CTLA4).

T2D is characterised by two metabolic abnormalities: (1) a decreased ability of peripheral tissues to respond to insulin (insulin resistance) and (2) beta cell dysfunction, which appears as insufficient insulin production in the face of insulin resistance and hyperglycaemia. Insulin resistance often develops before hyperglycaemia, and in the early stages of diabetes, it is frequently accompanied by compensatory beta cell hyperfunction and hyperinsulinemia.

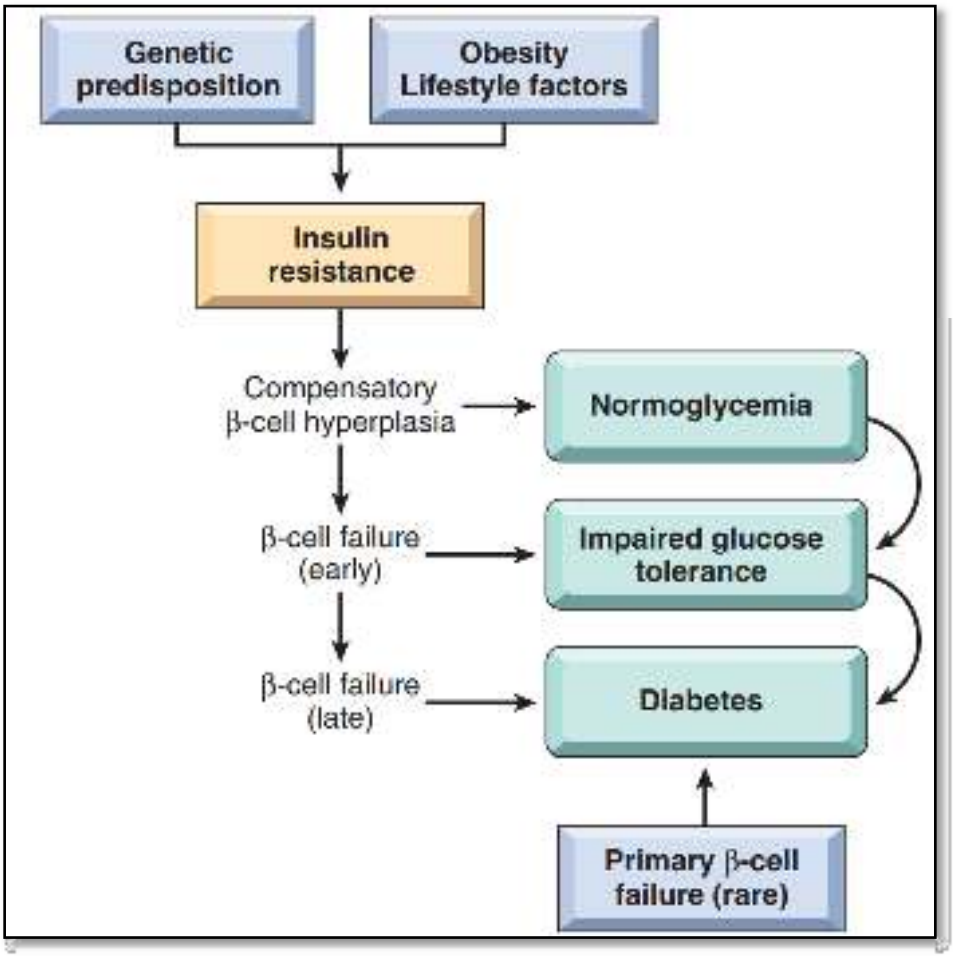


Figure 4: Pathogenesis of type 2 diabetes mellitus

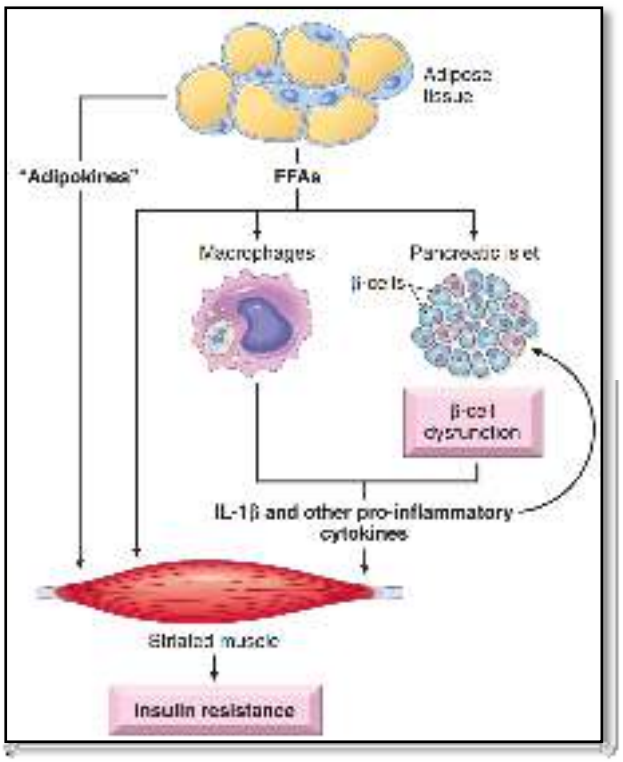


Figure 5: Mechanisms of β cell dysfunction and insulin resistance

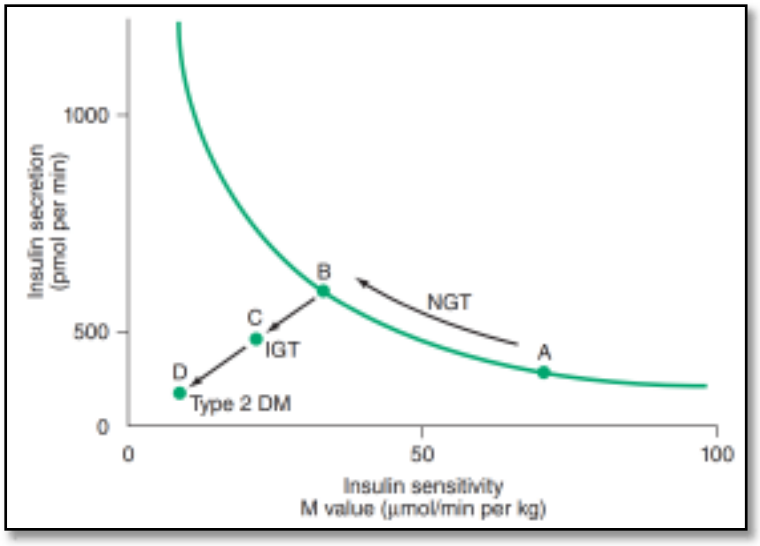


Figure 6: Metabolic changes during the development of T2DM[8]

Insulin secretion and insulin sensitivity are linked, because insulin secretion rises as an individual becomes more insulin resistant (by moving from point A to point B). Failure to compensate by increasing insulin secretion leads to impaired glucose tolerance (IGT; point C) and, eventually, T2DM (point D).

Table 1: Current Pharmacotherapy of T2DM[8]

Drug Class	Mechanism of Action	Example
Oral preparations		
Biguanides	Decreases hepatic glucose production	Metformin
Alpha-glucosidase inhibitors	Decreases gastrointestinal glucose absorption	Acarbose, miglitol, voglibose
Dipeptidyl peptidase IV (DPP-IV) inhibitors	Prolong endogenous GLP-1 action: -Increases insulin -Decreases glucagon	Alogliptin, linagliptin, saxagliptin, sitagliptin, vildagliptin
Insulin secretagogues: Sulfonylureas	Increases insulin secretion	Glibornuride, gliclazide, glimepiride, glipizide
Insulin secretagogues: Nonsulfonylureas	Increases insulin secretion	Mitiglinide, nateglinide, repaglinide
Sodium-glucose cotransporter 2 inhibitors	Increases renal glucose excretion	Canagliflozin, dapagliflozin, empagliflozin, ertugliflozin
Thiazolidinediones	Decreases insulin resistance, increases glucose utilization	Pioglitazone, rosiglitazone
Parenteral preparations		
Amylin agonists	Slow gastric emptying, decreases glucagon	Pramlintide
GLP-1 receptor agonists	Increases insulin, decreases glucagon, slow gastric emptying, satiety	Albiglutide, dulaglutide, exenatide, liraglutide
Insulin	Increase glucose utilization, decrease hepatic glucose production, and other anabolic actions	Aspart, glulisine, regular, detemir, glargine
Physical activity and Medical nutrition	Decrease insulin resistance, increase insulin secretion	Exercise, low-calorie, low-fat diet

3.4 Complications associated with T2DM[26]

The complications associated with T2D can be categorized as (1) acute and (2) chronic complications.

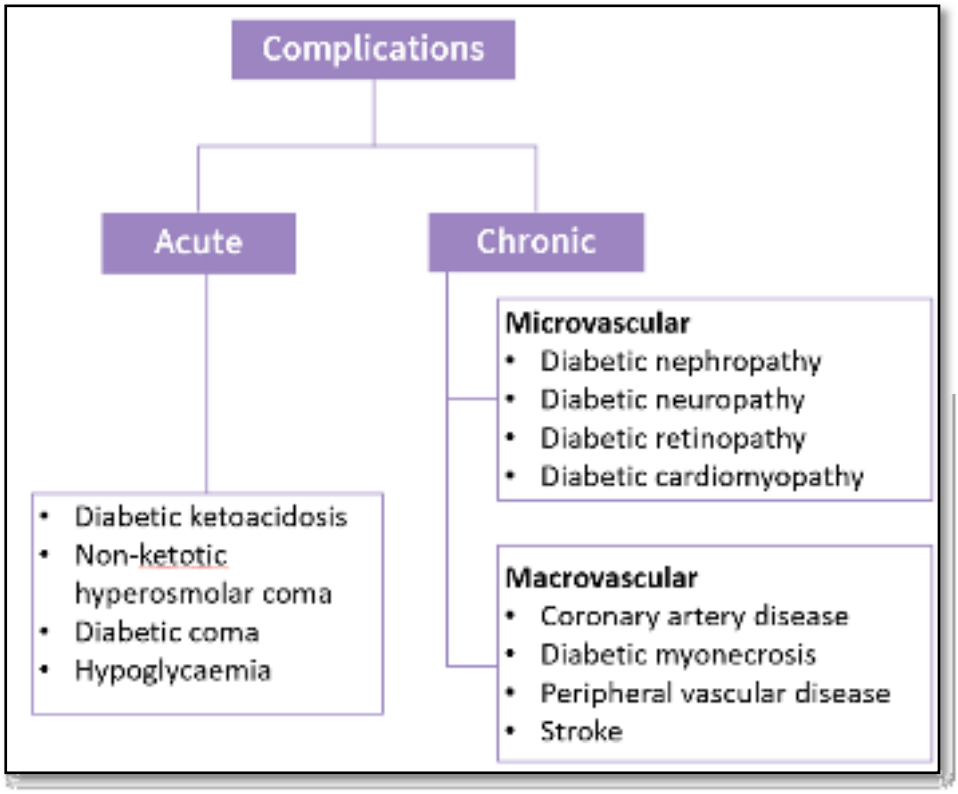


Figure 7: Complications of T2DM

3.5 Role of identified enzymes and selection of the genes

3.5.1 Pyruvate Kinase (Gene: L-PK)

Glycolytic enzymes are found in all mammalian cells or tissues because the glycolytic pathway is a crucial system for energy metabolism in organisms.[30] PK is a glycolytic enzyme that controls the rate at which pyruvate and ATP are produced from phosphoenolpyruvate and adenosine diphosphate (ADP). In mammals, PK is exists into four types: L-, R-, M1-, and M2-type isoenzymes. L-PK is primarily present in the liver, although it can also be found in the kidney, small intestine, and pancreatic -cells.[30,31] Fasting or diabetes reduces hepatic pyruvate kinase activity,

which is predominantly connected to the L-type isoenzyme, while a high carbohydrate meal or insulin treatment enhances it in diabetic rats.[32] The level of hepatic L-PK mRNA is also enhanced by a high-glucose diet, whereas it is lowered in streptozotocin-induced diabetic rats fed a high-glucose diet or in starved rats.[33,34] Metformin is known to suppress gluconeogenesis via lowering cellular ATP levels and indirectly increasing pyruvate kinase activity.[35] In contrast, another study found that activating AMPK reduced the transcriptional activity of the L-PK gene in hepatocytes.[36,37] As a result, pyruvate kinase still appears to be an essential therapeutic target in diabetic patients.

3.5.2 Glucose-6-phosphatase (Gene: G6pc)

One of the most important functions of the liver and, to a lesser extent, the kidney cortex is to provide glucose during times of hunger. In both tissues, glucose is produced from gluconeogenic precursors and additionally from glycogen in liver. It's also found in the -cells of pancreatic islets[38] and the intestinal mucosa of humans, mice, and rats,[39,40] especially in starvation and diabetes.[41] Both gluconeogenesis and glycogenolysis produce glucose-6-phosphate (Glc-6-P), which must be hydrolysed by glucose-6-phosphatase (G6Pase) before being released into the circulation as glucose. As a result, G6Pase plays a crucial role in blood glucose homeostasis.[42]

According to Cori et al., glucose is degraded through phosphorolysis to glucose-1-phosphate, which means the phosphate must be eliminated at a later step to generate free glucose.[43] According to de Duve and colleagues, the liver contains a phosphatase that can be extensively isolated by precipitation at pH 5 and acts exclusively on Glc-6-P.[42]

It has been postulated that G6Pase may additionally assist in phosphorylation of glucose when sugar concentrations are increased, as in diabetes, due to an extra phosphotransferase activity.[44] The fact that G6Pase depletion produces severe hypoglycaemia and that overexpression causes glucose intolerance shows that this enzyme is involved in glucose production in the body.[42]

Starvation and diabetes cause a 2–3 fold increase in G6Pase activity in the liver.[45–47] When glucocorticoids are administered *in vivo*, the activity of G6Pase in the livers of normal and adrenalectomized rats is enhanced to a small extent (by around 40%).[48,49] When the glucose concentration is elevated to 27.5 mM, G6Pase mRNA is increased 3-fold in Fao hepatoma cells and 20-fold in cultured hepatocytes.[50] In hepatoma cells overexpressing glucokinase, glucose boosts mRNA by up to 20-fold. In rats with diabetes caused by subtotal pancreatectomy, the effect of glucose *in vivo* was investigated. Normalizing glycaemia with insulin and phlorizin, which blocks tubular reabsorption and promotes urine excretion of glucose, can correct the 5-fold rise in G6Pase mRNA found in this scenario. These findings suggest that glucose may act as a direct inducer of G6Pase expression.[51] As a result, G6Pase appears to be an essential therapeutic target in diabetic patients.

3.5.3 Cholesterol-7α-hydroxylase (Gene: Cyp7a1)

Cholesterol is converted to bile acids in the liver.[52] Bile acids are physiological detergents that help with dietary lipid absorption and fat-soluble vitamin absorption, as well as the excretion and disposal of endogenous metabolites and xenobiotics through the biliary system. The principal bile acids produced in the human liver are cholic acid and chenodeoxycholic acid.[53] They're endogenous nuclear receptor ligands and signalling molecules that control lipid and glucose metabolism,

and they've been connected to fatty liver disease, obesity, and diabetes. Bile acids prevent the liver from producing triglycerides and gluconeogenesis.[54] These bile acid activities are predominantly mediated by the farnesoid X receptor (FXR), a bile acid-activated nuclear receptor. FXR activation by bile acids or synthetic ligands improves insulin sensitivity and hyperlipidemia in diabetic mice.[55]

In the liver, glucose appears to affect gene transcription. High blood sugar boosts bile acid synthesis and mRNA expression of the cholesterol-7α-hydroxylase (CYP7A1) gene, a key bile acid regulator. The AMP-activated protein kinase (AMPK) lowers CYP7A1 mRNA, hepatocyte nuclear factor 4 (HNF4) protein, and chromatin binding to the gene when it is activated. By raising ATP levels, glucose was discovered to inhibit AMPK and increase HNF4 to activate CYP7A1 gene transcription. Glucose enhances histone acetylation and lowers H3K9 di- and tri-methylation in CYP7A1 chromatin. Increased bile acid production in hyperglycaemia patients could be due to these epigenetic changes.[56]

The synthesis of bile acids in humans with diabetes is still poorly understood. The extensive metabolic changes that occur in diabetes patients, as well as the obvious species differences in bile acid production regulation in human and rodent models, make it difficult. However, a preliminary study discovered that the bile acid pool in diabetes patients with uncontrolled hyperglycaemia was significantly greater than in diabetic patients with regulated euglycemia. The increased de novo bile acid generation in hyperglycaemia people was assumed to be the cause of their larger bile acid pool.[57] Persistent epigenetic modifications caused by chronic exposure of hepatocytes to high glucose could be the reason of increased bile acid synthesis in hyperglycaemia, according to another study. Future clinical studies of hyperglycaemia

patients would provide additional insight into the mechanism of glucose and bile acid metabolism cross regulation in humans, due to the distinct species differences in bile acid synthesis regulation between humans and rodents, as well as the lack of a suitable *in vivo* model for studying glucose regulation of bile acid synthesis.[56]

Despite this, Cyp7a1 is a promising novel therapeutic target for the body's glucose balance. This could be another way by which specific medications aid in the regulation of insulin sensitivity and blood glucose in diabetic patients' bodies.

3.6 Plant profile: *Trigonella-foenum graecum* – also known as Fenugreek

Fenugreek has a long history in India, particularly in the Ayurveda and Unani traditions. Fenugreek seed powder taken orally may have hypoglycaemic and anti-hyperlipidemic characteristics, according to preliminary animal and human experiments. Fenugreek seeds contain 50% fibre (20% insoluble fibre and 30% soluble fibre), which can assist to slow down the rate of glucose absorption after a meal. This could be an unintended consequence of the hypoglycemic effect.[58] TFG has a wide range of therapeutic effects, as listed in the table below.

3.6.1 Various medicinal properties of TFG[59]

Anti-diabetic effect
Anti-inflammatory
Hypercholesterolaemic
Hypolipidemic effect
Antioxidant effect
Protective effect on GI tract & Liver
Immunomodulatory effect
Anti-hyperthyroidic effect
Digestive stimulant
Hepatoprotective
Galactogogue
Antibacterial & Antifungal
Antiulcer & Antilithigenic
Anticarcinogenic

3.6.2 Mechanism of blood glucose lowering property of TFG

Broca et al. discovered that 4-hydroxyisoleucine (4-OH-Ile), which an amino acid extracted and purified from TFG seeds, has *in vitro* insulinotropic activity, which is of considerable interest, and that its stimulating effect is related to glucose immolation in the medium, as demonstrated in isolated pancreatic beta cells.[60,61]

4-OH-Ile could be regarded a novel secretagogue with possible promise for the treatment of type 2 diabetes, a disease marked by defective insulin secretion and

various degrees of insulin resistance.[60,61] Streptozotocin-treated rats improved their diabetes state, according to Broca et al., at least in part due to a direct stimulating effect of 4-OH-Ile on beta cell function. These researchers showed that 4-OH-Ile can improve glucose tolerance in normal rats and dogs and raise insulin secretion *in vivo*, suggesting that 4-OH-Ile could be utilised to treat NIDDM.[60,61]

The TFG seed amino acid 4-OH-Ile increased glucose-induced insulin release in human and rat pancreas cells in vitro, according to Sauvaire et al. This amino acid appeared to act exclusively on pancreatic beta cells because somatostatin and glucose glucagon levels were unchanged.[22]

Insulin causes glucose transporter-4 (Glut-4) to translocate from an intracellular pool to the plasma membrane, promoting glucose uptake in muscle and adipose tissues. Glut-4 translocation is ineffective in diabetics due to a lack of insulin, and Glut-4 transporters stay inactive inside the cell. This causes a reduction in glucose absorption by muscle cells, contributing significantly to elevated blood glucose levels. As a result, restoring Glut-4 will result in normoglycemia. Anti-diabetic drugs such as insulin and TFG were used to successfully reverse the diabetes effect on the Glut-4 transporter to normal levels in experimental diabetes.[62]

In humans, fenugreek seeds have additional blood sugar lowering characteristics, such as reducing the activities of -amylase and sucrose.[63] They also lower blood levels of triglycerides, total cholesterol, and low-density lipoprotein cholesterol (LDL-C). Sapogenins, which enhance biliary cholesterol excretion in the liver, resulting in lower serum cholesterol levels, may be to responsible.[64,65] The lipid-lowering effect of fenugreek could be attributed to its oestrogenic component, which indirectly boosts thyroid hormone T4. As a result, dietary supplements that can

alter glucose homeostasis while also enhancing lipid indicators are intriguing. This is particularly true in people with metabolic syndrome who have diabetic markers. These people already have a problem with their glucose management and could benefit from a low-cost, food-based intervention aimed at bringing their metabolic environment back to normal. Fenugreek, a nutritional supplement, has been found to aid in this process.[28]

Table 2: Effect of TFG on diabetes mellitus in preclinical and clinical studies[59]

	Effect demonstrated
Animal models	
Rats	
Normal rats	• Hypoglycaemic effect • Prevention of diabetes induction • Anti-hyperglycaemia effect • Prevention of increase in glucose during glucose tolerance test
Diabetic rats	• Hypoglycaemic effect • Improved glucose tolerance • Anti-hyperglycaemia effect • Modulation of activities of gluconeogenic, glycolytic and lipogenic enzymes • Counteraction of hyperglycaemia, hyperinsulinemia, and glycated haemoglobin • Lowering of triglycerides and cholesterol
Mice	
Normal & Diabetic mice	• Hypoglycaemic effect
Rabbits	
Normal rabbits	• Hypoglycaemic effect
Diabetic rabbits	• Isolation of hypoglycaemic principle

	• Improved glucose tolerance, increased insulin levels, & hypolipidemic effect • Lowered fasting blood glucose & higher insulin secretion
Dogs	• Anti-diabetic effects
Others	
In vitro study	• Insulinotropic action of 4-hydroxyisoleucine
Human trials	
Normal & Diabetic humans	• Hypoglycaemic effect
NIDDM	• Hypoglycaemic effect, reduced insulin • Hypoglycaemic effect with no change in insulin • Improved glucose on glucose tolerance test • Reduced glucose excretion • Hypolipidemic effect • Reduction in glycated haemoglobin • Favourable effect on hypertriglyceridemia
IDDM	• Hypoglycaemic action • Improved glucose tolerance • Reduced glucose excretion • Hypolipidemic effect

Table 3: Effect of TFG treatment on physiological, biochemical and molecular parameters in diabetic rat tissue [28]

Parameters	**Diabetic**		**TFG**	
Body Weight	↓		↑	
Blood Glucose Level	↑		↓	
Insulin Level	↓		↑	
Carbohydrate metabolism	L	K	L	K
Glycolytic enzymes				
Hexokinase isoenzymes	↓	↑	↑	↓
Phosphofructokinase	↓	↑	↑	↓
Pyruvate Kinase	↓	↑	↑	↓
Lactate dehydrogenase	↓	↑	↑	↓
Gluconeogenesis				
Glucose-6-phosphatase	↑	↓	↓	↑
Fructose-1,6-bisphosphatase	↑	↓	↓	↑
PEPCK	↑	↓	↓	↑
Lipogenic				
G-6-P dehydrogenase	↓	↑	↑	↓
Malicenzyme	↓	↑	↑	↓
ICDH (NADP)	↓	↑	↑	↓
ATPCL	↓	↑	↑	↓
FAS	↓	↑	↑	↓
Antioxidant enzymes (Liver, brain, Muscle, Heart, Kidney)	↓		↑	

(a) Superoxide dismutase				
(b) Catalase				
(c) GPx				
(d) GR				
Lipid profile	↑	↑	↓	↓
Total lipids				
Triglyceride				
Cholesterol				
Lipid peroxidation (Liver, Kidney, Brain)	↑	↑	↓	↓
Malondialdehyde (MDA)				
Membrane fluidity	↓	↓	↑	↑
Membrane bound enzymes				
(a) Na^+K^+ATPase	↓	↑	↑	↓
(b) Ca^{2+}ATPase	↓	↑	↑	↓
Mitochondrial dehydrogenases				
ICDH-NAD	↑	↑	↓	↓
ICDH-NADP	↓	↑	↑	↓
MDH	↑	↑	↓	↓
GLDH	↑	↑	↓	↓
D-β-HBD	↓	↑	↑	↓
Membrane fluidity (Liver, brain, Muscle, Heart, Kidney)	↓		↑	
Glucose transporter-4 (Heart, Muscle, Brain)	↓		↑	

↑: increased; ↓: decreased

3.7 Animal Models of Type 2 Diabetes Mellitus

They can be broadly divided into 3 categories:

1. Chemically or Experimentally induced	• Adult STZ/Alloxan Models • Neonatal STZ/Alloxan Models • High Fat-diet Fed Models • Fat-Fed STZ Models • Nicotinamide STZ Models • Partial Pancreatectomised Models • Fructose Fed Models • Intrauterine Growth Retardation Models
2. Spontaneous or genetically derived animal models	• $Lep^{ob/ob}$ mouse • $Lepr^{db/db}$ mice • Zucker Fatty rats • Zucker Diabetic Fatty rats • OLETF rat • NZO mice • TallyHo/Jng mice • NoncNZO10/LtJ mice • Desert gerbil • Nile grass rat • GK rat • hIAPP mice • AKITA mice
3. Miscellaneous	• Steroid hormone induced • Drug induced

Table 4: Characteristics of T2D animal models

Model	Features
Adult STZ/Alloxan Models	Alloxan was initially used to induce T2D in 1943. Although it kills pancreatic beta cells, it has recognised side effects such as liver and kidney damage.[66,67] STZ is a naturally occurring antibiotic produced by the *Streptomyces achromogenes* bacterium. It is a structural analogue of N-acetyl glucosamine that acts as a strong alkylating agent, disrupting glucose transport and glucokinase activity while also causing DNA strands to break down. [67,68] Various doses of STZ or alloxan have been employed in the development of animal models over the years. From a single high-dose STZ injection (more than 65 mg/kg BW) to several low-dose STZ injections (35 mg/kg BW) have been used. In addition, STZ injection in conjunction with HFD is another variant. [69] STZ is typically given i.p. at different doses (35-65 mg/kg BW), whereas alloxan is injected at 40-200 mg/kg BW in rats. [70] Fasting or non-fasting hyperglycaemia, reduced serum insulin, and hyperlipidemia are the main characteristics.[70] Insulin resistance, on the other hand, is frequently absent, posing a restriction to this model.[70]
Neonatal STZ/Alloxan Models	Since the mid-1970s, neonatal alloxan and STZ animal models have been used. Following this, numerous attempts were made to refine the model, with the best success in using STZ at doses ranging from 25 to 50 mg/kg BW in male Spontaneous hypertensive rats (SHR). [71–73]

	STZ is administered intraperitoneally to newborn rats two days after birth. Until the fourth week, Fasting blood glucose (FBG) levels are normal to mildly hyperglycaemia. When the rats reach adulthood, their hyperglycaemia worsens. This results in adult-onset T2D mellitus. [74] There are variations in STZ dosages for different rodent strains, which must be recognised when using these models for research. [75,76] It has been reported that using alloxan is a superior model for maintaining diabetes conditions than the STZ – induced model.[76] The main characteristics include mild to severe hyperglycaemia, elevated A1C values, increased glucose excretion in urine, and increased food consumption.[73] This method's diabetes pathology has been found to be more sustainable over time (52 weeks). As a result, the pathophysiology of many diabetic issues can be studied in greater depth using these models.[73] However, because diabetes takes a longer period to develop (at least 12 weeks), this may not be suited for fast and routine pharmacological testing of anti-diabetic drugs or natural treatments. Another disadvantage is that these models have not been validated by anti-diabetic medicines, limiting their usefulness as T2D models.[70]
High Fat-Diet Fed models	This approach was originally used in the late 1980s. The premise of this model was that, because obesity is a major contributor to the development of T2D, feeding rodents a HFD (40–60% of total calories) would result in a comparable physiology. [77–79]

	C57BL/6 J mice were also used to develop this model; however, while insulin resistance develops, beta cell failure does not occur, resulting in a large increase in beta cell proliferation. [77–79] Overweight, obesity, impaired glucose tolerance, and insulin resistance are the main characteristics. [80] The amount and type of fat consumed, as well as the time of feeding, all influence the degree of hyperglycaemia. An extended period of HFD feeding improves the characteristics. [78,80] There are strain differences in rodents, with SD rats being more sensitive to HFDs causing insulin resistance and diabetes than other strains. [80,81] The length of time (> 10 weeks) necessary to produce the primary pathology of T2D makes this model inappropriate for many researchers because it raises the cost of the experiment. [69]
Fat-Fed STZ Models	This model was originally developed in 1947.[82] Animals are fed a high-fat diet to establish insulin resistance, followed by an injection of STZ to produce partial pancreatic beta cell failure.[83] The advantage of this model over genetic models is that it replicates natural pathophysiology while also exhibiting symptoms similar to T2D in humans. [83] Researchers used various approaches to establish the fat-fed-STZ rat paradigm, with varying percentages of total calories (from 30, 40, and 58 percent) in the diet and varying dosages of STZ (15, 25, 30, 35, 45, 50, and 55 mg/kg BW). [70]

	Insulin resistance, hyperglycaemia, poor glucose tolerance, hyperinsulinemia, and hyperlipidemia are the main symptoms. [70,84] Despite numerous adjustments, using an HFD with a low to moderate dose of STZ has demonstrated a higher overall success rate than many other strategies, making it a good model for researchers to induce T2D in rats. [85]
Nicotinamide STZ Models	The rationale behind this hypothesis is that STZ causes DNA damage, which activates DNA repair pathways that require significant levels of nicotinamide adenine dinucleotide. The administration of nicotinamide protects pancreatic beta cells from the damage caused by STZ. [86] The initial model used 230 mg/kg BW nicotinamide (i.p.) 15 minutes before administering 65 mg/kg BW STZ (i.p.) into 3-month-old male Wistar rats. [86] Non-fasting hyperglycaemia, impaired glucose tolerance, and insulin responses are the main characteristics. 40% of pancreatic insulin storage is preserved. A useful model for drug screening, particularly for non-obese T2D. [86,87]
Partial Pancreatectomised models	These models were created to help avoid alloxan-induced liver and kidney damage. [85] Main characteristics: mild to moderate hyperglycaemia after 4 days of surgery that can last up to 6 weeks. [86] The benefit of this model is that it mimics T2D by having a decreased pancreatic beta cell mass. [88]

	The main disadvantages of this model are (1) regeneration of the remaining pancreas, but for research into the adaptive processes of beta cells, this model may be very useful[86], and (2) the occurrence of digestive issues due to excision of the exocrine pancreas, resulting in an amylase enzyme deficiency. [88] Another significant constraint is the demand for significant technical and surgical expertise.
Intrauterine Growth Retardation (IUGR) model	IUGR has been linked to the onset of disease later in life, including obesity, hypertension, and T2D. [89,90] In newborns, IUGR causes significant losses in pancreatic beta cell mass, which does not recover in adulthood, resulting in impaired glucose tolerance and the development of T2D. [91] The advantage of this model is that it mimics the natural path of illness by causing hyperglycaemia and pancreatic beta cell loss early in life, which is preceded by insulin resistance. [91] This is caused by bilateral uterine artery ligation, which results in a partial reduction in blood flow to the foetus. [91] Insulin resistance, hyperglycaemia, hyperinsulinemia, obesity, and decreased beta cell mass are the main characteristics. [85] The following are significant limitations of this model: (1) other diabetes parameters such as lipid profile, kidney and liver function, and anti-diabetic treatment responses have not been recorded, putting its validity as a T2D model at jeopardy. (2) Effective surgery necessitates surgical and technical expertise. [85]

Zucker Fatty (ZF) / Zucker Diabetic Fatty (ZDF) rats	ZF rats were created in 1961 as a result of a cross between Merck M-strain and Sherman rats. [92] They were discovered to have a faulty leptin receptor, which resulted in hyperphagia and the formation of obese rats at roughly 4 weeks of age. [92] Rats develop hypertension, hyperinsulinemia, and hyperlipidemia as well. They also demonstrated a lack of glucose tolerance. [88,92] The ZDF strain was discovered after a mutation in the ZF strain. These rats are less obese but have increased insulin resistance. Diabetes develops in males between the ages of 8 and 10 weeks, whereas diabetes does not occur in females. [88,93]
Otsuka Long-Evans Tokushima Fat (OLETF) Rats	This rat was created after 18 weeks of selective breeding at Tokushima Research Institute from a naturally diabetic rat discovered in an outbred colony of Long Evans Rats in 1984. [94] Males are more likely than females to inherit diabetes. [94] There are three stages of histological alterations in pancreatic islets. [94] These rats exhibit renal complications as well. [95]
Nile Grass Rat	The Nile grass rat, *Arvicanthis niloticus*, has been recommended as a model for metabolic syndrome. [96] When fed a standard chow diet in captivity, these rats develop obesity, dyslipidemia, and hyperglycaemia by the first year of life. [97] Other symptoms include decreased beta cell mass, atherosclerosis, and liver steatosis. [97]
Goto–Kakizaki rats	A Japanese group developed GK rats by repeatedly breeding Wistar rats with the lowest glucose tolerance. [98]

	As a result, a slim model of T2D with glucose intolerance and poor glucose-induced insulin production was created. [98] In this paradigm, aberrant beta cell mass and/or function is assumed to be the primary cause of hyperglycaemia. [99,100] Researchers employ these rats in a variety of experiments ranging from T2D beta cell failure to diabetic consequences. [97]
Corticosteroid induced	When the adrenal cortex in rodents is stimulated by corticotrophin, it secretes large levels of steroid, culminating in the production of steroid diabetes. [101] Dexamethasone and prednisolone are the most prevalent glucocorticoids that cause steroid diabetes. [101] They enhance gluconeogenesis and inhibit insulin action, resulting in an increase in hepatic glucose production. Insulin resistance, hyperglycaemia, and hyperlipidemia are also present. [101]
Atypical antipsychotic induced diabetic model	When given for 60 days, atypical antipsychotics such as olanzapine (10 mg/kg i.p.) induce significant increases in blood glucose levels, LDL and total cholesterol in rats. [101]

Table 5: Classification of rodent models of T2DM[88,102]

Model Category	Obese	Non-Obese
Spontaneous or genetically derived diabetic animals	• ob/ob mouse • db/db mouse • KK mouse • KK/Ay mouse • NZO mouse • NONcNZO10 mouse • TSOD mouse • M16 mouse • Zucker fatty rat, • ZDF rat • SHR/N-cp rat • JCR/LA-cp rat • OLETF rat • Obese rhesus monkey • Bio breeding rats (BB) • WBN/KOB rats • WDF/TA-FA rat	• Cohen diabetic rat • GK rat • Torri rat Non obese C57BL/6 • (Akita) mutant mouse • ALS/Lt mouse
Diet/nutrition induced diabetic animals	• Sand rat • C57/BL 6J mouse • Spiny mouse	
Chemically induced diabetic animals	• GTG treated obese mice	• Low dose ALX or STZ adult rats, mice, etc. • Neonatal STZ rat
Surgical diabetic animals	• VMH lesioned dietary obese diabetic rat	• Partial pancreatectomized animals, e.g. dog, primate, pig & rats
Transgenic/knockout diabetic animals	• β3-receptor knockout mouse • Uncoupling protein	• Transgenic or knock out mice involving genes of insulin and insulin

	(UCP1) knock-out mouse	receptor and its components of downstream insulin signalling e.g. IRS-1, IRS-2, GLUT-4, PTP-1B and others • PPAR-g tissue specific knockout mouse, • Glucokinase or GLUT 2 gene knockout mice, • Human islet amyloid polypeptide overexpressed rat (HIP rat)
KK, Kuo Kondo; KK/Ay, yellow KK obese; VMH, ventromedial hypothalamus; ZDF, Zucker diabetic fatty; NZO, New Zealand obese; TSOD, Tsumara Suzuki obese diabetes; SHR/N-cp, spontaneously hypertensive rat/NIH-corpulent; JCR, James C Russel; OLETF, Otuska Long Evans Tokushima fatty; GTG, gold thioglucose; ALX, alloxan; STZ, streptozotocin; GLUT-, glucose transporter; IRS, insulin receptor substrate; GK, Goto-Kakizaki; PPAR, Peroxisome proliferator activated receptor, PTP, phosphotyrosine phosphotase; ALS, alloxan sensitive		

Table 6: Advantages and Disadvantages of different categories of T2DM animal models[88]

Model Category	Advantages	Disadvantages
1. Spontaneous diabetic animals	Type 2 diabetes development involves genetic variables and similarities to human T2DM The use of mostly inbred animal models, where the genetic background is homogeneous and environmental influences can be controlled, makes genetic dissection of this complex disease relatively simple. The variability of the results may be minimal, enabling a small sample size.	In contrast to human heterogeneity, diabetes has a strongly inbred, homogeneous, and primarily monogenic inheritance pattern, and its development is mostly genetically determined. For the diabetic study, there is a limited supply and it is costly. In animals with brittle pancreas (db/db, ZDF rat, etc.), mortality due to ketosis is significant, and survival requires insulin administration later on. Requires sophisticated upkeep
2. Diet/Nutrition induced diabetic animals	As in the diabesity syndrome of the human population, develop diabetes connected with obesity as a result of overnutrition. Chemical toxicity to other essential organs of the body can be avoided.	Dietary treatment is usually required for a long time. Simple dietary treatment causes no frank hyperglycaemia in genetically normal animals, making it unsuitable for evaluating antidiabetic drugs on the circulating glucose measure.
3. Chemical induced diabetic animals	Pancreatic beta cells are selectively lost (alloxan/STZ), leaving other pancreatic alpha and delta cells unaffected. Animals due to residual insulin secretion live longer without insulin therapy.	Rather than being a result of insulin resistance, hyperglycaemia is caused by direct cytotoxic impact on the beta cells and insulin insufficiency. Chemically induced diabetes is usually less persistent and, in

	Ketosis & resulting mortality is relatively less In comparison, it is less expensive to develop and maintain.	some cases, reversible due to beta cell regeneration. As a result, during long-term trials, attention must be made to assess pancreatic beta cell activity. Aside from its cytotoxic effect on beta cells, chemicals have harmful effects on other vital organs. The degree of variability in the results on the development of hyperglycaemia is perhaps substantial
4. Surgical diabetic animals	Chemical diabetogens' harmful effects on other body organs are avoided Because of the reduced islet beta cell mass, it resembles human type 2 diabetes	Complicated technical and post-operative procedures are involved. Other digestive issues (as a result of part of the exocrine component being removed – amylase enzyme insufficiency) Dissection of alpha islets (glucagon secreting cells) too along with beta cells can lead to issues in counter regulatory response to hypoglycaemia Mortality is comparatively higher
5. Transgenic/knock out diabetic animals	*In vivo* research can be done on the effects of a single gene or mutation on diabetes. Type 2 diabetes genetics becomes easier to dissect.	Production and maintenance procedures are extremely complex and expensive. Expensive for regular screening experiments

3.8 Streptozotocin induced type 2 Diabetes Mellitus

Of the no. of agents used for induction of DM listed above, STZ is one of the most widely used agent.

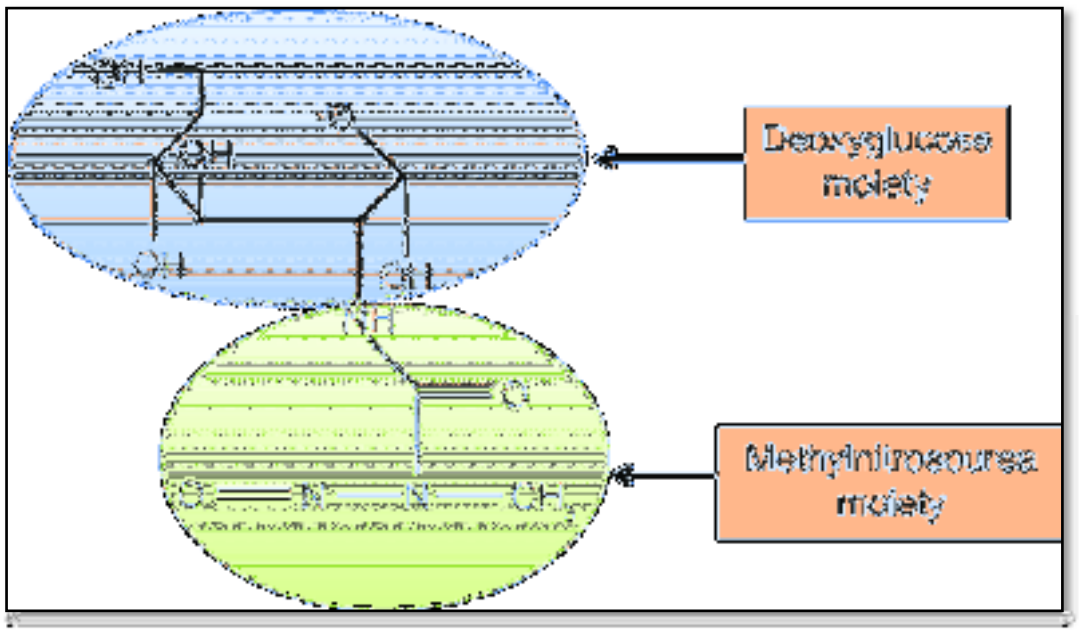


Figure 8: STZ molecule[103]

3.8.1 Mechanism of action of STZ:[104]

STZ [2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose] is taken up by the pancreatic β cells via glucose transporter GLUT2. STZ's intracellular activity causes DNA to be alkylated and fragmented. The nitrosourea moiety of STZ is responsible for its alkylating action, particularly at the O6 position of guanine. *Streptomycetes Achromogens* synthesise it.

The impact of streptozotocin on beta cells is characterised by changes in blood insulin and glucose concentrations. Hyperglycaemia is noticed two hours after injection, along with a reduction in blood insulin. Hypoglycemia develops six hours later as a result of the release of excessive levels of stored insulin into the

bloodstream. Finally, hyperglycaemia sets in, resulting in a drop in blood insulin levels.

The aberrations in cell activity are reflected in the variations in blood glucose and insulin concentrations.

3.8.2 Pathophysiology of STZ induced Diabetes Mellitus

GLUT-2 transports streptozotocin (STZ) into the pancreatic -cell, since it acts as a glucose analogue. It causes DNA alkylation and over-activation of poly-ADP ribose polymerase (PARP), resulting in NAD+ depletion, reduced cellular ATP, and compromising insulin.[105]

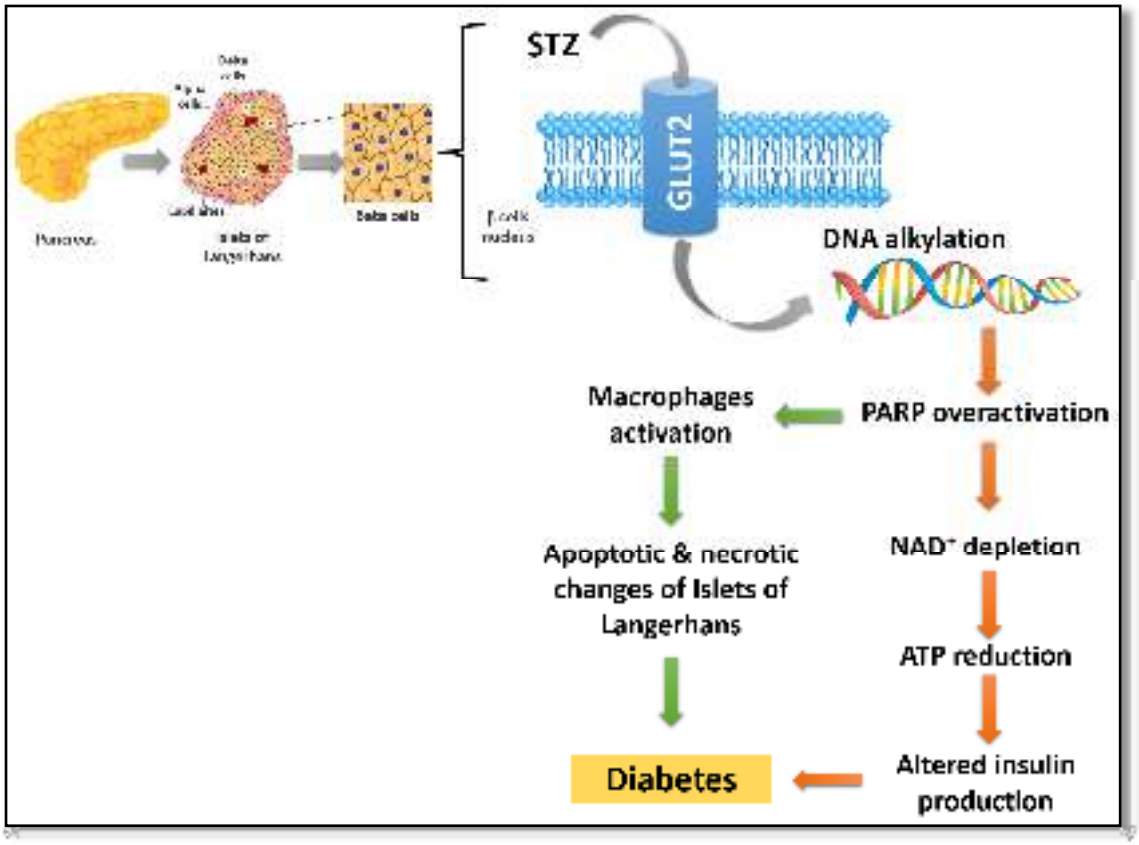


Figure 9: Pathophysiology of STZ induced DM

METHODOLOGY

Reporting of methodology is done as per the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines 2.0 for reporting preclinical research.[106]

4.1 Study Design:

This study was an experimental study done to determine the gene expression levels of selected genes in liver tissue of with 6 normal and 30 diabetic male Wistar rats receiving vehicle, metformin, TFG or metformin and TFG both.

Rats were divided in five groups randomly with a mean weight of 180 ± 20 gm.

4.2 Sample Size:

The sample size was calculated based on the 'resource equation' approach [107]:

$n = DF/k + 1$

DF – degrees of freedom

k = number of groups

n = number of subjects per group

Based on the acceptable range of the DF, viz. between 10 – 20,

Minimum number of animals per group → $n_{min} = 10/5 + 1 = 3$

Maximum number of animals per group → $n_{max} = 20/5 + 1 = 5$

Hence, for this study, between 3 and 5 animals per group were required. In other words, a total of 15 to 25 animals were required to keep the DF within the range of 10 to 20.

Furthermore, there are two more reasons why animal experiments on 5-6 rats per group are norm than exception. One, experimental animals are much more homogenous such as of same strain – thus inter-individual variability is small. Second, laboratory conditions are fairly well standardised, and the factors are under good control. Thus, any difference found between groups can be legitimately ascribed to the treatment.[108]

The final sample size was kept at 38 as sanctioned by the IAEC, with the following division per group owing to the mortality or non-induction of diabetes, if any, experienced by an animal during the study.

The division of groups with the doses of the respective treatments is given as follows:

Table 7: Division of Groups

Groups
Group 1: Normal Control (NC) (6)
Group 2: Diabetic Control (DC) (8)
Group 3: Standard Metformin (MET) (8)
Group 4: Test Fenugreek (TFG) (8)
Group 5: Test Combination (CBN) (8)

4.3 Inclusion and Exclusion criteria:

The animals were included in the study if they successfully developed diabetes, defined by blood sugar level of 200 mg/dl or more. [109–111]

The animals were excluded if they failed to develop diabetes. (blood glucose level of less than 200 mg/dl), or if the animal died prematurely, preventing the collection of further parameters (body weight and blood glucose level) and liver tissue at the end of the study.

Thirty-eight rats were randomized into the study. Out of this 6 were randomised in normal group and rest 32 underwent experimental induction of diabetes as per protocol. 26 rats were able to satisfy the inclusion and exclusion criteria and randomised into rest of the groups as follows:

DC: 6 rats; MET: 6 rats; TFG: 7 rats; CBN: 7 rats

Final statistical analysis was performed on a total of 29 rats as 3 rats died prematurely during the study: two from the TFG group and one from the CBN group.

4.4 Randomisation:

Randomisation was carried out as follows. Thirty-six 3-4 month old male Wistar rats, weighing 180 ± 20 gm, were obtained from Central Animal House of J. N. Medical College, Belagavi. At first 6 rats were randomly divided into one group as normal control. After successful induction of diabetes, 29 rats were then randomly divided into 4 groups (DC: 6 rats; MET: 6 rats; TFG: 7 rats; CBN: 7 rats). Random numbers were generated using computer based random number generator.

4.5 Blinding:

Blinding was introduced to eliminate observer bias. It was achieved by coding and masking of all the drugs used in the experiment by the guide before starting the experiments.

4.6 Outcome measures:

The following parameters were assessed:

- Random Blood Sugar (Day 1 and Day 30 – end of treatment)
- Body weight (Day 1 and Day 30 – end of treatment)
- Gene expression of L-PK gene in liver tissue
- Gene expression of G6PC gene in liver tissue
- Gene expression of Cyp7a1 gene in liver tissue

4.7 Statistical methods

Data was expressed as mean ± standard deviation (SD)

The level of significance was set at $p < 0.05$

Results were analyzed using GraphPad Prism Version 9 (San Diego, USA).

Student's paired *t* test was used to compare the following within each group:

1. RBS levels within each group on Day 1 (before the start of treatment) and Day 30 (end of treatment).
2. Body weight on Day 1 and Day 30.

Differences between various groups for the following parameters were analyzed using one-way Analysis of Variance (ANOVA) followed by post hoc Tukey's test.

1. RBS levels on Day 1 and Day 30
2. Body weight on day 1 and Body weight on day 30
3. Gene expression analysis for the selected genes

4.8 Experimental Animals:

3 – 4-month-old healthy male Wistar rats with a mean weight of 180 ± 20 gm were supplied by the Central Animal House of the institution.

Animals were housed under standard conditions and acclimatized to 12-h light/dark cycle for 10 days prior to the day of experimentation. They had free access to food and water ad libitum prior to the dietary manipulation.

The study was approved by the Institutional Animal Ethics Committee (IAEC) [Annexure 1] and conducted as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi for use, care and sacrificing of experimental animals.

4.9 Experimental Procedures:

4.9.1 Experimental induction of Diabetes:

Type-2 diabetes was induced in rats according to previous reports with slight modifications.[109,112,113]

Rats were given an HFD for two weeks before receiving a single low dosage (30 mg/kg) of STZ intraperitoneally. After diabetes induction, STZ-treated rats were

given 5% glucose instead of water for 24 hours to minimise hypoglycaemic shock-related mortality. To evaluate glucose levels, blood samples were taken from the tail vein 72 hours after STZ. Diabetic rats were defined as those with fasting blood glucose levels of more than 200 mg/dl[109–111] and were included in further study.

4.9.2 Preparation of STZ:[114]

40 mg STZ was weighed in a glass beaker and the beaker was covered with aluminium foil. Fresh citrate buffer of 0.05M at a pH of 4.5 was prepared immediately prior to injection.[115] (To prepare 0.05M citrate buffer – mix 0.05M sodium citrate & 0.05M citrate acid in a ratio of 2:3 → adjust the pH to 4.5). Immediately prior to injection, STZ was dissolved in the sodium citrate buffer to a final concentration of 40 mg in 7 ml.

The STZ solution was prepared immediately before injection and administered within 5 min of dissolution.

Using a 1-ml syringe and 23-G needle, STZ was injected intraperitoneally (i.p.) into the rats belonging to various experimental group at 30 mg/kg (5.25 ml/kg). An equal volume of citrate buffer was injected intraperitoneally into the control animals. 5% glucose water was given to rats for 24 hrs.

The body weight and blood glucose levels were measured at the beginning of study, onset of diabetes (Day 1) and at the end of study (Day 30). Body weight was measured using Essae Teraoka Pvt Ltd. digital weighing scale. Blood glucose was measured using Accu-Check Glucometer.

The composition of HFD is as follows:

Table 8: Composition of High Fat Diet [HFD]

Ingredients	Diet (g/kg)
Sodium Chloride	01
Yeast Powder	01
DL-Methionine	03
Vitamin & Mineral Mix	60
Cholesterol	10
Casein	250
Lard	310
Powdered Normal Pellet diet (NPD)	365

Figure 10: High-fat Diet fed to Diabetic Rats

4.9.3 Treatment Schedule

The confirmation day of diabetes was considered as day 1 of diabetes. Total of 32 rats were used and divided into five groups and received the following treatment according to Table 7.

Table 9: Number of rats per group with treatment schedule

Groups	Treatment	Dose
Group 1: Normal Control (6)	Vehicle only	0.5ml
Group 2: Diabetic Control (6)	Vehicle only	0.5ml
Group 3: Diabetic Rats + Metformin (6)	Metformin	180 mg/kg
Group 4: Diabetic Rats + Fenugreek (7)	Fenugreek	1 g/kg
Group 5: Diabetic Rats + Metformin + Fenugreek (7)	Metformin + Fenugreek	180 mg/kg + 1 g/kg

The dose of metformin was calculated based on the standard therapeutic human daily dose of 2g/day.[116] A dose of 1 g/kg of *Trigonella foenum-graecum* extract was administered to Groups 4 & 5.[117] All the drugs were administered orally as a single daily dose for a period of 30 days. All the experimental rats were given their respective diets (Normal – Group 1 or High fat – Groups 2 to 5).

Vehicle was distilled water. Metformin and TFG both were mixed in appropriate quantities of distilled water so as to administer their respective doses in 1 ml of formulation.

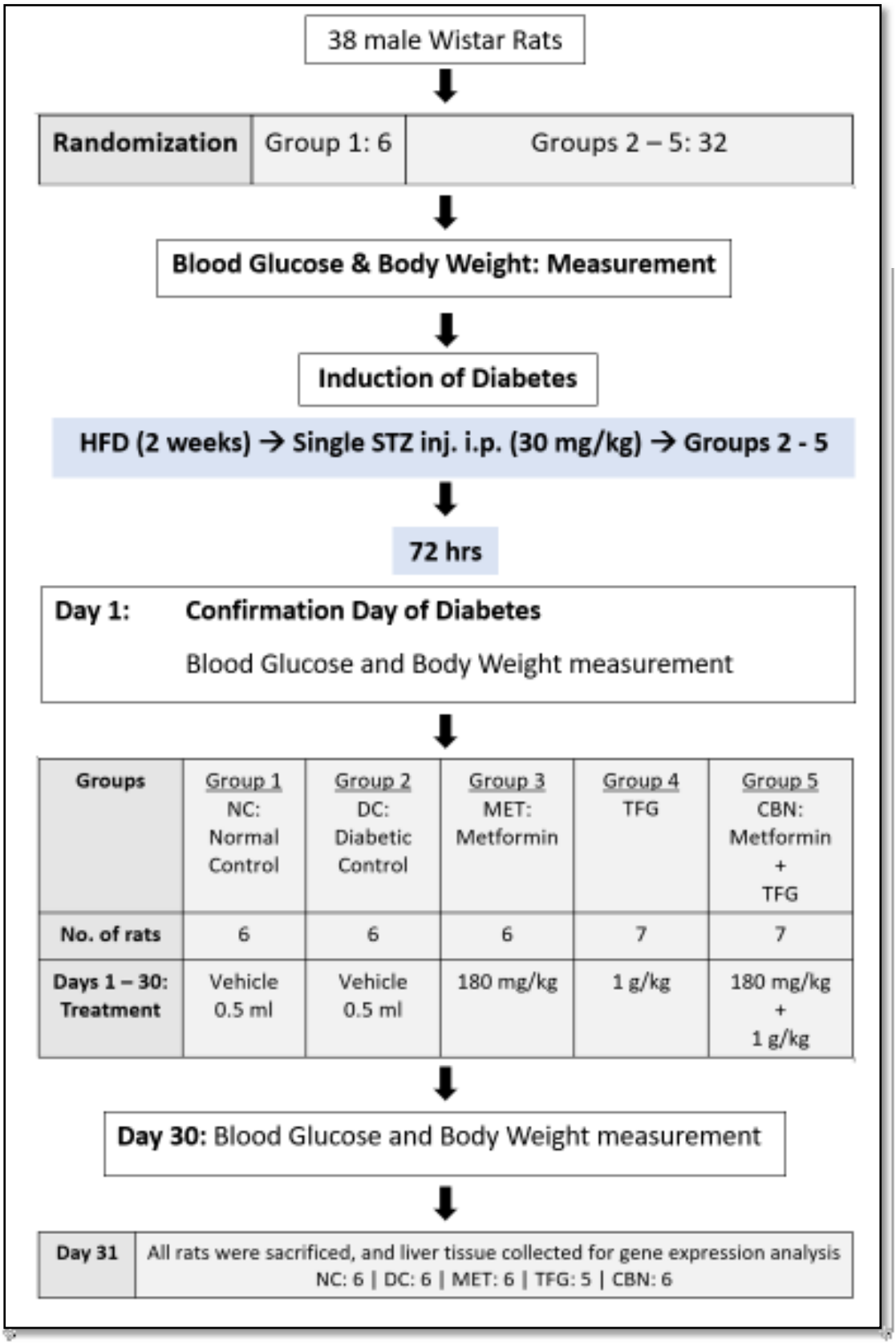


Figure 11: Design of the experimental study

4.9.4 Test Drugs, Reagents and Kits:

- Metformin and Thiopentone sodium were procured from the pharmacy of the hospital attached to the medical college.
- Streptozotocin (Cayman Chemical Company, CatLog no. 13104) was procured from Everon Lifesciences, New Delhi.
- TFG extract (60% hydro-alcoholic extract) was procured from Natural Remedies, Bangalore as a free sample.
- High-Fat Diet was purchased from VRK Nutritional Solutions, Sangli, Maharashtra.
- Accu-Chek Glucometer was purchased from hospital pharmacy.
- RNeasy Mini Kit (Qiagen - CatLog No. 74104), PrimeScript™ 1st strand cDNA Synthesis Kit (Takara - CatLog No. 6110A), SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara – CatLog No. RR820A) and Sample Protector for RNA/DNA (Takara – CatLog No. 9750) were procured from Juniper Lifesciences, Bangalore.
- Primers for the selected genes were procured from Bioserve biotechnologies India Pvt Ltd, Hyderabad.

Figure 12: Hydro-alcoholic extract of *Trigonella foenum-graecum* (60%)

Figure 13: Streptozotocin

4.9.5 Euthanasia: Animals were sacrificed using an overdose of anaesthesia as per the CPCSEA guidelines of euthanasia.[118] Thiopentone sodium at a dose of 120 mg/kg (3 – 4 times the normal anaesthetic dose) given as an intraperitoneal injection.[119] The animals underwent euthanasia on day 31 after completion of treatment schedule. Liver tissue was collected immediately after euthanasia from each animal and stored in sample protector for RNA/DNA until further analysis.

4.9.6 Gene Expression Analysis

RNA extraction: RNA was extracted from tissue specimen by using The RNeasy Mini Kit (Cat. no. 74104, Qiagen, Hilden, Germany). The stepwise protocol is as follows:

1. Not more than 30 mg tissue was used. The tissue was disrupted and lysate homogenized in 350 µl of Buffer RLT. The lysate was centrifuged for 3 min

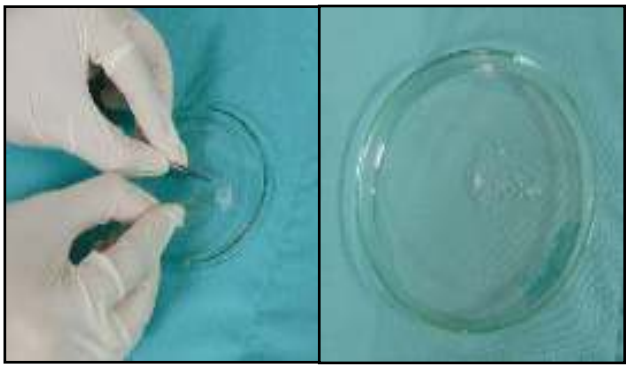
at maximum speed. The supernatant was carefully removed by pipetting, and used in step 2.

2. 1 volume of 70% ethanol was added to the lysate, and mixed well by pipetting. Immediately step 3 was undertaken.
3. Up to 700 µl of the sample was transferred, including any precipitate, to an RNeasy Mini spin column placed in a 2 ml collection tube . The lid was closed, and centrifuged for 15 s at ≥8000 x g. The flow-through was discarded.
4. 700 µl Buffer RW1 was added to the RNeasy spin column. The lid closed, and centrifuged for 15 s at ≥8000 x g. The flow-through was discarded.
5. 500 µl Buffer RPE was added to the RNeasy spin column. The lid closed, and centrifuged for 15 s at ≥8000 x g. The flow-through was discarded.
6. 500 µl Buffer RPE was added to the RNeasy spin column. The lid closed, and centrifuged for 2 min at ≥8000 x g.
7. The RNeasy spin column was placed in a new 1.5 ml collection tube. 30 µl RNase-free water was added directly to the spin column membrane. The lid was closed, and centrifuged for 1 min at ≥8000 x g to elute the RNA.

Figure 14: RNeasy Mini Kit (Cat. no. 74104, Qiagen, Hilden, Germany)

Figure 15: Microcentrifuge tube and Spin column

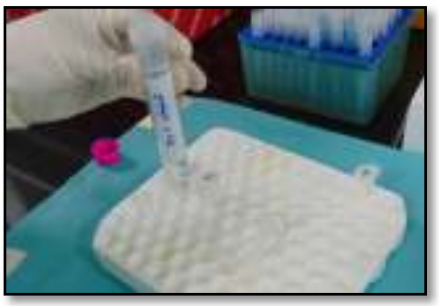
Figure 16: RNA extraction



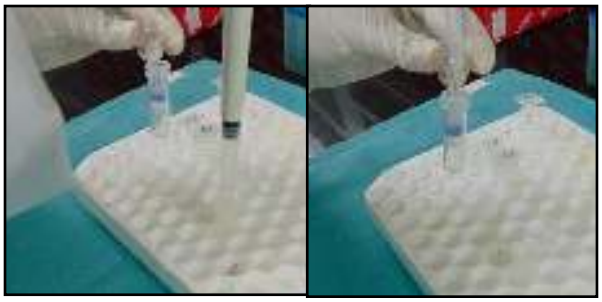
a) Tissue disruption and mincing

b) Addition of RLT Buffer

c) Transfer of homogenised tissue to microcentrifuge tube

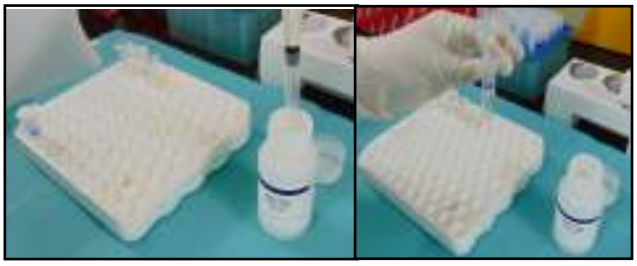


d) Addition of 70% ethanol



e) Transfer to RNeasy Mini Spin Column

f) Centrifugation



g) Addition of RW1

h) Addition of RPE

i) Addition of RNAse free water

cDNA conversion

cDNA conversion was done by using Primescript RT reagent kit (Cat No. RR037A, Takara, Japan)

The reaction mixture was prepared on ice.

Volume recommendations used per reaction are as follows:

Reagent	**Volume**	**Final concentration**
5X PrimeScript Buffer (for Real Time)	2 µl	1X
PrimeScript RT Enzyme Mix I	0.5 µl	
Oligo dT Primer (50 µM)	0.5 µl	25 pmol
RNase Free dH2O	1.5 µl	
RNA template	5 µl	
Total	10 µl	

The tube was centrifuged briefly, and then incubated using the following conditions:

37º for 15 min (Reverse transcription)

85º for 5 sec (Inactivation of reverse transcriptase with heat treatment)

4º (Storage)

cDNA samples were stored at -20ºC till further use in the PCR.

Figure 17: PrimeScript RT Reagent Kit (Cat No. RR037A, Takara, Japan).

Real time PCR:

Reference gene (internal control): β-actin

Target genes : L-PK, G6pc, Cyp7a1

Primers for the above genes were obtained from Bioserve biotechnologies India Pvt Ltd, Hyderabad.

β-actin primers: (120)

Forward primer: 5'- GCCCTGGCACCCAGCACAAT -3'

Reverse primer: 5'- GGAGGGGCCGGACTCGTCAT -3'

L-PK primers: (121)

L-PK Forward: 5'- GCAGAATCCATCGCCAAC -3'

L-PK Reverse: 5'- TCCTCGTGCCCAAGATAC -3'

G6pc primers: (122)

G6pc Forward: 5'- AGAGACTGTGGGCATCAATCT -3'

G6pc Reverse: 5'- CCGGAATCCATACGTTGATT -3'

Cyp7a1 primers: (123)

Cyp7a1Forward: 5'- GCATCTCAAGCAAACACCAT -3'

Cyp7a1 Reverse: 5'- TCCACTCACTTCTTCAGAGGC -3'

Real time PCR was carried out by using TB GreenPremix Ex Taq II (Tli RNaseH Plus) (RR820A, Takara, Kusatsu, Japan).

1) A plate layout was prepared in the Realpex software before preparation of the master mix.
2) Master mix was prepared on ice.

Volume recommendations used are per reaction are given below(single reaction).

Reagent	**Volume**	**Final concentration**
TB GreenPremix Ex Taq II (2X)	10 µl	1X
PCR Forward Primer (10 µM)	1.0 µl	0.4 µM
PCR Reverse Primer (10 µM)	1.0 µl	0.4 µM
Sterile purified water	6 µl	
cDNA template	2 µl	
Total	20 µl	

The tubes were centrifuged briefly and then placed in Realplex Master Cycler (Eppendorf, Hamburg, Germany).

Following PCR conditions were set up in the real-time master cycler (Eppendorf, Germany).

Initial denaturation was done at 95º for 30 sec

Followed by 40 cycles of

95º for 20 sec

60º for 30 sec

72º for 30 sec

Followed by melting curve (dissociation curve) from 60 ºC to 95 ºC for 20 minutes.

Figure 18: TB GreenPremix Ex Taq II

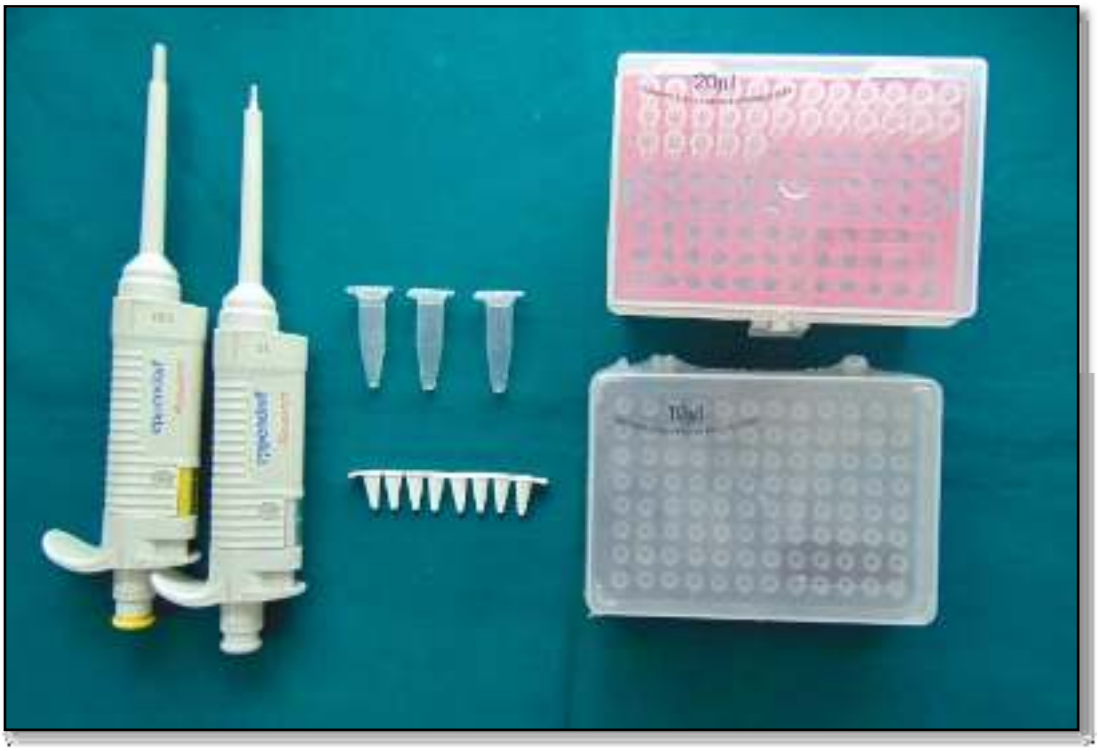


Figure 19: Micropipettes, Eppendorf tubes, PCR tubes and Microtips

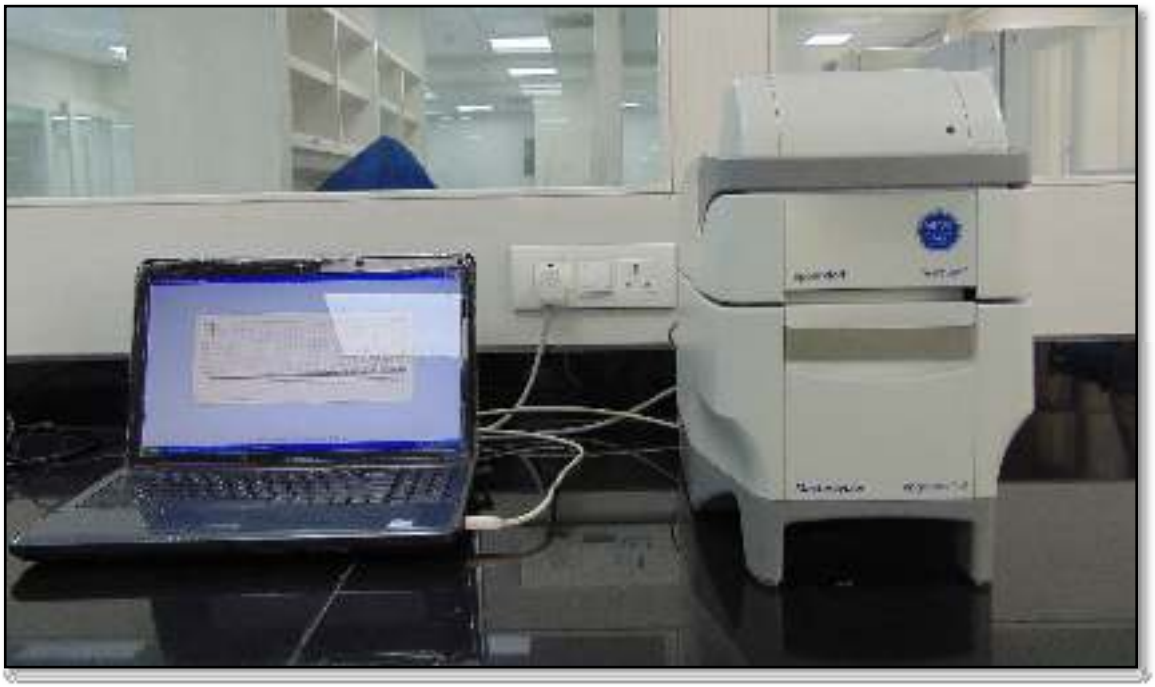


Figure 20: Real-time PCR Machine

Figure 21: L-PK Primer (forward & reverse)

Figure 22: G6pc Primer (forward & reverse)

Figure 23: Cyp7a1 Primer (forward & reverse)

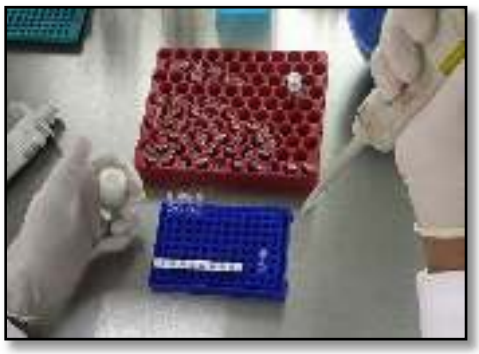
Figure 24: β-actin Primer (forward & reverse)

Figure 25: Real-time PCR

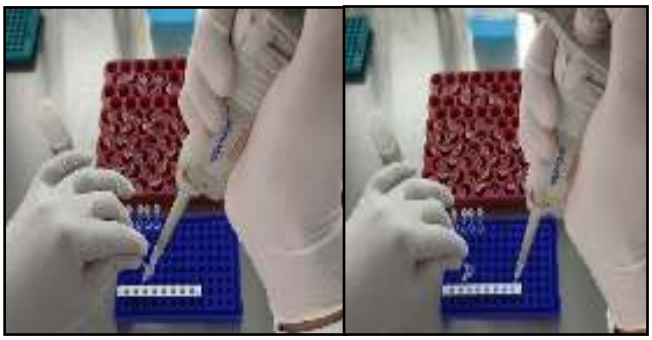
a) Preparation of plate layout in Realpex software

b) Addition of TB GreenPremix Ex Taq II

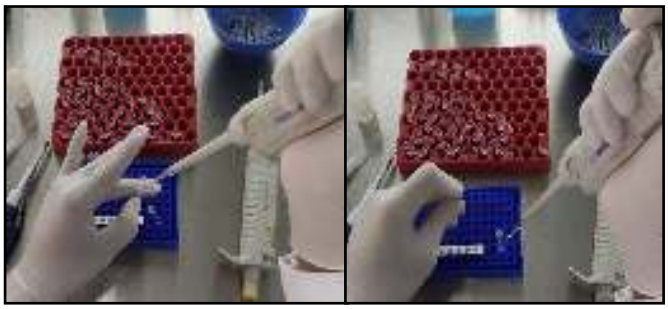
c) Addition of cDNA



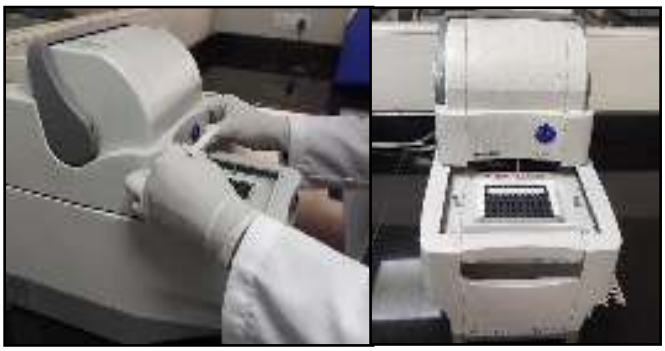
d) Addition of Sterile purified water



e) Addition of mix to PCR tubes



f) Addition of Primers



g) Placing PCR tubes in Realplex Master Cycler

All the reactions were run in duplicates. Positive reaction was detected by accumulation of a fluorescent signal in the form of amplification plot obtained in the Realplex software. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e., exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e., the lower the Ct level the greater the amount of target nucleic acid in the sample).

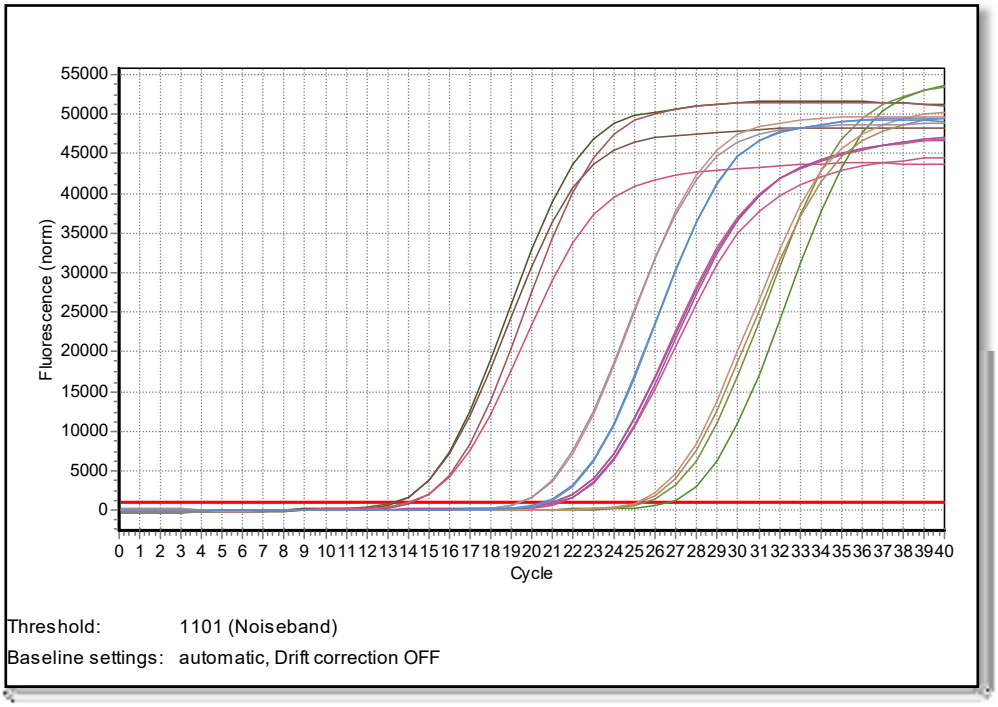


Figure 26: RT-PCR Amplification Plot (2nd Run)

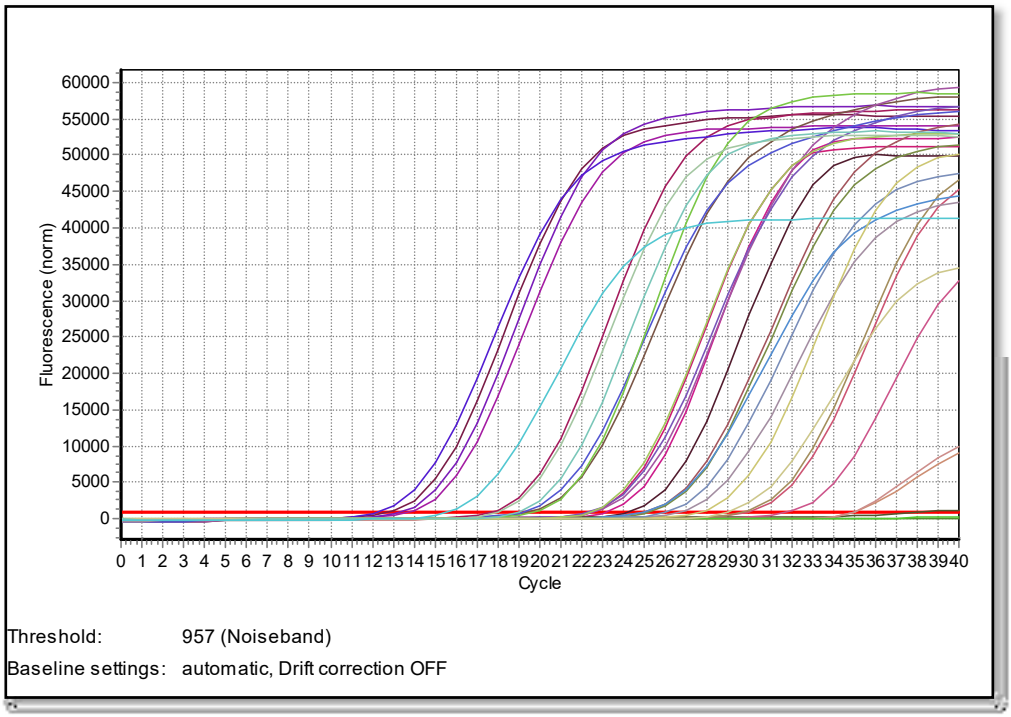


Figure 27: RT-PCR Amplification Plot (4th Run)

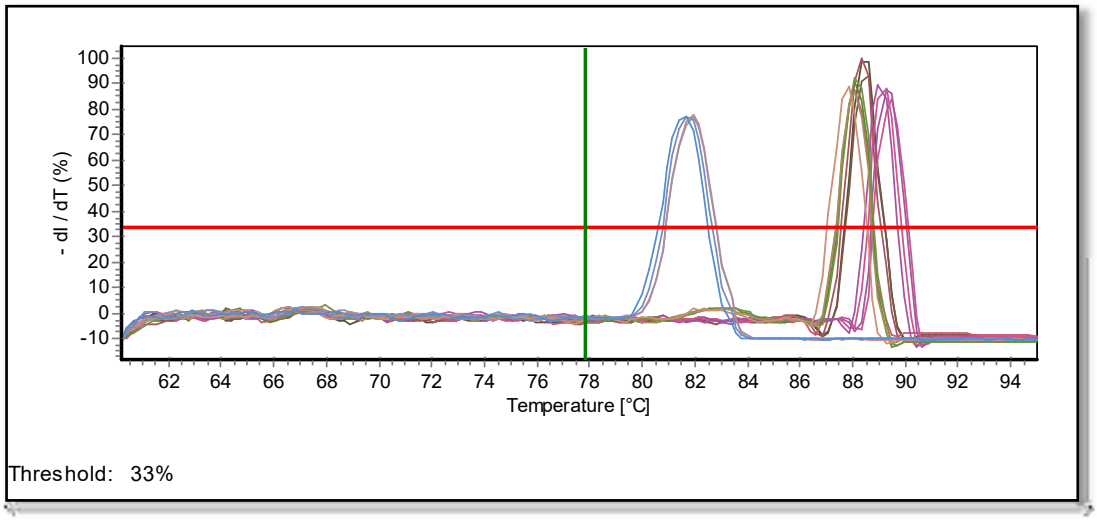


Figure 28: RT-PCR Melting Curve

Ct values for reference gene (β-actin gene) and target genes (L-PK, G6pc, Cyp7a1) were obtained. Relative gene expression was calculated by using $2^{-\Delta\Delta Ct}$ method.[124] Gene expression was calculated as fold change in increase/decrease in gene expression.

RESULTS

The results of the study are presented in form of Tables, Line graphs, and Bar Graphs. They are described under the heading of the respective parameters.

5.1 Random Blood Sugar

Random Blood Sugar levels in mg/dl were measured on Day 1 before the start of treatment and on Day 30 at the end of treatment.

5.1.1 One-way ANOVA followed by Tukey's multiple comparisons test was performed to compare the effect of metformin, TFG and combination of metformin and TFG on RBS levels on Day 1 and Day 30.

Day 1

A one-way ANOVA revealed that there was a statistically significant difference in RBS levels between at least two study groups. [Table 10, Graph 1]

Tukey's Test for multiple comparisons found that the mean RBS level was significantly different between NC vs. DC, NC vs. MET, NC vs. TFG, NC vs. CBN, DC vs. TFG, DC vs. CBN, MET vs. TFG, and MET vs. CBN groups. [Table 11, Graph 1]

There was no statistically significant difference in mean RBS levels between DC vs. MET or between TFG vs. CBN groups. [Table 11, Graph 1]

Day 30

A one-way ANOVA revealed that there was a statistically significant difference in RBS levels between at least two study groups. [Table 10, Graph 2]

Tukey's Test for multiple comparisons found that the mean RBS level was significantly different between NC vs. DC, DC vs. MET, DC vs. TFG, and DC vs. CBN groups. [Table 12, Graph 2]

There was no statistically significant difference in mean RBS levels between NC vs. MET, NC vs. TFG, NC vs. CBN, MET vs. TFG, MET vs. CBN, and TFG vs. CBN groups. [Table 12, Graph 2]

5.1.2 Student's paired *t* test was performed to compare RBS levels before and after treatment within each respective group. [Table 13]

There was a significant increase in the RBS level after 30 days of administration of vehicle compared to the start of the study but rats did not become diabetic / no clinical significance. [Graphs 3 & 4]

There was no significant difference in the RBS level after 30 days of administration of vehicle in diabetic rats compared to the start of the study. [Graphs 3 & 4]

There was a significant decrease in the RBS level after 30 days of treatment with metformin compared to the start of the treatment. [Graphs 3 & 4]

There was a significant decrease in the RBS level after 30 days of treatment with TFG compared to the start of the treatment. [Graphs 3 & 4]

There was a significant decrease in the RBS level after 30 days of treatment with combination of metformin + TFG compared to the start of the treatment. [Graphs 3 & 4]

Table 10: Analysis of Blood glucose levels using ANOVA

	RBS (Mean ± SD mg/dl)					ANOVA Result		
Groups	**NC**	**DC**	**MET**	**TFG**	**CBN**	**$F_{4,24}$**	**p - value**	**Significant**
Day 1	112.67 ± 9.37	509.83 ± 92.86	515.83 ± 95.51	371.60 ± 99.57	334.00 ± 42.46	28.29	< 0.0001	Yes****
Day 30	150.83 ± 22.98	401.67 ± 157.10	203.50 ± 40.90	211.20 ± 59.10	254.00 ± 22.32	8.63	0.0002	Yes***

$p < 0.05$: statistically significant; ***$p < 0.001$; ****$p < 0.0001$; Abbreviations – NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; SD: standard deviation; RBS: random blood sugar; ANOVA: Analysis of Variance

Table 11: Comparison of Blood glucose level between different groups using Tukey's multiple comparisons test (Day 1)

Group Comparisons	Mean diff.	95.00% CI of diff.	Adjusted P Value	Summary
NC vs. DC	-397.2	-526.1 to -268.3	<0.0001	****
NC vs. MET	-403.2	-532.1 to -274.3	<0.0001	****
NC vs. TFG	-258.9	-394.1 to -123.7	<0.0001	****
NC vs. CBN	-221.3	-350.2 to -92.43	0.0003	***
DC vs. MET	-6.000	-134.9 to 122.9	>0.9999	ns
DC vs. TFG	138.2	3.042 to 273.4	0.0433	*
DC vs. CBN	175.8	46.93 to 304.7	0.0042	**
MET vs. TFG	144.2	9.042 to 279.4	0.0325	*
MET vs. CBN	181.8	52.93 to 310.7	0.0030	**
TFG vs. CBN	37.60	-97.59 to 172.8	0.9221	ns

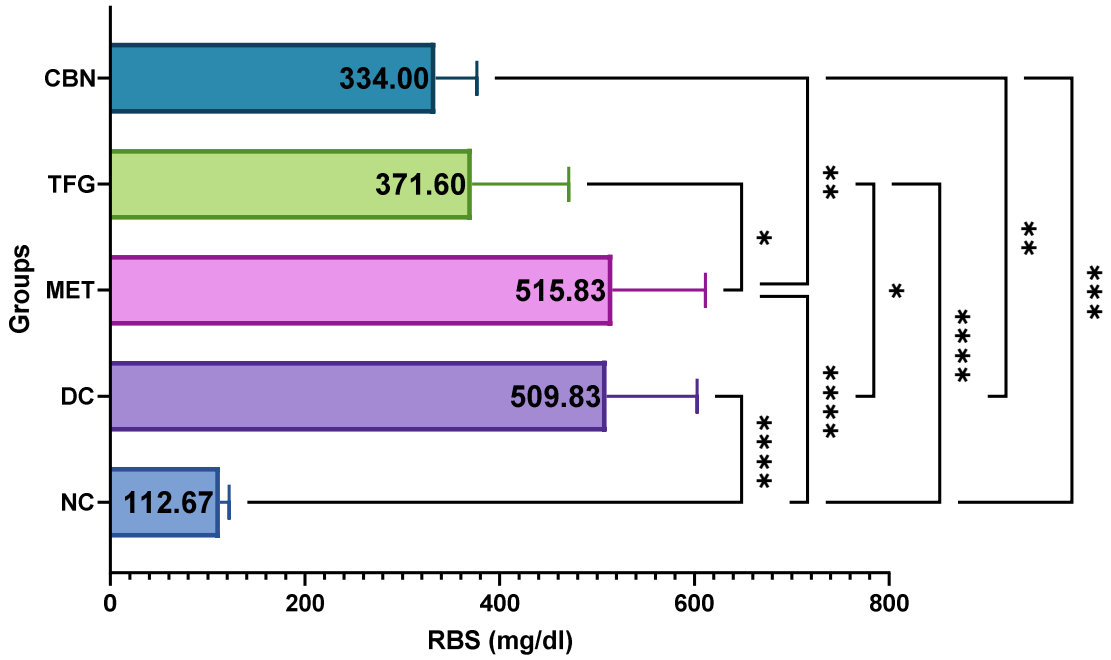
$p < 0.05$: statistically significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; Abbreviations – ns: not significant; NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; SD: standard deviation; RBS: random blood sugar

Table 12: Comparison of Blood glucose level between different groups using Tukey's multiple comparisons test (Day 30)

Group Comparisons	Mean diff.	95.00% CI of diff.	Adjusted P Value	Summary
NC vs. DC	-250.8	-385.7 to -116.0	0.0001	***
NC vs. MET	-52.67	-187.5 to 82.20	0.7784	ns
NC vs. TFG	-60.37	-201.8 to 81.08	0.7187	ns
NC vs. CBN	-103.2	-238.0 to 31.70	0.1948	ns
DC vs. MET	198.2	63.30 to 333.0	0.0019	**
DC vs. TFG	190.5	49.02 to 331.9	0.0047	**
DC vs. CBN	147.7	12.80 to 282.5	0.0270	*
MET vs. TFG	-7.700	-149.1 to 133.7	0.9998	ns
MET vs. CBN	-50.50	-185.4 to 84.36	0.8032	ns
TFG vs. CBN	-42.80	-184.2 to 98.64	0.8973	ns

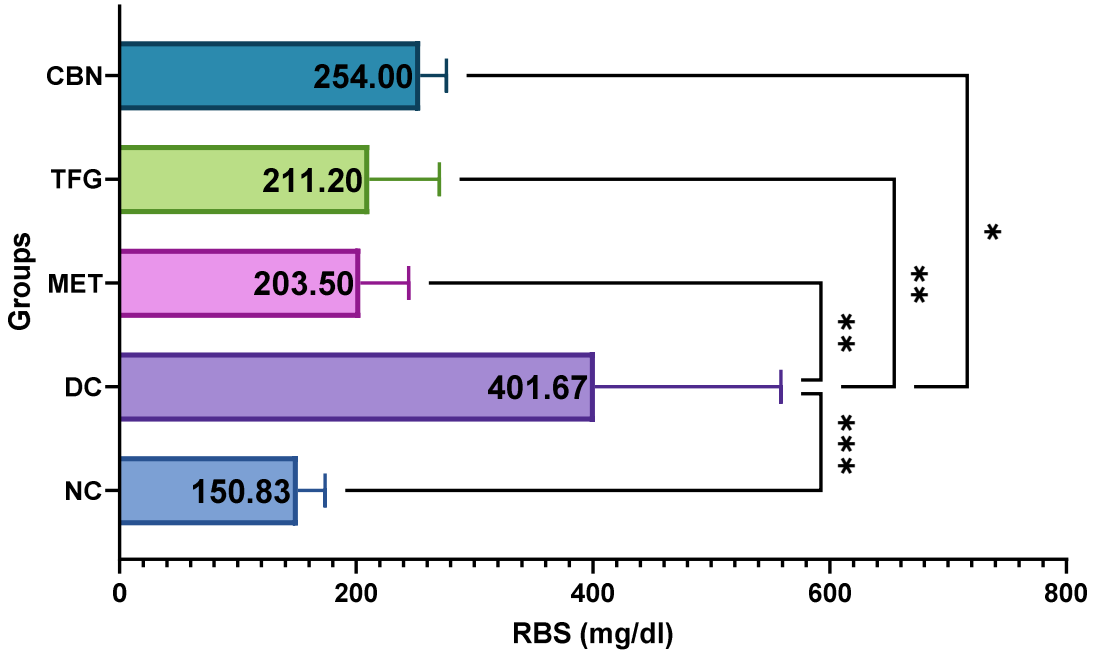
$p < 0.05$: statistically significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; Abbreviations – ns: not significant; NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; SD: standard deviation; RBS: random blood sugar; CI: confidence interval

Graph 1: RBS – Day 1 (ANOVA followed by Tukey's multiple comparisons test)



$p < 0.05$: statistically significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; Abbreviations – NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; RBS: random blood sugar; ANOVA: Analysis of Variance; Asterisks indicate p value generated by Tukey's test

Graph 2: RBS – Day 30 (ANOVA followed by Tukey's multiple comparisons test)



$p < 0.05$: statistically significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; Abbreviations – NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; RBS: random blood sugar; ANOVA: Analysis of Variance; Asterisks indicate p value generated by Tukey's test

Table 13: Comparison of baseline and final blood glucose levels using Student's paired *t* test

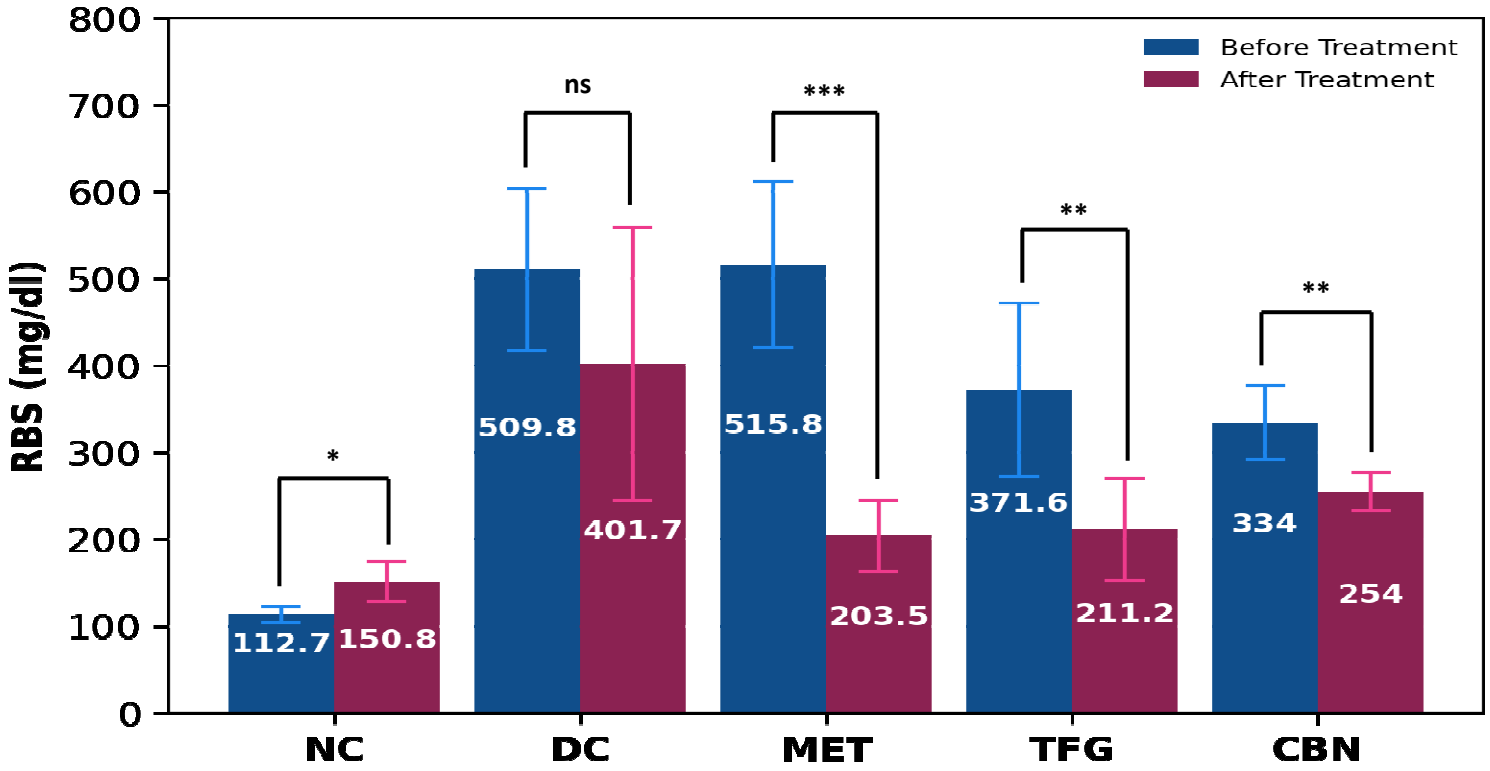
Groups	RBS (mg/dl)			Paired *t* test			
	Mean	**SD**	**SEM**	***t* value**	**df**	**p-value (2-tailed)**	**Significant**
NC							
Before	112.7	9.374	3.827	3.104	5	0.027	Yes*
After	150.8	22.98	9.382				
DC							
Before	509.8	92.86	37.91	1.229	5	0.274	ns
After	401.7	157.1	64.14				
MET							
Before	515.8	95.51	38.99	11.02	5	0.0001	Yes***
After	203.5	40.90	16.70				
TFG							
Before	371.6	99.57	44.53	4.69	4	0.009	Yes**
After	211.2	59.10	26.43				
CBN							
Before	334.0	42.46	17.33	5.738	5	0.002	Yes**
After	254.0	22.32	9.114				

$p < 0.05$: statistically significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; Abbreviations – ns: not significant; NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; SD: standard deviation; RBS: random blood sugar

Graph 3: Random Blood Sugar (mg/dl) – Baseline & Final

Abbreviations – NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; RBS: random blood sugar

Graph 4: Random Blood Sugar – Before & After Treatment (Student's paired *t* test)



p < 0.05: statistically significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

ns: not significant; NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; RBS: random blood sugar

5.2 Body weight

Body weight in grams was measured on Day 1 before the start of treatment and on Day 30 at the end of treatment.

5.2.1 One-way ANOVA followed by Tukey's multiple comparisons test was performed to compare the effect of metformin, TFG and combination of metformin and TFG on body weight on Day 1 and Day 30.

Day 1

A one-way ANOVA revealed that there was a statistically significant difference in body weight between at least two study groups. [Table 14, Graph 5]

Tukey's Test for multiple comparisons found that the mean body weight was significantly different between NC vs. MET, NC vs. TFG, and NC vs. CBN. [Table 15, Graph 5]

There was no statistically significant difference in mean body weight between NC vs. DC, DC vs. MET, DC vs. TFG, DC vs. CBN, MET vs. TFG, MET vs. CBN, or between TFG vs. CBN groups. [Table 15, Graph 5]

Day 30

A one-way ANOVA revealed that there was no statistically significant difference in body weight between at least two groups. [Table 14 and Graph 6]

Tukey's Test for multiple comparisons found that the mean body weight was not significantly different between either of the groups. [Table 16, Graph 6]

5.2.2 Student's paired *t* test was performed to compare the body weight before and after treatment within each respective group. [Table 17]

There was a significant increase in the body weight of normal rats after 30 days of administration of vehicle compared to the start of the study. [Graphs 7 & 8]

There was no significant difference in the body weight of diabetic rats after 30 days of administration of vehicle compared to the start of the study. [Graphs 7 & 8]

There was no significant difference in the body weight of diabetic rats after 30 days of treatment with metformin compared to the start of the treatment. [Graphs 7 & 8]

There was no significant difference in the body weight of diabetic rats after 30 days of treatment with TFG compared to the start of the treatment. [Graphs 7 & 8]

There was no significant difference in the body weight of diabetic rats after 30 days of treatment with combination of metformin + TFG compared to the start of the treatment. [Graphs 7 & 8]

Table 14: Analysis of Body weight using ANOVA

	BW (Mean ± SD gm)					ANOVA Result		
Groups	**NC**	**DC**	**MET**	**TFG**	**CBN**	**$F_{4,24}$**	**p - value**	**Significant**
Day 1	211.5 ± 7.97	257.2 ± 33.54	271.7 ± 44.65	278 ± 17.49	275 ± 30.17	4.93	0.0048	Yes**
Day 30	282.2 ± 20.54	277.2 ± 54.84	278.5 ± 53.15	278.8 ± 34.17	299.5 ± 50	0.25	0.905	ns

$p < 0.05$: statistically significant; ***$p < 0.001$; ****$p < 0.0001$; Abbreviations – ns: not significant; NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; SD: standard deviation; BW: body weight

Table 15: Comparison of Body weight between different groups using Tukey's multiple comparisons test (Day 1)

Group Comparisons	Mean diff.	95.00% CI of diff.	Adjusted P Value	Summary
NC vs. DC	-45.67	-96.79 to 5.461	0.0959	ns
NC vs. MET	-60.17	-111.3 to -9.039	0.0155	*
NC vs. TFG	-66.50	-120.1 to -12.88	0.0100	*
NC vs. CBN	-63.50	-114.6 to -12.37	0.0099	**
DC vs. MET	-14.50	-65.63 to 36.63	0.9168	ns
DC vs. TFG	-20.83	-74.46 to 32.79	0.7816	ns
DC vs. CBN	-17.83	-68.96 to 33.29	0.8402	ns
MET vs. TFG	-6.333	-59.96 to 47.29	0.9966	ns
MET vs. CBN	-3.333	-54.46 to 47.79	0.9997	ns
TFG vs. CBN	3.000	-50.62 to 56.62	0.9998	ns

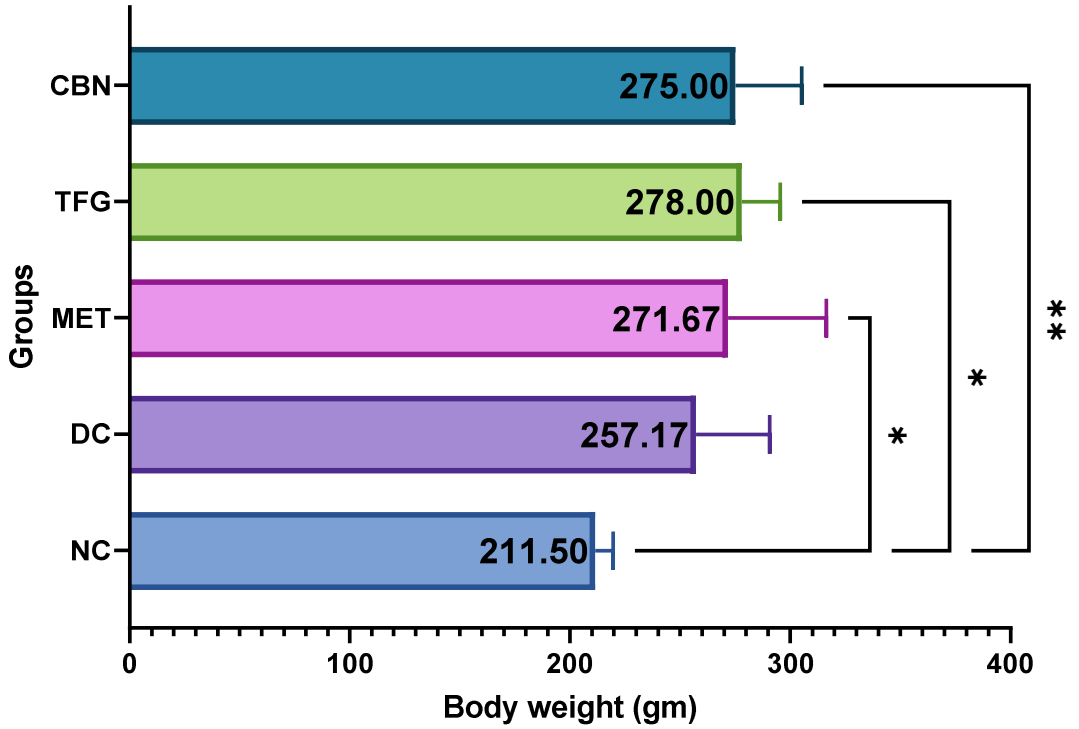
$p < 0.05$: statistically significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; Abbreviations – ns: not significant; NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; SD: standard deviation; CI: confidence interval

Table 16: Comparison of Body weight between different groups using Tukey's multiple comparisons test (Day 30)

Group Comparisons	Mean diff.	95.00% CI of diff.	Adjusted P Value	Summary
NC vs. DC	5.000	-71.42 to 81.42	0.9997	ns
NC vs. MET	3.667	-72.75 to 80.08	>0.9999	ns
NC vs. TFG	3.367	-76.78 to 83.51	>0.9999	ns
NC vs. CBN	-17.33	-93.75 to 59.08	0.9614	ns
DC vs. MET	-1.333	-77.75 to 75.08	>0.9999	ns
DC vs. TFG	-1.633	-81.78 to 78.51	>0.9999	ns
DC vs. CBN	-22.33	-98.75 to 54.08	0.9082	ns
MET vs. TFG	-0.3000	-80.44 to 79.84	>0.9999	ns
MET vs. CBN	-21.00	-97.42 to 55.42	0.9251	ns
TFG vs. CBN	-20.70	-100.8 to 59.44	0.9393	ns

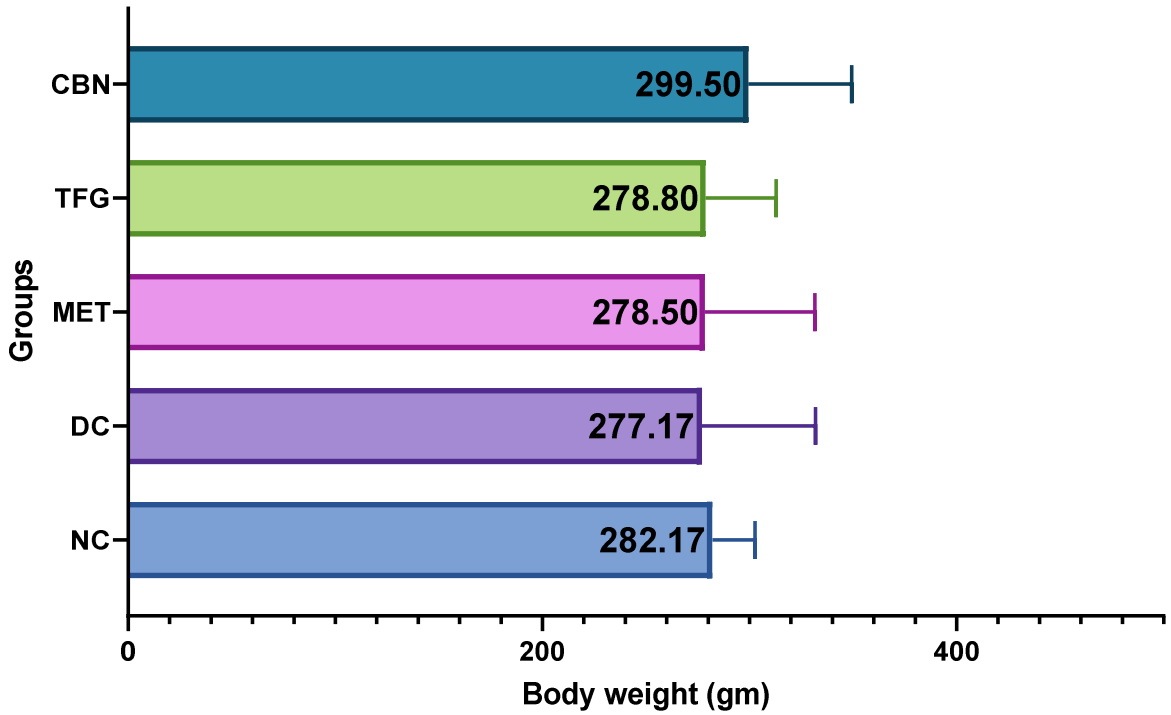
$p < 0.05$: statistically significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; Abbreviations – ns: not significant; NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; SD: standard deviation; BW: body weightr; CI: confidence interval

Graph 5: BW – Day 1 (ANOVA followed by Tukey's multiple comparisons test)



p < 0.05: statistically significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; Abbreviations – NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; BW: body weight; ANOVA: Analysis of Variance; Asterisks indicate p value generated by Tukey's test

Graph 6: BW – Day 30 (ANOVA followed by Tukey's multiple comparisons test)



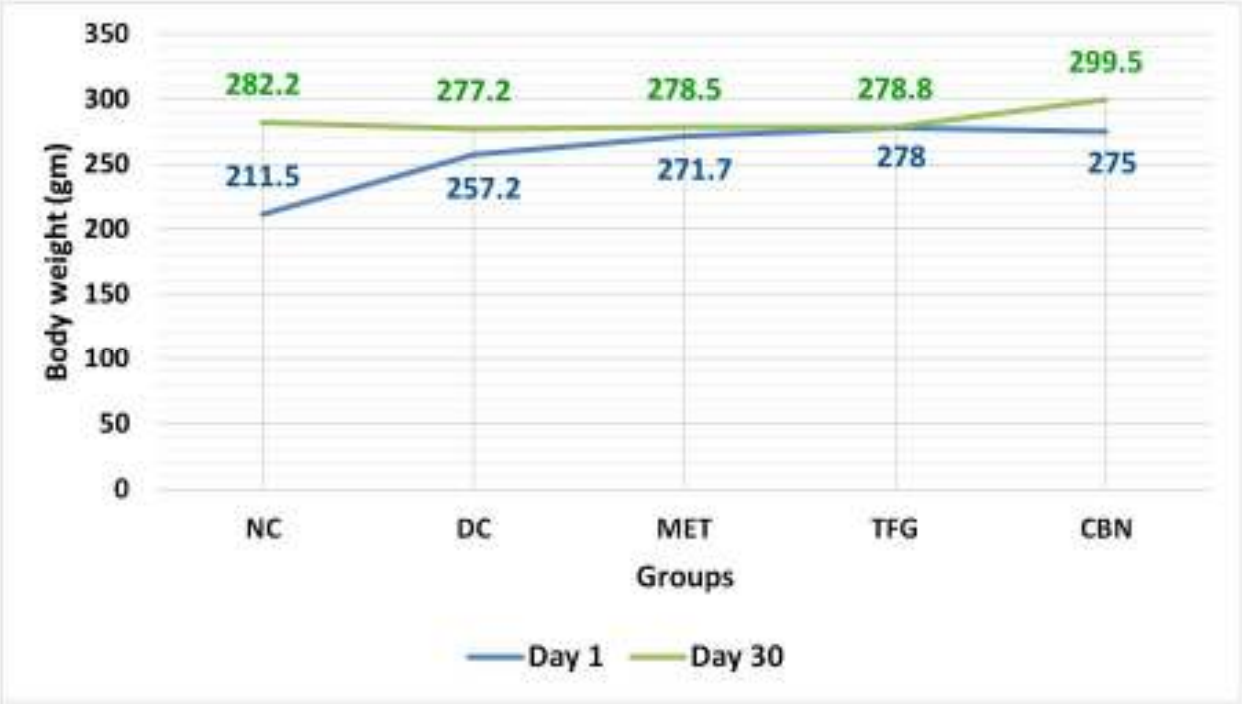
p < 0.05: statistically significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; Abbreviations – NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; BW: body weight; ANOVA: Analysis of Variance; Asterisks indicate p value generated by Tukey's test

Table 17: Comparison of baseline and final body weight using Student's paired *t* test

Groups	Body weight (gm)			Paired *t* test			
	Mean	SD	SEM	*t* value	df	p-value (2-tailed)	Significant
NC							
Before	211.5	7.97	3.25	9.135	5	0.0003	Yes***
After	282.2	20.54	8.38				
DC							
Before	257.2	33.54	13.69	1.325	5	0.2426	ns
After	277.2	54.84	22.39				
MET							
Before	271.7	44.65	18.23	1.386	5	0.2243	ns
After	278.5	53.15	21.70				
TFG							
Before	278	17.49	7.82	0.047	4	0.9648	ns
After	278.8	34.17	15.28				
CBN							
Before	275	30.17	12.32	1.957	5	0.1077	ns
After	299.5	50	20.41				

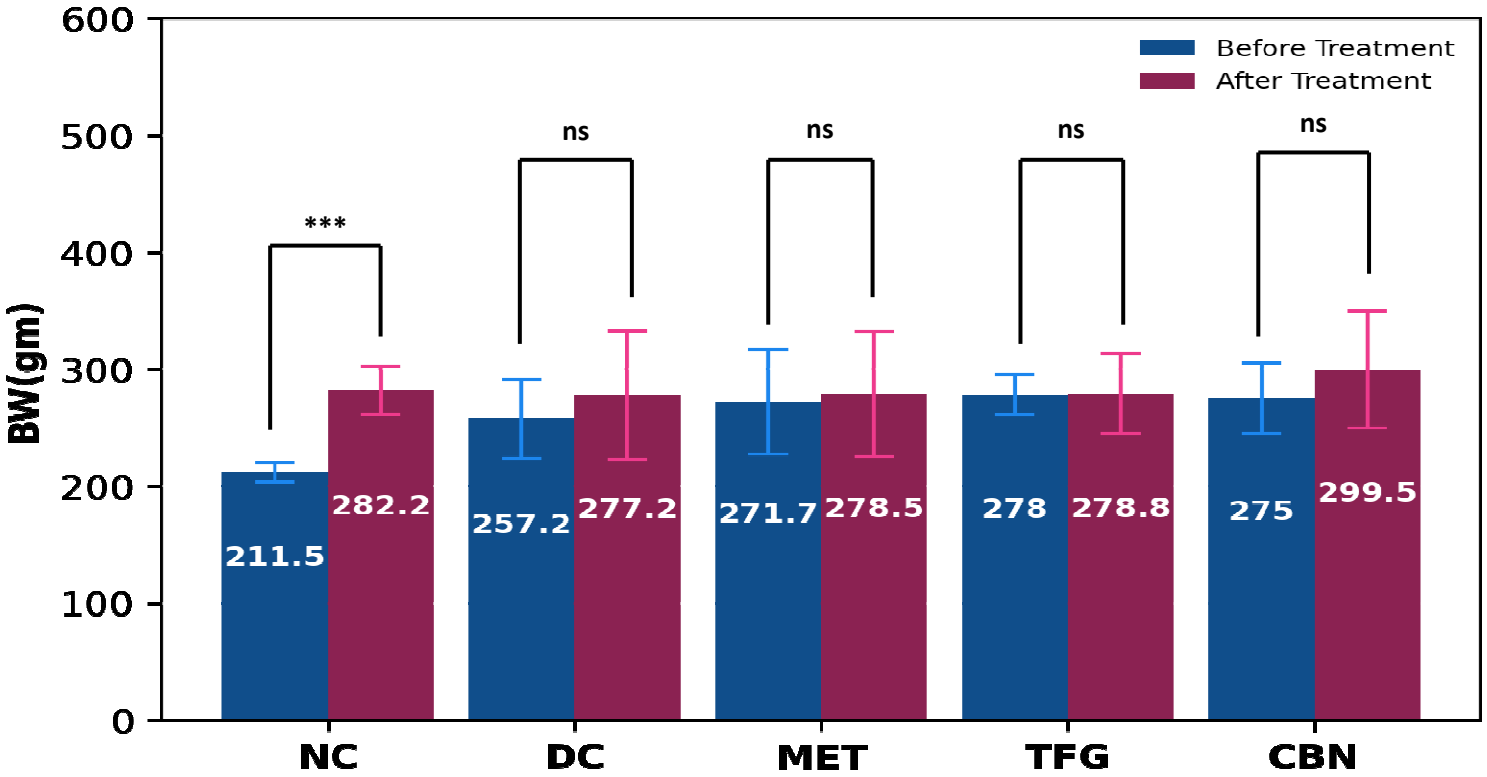
$p < 0.05$: statistically significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; Abbreviations – ns: not significant; NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; SD: standard deviation; BW: body weight

Graph 7: Body Weight (gm) – Baseline & Final



Abbreviations – NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; BW: body weight

Graph 8: Body weight – Before & After Treatment (Student's paired *t* test)



p < 0.05: statistically significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

ns: not significant; NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; BW: body weight

5.3 L-PK Gene expression

L-PK gene expression levels were measured by Real-time PCR at the end of treatment from the liver tissue of diabetic rats.

5.3.1 One-way ANOVA followed by Tukey's multiple comparisons test was performed to compare the effect of metformin, TFG and combination of metformin and TFG on L-PK gene expression levels.

A one-way ANOVA revealed that there was no statistically significant difference in L-PK gene expression levels between at least two study groups. [Table 19, Graph 9]

Table 18: Descriptive Statistics of expression of L-PK Gene

Groups	Mean	SD	SEM	95% CI		Fold Change	↑/↓
				Lower	Upper		
NC	1.08	0.40	0.16	0.65	1.50	1.08	↑
DC	0.66	0.71	0.29	-0.07	1.41	1.50	↓
MET	0.29	0.32	0.13	-0.05	0.62	3.47	↓
TFG	37.97	82.46	36.88	-64.41	140.4	37.97	↑
CBN	1.34	1.5	0.61	-0.23	2.92	1.34	↑

Abbreviations – NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; SD: standard deviation; SEM: standard error of mean; CI: confidence interval; ↓: downregulation; ↑: upregulation

Table 19: Analysis of Expression of L-PK Gene using ANOVA

Groups	NC	DC	MET	TFG	CBN	ANOVA Result		
						$F_{4,24}$	p - value	Significant
Mean ± SD	1.077 ± 0.40	0.66 ± 0.71	0.29 ± 0.32	37.97 ± 82.46	1.34 ± 1.5	1.258	0.3135	ns

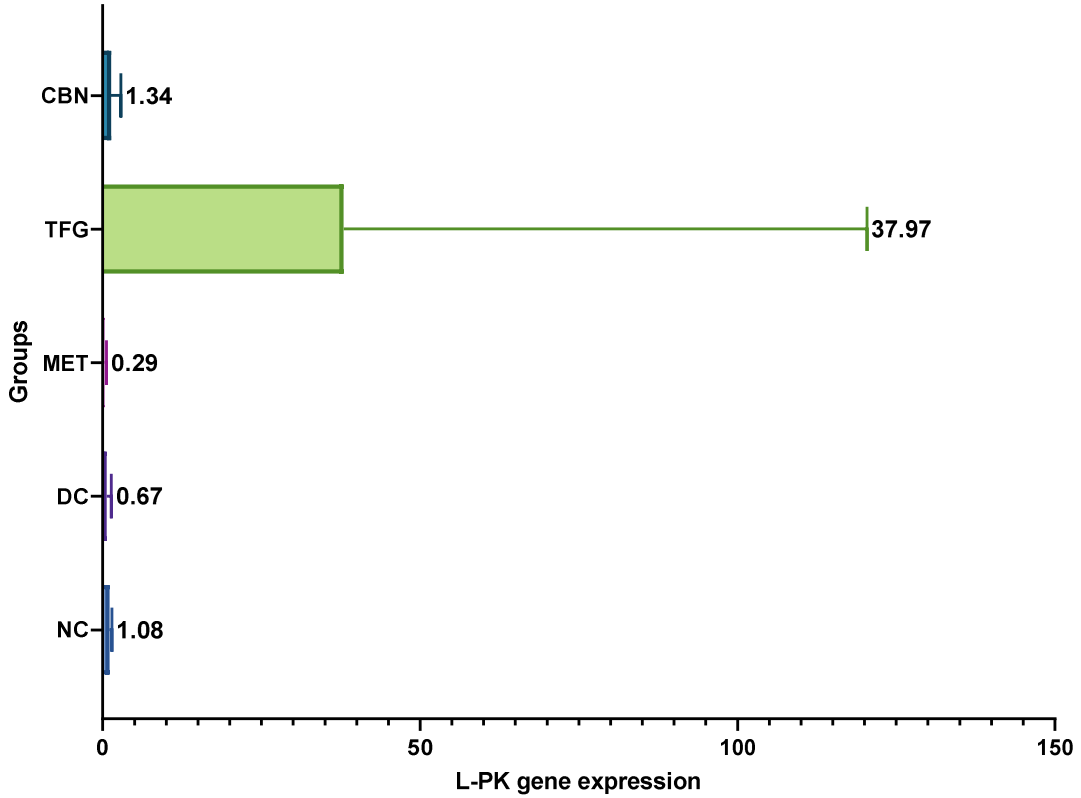
$p < 0.05$: statistically significant; Abbreviations – ns: not significant; NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; SD: standard deviation;

Table 20: Comparison of L-PK gene expression between different groups using Tukey's multiple comparisons test

Group Comparisons	Mean diff.	95.00% CI of diff.	Adjusted P Value	Summary
NC vs. DC	0.4114	-56.86 to 57.68	>0.9999	ns
NC vs. MET	0.7895	-56.48 to 58.06	>0.9999	ns
NC vs. TFG	-36.89	-96.96 to 23.17	0.3915	ns
NC vs. CBN	-0.2651	-57.54 to 57.01	>0.9999	ns
DC vs. MET	0.3780	-56.89 to 57.65	>0.9999	ns
DC vs. TFG	-37.30	-97.37 to 22.76	0.3807	ns
DC vs. CBN	-0.6765	-57.95 to 56.60	>0.9999	ns
MET vs. TFG	-37.68	-97.75 to 22.39	0.3709	ns
MET vs. CBN	-1.055	-58.33 to 56.22	>0.9999	ns
TFG vs. CBN	36.63	-23.44 to 96.69	0.3986	ns

$p < 0.05$: statistically significant; Abbreviations – ns: not significant; NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; SD: standard deviation; CI: confidence interval

Graph 9: L-PK Gene expression (ANOVA followed by Tukey's multiple comparisons test)



$p < 0.05$: statistically significant; Abbreviations – NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; ANOVA: Analysis of Variance

5.4 G6pc Gene expression

G6pc gene expression levels were measured by Real-time PCR at the end of treatment from the liver tissue of diabetic rats.

5.4.1 One-way ANOVA followed by Tukey's multiple comparisons test was performed to compare the effect of metformin, TFG and combination of metformin and TFG on G6pc gene expression levels.

A one-way ANOVA revealed that there was no statistically significant difference in G6pc gene expression levels between at least two study groups. [Table 22, Graph 10]

Table 21: Descriptive Statistics of expression of G6pc Gene

Groups	Mean	SD	SEM	95% CI		Fold Change	↑/↓
				Lower	Upper		
NC	2.21	2.32	0.95	-0.22	4.65	2.21	↑
DC	24.15	38.65	15.78	-16.41	64.71	24.15	↑
MET	9.63	20.77	8.48	-12.17	31.43	9.63	↑
TFG	16.59	32.55	14.55	-23.82	57	16.59	↑
CBN	17.97	31.19	12.73	-14.77	50.71	17.97	↑

Abbreviations – NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; SD: standard deviation; SEM: standard error of mean; CI: confidence interval; ↓: downregulation; ↑: upregulation

Table 22: Analysis of Expression of G6pc Gene using ANOVA

Groups	NC	DC	MET	TFG	CBN	ANOVA Result		
						$F_{4,24}$	p - value	Significant
Mean ± SD	2.21 ± 2.32	24.15 ± 38.65	9.63 ± 20.77	16.59 ± 32.55	17.97 ± 31.19	0.542	0.7063	ns

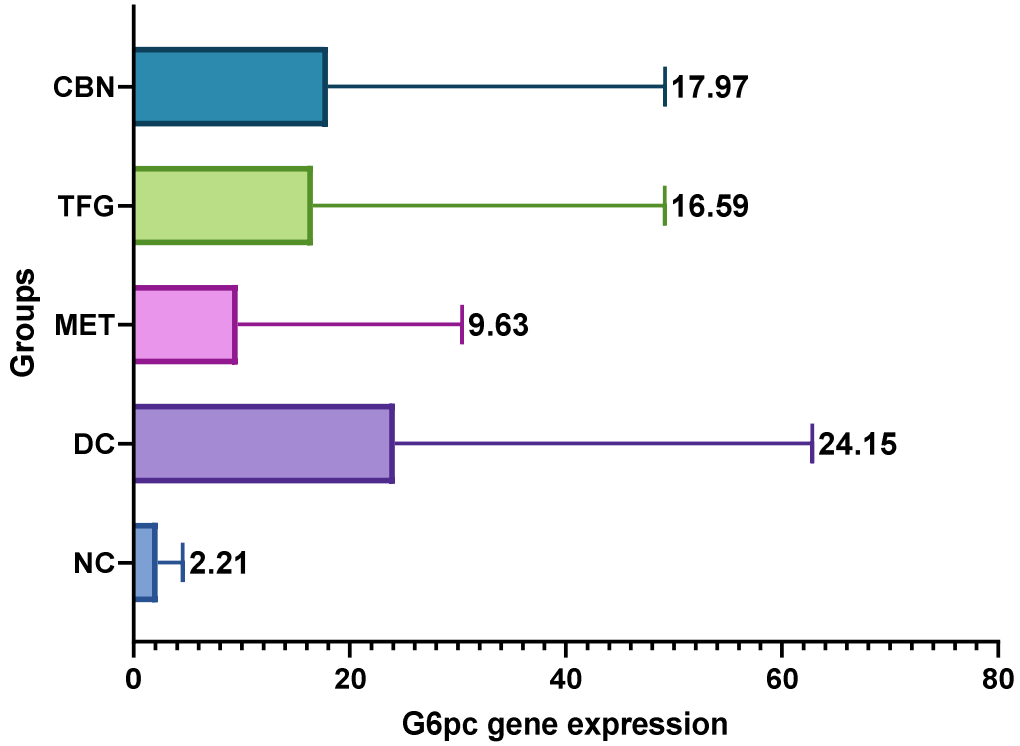
$p < 0.05$: statistically significant; Abbreviations – ns: not significant; NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; SD: standard deviation;

Table 23: Comparison of G6pc gene expression between different groups using Tukey's multiple comparisons test

Group Comparisons	Mean diff.	95.00% CI of diff.	Adjusted P Value	Summary
NC vs. DC	-21.94	-69.49 to 25.61	0.6583	ns
NC vs. MET	-7.419	-54.97 to 40.13	0.9902	ns
NC vs. TFG	-14.38	-64.25 to 35.49	0.9122	ns
NC vs. CBN	-15.76	-63.31 to 31.79	0.8631	ns
DC vs. MET	14.52	-33.03 to 62.07	0.8942	ns
DC vs. TFG	7.560	-42.31 to 57.43	0.9912	ns
DC vs. CBN	6.177	-41.37 to 53.73	0.9951	ns
MET vs. TFG	-6.958	-56.83 to 42.91	0.9936	ns
MET vs. CBN	-8.341	-55.89 to 39.21	0.9848	ns
TFG vs. CBN	-1.383	-51.25 to 48.49	>0.9999	ns

$p < 0.05$: statistically significant; Abbreviations – ns: not significant; NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; SD: standard deviation; CI: confidence interval

Graph 10: G6pc Gene expression (ANOVA followed by Tukey's multiple comparisons test)



$p < 0.05$: statistically significant; Abbreviations – NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; ANOVA: Analysis of Variance

5.5 Cyp7a1 Gene expression

Cyp7a1 gene expression levels were measured by Real-time PCR at the end of treatment from the liver tissue of diabetic rats.

5.5.1 One-way ANOVA followed by Tukey's multiple comparisons test was performed to compare the effect of metformin, TFG and combination of metformin and TFG on Cyp7a1 gene expression levels.

A one-way ANOVA revealed that there no statistically significant difference in Cyp7a1 gene expression levels between at least two study groups. [Table 25, Graph 11]

Table 24: Descriptive Statistics of expression of Cyp7a1 Gene

Groups	Mean	SD	SEM	95% CI		Fold Change	↑/↓
				Lower	Upper		
NC	1.02	0.26	0.10	0.75	1.30	1.02	↑
DC	81.88	125	51.01	-49.25	213	81.88	↑
MET	4.85	6.73	2.75	-2.21	11.91	4.85	↑
TFG	50.20	101.4	45.35	-75.73	176.1	50.20	↑
CBN	50.37	116.4	47.53	-71.81	172.6	50.37	↑

Abbreviations – NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; SD: standard deviation; SEM: standard error of mean; CI: confidence interval; ↓: downregulation; ↑: upregulation

Table 25: Analysis of Expression of Cyp7a1 Gene using ANOVA

Groups	NC	DC	MET	TFG	CBN	ANOVA Result		
						$F_{4,24}$	p - value	Significant
Mean ± SD	1.02 ± 0.26	81.88 ± 125	4.85 ± 6.73	50.20 ± 101.4	50.37 ± 116.4	0.90	0.4809	ns

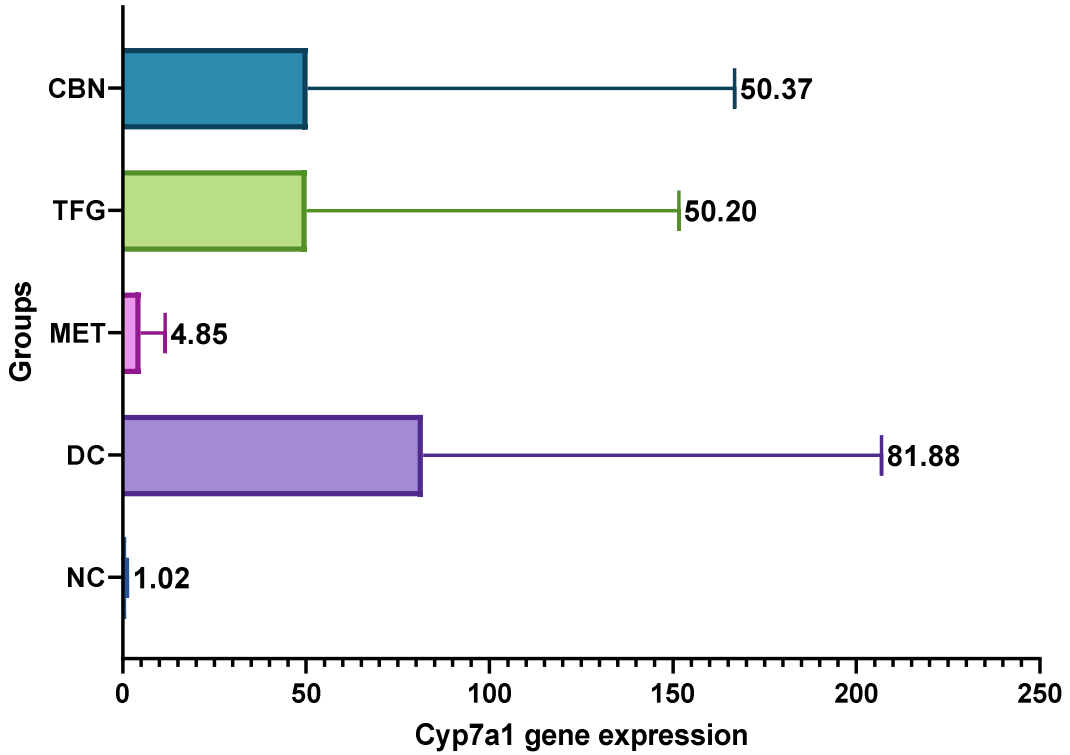
$p < 0.05$: statistically significant; Abbreviations – ns: not significant; NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; SD: standard deviation;

Table 26: Comparison of Cyp7a1 gene expression between different groups using Tukey's multiple comparisons test

Group Comparisons	Mean diff.	95.00% CI of diff.	Adjusted P Value	Summary
NC vs. DC	-80.86	-231.1 to 69.36	0.5202	ns
NC vs. MET	-3.826	-154.0 to 146.4	>0.9999	ns
NC vs. TFG	-49.17	-206.7 to 108.4	0.8866	ns
NC vs. CBN	-49.35	-199.6 to 100.9	0.8668	ns
DC vs. MET	77.04	-73.19 to 227.3	0.5657	ns
DC vs. TFG	31.69	-125.9 to 189.2	0.9749	ns
DC vs. CBN	31.51	-118.7 to 181.7	0.9708	ns
MET vs. TFG	-45.35	-202.9 to 112.2	0.9127	ns
MET vs. CBN	-45.53	-195.8 to 104.7	0.8968	ns
TFG vs. CBN	-0.1773	-157.7 to 157.4	>0.9999	ns

$p < 0.05$: statistically significant; Abbreviations – ns: not significant; NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; SD: standard deviation; CI: confidence interval

Graph 11: Cyp7a1 Gene expression (ANOVA followed by Tukey's multiple comparisons test)



$p < 0.05$: statistically significant; Abbreviations – NC: normal control; DC: diabetic control; MET: metformin; TFG: *Trigonella-foenum graecum*; CBN: combination; ANOVA: Analysis of Variance.

DISCUSSION

There is no doubt that diabetes mellitus is on the rise in the world. The burden of the disease keeps rising by the year. We have come a long way in the way this disease is approached and managed.

The present study was conducted to evaluate the effect of *Trigonella-foenum graecum* (TFG) seed extract on gene expression of selected genes in the liver tissue of male Wistar rats in which diabetes was induced by a combination of STZ and HFD. The secondary objective was to the study the interaction between TFG and metformin at the genetic level by administering them in combination to diabetic rats.

In the model of diabetes used in the present study, animals were fed a high-fat diet to establish insulin resistance, followed by a low dose of STZ injection to cause partial pancreatic beta cell failure. This model has a significant benefit over genetic models in that it replicates natural pathogenesis while also displaying some features of T2D in humans.[85] Furthermore, STZ seems to be more specific compared to Alloxan as there is uptake by beta cell specific GLUT2, but not by other glucose transporters. Alloxan not only has toxic effects on islets of Langerhans, but also affects other body organs like liver and kidney. It usually produces severe diabetes. STZ has a higher chemical stability & a lower toxicity which allows for easier manipulation and flexible dosing. It also has a higher rate of induction of diabetes. Mortality rate of animals is found to be higher by Alloxan than STZ due to a high loss in body weight.[104,125]

Trigonella foenum-graecum, commonly known as fenugreek, is a plant whose seeds, leaves, and extracts have been extensively employed as a source of antidiabetic

compounds in several model systems.[59,126,127] Fenugreek has a long history in India, particularly in the Ayurvedic and Unani traditions.[58,59] Fenugreek seed powder taken orally appears to have hypoglycaemic and anti-hyperlipidaemic effects, according to preliminary animal and human experiments. Fenugreek seeds contain 50% fibre which can help to reduce the pace of post-prandial glucose absorption. This could be a secondary mechanism of the hypoglycaemic impact.[28]

Though there is substantial evidence that TFG has anti-diabetic effect,[59] there is a significant gap in understanding the processes involved. The rationale for this study was to go into greater detail and attempt to determine whether TFG impacts specific genes that code for enzymes that are actively involved in glucose metabolism and also influence the expression of a gene involved in bile acid production. As fundamental parameters, blood sugar level and body weight at the end of study were compared with baseline levels.

The gene expression of genes encoding pyruvate kinase – liver type and glucose-6-phosphatase was analyzed in the current study. Furthermore, gene expression analysis of the Cyp7a1 gene was analyzed, which has been identified as a novel therapeutic target for anti-diabetic effect of metformin.[123]

Effect on Blood sugar level:

In the current study, the levels of blood glucose in the diabetic group remained elevated throughout the study period. Metformin and TFG administration independently were found to significantly lower blood sugar levels after 30 days of daily oral dosing. Furthermore, the combination of metformin and TFG resulted in a significant reduction in RBS at the end of treatment. This was, however, less than when both compounds were given separately. These findings are consistent with the

findings of other preclinical studies which used metformin as a standard group in their respective studies as well.[128,129] TFG has a comparable effect to previous investigations on alloxan-induced diabetic rats, where Raju et al.[126] and Khosla et al.[130] observed a significant reduction in FBS after 21 days and 14 days of treatment with TFG seed powder, respectively. Joshi et al.[117] also found a significant reduction in BSL after 31 days of treatment with hydroalcoholic extract of TFG at a dose of 1g/kg.

The CBN group data is relatively new to this study because there haven't been many studies examining the co-administration of metformin with TFG. Although, a study found that metformin and TFG have synergistic effects on lipid profiles in T2DM patients.[131] The efficacy and safety of a fenugreek-based medication (Fenfuro®) were examined in a clinical trial. According to their findings, Fenfuro in combination with Metformin produced greater effects than Metformin alone.[132]

Effect on Body weight:

There was a significant increase in body weight of diabetic rats compared to normal rats on day 1 in the current study. The diabetic rats' consumption of a high-fat diet compared to normal rat chow diet by normal rats for 2 weeks may be partly responsible for this substantial rise.

However, after 30 days of daily treatment, the effect of the standard drug metformin, TFG, or a combination of both did not demonstrate a significant difference in the body weight of diabetic rats. Rather, when the groups were analysed separately, it was discovered that the MET, TFG, and CBN groups maintained the body weight of diabetic rats regardless of HFD ingestion on a regular basis. The effects of metformin on the body weight of diabetic rats are consistent with the

evidence that metformin is a weight-neutral or weight-sparing medication, as opposed to other anti-diabetic medicines that cause weight gain.[133] The body weight results of the TFG group differ from the earlier work done by Joshi et al.[117] and Siddiqui et al.[134] Joshi et al. found a substantial decrease in the body weight of diabetic rats following 31 days of oral therapy with different doses of TFG. Furthermore, Siddiqui et al. found that body weight of diabetic rats increased after 21 days of therapy with TFG seed powder.

The CBN group is a novel discovery because there has been little investigation into the effect of co-administration of metformin and TFG on the body weight of diabetic rats. The normal control rats showed a significant gain in body weight consistent with an increase in age, similar to prior study results by Joshi et al. where the normal rats.[117] In contrast to the finding of decrease in body weight in diabetic rats in earlier studies, the present study showed no significant difference in body weight from baseline in the diabetic rats.[117,134] The possible reason for no significant decrease in weight in our study could be STZ having been administered in a low dose as compared to alloxan used in other studies.

Effect on L-PK gene expression:

PK is a glycolytic enzyme that regulates the rate of pyruvate and ATP production from phosphoenolpyruvate and ADP. Hepatic pyruvate kinase activity, which is primarily related to the L-type isoenzyme, reduces with fasting or diabetes and increases with a high carbohydrate diet or insulin treatment in diabetic rats.

The present study is in line with the previous research [32,62] that showed a decrease in pyruvate kinase activity in diabetic states. Our results also showed a downregulation of L-PK gene by 1.5-fold in the diabetic control group. The effect of

metformin on L-PK also showed a downregulation by 3.47-fold. This is similar to the results of the research done by Leclerc et al.[36] and Foretz et al.[37] which showed that activation of AMPK reduced the transcriptional activity of L-PK gene in hepatocytes. Since metformin is known to activate AMPK, our results align with these studies. In contrast, there is another study done by Argaud et al.[35] which showed that metformin indirectly increased the activity of pyruvate kinase by increasing the level of ATP. Since there is an indirect component, the downregulation of L-PK gene by metformin in the present study favours that conclusion also.

There have been very few research studies that have investigated the influence of TFG on glucose metabolism. One of these concluded that a moderate to high dose of TFG greatly boosted the activity of the pyruvate kinase enzyme, which is normally reduced in diabetics.[135] Studies conducted by Mohammad et al.[136] and Preet et al.[137] reached similar outcomes. The present study produced similar results [62] at the genetic level as well. TFG considerably upregulated the level of the L-PK gene by 37-fold, albeit not significantly.

In the combination group, there was an upregulation of the L-PK gene level by 1.34-fold. This could imply that metformin interferes with TFG's ability to exert its anti-diabetic activity through the L-PK gene. There is also a possibility of interaction with activation of AMPK by metformin.

Effect on G6PC gene expression:

G6Pase is important for maintaining blood glucose homeostasis.[42] G6Pase is encoded by the gene G6pc. In diabetic conditions, activity of G6Pase enzyme is enhanced.[135] This is in line with the results of our study that found a 24.15-fold increase in the expression of G6pc gene in the diabetic control group. Metformin was

able to control the upregulation of G6pc gene as there is a substantial difference in the upregulation of G6pc by metformin by only 9.63-fold. This confirms the effect of metformin on G6pc gene expression as concluded by Mues et al.[138] and Moonira et al.[139] This effect is due to both AMPK-dependent regulation[138] and AMPK-independent mechanisms.[139] TFG was also found to considerably inhibit the activity of the glucose-6-phosphatase enzyme by limiting the upregulation of G6pc gene by 16.59-fold. This was similar to the results concluded by other studies on effect of TFG on glucose-6-phosphatase enzyme.[136,137] Though there was a partial restriction of upregulation of G6pc in the combination group with 17.97-fold, it was less than in the groups treated with metformin or TFG alone. This could imply that TFG and metformin are both interfering in each other's mechanisms that causes a drop in the level of the glucose-6-phosphatase enzyme.

Effect on Cyp7a1 gene expression:

Cyp7a1 gene encodes for cholesterol-7α-hydroxylase enzyme. This is the rate limiting enzyme for bile acid synthesis and is found to serve an important role in diabetics.[123] Farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily of ligand-activated transcription factors that has been linked to glucose metabolism in addition to its documented role in bile acid (BA) and lipid metabolism.[140,141] Bile acids are endogenous ligands for the FXR receptor, which is found in the liver, intestine, kidney, and adrenal gland at the highest levels.[142] Although the mechanisms involving changes in FXR gene expression are unclear and presumably complex, but it appears that glucose homeostasis and FXR expression are linked.[140]

In this study, Cyp7a1 was upregulated in diabetic rats by 81.8-fold, but it was not statistically significant. This upregulation could be due to decreased expression of FXR receptors caused by streptozotocin induction.[143] Also, there was no downregulation of BA synthesis due to negative feedback regulation.[143] On the other hand, it is likely that metformin was able to restore FXR expression due to high glucose, and hence the elevation of Cyp7a1 found in that group was not as much as in the diabetic group with an upregulation of 4.8-fold.

Another likely explanation for metformin's modest upregulation of Cyp7a1 is its mechanism based on AMPK activation. AMPK has been demonstrated to phosphorylate FXR downstream of SIRT1.[144] AMPK subunits bind to FXR directly, and the AMPK complex works as an FXR repressor by direct phosphorylation. This inhibits FXR target genes, resulting in an impact comparable to that reported in FXR defective mice, viz increased bile acid production.[145] As a result, there is an increase in cholesterol-7-hydroxylase, which would explain the upregulation of Cyp7a1.

In the present study, TFG upregulated Cyp7a1 gene. This finding could be explained by the hypothesis that, TFG has been proven to promote BA excretion in the faeces.[146] This could imply that it reduces the body's BA pool by eliminating them from the enterohepatic circulation. As a result, the body would produce more BA from endogenous cholesterol in order to maintain the BA pool. This would infer an increase in cholesterol-7-hydroxylase, which would be caused by Cyp7a1 overexpression similar to the effect of BA sequestrants.[140] Hence, this could explain the results of the present study where TFG upregulated Cyp7a1 gene by approximately 50.2-fold. As with the combination group, it is possible that the

upregulation impact of metformin and TFG on Cyp7a1 was cumulative, resulting in a 50.4-fold increase.

Strengths:

- Improvement in the understanding of a potential therapeutic target (Cyp7a1) for future anti-diabetic research. As per recent research, Cyp7a1 has been identified as a new target for antidiabetic drugs. The present study is the first of its kind to have studied the effect of TFG on gene expression of Cyp7a1.
- One of the few experimental studies which has evaluated the effect of TFG at the genetic level in order to better understand the mechanism of its anti-diabetic action.
- A one-of-a-kind study investigating the interaction between metformin and TFG at the genetic level.

Limitations:

- Parameters related to the function of Cyp7a1 such as bile acid levels may have been investigated at the end of the study to see if they correlated with Cyp7a1 gene expression levels.
- HbA1c levels should have been studied in order to correlate them with random blood sugar levels and get an understanding of the blood glucose control over a fairly long period.
- Outliers in the groups may have skewed the results; therefore, further such studies on gene expression levels should be done to confirm the influence of these drugs on selected genes.

CONCLUSION

In conclusion, this study has improved our understanding of TFG's anti-diabetic activity, and the possible mechanisms involved.

Furthermore, the effect of co-administration of metformin and TFG on glucose metabolism and bile acid production may not be synergistic. This pharmacological interaction at the genetic level becomes crucial for patients intending to take metformin and TFG for supposedly better control of blood glucose. In India, it is a common practice for diabetic patients who are on allopathic drugs to be consuming TFG since methi seeds are widely available and are used in almost every household as a culinary spice. Based on the findings of the present study, a combination of metformin with TFG would be best avoided. As mentioned earlier, further studies would be required to substantiate the evidence generated from the present study. Moreover, because of its involvement with bile acid production and anti-diabetic impact, Cyp7a1 has proven to be an important therapeutic target for both metformin and TFG as shown by the current study.

SUMMARY

The purpose of this study was to observe the effect of TFG on gene expression of the L-PK, G6pc, and Cyp7a1 genes in the liver tissue of diabetic rats in which DM was induced by a combination of low dose STZ with HFD. The secondary objective was to study the interaction of TFG and metformin based on their effect on expression of the same genes.

The rats were divided into five groups with eight rats in all groups except the normal control group which had six rats. Groups: normal control (NC), diabetic control (DC), metformin (MET), TFG and combination of metformin and TFG (CBN). Diabetes was induced in 32 rats by a combination of HFD given for two weeks & single low-dose intraperitoneal injection of Streptozotocin (STZ) of 30 mg/kg. Rats with random blood sugar (RBS) levels > 200 mg/dl after 72 hours of STZ injection were included in the study. Respective diets were continued throughout the study. Test compounds or vehicle were administered for 30 days by oral route. RBS and body weight were tested on day 1 and day 30 of the experiment. At the end of the study, all rats were euthanized and liver tissue was harvested for gene expression analysis. The Real-time PCR technique was used to analyse gene the expression.

We found that TFG promotes glycolysis by upregulating the L-PK gene. It also produces a controlled overexpression of the G6pc gene, meaning that it regulates glucose synthesis in the body, which is advantageous to diabetics. It resulted in a significant overexpression of the Cyp7a1 gene, which may be related to its role in bile acid excretion in the faeces. This provides a modest insight into the mechanism underlying TFG's long-standing anti-diabetic effect, as well as a need for further research in understanding its mechanisms.

We also discovered a putative genetic connection between TFG and metformin based on the expression of all three identified genes. It has given us an insight into the non-synergistic activities of both these agents when administered in conjunction.

Hence, it can be concluded that, administration of metformin and TFG in combination has an undesirable pharmacological interaction on glucose metabolism and bile acid synthesis at the genetic level. This information is critical for people who wish to take metformin with TFG for ostensibly better blood glucose management and it is advisable to avoid combining metformin and TFG. Additional research would be required to substantiate the data obtained by the current study. Also, the current investigation has proven Cyp7a1 to be an essential therapeutic target for both metformin and TFG due to its involvement in both, bile acid synthesis and anti-diabetic effect.

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ANNEXURE I- IAEC APPROVAL CERTIFICATE

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH
(Deemed to be University)
JAWAHARLAL NEHRU MEDICAL COLLEGE,
NEHRU NAGAR, BELAGAVI - 590010, (KARNATAKA).
INSTITUTIONAL ANIMAL ETHICS COMMITTEE.
Phone No. JNMC (0831) 2444040

Dr.(Mrs)P.P.Patil Chairperson, IAEC. Prof & Head Physiology, J.N.Medical College, Belagavi	Dr.P.A.Patil Main Nominee - CPCSEA Prof & Head of Pharmacology, USM-KLE, IMP, Belagavi	Dr.(Mrs)Rekha Nayaka M.R Member - Secretary IAEC Asso Prof of Pharmacology J.N.Medical College, Belagavi

CPCSEA Reg.No.: 627/PO/Re/S/02/CPCSEA

MEMBERS:

Dr Ramappa Digge
Scientist D, RMRC,
ICMR, Belagavi.

Smt Sunil K.Patil
Non-scientific Social worker,
Nidasoshi.

Dr. Sudha Devarshidy,
Hon.Veterinarian,
Belagavi

Dr. (Mrs)S.A.Hogade,
Officer Incharge,
Central Animal House,
JNMC, Belagavi

Dr. (Mrs)S.M.Bhimalli,
Prof of Anatomy
JNMC,Belagavi

Dr. Vishwanatha Swamy
AEM
Link Nominee CPCSEA
Dept of Pharmacology &
Toxicology
KLE's Coll Of Pharmacy,
Hubballi

CERTIFICATE

This is to certify that the M.D/ M.D.S/ Ph.D/ Research project Entitled "Effect of *Trigonella foenum-graecum* on gene expression in liver tissue of experimentally induced diabetic rats"

Submitted by- PG Pharmacology, JNMC.

Has been approved by the Institutional Animal Ethical Committee

Meeting held on 22-2-20 vide Resolution No. 12/1

For sanction of 38 Male Wistar Rats

(Dr. P.A. Patil)
Signature and Name:
CPCSEA-Main Nominee

Dr. Rekha Naya
Signature and Name:
Chairman/Mem.Secretary

ANNEXURE - II - CPCSEA REGISTRATION & RENEWAL

No. [illegible] - AWD (P1)
Government of India
Ministry of Statistics & Programme Implementation
(Committee for the Purpose of Control and Supervision of Experiments on Animals)

Shastri Bhawan, New Delhi-110001
Dated the 19th June 2002.

To

The Principal/Director/Dean
K.L.E. Society's Jawaharlal Nehru Medical College
Nehru Nagar
Belgaum
Karnataka

Subject: Registration of Establishments [illegible] "Breeding of and Experiments on Animals (Control and Supervision) Rules 1998".

Sir/Madam,

[illegible] Registration Number is [illegible] CPCSEA [illegible]

1. You are requested to quote the above Registration Number in all your future correspondence with the Committee. [illegible]
2. [illegible]
3. [illegible]

[illegible]

Yours faithfully,

MEMBER SECRETARY (CPCSEA) / DIRECTOR [illegible]

Copy to: [illegible]

F. No. 25/373/2010-AWD
Government of India
Ministry of Environment, Forest & Climate Change
Animal Welfare Division
O/o Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)
******
5th Floor, Vayu Block, Indira Paryavaran Bhawan,
Jor Bagh Road, New Delhi - 110003
28/12/2017

To

Dr. Parwati Patil, Chairperson, IAEC
K.L.E.Society's Jawaharlal Nehru Medical College
Nehru Nagar, Belgaum - 590 010 KarnatakaTel:
0831-2471701/02
Email:docparwati@yahoo.co.in
Mobile:9449018436

Subject: Renewal of Registration and Reconstitution of Institutional Animals Ethics Committee (IAEC)-regarding
Madam,

The registration of Animal House Facility of your establishment with CPCSEA has been renewed **for a period of five years from the date of issue of this letter.**

- The new registration number of Animal House Facility of your establishment is ***627/PO/Re/S/02/CPCSEA for Research for Education Purpose on small animals.*** Henceforth, the new registration number may kindly be quoted in all your future correspondence with this office.
- The CPCSEA has accepted the following members recommended by the establishment:

S.No.	Name of the IAEC Members	Designation in IAEC
1	Dr Parwati Patil	Biological Scientist, Chairperson
2	Dr Rekha M.R. Nayaka	Scientist from different discipline, Member Secretary
3	Dr. Sumati A Hogade	Scientist Incharge of Animal House Facility
4	Dr. Shilpa M. Bhimalli	Scientist from different discipline
5	Dr. Sudha Devareddy	Veterinarian

- CPCSEA hereby nominates the following members to the Institutional Animals Ethics Committee (IAEC) of your establishment:

S.No.	Name	Nominated as
1	**Dr. P.A. Patil** 'Vishag' 23-A, II Main, II Cross, Bauxite Road, Belgaum - 590010, Karnataka Contact No :9448089510 Email : drpapatil@yahoo.co.in	Main Nominee
2	**Dr. Viswanatha Swamy A.H.M.** Associate Professor, Dept. Of Pharmacology & Toxicology, Karnataka Lingayat Education Society's College of Pharmacy, Vidyanagar, Hubli - 580 031, Karnataka Contact No :9449667355 Email: [illegible]2004@yahoo.com	Link Nominee
3	**Dr. Banappa S Unger** Scientist -D (Pharmacology), Regional Medical Research Centre, Indian Council of Medical Research, Nehru Nagar, Belgaum-590010, Karnataka Contact No :9916373018 Email : banappa[illegible]@gmail.com	Scientist from outside the Institute
4	**Shri. Sunil R Patil** At/po: Nidasoshi, Tq: Hukkeri, Dist: Belgaum, Karnataka - 591236 Contact No :9902443037 Email : [illegible]@rediffmail.com	Socially Aware Nominee

(Please note that any change in IAEC members can be made only with prior approval of CPCSEA.)

- The IAEC is valid for a period of five years and is coterminous with renewed period of registration. IAEC is required to be reconstituted at the time of renewal of registration as per CPCSEA guidelines [illegible]

same on the website of the CPCSEA.

- **It is stated that only above approved IAEC members shall sign, with date, on the attendance sheet of the IAEC meetings, and decisions will be taken only in meetings where quorum is complete. The quorum for holding IAEC meeting is six (6), and CPCSEA Nominees must be present in such meetings .Link Nominee can attend in case main nominee conveys his unavailability in writing to the chairman IAEC.Socially aware member's presence is compulsory in cases referred to CPCSEA and atleast in one meeting in a calendar year.Any decision taken in the meetings of IAEC without quorum shall be considered invalid.**
- It is also to inform you that before commencing any research on large animals you are required to send research protocols with due recommendation of IAEC to CPCSEA for further approval (procedure for submission of Research Protocols is available on the website of CPCSEA).

Yours faithfully,

(S. Gowri Shankar)
Deputy Secretary (AW) & Member Secretary (CPCSEA)

Copy for necessary action to: Nominees of CPCSEA.
The Main Nominee is requested to ensure that the IAEC meetings are held regularly as stipulated in the SOP of CPCSEA and submit the Annual Inspection Reports of the Animal House Facility regularly on the Website of CPCSEA.

ANNEXURE - III - MASTER CHART

L-PK		NC	DC	MET	TFG	CBN
	1	0.85	0.09	0.84	0.55	0.08
	2	1.01	0.18	0.04	0.46	0.05
	3	1.59	0.01	0.53	2.20	3.24
	4	0.44	1.54	0.12	185.46	3.00
	5	1.30	1.54	0.08	1.18	1.61
	6	1.27	0.63	0.12		0.08
	Mean	1.08	0.67	0.29	37.97	1.34
	Fold change	1.077431957	1.501540671	3.472779609	37.9695689	1.342503679
	Up/Down	↑	↓	↓	↑	↑
	SD	0.401909505	0.709733324	0.324546361	82.45513516	1.501329014

G6pc		NC	DC	MET	TFG	CBN
	1	0.203063099	2.084931522	2.848100391	1.624504793	17.26765178
	2	1.853176124	35.50622311	0.550952558	1.892115293	2.114036081
	3	5.958709852	98.3600116	0.38958229	1.853176124	3.732131966
	4	0.094732285	2.948538435	51.98415337	74.80174391	3.784230587
	5	4	2.181015465	0.450625231	2.770218936	0.47963206
	6	1.156688184	3.810551992	1.558329159		80.44885597
	Mean	2.211061591	24.14854535	9.630290499	16.58835181	17.97108974
	Fold change	2.211061591	24.14854535	9.630290499	16.58835181	17.97108974
	Up/Down	↑	↑	↑	↑	↑
	SD	2.32361189	38.64916107	20.77063159	32.54520435	31.19416045

Cyp7a1		NC	DC	MET	TFG	CBN
	1	1.117287138	3.61751751	3.91768119	5.241573615	2.620786808
	2	1.469168633	280.1391875	1.931872658	1.958840595	4.723970646
	3	1.049716684	199.4661324	4.907539909	10.056107	1.337927555
	4	0.901250463	3.60500185	0.035648866	231.5211207	2.361985323
	5	0.773782497	2.685145006	0.291183397	2.203810232	3.182145935
	6	0.817902059	1.790050142	18.00093576		288.0149721
	Mean	1.021517912	81.88383907	4.847476962	50.19629042	50.37363139
	Fold change	1.021517912	81.88383907	4.847476962	50.19629042	50.37363139
	Up/Down	↑	↑	↑	↑	↑
	SD	0.255990076	124.9571291	6.727493665	101.4162027	116.4253299

RBS (1)	Rats	NC	DC	MET	TFG	CBN
	1	114	455	503	458	355
	2	109	556	599	393	294
	3	98	591	542	333	388
	4	126	599	599	219	358
	5	111	357	512	455	275
	6	118	501	340		334
	Mean	112.6666667	509.8333333	515.8333333	371.6	334
	SD	9.373722135	92.85777655	95.51422233	99.56806717	42.45939236

RBS (30)	Rats	NC	DC	MET	TFG	CBN
	1	135	216	210	300	267
	2	133	472	186	241	233
	3	186	362	223	185	254
	4	127	231	265	154	257
	5	159	576	195	176	226
	6	165	553	142		287
	Mean	150.8333333	401.6666667	203.5	211.2	254
	SD	22.98187692	157.1071821	40.90354508	59.09906937	22.32487402

BW (1)	Rats	NC	DC	MET	TFG	CBN
	1	210	190	237	299	267
	2	200	268	310	269	286
	3	205	276	239	255	221
	4	215	280	337	276	290
	5	220	262	279	291	276
	6	219	267	228		310
	Mean	211.5	257.1666667	271.6666667	278	275
	SD	7.968688725	33.54052275	44.64825491	17.49285568	30.17283547

BW (30)	Rats	NC	DC	MET	TFG	CBN
	1	290	203	225	255	236
	2	248	254	316	232	303
	3	305	285	247	290	245
	4	272	370	355	305	346
	5	298	288	300	312	311
	6	280	263	228		356
	Mean	282.1666667	277.1666667	278.5	278.8	299.5
	SD	20.53695855	54.83581555	53.15166978	34.17162566	50.00299991