INVESTIGATION OF THE EFFECT OF QUERCETIN ON NEUROLOGICAL ALTERATIONS DURING TYPE 2 DIABETES MELLITUS IN SWISS ALBINO MICE

Thesis submitted in fulfillment of the requirements for the Degree of
DOCTOR OF PHILOSOPHY
IN
PHARMACEUTICAL SCIENCES

BY

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ENROLLMENT NO. 136755

DEPARTMENT OF PHARMACY
JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY
WAKNAGHAT, DISTRICT SOLAN, H.P., INDIA
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MAY 2019
Dedicated to my beloved grandparents

Mr. and Mrs. B. R. Mehta

&

Late Mr. and Mrs. Mangat R. Mehta

And my beloved Father

Late. Mr. (NK) Jiwan Lal Mehta
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DECLARATION BY THE SCHOLAR

I, Vineet Mehta, hereby declare that the work reported in the Ph.D. thesis entitled “Investigation of the effect of quercetin on neurological alterations during Type 2 Diabetes mellitus in Swiss Albino mice” submitted at the Jaypee University of Information Technology, Waknaghat, India, is an authentic record of my work carried out under the supervision of Dr. Udayabanu Malairaman. I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my Ph.D. thesis.

Vineet Mehta
Department of Pharmacy
Jaypee University of Information Technology, Waknaghat, India
SUPERVISOR’S CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled “Investigation of the effect of quercetin on neurological alterations during Type 2 Diabetes mellitus in Swiss Albino mice”, submitted by Vineet Mehta at the Jaypee University of Information Technology, Waknaghat, India, is a bonafide record of his original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

Dr. Udayabanu Malairaman
Assistant Professor
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Vineet Mehta
ABSTRACT

Diabetes mellitus (DM) is a complex progressive metabolic disorder arising from variety of pathogenic mechanisms, genetic or environmental, resulting in hyperglycemia. Hyperglycemia leads to both acute symptoms and chronic abnormalities, amongst which neuropathy, is one of the major causes of morbidity. In the present study, we screened 267 natural molecules for their antidiabetic potential and associated neurological complications through docking studies, followed by in-vitro assays. Our results demonstrated quercetin to be most efficient molecule and hence we further investigated its potential to modulate type II diabetes (T2DM) and associated neurological complications in animal model of experimental diabetes by using multiple low dose streptozotocin (STZ) injection in Swiss albino mice. Further, to get a better insight into the mechanism of quercetin action, we induced insulin resistant state in mice by subjecting them to 21 day chromic unpredicted stress (CUS) and investigated the neuromodulatory effect of quercetin treatment.

Diabetes was allowed to progress for 8 weeks during which animals were treated with 15 mg/kg quercetin and 5 mg/kg rosiglitazone. STZ treated animals showed steady increase in the blood glucose levels, development of glucose intolerance and insulin resistance (IR). Diabetic animals were highly anxious, depressed and showed marked learning and memory dysfunction. Quercetin and rosiglitazone treatment improved hyperglycemia, glucose intolerance, attenuated IR and behavioral dysfunction. Behavioral deficits in diabetic animals may be attributed to hyperglycemia mediated enhanced hippocampal oxidative stress, neurodegeneration and impaired neuronal insulin signaling, which were attenuated by quercetin and rosiglitazone treatment.

Further, CUS induced pre-diabetic state, IR and behavioral dysfunctions in mice, which were associated with enhanced hippocampal oxidative stress, neurodegeneration and impaired hippocampal insulin signaling. Quercetin treatment (30 mg/kg; po; od) during CUS improved glucose homeostasis, alleviated IR and behavioral dysfunctions, which may be attributed to its potential to attenuate hippocampal oxidative stress, enhance neurogenesis and by improving insulin signaling pathway.
Further, we confirmed the mechanism of quercetin action through cell line based studies. We cultured L6 muscle cells with quercetin and insulin in the presence and absence of insulin signaling inhibitor, LY294002, and determined the glucose uptake through fluorescent glucose (2NBDG) uptake assay. Quercetin and insulin upregulated 2NBDG uptake in control cells but failed when cells were treated with LY294002, suggesting that quercetin acts through insulin signaling pathway to exert its pharmacological effects.

Mechanistically, quercetin upregulated the neuronal insulin signaling pathway and enhanced GLUT4 expression, independent of insulin and InR. In conclusion, quercetin may find a clinical application in managing neurological complications associated with T2DM and IR by activating neuronal insulin signaling pathway, irrespective of insulin or InR expression.
LIST OF ABBREVIATIONS

% Percent

⁰C Degree Celsius

μg Microgram

μl Microliter

μM Micromole

ml Milliliter

Aβ Amyloid beta

AGE Advanced Glycosylated End products

ALX Alloxan

ANOVA Analysis of variance

BBB Blood Brain Barrier

BCL2 β-Cell Lymphoma 2

BSA Bovine Serum Albumin

CA1 Cornu Ammonis 1

CA2 Cornu Ammonis 2

CA3 Cornu Ammonis 3

CNS Central Nervous System

CPCSEA Committee for the Purpose of Control and Supervision of Experiments on Animals

CTCF Corrected Total Cell Fluorescence

CTRL Control

CTRL + Q Control + quercetin

CTRL + ROSI Control + rosiglitazone

CUS Chronic Unpredicted Stress

CUS + Q Chronic Unpredicted Stress + quercetin

DAB 3,3’-diaminobenzidine

DAPI 4’,6-diamidino-2-phenylindole
DCX  Doublecortin
DG     Dentate gyrus
DM     Diabetes Mellitus
DMEM   Dulbecco's Modified Eagle's medium
DPP-IV Dipeptidyl peptidase-4
DPPH   2,2-diphenyl-1-picrylhydrazyl
EPM    Elevated Plus Maze
FBG    Fasting Blood Glucose
FBS    Fetal Bovine Serum
FITC   Fluorescein isothiocyanate
FS     Foot Shock
FST    Force Swim Test
FWD    Food and Water Deprivation
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
GC     Glucocorticoid
GD     Gestational Diabetes
GIP    Glucose-Dependent Insulinotropic Polypeptide
GLP-1  Glucagon-Like Peptide-1
GLUT   Glucose Transporter
GLUT2  Glucose Transporter 2
GLUT4  Glucose Transporter 4
GTG    Goldthioglucose
H₂O₂   Hydrogen Peroxide
HbA1c  Glycated Haemoglobin
HOMA-IR Homeostatic Model Assessment for Insulin Resistance
HPA    Hypothalamic-Pituitary-Adrenal axis
IAEC   Institute Animal Ethics Committee
<table>
<thead>
<tr>
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<tr>
<td>IDE</td>
<td>Insulin Degrading Enzyme</td>
</tr>
<tr>
<td>InR</td>
<td>Insulin Receptor</td>
</tr>
<tr>
<td>INSG1</td>
<td>Insulin Induced Gene 1</td>
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<td>i.p.</td>
<td>Intraperitoneal</td>
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<td>IR</td>
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<td>NOR</td>
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<td>PBS</td>
<td>Phosphate Buffer Saline</td>
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<tr>
<td>PBST</td>
<td>Phosphate Buffer Saline having 0.1% Tween-20</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
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<tr>
<td>p. o.</td>
<td>Oral route of drug administration</td>
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<tr>
<td>PPAR-γ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>PSARL</td>
<td>Presenilins-Associated Rhomboid-Like Protein</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive Nitrogen Species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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ROSI  Rosiglitazone  
SD  Standard Deviation  
SDS-PAGE  Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis  
SGCT2  Sodium Glucose Co-transporter 2  
SOD  Superoxide Dismutase  
SPT  Sucrose Preference Test  
STZ  Streptozotocin  
STZ + Q  Streptozotocin + quercetin  
STZ + ROSI  Streptozotocin + rosiglitazone  
T1DM  Type 1 Diabetes Mellitus  
T2DM  Type 2 Diabetes Mellitus  
TBARS  Thiobarbituric Acid Reactive Substances  
TC  Tilt Cage  
TCF  Total Cell Fluorescence  
TRITC  Tetramethylrhodamine  
TST  Tail Suspension Test  
TTH  Total thiol  
UCP1  Uncoupling Protein  
WHO  World Health Organization
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CHAPTER 1

INTRODUCTION
1. INTRODUCTION

Diabetes mellitus (DM) is recognized as a life-style disorder affecting the glucose metabolism of our body. It is characterized by chronic and persistent hyperglycemic state, which may arise from variety of genetic and environmental factors [1]. Hyperglycemic state may arise either due to the destruction of insulin secreting pancreatic β-cells, resulting in accumulation of glucose in the circulation due to lack of insulin (Diabetes Mellitus Type-I; T1DM), or it may arise because of the reduced sensitivity of target cells towards the biological action of insulin, known as insulin resistant state (Diabetes Mellitus Type-II; T2DM). [2]. Clinically, polydipsia, polyuria, polyphagia, weight loss, fatigue and numbness in the extremities are the major symptoms of DM [3]. In the year 2015, diabetes affected 415 million people worldwide and was having a prevalence rate of 8.5% in the adult population, besides, approximately 46% cases of T2DM remained clinically undiagnosed. Prevalence rate of DM had quadrupled since 1980. It is estimated that by the year 2040, DM will be the 6th leading cause of death and there will be 642 million people living with DM. In the year 2012, 3.7 million people died as a consequence of DM, amongst which 1.5 million deaths were directly attributed to DM and 2.2 million additional mortality resulted from hyperglycemic related complications. Following China, India is home to second largest diabetic population in the world (69.2 million) with the prevalence rate of 8.7% in 2015 and approximately 52% cases of T2DM remained undiagnosed [4, 5]. Diabetes has become epidemic in recent years and the risk factors responsible for the development of DM are also increasing at alarming rate. Obesity, overweight and physical inactivity are the prime risk factors leading towards the development of hyperglycemia. According to a report of WHO (2016), 84% of girls and 78% of boys were short of the physical activity standards for their age group, 1 in every 3 adults was observed to be overweight and 1 in every 10 individual was categorized as obese [5]. These disturbing figures clearly indicate the need to look for the counter measures against DM and its associated secondary complications.

The mortality and morbidity associated with DM can be attributed to the secondary diabetic complications, amongst which cardiovascular complications, neuropathy, nephropathy and retinopathy are well documented. Moreover, currently available antidiabetic therapeutics is focused on eliminating excessive glucose from the blood circulation, and therefore, provides only symptomatic relief from the disorder, without modulating the causative factors for DM or
its complications. Neurological complications of diabetes are highly devastating and are least understood. Despite enormous healthcare advancement and regular therapy, DM and its associated neurological complications keep on progressing day by day, which enormously impairs the quality of life [6]. Thus, the demand of the present day is to search for suitable alternatives that could halt the diabetic progression along with central nervous system (CNS) abnormalities that have already developed.

Hyperglycemia induces several structural, chemical and functional irregularities in the CNS. Neurodegeneration, memory impairment, neurochemical changes, blood brain barrier (BBB) destruction, hippocampus and cortex and ultrastructure abnormalities of mitochondria, endoplasmic reticulum, microtubule, and chromatin mainly contributes to diabetic CNS complications [7]. Mechanisms leading to the diabetic neuropathy are linked to the reactive oxygen (ROS) and nitrogen species (RNS) generation, either directly or indirectly, followed by mitochondrial dysfunction, enhanced oxidative and inflammatory stress. Hyperglycemia increases the load of ROS/RNS through advanced glycation end-products (AGE), polyol, poly ADP ribose polymerase (PARP), hexosamine and oxidative stress pathways leading to saturation of protective enzymes, catalase and superoxide dismutase (SOD), responsible for neutralization of free radicles. ROS/RNS, in turn, leads to the destruction of neurons, especially axon where blood supply and mitochondria are present in an abundant amount [6, 8, 9]. Neurogenesis in the adult mammalian brain occurs in the subgranular zone of the dentate gyrus in the hippocampus, which is actively involved in the learning and memory functions. Thus, regulation of hippocampal neurogenesis modulates the learning and memory functions of the individual [10].

Previously reported studies associates both, T1DM and T2DM with reduced hippocampal neurogenesis [11-14]. Biological activity of the peripherally synthesized insulin in the brain is a debatable question since decades. Several researchers have reported that the peripheral insulin can cross BBB to exert its biological action, whereas conflicting reports suggest that insulin is indigenously synthesized in the brain [15-18]. The mammalian brain lacks its own fuel stores and it utilizes circulating glucose as its primary source of energy. Adult brain compromises approximately 2% of the body weight and it utilizes up to 60% of body's glucose (approximately 120g glucose/day), making it the major consumer of the glucose. Glucose metabolism in the brain provide fuel for maintaining normal physiological functions through the generation of the ATP and neurotransmitters and is essential for neuronal and non-neuronal cellular maintenance.
and functioning. Therefore, uninterrupted supply of glucose in the CNS is critical for regulating regular activities and functions of the brain. Glycemic state in the CNS regulates the energy metabolism of the entire body and any disturbance in the glucose metabolism in CNS adversely affects the healthy state of the brain and individual [19-22]. Insulin act on its receptors located in hippocampus and cortex region of the brain. These areas are having a large number of insulin receptors (InR), which on activation enhance expression of insulin responsive glucose transporters (GLUT) necessary for maintaining energy homeostasis of the neuron [23]. Within the brain, insulin is degraded by the enzyme known as insulin degrading enzyme (IDE), which is also known to clear amyloid-β (Aβ) protein from the brain. During T2DM, high blood glucose level induces hyperinsulinemia which downregulates insulin uptake by BBB thereby reducing its concentration in hippocampus and cortex, besides insulin resistance (IR) results in lower insulin levels through feedback mechanisms inside the brain. Lack of insulin in turn downregulates IDE and thus clearance of Aβ protein is reduced. Aβ deposits in hippocampus forming amyloid plaques leading to neurodegeneration, the decline in cognitive functions, dementia and late onset Alzheimer’s disease (LOAD). Further, IR developed during T2DM lowers glucose uptake into neurons, leading to neuronal starvation, degeneration, and alterations on behavioral and CNS functions. In T2DM all these factors combine together and contribute towards the decline in cognitive functions [24, 25].

Our lifestyle has changed drastically in recent years along with the technological development. T2DM is considered as one of the lifestyle disorders that arise from several environmental risk factors, of which obesity, overweight, lack of physical activity, unhealthy diet and daily life stress are the major contributing factors, besides genetic factors [1-5]. In recent years, several studies conducted on clinical subjects and experimental animals have revealed that environmental, emotional and other type of psychological stress is associated with the genesis of T2DM and demonstrate a significant comorbidity of stress with IR and T2DM [26-28]. Stress is an essential defense mechanism which prepares an organism, both physically and mentally, to manage the stressful circumstances faced regularly in daily life. However, chronic and prolonged stress has a deleterious effect on the body, especially in the CNS. Chronic stress results in prolonged activation of hypothalamic-pituitary-adrenal (HPA) axis, inducing adrenal hypertrophy and thereby excessive production of glucocorticoids (GC). GCs freely cross the BBB and promote hippocampal atrophy and functional decline by lowering glucose uptake and

3
ATP generation, elevating oxidative stress and by impairing reuptake of glutamate from hippocampal synapse resulting in prolonged excitotoxicity [29]. More importantly, the hippocampus has a high density of corticosterone receptors and therefore, hippocampal neurons are highly vulnerable to GC mediated toxicity. Chronic stress disrupts the hippocampal neuronal plasticity and induces hypercortisolemia, neurodegeneration and behavioral dysfunction such as depression, and cognitive impairments [30-35]. Hypercortisolemia is associated with the development of IR within the body, reduced pancreatic insulin secretion and impaired glucose homeostasis, thereby resulting in the development of T2DM like state [36-40]. It is now evident from the recent findings that both, chronic stress and T2DM, inflicts memory impairment, cognitive dysfunction, and other neurological disorders like Alzheimer's disease etc. [39-43]. In a separate study, it was reported that impaired cognitive function, characterized by low episodic memory performance, precedes stress and depression [44]. GCs further impair cognitive function by elevating the Aβ protein level in the hippocampus, reducing the size of the hippocampus, prefrontal cortex and other areas responsible for mood and cognition [45-47]. Further, hypercortisolemia has been reported to promote fat deposits viscerally which in turn cause inhibition of InR and affects insulin sensitivity levels in such patients [48] causing IR, and thus T2DM by antagonizing the hypoglycemic effect of insulin [49].

In past few decades, extensive research on insulin reports its presence in CNS. However, the exact role and mechanism by which it operates in the CNS still remain unclear to large extent. Although, locally synthesized insulin in the brain has been reported [50-52], no conclusive evidence is yet available whether it is produced by the brain itself or is transported across BBB from the peripheral system. Insulin/insulin signaling has neuromodulatory and neurotrophic effect on the brain and is necessary for maintaining normal cognitive functioning and has been reported to improve learning & memory and for maintaining synaptic plasticity [23, 53, 54]. Abnormal insulin/ InR activities are associated with cognitive dysfunction [55] and chronic stress [48]. Further, central IR impairs neuronal plasticity and intensifies neuronal oxidative stress [56], cognitive dysfunction [57] and enhance vulnerability to neurodegenerative ailments [58]. Furthermore, hippocampal expression of glucose transporter 4 (GLUT4) on neurons is insulin dependent and may play important role in learning and memory acquisition [59]. Accumulated evidence from past suggests that insulin signaling in the CNS may play a crucial
part in the development and progression of T2DM as well as behavioral dysfunctions associated with it and chronic stress.

Keeping in mind the side effects and ineffectiveness of clinical anti-diabetic therapeutic to prevent the progression of neurological complications associated with diabetes, research work in past have been focused on finding some alternative and safe therapeutic strategies, which not only prevent the progression of T2DM, but also alleviate the complications that had already set in. Further, WHO reported that 90% cases of T2DM can be prevented by making necessary lifestyle and dietary changes [60], indicating the impact of herbal therapeutic and dietary management of DM. Flavonoids are regularly consumed in the diet in form of fruits, vegetables, beverages etc., are well-known antioxidants and are known to lower the oxidative stress in the body. Clinically, enhanced dietary flavonoids consumption reduces the risk of T2DM and inhibit oxidative DNA damage in the diabetic patients [61, 62]. Plants and their preparations such as Gingo biloba [63], sweetened dried and raw cranberries [64], green tea, grapes [65, 66], Dodonaea viscosa [67], Cecropia pachystachya [68], Vaccinium angustifolium [69], Helichrysum plicatum [70], Urtica dioica [41] etc. are known to possess high percentage of flavonoids and are reported to possess antidiabetic potential in various experimental animal models.

Present research work was aimed to investigate how T2DM or insulin signaling and its impairment in CNS affects behavior such as depression, cognitive abilities, learning and memory. Moreover, we aimed to investigate whether or not quercetin is having any beneficial effect in modulating behavioral complications associated with T2DM and to elucidate its mechanism of action. In the present study, we screened 267 small molecules of natural origin, through in-silico and in-vitro experimentation, for their potential against T2DM and its associated CNS complications, and concluded quercetin to be the most promising molecule. We further investigated the mechanism through which T2DM inflicts neuronal deficit and behavioral dysfunction by using mice model of experimental diabetes and chronic stress and evaluated the effect of quercetin on it. The mechanism of quercetin action was then confirmed through cell line based assays and in-silico docking studies.
CHAPTER 2

REVIEW OF LITERATURE
2. REVIEW OF LITERATURE

2.1. Diabetes Mellitus (DM)

DM is characterized by impaired glucose homeostasis and persistent hyperglycemia. DM is a complex condition that arises because of either body is not able to produce enough insulin to dispose of the glucose in the circulation or body is not able to utilize insulin efficiently, thereby continuously accumulating glucose in the circulation [2, 4]. Symptomatically, DM is associated with polyphagia, polydipsia, and polyuria, besides tingling sensation and numbness in the extremities is usually observed in advanced stages of the disorder as a result of peripheral neuropathy. Clinically, DM is diagnosed by estimating fasting blood glucose (FBG) and glycated hemoglobin levels (HbA1c), which divides population into three categories; viz. normal (FBG ≤ 100 mg/dl or HbA1c levels ≤ 5.6%), prediabetic (FBG 101-124 mg/dl or HbA1c levels 5.7-6.4%) and diabetic (FBG ≥ 125 mg/dl or HbA1c ≥ 6.5%) [1, 2, 4, 5]. DM in itself is not a single disorder but it is a result of a combined effect of etiologically different mechanisms that result in a common phenotype of impaired glucose homeostasis and hyperglycemia. These etiological factors are associated with impaired secretion of insulin from the pancreases, reduced glucose utilization and increased dietary uptake, besides inflicting IR state in the entire body, resulting in the development of hyperglycemia [1, 71]. Impaired glucose homeostasis and persistent hyperglycemic state interfere with the normal physiology and functioning of different organs and organ systems, thereby leading to several complications which compromise healthy state and the quality of living [1, 6, 71].

2.2. Classification of Diabetes Mellitus

2.2.1. Type I Diabetes Mellitus (T1DM)

T1DM is also known as juvenile onset diabetes or insulin dependent DM. It appears suddenly in the early childhood because of the lack of insulin within the body as a result of pancreatic β-cells destruction, which may be a result of autoimmune, genetic or environmental causative factors. This condition is incurable till date and the patient is entirely dependent on insulin injections, without which it cannot survive [1, 2, 4, 71].

2.2.2. Type II Diabetes Mellitus (T2DM)

T2DM appears in the later stages of life and is known as late onset or insulin independent DM. It usually remain undiagnosed for several years. It is a most prevalent form of DM and T2DM
alone account for approximately 90% cases of DM. It is generally attributed as life style disorder originating primarily from unhealthy diet, lack of physical activity and overweight. No permanent cure is available for T2DM till now, however, certain dietary and lifestyle changes may help in the management along with antidiabetic drugs [1, 2, 4, 71].

2.2.3. Gestational Diabetes (GD)
GD appears during the third trimester of the pregnancy and it can adversely affect both mother and fetus. It has to be managed during pregnancy by several drugs like insulin and metformin and its symptoms usually disappear after pregnancy. However, both mother and child remain at higher risk of the development of T2DM in future [1, 71].

2.3. Epidemiology
With the advancement of technology and modern lifestyle, the prevalence of DM has consistently increased in past few decades. The number of diabetic patients has increased to 415 million in 2015 from just 108 million in 1980 and it is estimated that by the year 2040 there will be 642 million diabetes patients. Prevalence of diabetes in the adult population (>18 years) have shown marked increase in past few decades, with the prevalence rate of 4.7% in the year 1980 and 8.8% in the year 2015. In the year 2015, diabetes accounted for 5 million deaths globally and inflicted a burden of 673 billion USD for healthcare management. Further, 16.2% (20.9 million) live births were affected by diabetes in 2015 and 318 million people were diagnosed with impaired glucose tolerance. Further, International Diabetes Federation estimates that one in eleven individuals have diabetes, almost 50% cases of T2DM remain undiagnosed, 12% of global healthcare budget is spent on diabetes alone, every six seconds a life is lost due to diabetes and one in every seven live births are affected by diabetes.

Prevalence of diabetes is rising more rapidly in the countries of low- and middle-income zone. India is home to 69.2 million diabetic patients and spends only 3% of global expenditure on diabetes management (23 billion USD). It is estimated that by the year 2040 India will be home to 103.5 million diabetic patients, which does not include the population which remains undiagnosed (almost 46%).

Further, India ranks first in terms of being home to a population having impaired glucose tolerance (36.5 million) and is estimated that this number will go up to 63.6% by the year 2040.
To add further, India is home to a second largest population of children (0-14 years) having T1DM (70,200) [2-4].

2.4. Risk Factors

The exact cause of the development of T1DM is not known, however, the complex genetic-environmental interaction is believed to be prime factor. Though no strong evidence for the involvement of any environmental factor is yet established, the most accepted hypothesis is that development of T1DM is governed by autoimmune dysfunction, where antibodies are directed against the pancreatic β-cells, thereby compromising the insulin producing capacity of the body and ultimately leading to hyperglycemia. For this reason management of T1DM is entirely based on the external supply of insulin.

T2DM, on the other hand, is the predominant form of DM accounting for 90% diabetic cases. It is a consequence of several environmental factors, besides, the family history of diabetes also is a critical factor. Overweight, unhealthy diet, lack of exercise, impaired glucose homeostasis and reduced physical activity are the prime risk factors responsible for the development of T2DM. Further, family history, increasing age, gestational diabetes and poor nutrition during pregnancy also contributes significantly towards T2DM (Figure 2.2A). Globally, more than one in three adults were diagnosed overweight and more than one in every 10 individual were diagnosed to be obese in 2014 [2, 4, 5]. Interestingly, Neel and Sargis (2011) have reported a strong correlation between prevalence of T2DM and industrial chemical production (Figure 2.2B). This report suggests that industrial chemicals which are consumed regularly by humans throughout
the world, such as artificial preservatives, flavoring agents, coloring agents, antimicrobials, taste enhancers etc. used in beverages and packed food greatly increases the risk for the development of T2DM [72].

![Figure 2.2: Risk factors for the development of T2DM (A) [2, 4, 5]; Correlation between industrial chemical production and prevalence of T2DM (B) [72]

India, being a developing nation, is comparatively at the higher risk for the development of T2DM based on the facts which demonstrate that 73% adult population of India is overweight and one in every two individual is obese, with maximum risk age between 28-38 years (Figure 2.3A). Further, 269.7 million diabetic people in India resides in the urban area. Urbanization is estimated to cause a drastic increase in diabetic load by the year 2040, the diabetic population will almost double by then and reach up to 313.3 million. In contrary rural population is estimated to remain somewhat stable and will show an only slight increase in diabetic population (145.1 million in 2015 and 163.9 million in 2040) (Figure 2.3B).

2.5. Complications

Without proper management, diabetes adversely affects the normal functioning and physiology of various organs and organ systems of the body. These complications, individually as well as combined, significantly compromise the healthy living and are primarily responsible for the morbidity and mortality in diabetic patients. Hyperglycemia can have a devastating effect which may be life threatening if it triggers conditions such as diabetic ketoacidosis and hyperosmolar coma, which is more prominent in T2DM. Diabetic problems are
not always associated with hyperglycemia, but occasionally abnormally low blood glucose have been observed in diabetic patients, especially if patient skips meals, take an overdose of antidiabetic or after physical exertion. This condition may lead to unconsciousness and occasional seizures may be precipitated [1, 6].

Diabetes inflicts maximum damage in the nervous system, cardiovascular system, kidney, and eyes. Excessive ROS, RNS, and AGE produced during hyperglycemic state interact with the biomolecules, damages blood capillaries and blood vessels and increases the risk of heart disorders, hypertension, stroke etc. It further produces HbA1c, which compromises the oxygen carrying capacity of the hemoglobin and thereby generating ischemic like state in the body. This, in addition to reduced blood flow, especially to the extremities, results in nerve damage or neuropathy. Peripheral diabetic neuropathy is responsible for foot ulcers, infections and increases the chances of leg amputation [1, 6]. Central neuropathy is less understood, however, accumulated evidence from past suggest that diabetes increases neurodegeneration in the CNS.
and is having a strong association with neurological complications such as learning and memory dysfunction, cognitive impairments, depression, Alzheimer’s disease, Parkinsonism etc. [73, 74]. Diabetes mediate damage of retinal small blood vessels, reduced blood flow, oxygen and nutrient supply to the retina, leading to diabetic retinopathy, which is one of the major cause of blindness. Further, nephrons are highly sensitive to hyperglycemic damage and diabetes is one of the major cause of kidney failure, and this condition is known as diabetic nephropathy. During pregnancy, diabetes has deteriorating effects on both fetus and mother. DM markedly enhance the risk of congenital malformations, delayed development, obstetric complications, c-sectioning, stillbirth, perinatal death, and maternal morbidity and mortality, besides increasing the risk for developmental disorders, such as autism, in children [1, 6, 75]. Consideration the marked increase in the global prevalence of diabetes along with advancement in the healthcare sector, which have significantly increased the life expectancy in diabetic patients, continuous therapy, social stress and increased lifespan have modified the spectrum of secondary diabetic complications and associated morbidity [1, 2, 4-6].

2.6. Management of Diabetes

It is now evident that about 46% cases of T2DM remain undiagnosed for a long time and by the time clinical symptoms appears, enormous damage in the biological system has already occurred, which is mostly not reversible. Therefore, the first step in the management of diabetes is the early detection, longer it remains undiagnosed, more severe will be diabetic complications [2, 4-6]. Further, till now there is no permanent cure for this disorder and best possible management is through diabetes prevention, which needs to eliminate risk factors leading to diabetes way before diabetes is detected clinically. Clinical or drug management is a need in cases where generally long standing diabetes exists, which is difficult to control. Diabetes is a progressive disorder and despite continuous therapeutic management, it steadily progresses and so does its complications, although progression rate could be delayed with proper management. Therefore, continuous monitoring and management can improve life expectancy and quality of life, but it fail to treat this disorder and its secondary complications that have already set-in [76].

Recent reports demonstrated that incidence of T2DM can be reduced by following necessary dietary modifications and life style changes. Eating healthy food, fibers, regular exercise, maintaining normal mass index (25 kilogram/m$^2$) and avoiding smoking, alcohol, saturated and
trans-fats rich diet has resulted in reduction on the incidences of T2DM. Patients suffering from T2DM need to be continuously monitored for drugs, diet and physical activity for better results [60, 76].

2.6.1. Pharmacological Interventions

Pharmacological interventions that are commonly used in the management of T2DM are listed in Table 2.1.

Table 2.1: List of drugs used clinically in the management of DM

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Class</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sulfonylureas</td>
<td>Tolbutamide, Acetohexamide, Tolazamide, Glipizide, Glyburide, Glimepiride, Gliclazide, Gliquidone, Chlorpropamide,</td>
</tr>
<tr>
<td>2.</td>
<td>Biguanides</td>
<td>Buformin, Metformin</td>
</tr>
<tr>
<td>3.</td>
<td>Thiazolidinediones</td>
<td>Rosiglitazone, Pioglitazone, Troglitazone</td>
</tr>
<tr>
<td>4.</td>
<td>Meglitinides</td>
<td>Repaglinide, Nateglinide</td>
</tr>
<tr>
<td>5.</td>
<td>α-glucosidase inhibitors</td>
<td>Acarbose, Miglitol, Voglibose</td>
</tr>
<tr>
<td>6.</td>
<td>Incretin-based therapies</td>
<td>Exenatide and Liraglutide</td>
</tr>
<tr>
<td>7.</td>
<td>Insulin and analogues</td>
<td>Insulin, NPH insulin, Lente insulin, Ultralente insulin, etc.</td>
</tr>
<tr>
<td>8.</td>
<td>DPP-IV inhibitors</td>
<td>Vildagliptin, Sitagliptin, Saxagliptin, Linagliptin</td>
</tr>
<tr>
<td>9.</td>
<td>Others</td>
<td>Anagliflozin, Dapagliflozin, Empagliflozin, Exenatide, Liraglutide, Taspoglutide, Lixisenatide,</td>
</tr>
</tbody>
</table>

2.6.1.1. Sulfonylureas

Sulfonylureas are divided into two subclasses of which tolbutamide and chlorpropamide are considered as first generation sulfonylureas. Acetohexamide, tolazamide, glipizide, glyburide, glimepiride, gliclazide, and gliquidone are classified as the second generation sulfonylureas. Sulfonylureas are well tolerated by the body and act by enhancing pancreatic insulin secretion. Mechanistically, sulfonylureas act on its receptors located on the membrane of β-cell and induce depolarization through downregulation of ATP-sensitive potassium channels, which in turn enhances influx of Ca\textsuperscript{2+} leading to degranulation of insulin storing vesicles and stored insulin is released into the circulation, irrespective of the glycemic state. Sulfonylureas modulates the
second phase of insulin secretion, without affecting the first phase [71]. Sulfonylureas are associated with hypoglycemia and overdose may lead to unconsciousness and seizures. Elderly patients are at higher risk of development of hypoglycemia [77]. Long acting sulfonylureas such as glyburide are commonly associated with hypoglycemia which is generally precipitated because of impaired renal functions, age, insulin and insulin sensitizers use, reduced diet, excessive exercise and physical activity, alcohol abuse and drug interaction. Therefore, use of short acting sulfonylureas such as gliclazide and glipizide is recommended in elderly patients [78-80].

2.6.1.2. Biguanides

Biguanides includes metformin, phenformin, and buformin. Metformin is the most commonly prescribed drug from this class. Biguanides are used for the management of DM in patients who are generally obese and overweight. Biguanides act through multiple mechanisms which includes suppressing glycogenolysis, sensitizing cells towards insulin, increasing glucose uptake by elevating cellular GLUTs expression, reducing dietary uptake of glucose and by increasing fatty acid oxidation [81]. Further, recent findings demonstrated that metformin interferes with the expression of the gluconeogenic gene in the liver, thereby promoting gluconeogenesis and disposing glucose from blood [82]. Hypoglycemia is usually not associated with biguanides, however, may occasionally precipitate low blood glucose levels, besides needs continuous monitoring for lactic acidosis, which limits its clinical use especially in elderly, during pregnancy and kidney failure [81].

2.6.1.3. Meglitinides

Repaglinide and nateglinide come under meglitinides category of oral hypoglycemics. These drugs are non-sulfonylurea secretagogues and acts by increasing insulin secretion from pancreas through a mechanism similar to sulfonylureas. They act by binding on to sulfonylurea receptor as well as to other receptors [71, 83]. Meglitinides are given just before meals to control postprandial glucose rise and they are short acting drugs (4-6 h), therefore did not possess potential to induce hypoglycemia [84]. These drugs are metabolized in the liver as well as in kidney, therefore, continuous patient monitoring is necessary if used in elderly or patients with compromised liver and kidney functions [83].
2.6.1.4. Thiazolidinedione

Rosiglitazone, pioglitazone, and troglitazone come under this class. These drugs are very potent insulin sensitizers, hence commonly prescribed for the management of IR and T2DM. These drugs act through activation of peroxisomes proliferator activated receptor-γ (PPAR-γ). PPAR-γ interact with the retinoid-X receptor forming a complex, which enters the nucleus and activates PPAR-γ sensitive gene elements leading to transcription of genes responsible for insulin sensitization [6, 71, 85]. Thiazolidinediones are not associated with hypoglycemia since they are insulin sensitizing agents and therefore are frequently used in patients with compromised renal functions, however, rosiglitazone is associated with cardiovascular toxicity and therefore finds limited clinical use [86]. Further, these drugs are known to cause peripheral edema, fluid retention, weakens bones, increase fracture risk, and therefore their use is limited in women and elderly patients. These drugs are contraindicated in patients having congestive heart failure and those who suffered from a heart attack [87].

2.6.1.5. α-glucosidase Inhibitors

α-glucosidase inhibitors class of oral hypoglycemic comprises acarbose, miglitol, and voglibose. As the name suggests, these drugs act by limiting the dietary inflow of glucose by inhibiting the activity of the α-glucosidase enzyme, which is involved in the carbohydrate metabolism and digestion. These drugs are not commonly used but find some clinical applications in the management of postprandial glucose surge in obese patients. These drugs are contraindicated during renal complications and are associated with occasional diarrhoea and flatulence [78].

2.6.1.6. Incretin-Based Therapies

Incretin is referred to the hormone/s which stimulate insulin release from the pancreas in response to meals. Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulino tropic polypeptide (GIP) are two incretins present in our body. Drugs such as exenatide and liraglutide are classified as incretin-based antidiabetics. These drugs improve glucose homeostasis and have proved effective in the management of weight. These drugs are free from the risk of hypoglycemia and are generally prescribed as an adjuvant therapy along with oral hypoglycemic agents, exercise, and dietary management. Further, recent literature suggests that these drugs may be beneficial during inflammatory stress and cardiovascular complications, besides having CNS stimulating effect [78, 88].
2.6.1.7. Dipeptidyl Peptidase-IV (DPP-IV) Inhibitors

DPP-IV is an enzyme which is known to inhibit the activity of naturally occurring incretin hormones. Therefore, DPP-IV inhibitors increase incretin hormones in our body and thereby improve glucose homeostasis during T2DM. DPP-IV inhibitors are the comparatively new class of antidiabetic drugs and consists of vildagliptin, sitagliptin, saxagliptin, and linagliptin. These drugs are very efficient in managing diabetes as a monotherapy, especially in those patients where dietary and exercise measures failed to produce efficient results. There are occasionally prescribed along with other hypoglycemic such as insulin and thiazolidinedione to generate better glycemic control and to reduce dose and adverse effects of oral hypoglycemics. These drugs are non-hypoglycemic but are expensive, besides having a limited potency which cannot control extreme diabetic cases [89, 90].

2.6.1.8. Insulin and Insulin Analogues

Insulin is a well-tolerated and gold standard drug for the management of diabetes. It is mostly free from any side effects, however, dosage has to be strictly monitored. Insulin therapy suffers from several drawbacks. Like others it also provides only symptomatic relief without any effect of progression of diabetes and its complications. With time body develops resistance to insulin and its dose has to be increased with time. Moreover insulin is very short acting and therefore has to be taken before every meal which in itself exerts lots of stress. Occasionally hypoglycemia has been reported with the use of short acting insulin, while long acting insulin is safer and is generally used in combination with insulin sensitizers for the management of T2DM. Another drawback associated with these formulations is that their absorption pattern varies from patient to patient and therefore there are chances of hypoglycemia (if rapidly absorbed and released) or inadequate control (if release is delayed). Another formulation of insulin comes in the form of inhalation, and it has been approved by FDA and European Medicines Evaluation Agency for the management of T1DM and T2DM. This formulation delivers insulin directly to lungs and it is rarely used clinically since it provides no additional advantage over other rapid acting formulations [76].

2.6.1.9. Others

Recently, dopamine agonist bromocriptine has shown some promising results in the management of T2DM. The underlying mechanism of bromocriptine action is not fully established, however,
it is reported to normalize HbA1c levels after 24-week therapy [91]. Sodium-glucose cotransporter 2 (SGCT-2) inhibitors, such as anagliflozin, dapagliflozin, and empagliflozin, are used in the symptomatic management of hyperglycemia. SGCT-2 is known to reabsorb 70-80% of glucose filtered from glomerulus in the nephron. Therefore, SGCT-2 inhibitors prevent this reuptake and lowers blood glucose levels by excretion of glucose in urine [92].

2.7. Limitations of Current Therapeutics

As discussed above, diabetic therapeutics is entirely based on eliminating glucose from the blood circulation, either through limiting dietary glucose input (α-glucosidase inhibitors, biguanides), disposing glucose into the cells (insulin, insulin analogs, thiazolidinedione, incretins, biguanides, sulfonylureas etc.) or by eliminating excessive glucose (SGCT-2). These therapeutic approaches are very efficient in limiting the blood glucose levels in safe range, however, they suffer from serious drawback such as hypoglycemia, cardiovascular complications, edema etc. These medications have to be taken daily, probably before every meal, and are to be continued throughout the life, which in itself exerts lots of financial burden and psychological stress to the patients and their attendants. Further, with time and continuous medications resistance develops against these medications, especially insulin, and the dose has to be increased regularly. These drugs are accompanied by serious side effects such as hypoglycemia, congenital damage, preeclampsia, eclampsia, prenatal death, cardiovascular risks, and can even precipitate seizures, besides, most of them are contraindicated in patients who are compromised with kidney and liver functions and in patients having history of cardiac disorders [4-6, 76]. More importantly, it is well established that about half of T2DM cases remain undiagnosed for a long time. By the time symptoms develops and disorder is clinically diagnosed, a large amount of damage in the biological system has occurred. Besides, diabetes and these complications, especially neuropathy, progress continuously despite regular drug treatments. Current therapeutics is not capable of preventing the development and progress of diabetes and diabetic complications, although they may slow down the rate of progression. Further, current therapeutics are not able to reverse the damage that has occurred due to diabetes at any stage. Diabetic complications, especially CNS neuropathy, is highly devastating and it markedly compromises quality and state of healthy living. T2DM exaggerate neuronal oxidative/inflammatory stress, cognitive impairments, learning & memory dysfunction,
depression, anxiety, autism and neurological disorders like Alzheimer’s disease and Parkinsonism. T2DM induces neurodegeneration and it interferes with neurogenesis, neuronal synaptic plasticity and survival, thereby leads to the development of several life threatening complications [4-6, 76]. None of the current therapeutics have shown potential to either halt or reverse CNS complications associated with diabetes. Therefore, it is necessary to develop some alternative treatment stratagems which may halt the progression of diabetes and associated complications.

2.8. Diabetes and Neurological Complications

Diabetes or hyperglycemia inflicts severe damage to the nerves in both peripheral and CNS, thereby precipitating a variety of complications. Combined, these complications are referred to as neurological complications associated with diabetes. In the CNS, these complications primarily include neurodegeneration, learning and memory dysfunction, depression, anxiety, autism, phobias and anorexia [93-95]. DM is recognized as one of the prime underling cause for the development of cognitive deficit, dementia, and depression in clinical as well as in experimental settings [93, 94, 96]. Compared to healthy population, patients suffering from diabetes are at greater risk of developing these complications [93, 94]. Neurological complications associated with the diabetes are linked to the development of excessive oxidative and inflammatory stress in the CNS, which induces neurodegeneration and thereby compromises neuronal physiology and functioning [97-99].

Clinical reports suggest the strong interplay between DM, depression and cognitive dysfunction [93]. The mechanisms leading to the development of these complications are very complex, and are governed by more than one independent mechanisms. Development of these disorders depends on several environmental as well as genetic factors which adds to the complexity of understanding the pathophysiology involved in it. A meta-analysis report including 21,351 adult subjects from 42 different studies demonstrated that prevalence of diabetes associated depression was 28% in women suffering from DM, 18% in males having DM, 21% in controlled studies, 30% in uncontrolled studies, 20% in community samples and 32% in clinical samples. Also, comorbididity was assessed to be higher (31%) in self-report questionnaires than diagnostic counselling (11%) [93, 96]. Global prevalence of comorbid diabetes and depression is also governed by demographical factors and prevalence greatly varies between economically different
nations, diabetes stage and type of diabetes, which make it very complex to get a generalized outcome. HbA1c is formed by a chemical reaction between hemoglobin and excessive blood glucose. It gives an average data of diabetes progression of past three months and in a study it was demonstrated that higher HbA1c levels show great correlation with the clinically diagnosed depression (Figure 2.4), suggesting that diabetic subjects are at higher risk of the development of depression [93]. Further, it is now established that diabetes, as well as depression, inflicts cognitive dysfunction, learning & memory impairments and dementia.

Hyperglycemia induces oxidative/inflammatory stress, microangiopathy and endothelial alterations, which contributes in the development of cognitive impairments. Further, depression, aging IR, insulin deficiency, hypoglycemia, hypertension, dyslipidemia etc. are known to exaggerate development of cognitive impairments and dementia. All these pathophysiological pathways have been summarized in Figure 2.5 below. Further, experimental in-vivo experimentation on rodents has demonstrated the correlation between experimental diabetes and depression. Further, the bidirectional relation between depression and cognitive dysfunction, diabetes and cognitive dysfunction and other behavioral dysfunction is also established [99-101].
Role of the brain in the management of blood glucose levels is not well established. However, considering the fact that brain compromises approximately 2% of total body's weight and utilizes 60-70% of body's total glucose, suggests its role in the regulation of overall glycemic state in the body. Further, presence of high density of GLUTs in the brain further indicate the involvement of the brain in maintaining overall glucose homeostasis in the body. Neurons are highly susceptible to the diabetes mediated damage. During T2DM, IR develops in peripheral as well as in CNS, thereby limiting the amount of glucose uptake by brain leading to neuronal starvation [19-22]. Further, insulin signaling dysregulation in the brain is associated with neurodegeneration, impairs plasticity and disrupts neurogenesis, which further exaggerate diabetes mediated behavioral dysfunctions [56-58].

Diabetes induces neuronal damage in the hippocampus via inflicting severe oxidative and inflammatory stress [97]. During oxidative stress, levels of nitric oxide (NO), hydroxyl (OH⁻) radicals, superoxide anion (O²⁻), and hydrogen peroxide (H₂O₂) and commonly attributed as ROS, rises significantly and damages the proteins and other biomolecules. Further, excessive production of ROS saturates antioxidant defense enzymes, which render neurons exposed to oxidative damage [103]. Further, NO induces inflammatory stress in the hippocampus [104] and is associated with neurodegeneration and depressive state in rodents [105].
2.9. Insulin and its role in CNS

Insulin is a pancreatic peptide hormone, which was discovered in 1921 by Frederick Banting and Charles H. Best. They demonstrated that insulin controls glycemic state in the body when they observed the hypoglycemic effect of the pancreatic extract of dogs. It was obtained in the pure crystalline form in 1926 and its chemical structure was fully characterized in the year 1956 by Sanger. Structurally, insulin is a 51 amino acids (5808 Da) hormone, having two chains, 21 amino acids join to forms the A-chain, while B-chain is composed of 30 amino acids. Both peptide chains interconnected by disulfide bonds formed by cysteine residues located at the 7\textsuperscript{th} position of A-chain and 7\textsuperscript{th} of the B-chain and 20\textsuperscript{th} position of A-chain and 19\textsuperscript{th} position of B-chain. Initially, insulin is synthesized as a single 110 amino acid peptide known as preproinsulin. Initially, 24 amino acids are cleaved off to produce 86 amino acid proinsulin peptide, followed by cleavage of 35 amino acid connecting chain (C peptide) inside Golgi apparatus through proteolysis to give bioactive insulin peptide and C-peptide chain. Both of these peptides are stored in the insulin storage vesicles and are secreted into circulation on activation [71].

Insulin secretion is controlled by a combination of neuronal, chemical, and hormonal mechanisms. Under basal conditions, human pancreas recreates approximately 1U insulin/h to maintain basal glucose level in the circulation. The release of the insulin from pancreas depends upon the activation of glucose sensing mechanism in the beta cells. Glucose enters β-cells through GLUT-2 located on their cell wall and gets phosphorylated by the glucokinase enzyme. This, in turn, activates glucoreceptor and inhibit ATP sensitive potassium channels leading to a partial depolarized state of β-cells. This increases intracellular levels of Ca\textsuperscript{2+} ions result in fusion of the membranes of β-cells and insulin storage vesicles and the stored insulin, along with C-peptide, is released into the circulation. Two hormones, namely glucagon, and somatostatin, also controls the rate and amount of insulin secretion by β-cells. Glucagon, secreted by pancreatic α-cells, evokes the release of insulin whereas somatostatin (secreted from the D-cells) has an inhibitory effect on insulin release. Further, neuronal mechanisms also control insulin secretion. Activation of cholinergic signaling and β\textsubscript{2}-adrenergic receptors increase insulin secretion and activation of α\textsubscript{2}-adrenergic receptor inhibit it [1, 71].

Mechanism of insulin action is governed through activation of InR which is present universally on every cell, however, the density may vary. InR is a heterotetrameric glycoprotein, which
consists of two extracellular α subunits and two transmembrane β subunits. InR on activation by insulin, result in phosphorylation and activation of tyrosine kinase residues located on the β-subunit. This activates the downstream signaling cascade which ultimately leads to the translocation of GLUT (stored in vesicles within the cytoplasm) to the cell membrane via phosphorylation of Akt, and thereby glucose is able to freely enter cells through GLUT [1, 6, 71]. A complete illustration of the mechanism of insulin signaling has been depicted in Figure 2.6 below.

![Figure 2.6: Schematic representation of Insulin signaling cascade. IRS1: insulin receptor substrate 1; IRS2: insulin receptor substrate 2; PI3K: phosphatidylinositol 3 kinase; GLUT4: glucose transporter type 4; INSG1: insulin induced gene 1; PPAR-γ: peroxisome proliferator activated receptor-γ; MAPK-1: mitogen activated protein kinase 1; PKB: protein kinase B; BCL-2: β-cell lymphoma 2 [6]
](image)

Role of insulin in the CNS is still a debatable question. There is a set of reports which suggest that neuronal glucose utilization is independent of insulin actions, while others contradict it. Further, the presence of a large number of InR and GLUT in the hippocampus and other parts of the brain suggest that insulin may be actively involved in glucose uptake and utilization in the brain [15-18]. Insulin action is very quick and GLUT4 translocation on to the hippocampal neuronal membrane and hippocampal glucose utilization has been associated with improving hippocampal neuronal activity and hippocampal dependent learning and memory formation [59]. Impairment in hippocampal insulin signaling contributes in the genesis of cognitive dysfunction
and the drugs which upregulate central insulin signaling are reported to improve cognitive functions [41]. Further central IR has been associated with behavioral dysfunction, impaired synaptic plasticity and neuronal damage [56-58].

2.10. Chronic stress and neurological complications

Acute stress is a common part of the day to day life and it is considered as an essential defense mechanism to prepare body physically as well as mentally to deal with a variety of stressors faced daily. However, chronic stress is undesirable and has a deleterious effect on the body. Chronic stress induces a variety of complications throughout the body. Pathophysiology of neurological complications associated with stress is not well understood yet [107, 108]. The stress response is mediated through HPA axis, which upon activation upregulate corticosterone levels in the blood. These corticosterone acts on the GC and mineralocorticoid receptors and prepares body response against the stressors. During chronic stress, prolonged activation of HPA axis occurs, resulting in hypercortisolemia. Excessive levels of circulating GCs induces GC toxicity throughout the body. GCs can freely enter the brain through the BBB and interact with the specific GC receptors located abundantly in the cortex and hippocampus. In hippocampus, hypercortisolemia induces neurodegeneration [109], reduces dendritic complexity [110], impairs synaptic plasticity [35, 111, 112], alter neuronal excitation process [35, 112], reduces hippocampal volume [108, 113], and induces behavioral dysfunction [30, 39, 40, 114, 115].

Mechanisms through which these complications develop are not fully known, however, stress mediated oxidative and inflammatory stress contributes markedly in the development and progression of these complications. Like DM, chronic stress inflicts oxidative stress by generating excessive ROS/RNS and depletion of neuronal antioxidants [39, 116, 117]. Enhanced oxidative stress during chronic stress is known to induce neurodegeneration in different regions of the brain [118], cognitive dysfunction [39-41, 114], depression [39, 40], anxiety disorder [119] and other psychiatric ailments [118, 120, 121]. Recent findings suggest that oxidative and inflammatory stress exist comorbidity in the brain and their interplay has been associated with marked impairment of neuronal plasticity and exaggeration of several neurodegenerative ailments viz. cognitive impairment, depressive behavior and Alzheimer's disease [116, 122-124].

Inflammatory stress further modulates pathophysiology of psychic disorders and down regulates signaling cascades which regulate mood and behavior of an individual in both humans as well as in animal models [123-127]. Further, these findings are strongly justified by studies where
alleviating oxidative and inflammatory stress in the different regions of the brain has shown remarkable improvement in the stress mediated behavioral alterations [124, 128, 129].

2.11. Comorbidity of Stress/Depression, T2DM and cognitive dysfunction

Under stressful conditions, cortisol provides our body with the extra amount of glucose through gluconeogenesis to be utilized in coping up with stressors. This energy is utilized for the fight-fight-fright response against stressors. Prolonged hypercortisolemia produces enough glucose to result in hyperglycemia. The actual mechanism underlying the development of stress mediated IR is unknown, however, it is established that cortisol suppresses the functioning of insulin and render cells resistant towards insulin action. During chronic stress whole body remain in the insulin resistant state. Continuously rising glucose levels exerts pressure on the pancreas to produce insulin. Eventually, with time they struggle to meet the increasing demand for insulin, glucose remains in circulation and cells starve to death in limited glucose availability due to IR [130]. Cortisol is associated with weight gain and is known to mobilize triglycerides to visceral fat deposits. Cortisol aids in the adipogenesis and maturation. Further, due to cell starvation (as a result of IR), brain continuously receive hunger signals leading to polyphagia. Excessive calories are stored as fat and thereafter obesity follows [130-132]. In recent year, accumulated evidence suggests that high levels of GCs in the circulation produce several physiological responses that is characteristic to T2DM. In experimental animals, peripheral administration of corticosterone has been demonstrated to impair both central as well as peripheral insulin signaling, leading to improper glucose homeostasis [133]. T2DM is known to induce hypercortisolema and a typical hypercortisolemic/hyperglycemic states exist in T2DM. Further, it is evident that hypercortisolema observed during T2DM adversely affects cognitive and memory functions [24]. Both, chronic stress and IR contributes in the hippocampal neuronal loss [134, 135] and stress and GCs are known to exacerbate pathogenesis of Alzheimer’s disease [133, 135].

Stress, anxiety, and depression share common pathophysiology and generally occurs together. Depression could be considered as the later stage of prolonged stress. Clinically, anxiolytics are commonly used in the management of depressive like behavior and has shown some promising results [136, 137]. Major depression results in serious deteriorative effect in the CNS and the volume/s of hippocampus, cortex, and amygdala are significantly reduced, which is primarily associated with high GC levels in the circulation, similar to that observed during exposure to
chronic stress [136]. Research work focused on studying the impact of GC on the neuroanatomical alterations in the CNS suggest that beyond certain limits of GCs in the brain, GC receptors present on the hippocampal neurons are overexcited, which render these neurons vulnerable to excitotoxicity, resulting in neuronal degeneration, hippocampal neuronal loss, reduced hippocampal volume and compromises hippocampus based functioning [34, 35]. Insulin is one of the prime factor regulating neuronal differentiation and neurogenesis in the hippocampus. These functions are antagonized by excessive circulating GCs, which causes a reduction in insulin secretion from the pancreas, increased glucagon levels, stimulates gluconeogenesis and induces T2DM like state in the body [37, 38].

Comorbidity of depression and diabetes is well documented, however, it is not yet clear that which of this conditions is the causative factor for the other. Diabetes greatly increases the risk of the development of depression. Population based studies demonstrated that prevalence of depressive like behavior is high in diabetic patients (8-25%) and even higher in patients where diabetes is accompanied with complications (40-80%). Further, these studies also demonstrated that incidences of hyperinsulinemia and hyperglycemia are higher in depressed individuals. Diabetes not only increases the risk of the development of depression but also worsen the symptoms of depression [138]. Meanwhile, depression compromises the normal physiology and functioning. Depression not only enhances the risk of T2DM development but also exacerbate the symptoms of diabetes. People having depression and diabetes showed more severe symptoms of diabetes than nondiabetic patients [37, 38, 139]. Increased risk of the development of depression in patients with T2DM has been associated with increased counter regulatory hormone mechanism, glucose intolerance and inflammatory stress [49]. Clinically, depression induces prediabetic like state in patients, which ultimately get transformed to T2DM if left unattended [138, 140]. In one of the cohort study, 11,615 nondiabetic patients with depressive like symptoms, such as a feeling of worthlessness, sleep dysfunction, loss of libido, suicidal tendencies, and increased irritability, were selected and monitored up to 6 years. Findings of these studies revealed that depression and its peculiar symptoms increase the incidence of T2DM, which could be explained through demographic, metabolic and lifestyle factors [138]. In another set of population based study, 2,127 middle aged men and 3,100 middle aged women suffering from psychological stress, anxiety, and depression were followed for more than 8 years (8-10 years). Results of this study revealed that physiological distress greatly increase the risk of
T2DM and it was observed that out of 2,127 men 245 were pre-diabetic and 103 had already developed T2DM. Further, out of 3,100 women studied, 177 were found to be pre-diabetic and 57 were diagnosed positive for T2DM. This report also concluded the higher susceptibility of males for the development of depression/stress mediated diabetes as compared to women [141]. Further, hazard ratio in comorbid depression and diabetes is higher than depression and diabetes alone (Figure 2.7).

![Figure 2.7: Effect of depression on all-cause mortality in patients with diabetes [93, 141]](image)

Observations from the clinical settings have been replicated successfully in animal models also. In rodent models, depression and chronic stress induced significant levels of hyperglycemia, impaired glucose homeostasis, hypercortisolemia, cognitive dysfunction, immunosuppression and hypoinsulinemia [142, 143]. Hyperglycemia results in several neurological dysfunctions such as depression, anxiety and cognitive dysfunction [97]. Both hyperglycemia and depression inflict neuronal oxidative stress, which is one of the mechanisms behind development of neurological complications [144]. Accumulated evidence suggests that hypercortisolemia, observed during chronic stress, depression, and diabetes, disrupts insulin mediated translocation of GLUT4 to the neuronal membrane and impairs signaling cascades, which results in reduced metabolic activities and neuronal plasticity in the hippocampus. All these factors combine together to generate a physiological impaired process resulting in neurodegeneration and cognitive dysfunction [33, 59, 145]. Chronic unpredicted stress (CUS) model in animals has been widely exploited to explore stress/depression induced neurological complications. Animals subjected to CUS mimic physiological stress observed in clinical settings through hyper activation of HPA axis. These animals exhibit IR, glucose intolerance and alter plasma insulin
levels [143, 146]. In rodent brain, insulin is known to act as a neuroprotective agent, improves dendritic sprouting, neuronal regeneration and supports neurogenesis. Impaired hippocampal insulin signaling induces memory dysfunction and pathophysiological condition that is similar to Alzheimer’s disease [147]. Pathogenesis of cognitive impairment is a very complex process, especially during the comorbid depression and diabetes (Figure 2.8). Development of neurological complications during chronic stress has been associated with the enhanced oxidative and inflammatory stress, directly or indirectly. Several anti-inflammatory agents have shown potential to alleviate stress and anxiety [116]. Hippocampal atrophy and hippocampal based functions have shown some improvement by intervening stress-free period [148].

![Figure 2.8: Pathophysiology of cognitive dysfunction during comorbidity of diabetes and depression](image)

Literature is filled with numerous reports which try to unwind the complex connection between the high levels of blood cortisol, depression, hyperglycemia and cognitive dysfunction. Patients receiving GC therapy has shown severe symptoms of depression and cognitive dysfunction [149-151]. Corticosteroids modulate neuronal plasticity. The pattern of hippocampal long term potentiation (LTP) was markedly altered upon enhancing circulating GC levels or when GC receptors were occupied (as observed during chronic stress, depression and T2DM), which generally follow a specific pattern at normal corticosterone levels in the blood [35, 152]. LTP potentiation is an important process in the hippocampal neurons which is essential for acquiring and retaining memory. Further, integrity of hippocampal cholinergic signaling is essential for learning functions and memory formation, and modulation of muscarinic receptors is known to alter cognition. Alterations in the muscarinic signaling have resulted in behavioral dysfunction like depression, learning and memory impairment and cognitive dysfunction. Both, hyperglycemia and chronic stress (or depression) are well documented to impair hippocampus based cholinergic signaling and induce behavioral alterations. These findings are supported by
the findings where improvement of hippocampal muscarinic signaling has improved behavioral alterations. For example, blocking of mAChR4 in the hippocampus result in presynaptic desensitization of autoreceptor and is one of the therapeutic targets in managing cognitive functions [104, 153-156].

Further, elevated levels of corticosterone downregulates hippocampal neurogenesis. Depression induces neurodegeneration, memory dysfunction, reduces hippocampal neurogenesis and volume [157]. GCs arrests natural cell differentiation and proliferation [158], induces apoptosis in mature and progenitor neurons [159], adversely affects synaptic plasticity, inflicts dendritic atrophy and result in the loss of synaptic activity in the hippocampus [148, 160, 161], adversely affecting mood, cognition and memory functions [34, 162, 163].

2.12. Experimental models of T2DM and stress

Experimental animal models are necessary to evaluate pathogenesis of any disease/disorder and the secondary complications associated with them. In a drug designing and evaluation process, these animal based studies are termed as preclinical studies prior to evaluation on human subject in clinical trials. Majority of procedures used to explore pathogenesis of T2DM, stress and IR and related secondary complications in pre-clinical or research studies are rodents (rat and mice) based, since their physiology and therapeutic response is similar to humans up to a great extent. Therefore, in the current section animal models which are widely used to evaluate T2DM and stress as well as its associated complications are discussed below.

2.12.1. Spontaneous/genetic models of T2DM

2.12.1.1. ob/ob mouse

It is an obese mouse model of T2DM and it is also known as Lep<sup>ob</sup> model. ob/ob mouse is derived from the C57BL/6J mouse strain through recessive monogenic autosomal mutation on chromosome number 6. It was first derived in the Bar Harbor, Jackson laboratory [164] and is one of the most popular genetic models to evaluate T2DM and associated complications. Mutation on chromosome 6 corresponds to leptin gene and mouse rapidly gains weight which may increase by 3 time than control wild-types. Leptin acts as a hormone and regulates brain centers involved in the feed intake, fat deposition, and energy utilization. Therefore, this mutation results in impaired leptin functioning and eventually alters energy metabolism, diet, and endocrine functions, leading to the development of hyperglycemia. Animals showed marked
polyphagia, which along with reduced energy expenditure results in obesity which become evident around 4th week of age. By the week 14, symptoms of T2DM become evident and animals develop hyperglycemia, impaired glucose tolerance, hyperinsulinemia, sub-fertility, delayed wound healing etc. Gradually animals become severely diabetic and die early [164, 165]. IR develops and is associated with gluconeogenesis, glycogenolysis, impaired gluconeogenesis, decreased enzymatic activity of enzymes involved in glycolytic pathway and glycogen synthesis as well as increased hepatic lipogenesis. This IR is observed due to reduced binding of insulin-InR interaction, impaired autophosphorylation of InR and downstream signaling cascade [166].

2.12.1.2. db/db mouse

$db/db$ (Lep$^{db}$) model was derived from the recessive autosomal mutation on chromosome number 4 in C57BL/KsJ stain by Bar Harbor, Maine [164]. This mutation on the chromosome 4 corresponds leptin receptor. As per the leptin mechanism explained above, a mutation in this gene results in spontaneous obesity, hyperphagia, hyperglycemia, IR and hyperinsulinemia within 12 months of age. Further, few animals develop ketosis and those animals die early and do not survive more than 10 weeks. Diabetic symptoms observed in this model are somewhat similar to ob/ob mouse. Unlike Lep$^{ob}$, administration of exogenous leptin does not relieve hyperphagia, obesity and body weight [166]. This model is used to evaluate pathophysiology of T2DM and screening of insulin mimetics and insulin sensitizing drugs [167-174].

2.12.1.3. Kuo Kondo (KK) mouse

KK mouse is a Japanese polygenic model to evaluate obesity and T2DM. It was developed by selectively inbreeding animals with large body size [134, 175]. These animals start to show obesity by 8th week and attain maximum weight between 16-20 weeks. These animals gradually develops hyperphagia, hyperinsulinemia and IR. In this model, IR develops earlier than obesity and animals show greater insulin content, which is a result of degranulation, hyperplasia, and hypertrophy in insulin producing pancreatic β-cells [134, 175]. Insulin fails to inhibit gluconeogenesis and activate glycolysis and lipogenesis pathways, as observed in Lep$^{db}$ animals.

2.12.1.4. Yellow KK obese (KK/A$^{y}$) mouse

KK/A$^{y}$ mouse is having two critical genes, yellow obese (Ay) and diabetic gene. Ay gene is lethal and animals homozygous for the yellow spontaneous mutation die early after
implementation. Heterozygous animals (KK/Ay mouse) develops obesity, hyperglycemia, hyperinsulinemia and impaired glucose tolerance by the 2 months of age. This model is used extensively for screening antidiabetic drugs of various classes [167, 170, 171, 176, 177]. Male animals are more sensitive and develop more severe obesity, hyperphagia, and diabetes [178]. These animals show a gradual decrease in insulin sensitivity and hypertrophy of pancreatic β-cells, which also appears to be degranulated [179]. Accumulated evidence suggests that IR in these animals arise as a result of defective InR and downstream signaling, along with defective glucose oxidation pathways [166].

2.12.1.5. New Zealand Obese (NZO) mouse
The NZO mouse is developed by inbreeding several generations with their parents. It is polygenic mouse model widely used to evaluate and screen drugs against obesity and diabetes. During first 2 months of life, the body weight of these animals rise rapidly, animals become obese and show severe hyperphagia, hyperinsulinemia and glucose intolerance. Hyperphagia is a prime cause of the development of obesity and diabetic like state in these animals and this condition arises primarily due to the hyperleptinaemia and leptin resistance in these animals [166]. Diabetes continuously progresses to severity and pancreas struggle to cope up with the hyperglycemia. To maintain increasing insulin demand, pancreas undergoes hypertrophy and hyperplasia to an extent such that approximately 90% cell population of the pancreas are composed of insulin producing β-cells [165]. Hyperglycemia is further exaggerated by impaired glucose metabolizing pathways and overactive gluconeogenesis pathway in different parts of the body, especially liver [180-182]. NZO mice become insulin resistant from early stages of life and are highly susceptible to develop an autoimmune disorder. Therefore, these animals serve as a useful tool to evaluate inter-relation between autoimmunity, T2DM and obesity [179].

2.12.1.6. Zucker fatty rat
In 1961, Zucker, Harriet Bird Memorial Laboratory, USA spotted a spontaneous mutation in the Sherman and Merck rat stock which lead to severe obesity in these animals. Zucker fatty rat (fa/fa) are also known as an obese rat and are now labeled as Lepr\(^{fa}\). These animals develop from the recessive autosomal mutation on the \(fa\) gene located on chromosome number 5. In first 2 months of age, animals showed marked hyperphagia and consequently, obesity develops with increased subcutaneous fat depositions. Subsequently, hyperglycemia, hyperinsulinemia, IR and
glucose intolerance develops in these animals [164, 183]. Like mouse models, here also defective leptin receptor in hypothalamus impairs leptin signaling and induces leptin resistance and subsequent complications [164, 166]. Diabetic condition is further exaggerated by impaired glucose metabolic pathways and enhanced gluconeogenesis. This model is used widely to screen insulin mimetic, insulin sensitizers and antiobesity agents [167, 170, 171].

2.12.1.7. Zucker diabetic fatty rat (ZDFR)

This model was developed specifically to study T2DM and is a modification of selectively inbred Zucker fatty rats. ZDFR are less obese and show a higher degree of IR, when compared to fatty rats. Male animals develop T2DM easily and symptoms start to appear by 5th week of age. Female counterparts are obese and show some degree of IR, however, do not show the complete development of diabetes and therefore are used as controls. These animals are not able to counter hyperglycemia by inducing hypertrophy and hyperplasia in the pancreas and β-cells appears to be brittle and quickly succumbs to insulin over-secretion pressure and are highly prone to apoptosis [184]. Like the human diabetic state, β-cells of these animals show impaired sensitivity towards blood glucose and automatically reducing insulin secretion, However, sensitivity towards secretogogues remain intact, thereby mimicking human T2DM. Hyperglycemia in these animals has downregulates GLUT2 activity in β-cells and GLUT4 in the adipose and muscle tissue [185]. These animals serve as a key model to investigate IR and beta cell dysfunction during T2DM, besides, is efficient for screening of insulin mimetic and insulin sensitizing molecules [167, 171].

2.12.1.8. Spontaneously hypertensive rat/NIH-corpulent (SHR/N-cp) rat

SHR/N-cp rat has been derived from inbreeding SHR/N strain, and serves as a good model to investigate T2DM and obesity associated hypertension. Males are more susceptible to the development of obesity and develop hyperphagia, hyperglycemia, hyperinsulinemia, IR, dyslipidemia, hyperleptinemia, glucose intolerance and hypertension [186]. These animals are useful in investigating obesity and T2DM and associated hypertension, besides serving as a useful model for investigating the role of dietary supplements during T2DM.

2.12.1.9. James C Russel:LA-cp (JCR/LA-cp) rat

JCR/LA-cp rats were developed by backcross of LA/N-cp males with hooded rat species with 3% involvement if SHR gene and presence of fa allele. Heterozygous (+/cp) and dominant
homozygous (+/+) animals are metabolically normal. However, animals recessive for the \( cp/cp \) gene develops obesity, \( \beta \)-cell hyperplasia, IR, hyperinsulinemia, hyperglycemia, glucose intolerance, and hyperlipidemia. \( cp \) genes produce a nonfunctional leptin receptor protein which induces a leptin deficient state in these animals and thereby producing abnormalities similar to those observed in genetic mouse models. FBG levels in these animals remain normal, which limit the use of these animals in the T2DM research [187]. These animals show several cardiovascular complications, such as vasculopathy, and therefore serves as a good tool to evaluate diabetes mediated cardiovascular complications and other related disorders.

**2.12.2. Diet induced T2DM**

There are few animal species, such as Sand rat, Tuco-tuco and Spiny mouse, in which diabetes cannot be induced chemically or genetically. In humans, unhealthy diet is one of the major reason for the development of T2DM and hence these animals prove to be beneficial in evaluating diet-induced diabetes as they are highly susceptible for diet-induced obesity and T2DM [164].

**2.12.2.1. Sand Rat**

Sand rat (\( Psammomys obesus \)) is a wild animal which remain healthy and non-obese in wild but in captivity it gradually develops obesity and T2DM when given standard high energy laboratory chaw [185, 188]. Sand rat gradually develops hyperphagia and thereby become obese and diabetic. Animals show severe hyperinsulinemia, glucose intolerance, degeneration of pancreatic \( \beta \)-cells followed by severe insulin deficiency and ketosis, leading to death of these animals [189]. In this model, IR develops quickly and GLUT4 levels remain very low, resulting in glucose deprivation of cells, especially in skeletal muscles, despite of excessive insulin available in the blood. In later stages, insulin deficiency develops as a consequence of degenerating \( \beta \)-cells resulting in increased proinsulin to insulin ratio. This symptom is characteristic to clinical pathophysiology of human T2DM [190]. Further, natural ability of body to produce insulin is severely reduced, adipose tissue is diminished and protein breakdown start. At this stage animals are totally dependent on external supply of insulin or else they die. These animals are used to evaluate insulin mimetic and insulin sensitizers [191, 192]. In recent years, research work on these animals has given few novel biomolecules such as ‘beacon gene’ (which positively correlates with the body fat of these animals), ‘Tanis protein’ (which is produced in liver and
serves as a connecting link between T2DM, inflammation and vascular abnormalities) and ‘PSARL protein’ (which is expressed in the skeletal muscles and it show correlation between IR and T2DM) [193, 194]. In captivity, PSARL protein is significantly reduced leading to the development of obesity and IR, however IR get abolished on restoration of this protein through exercise and diet. Interestingly, in wild, this protein is expressed in good amount [195].

2.12.2.2. C57BL/6J mouse (High fat diet model)
C57BL/6J mouse is non-obese, however feeding it with diet rich in fat result in marked obesity and T2DM in these animals. It was first developed in Japan, is now one of the best model to study diabetes and obesity. It is available at Jackson Laboratory, Bar Harbor. After feeding high fat diet, these animals become severely obese and show marked hyperinsulinemia, hyperglycemia, IR and impaired glucose tolerance [196]. These animals show chronically elevated glucose levels in both fasting and non-fasting state and therefore serves as a better model than C57BL/6J (ob/ob) model, where FBG levels is normal. These animals inherit most of the parent’s characters with genetic predisposition and develop T2DM after obesity. This model is an outcome of the genetic and environmental risk factors in combination and therefore it is considered superior over genetic models and best represent clinical pathology of human T2DM. However, it suffer from a drawback that degree of IR or T2DM induced varies amongst animals which limit its use [197].

2.12.2.3. Spiny mouse
*Acomys calirinus* (Spiny mouse) is found in the semi-desert regions of eastern Mediterranean area. These animals has low insulin levels in natural environment and remain non-obese. However, in captivity when fed with high-energy regular laboratory mouse chaw, they become obese and develop T2DM. These animals show marked hyperplasia and hypertrophy in pancreatic β-cells and thereby pancreatic insulin content is increased. However, their insulin-secreting mechanism is significantly impaired and blood levels of insulin remains low [198].

2.12.3. Chemical induced T2DM
Chemical induced diabetes are the most commonly used models to evaluate pathophysiology of T2DM, development of various complications and to screen various anti-diabetic drugs. These models mimic environmental factors responsible for the development of T2DM and produces hyperglycemic state in the animals with high degree of reliability and uniformity. Further, these
models offers an additive advantage that the severity of the diabetes can be controlled by varying the dose of chemicals.

2.12.3.1. Goldthioglucose obese diabetic mouse
Goldthioglucose (GTG) is a chemical which induces T2DM in rodents when 150-350 mg/kg dose is injected through intraperitoneal route. After 16-20 week of injection, animals become obese and diabetic with pronounced hyperglycemia, hyperinsulinemia and IR [199]. Mechanistically, GTG is transported to ventromedial hypothalamus where it produces toxicity through necrosis. Compromised functioning of the hypothalamus result in hyperphagia and obesity. This in contrast elevates the levels of body lipids, enhances the lipogenesis and triglycerides secretion along with reduced glucose utilization in the muscle. These symptoms mimic clinical scenario and resemble those observed in ob/ob mice. Further, insulin signaling cascade is disrupted resulted in impaired insulin response and hyperglycemia [200]. This model suffers from a disadvantage that it take long time to develop T2DM and obesity, besides, it is associated with high mortality, which limit its use for the research goals.

2.12.3.2. Alloxan and Streptozotocin-induced diabetes in rodents
Alloxan (ALX) and Streptozotocin (STZ) are having specific toxicity towards pancreatic β-cells. These compounds are most widely used in research to induce experimental T1DM and T2DM in animals, especially rodents. Ability of ALX to induce pancreatic β-cells necrosis was discovered in the year 1943 when tested on rabbits. Since then, it has been used regularly to investigate pathophysiology of diabetes and screening of anti-diabetic drugs. ALX is a uric acid derivative, having maximum stability at pH 3. To induced experimental diabetes, ALX is injected through intraperitoneal or intravenous route to the animals at a dose varying between 80-200 mg/kg, depending upon the severity of diabetes needed. It must be noted that it should be prepared fresh before every use and used within 1 h due to its high instability in the solution form. It radially enters pancreatic β-cells through GLUT2 and generates dialuric acid along with ROS in the presence of thiols. Oxidative stress degenerates pancreatic β-cells and thereby reducing the amount of insulin produced by these cells. Within 4-5 days of ALX injection animals become diabetic, which continues to grow uncontrolled with severe symptoms like, hyperglycemia, glucosuria, polydipsia, polyphagia, hyperlipidemia and animals develops marked neuropathy, retinopathy, cardiovascular complications and nephropathy if left untreated for longer duration.
This model suffers from three major drawbacks which limits its use in the research field. Firstly, the degree of diabetes-induced in animals varies from animal to animal and is not proportionate to the dose of ALX given. Secondly, ketosis and mortality associated with ALX injection is very high. Finally, diabetes-induced by ALX injection is basically T1DM, and its potential to induce T2DM is still debatable.

STZ was originally derived from *Streptomyces achromogenes* as an antibiotic. Potential of STZ to induce diabetes was demonstrated in dogs by Rakieten et al. in 1963 [204]. GLUT2, present on the β-cells membrane, selectively uptakes STZ, where it is degraded to nitrosourea, which then alkylates DNA and activate poly ADP-ribosylation (PAR). Activation of PAR results in the depletion of cellular NAD$^+$ and ATP. Together, DNA methylation and PAR activation result in the necrosis of β-cells, thereby compromising their insulin producing capacity. Further, to enhance pancreatic damage, STZ inflicts oxidative and nitrative stress in the pancreas and thereby resulting in the formation of highly reactive O$_2^-$ free radicals, NO, H$_2$O$_2$ and OH$^-$, which further damages the pancreas [205-207]. To induce diabetes, STZ is injected through intraperitoneal or intravenous route at a dose varying from 50-200 mg/kg. Low dose or multiple low-dose STZ injection are used to induce T2DM whereas high doses are known to induce T1DM. The sensitivity of the animals towards STZ depend upon the age, species, and strain of animals, and therefore the degree of diabetes induced and its severity can be controlled [208].

In research work, ALX and STZ models are widely used to screen molecules/compounds of synthetic and natural origin for their antidiabetic, insulinomimetic and insulinotropic potential. Apart from the basic model of ALX and STZ administration, several new models have been developed which are a modification of these basic models.

### 2.12.3.3. Streptozocin- nicotinamide (STZ-NAD) induced diabetes

This model is a modification of the standard STZ model of diabetes. In this model, animals (rats or mice) are injected 230 mg/kg of NAD 15 minutes before injecting 60-70 mg/kg STZ through intravenous route. This treatment results in the development of stable diabetes without affecting circulating fasting insulin levels. Mechanistically, NAD inhibit PARP and is a strong antioxidant and limits the damaging effect of STZ on the pancreas to DNA methylation. To be more specific, NAD neutralizes the oxidative and nitrative stress induced by STZ, and thus damage mediated through oxidative and nitrative stress is prevented, resulting in minor damage in the pancreatic β-
cells and the development of T2DM. Therefore, this model is beneficial in evaluating the complications associated with T2DM and for screening molecules/compounds for their insulin mimetic and insulinotropic actions. Recently, this model successfully induced T2DM in a non-rodent model of Gottingen pig, which provided good opportunity to study T2DM in closest possible similarity to human pathology [209, 210].

2.12.3.4. Neonatal streptozotocin induced diabetes in rats

Single high dose injection of STZ to induce diabetes suffers from a drawback that it may produce T1DM instead of T2DM. Therefore, a modification in this model was made to develop a model for T2DM by single STZ injection given to rat pups immediately after birth. This model is known as Neonatal STZ induced diabetes in rats. In this model single STZ injection of 80-100 mg/kg is given through intraperitoneal, intravenous or subcutaneous route to the 2 or 5 day born pups. These rats, usually Wistar or Sprague-Dawley, develop T2DM in the adulthood [211, 212]. This model is best suited for the investigation of the regeneration of pancreatic β-cells and their insulin secreting defects [211, 212]. Further, there are reports in which researchers have induced T2DM in rats by injecting 200 mg/kg ALX intraperitoneally to 2 or 6 day old rats and produced a model more suitable to study long term complications associated with T2DM [213]. It can be concluded from the above discussion that STZ and ALX produce diabetes through the destruction of pancreatic β-cells and thereby generating an insulin deficit state in the body.

There are several reports in which researchers have attempted to mimic human diabetes like condition in the animal models by injecting STZ to the genetically insulin resistant animals such as SHR, Zucker fatty rat etc., high fat fed animals and high fructose diet fed animals. [214]. Through these models, initial hyperinsulinemia and IR is developed by feeding animals with special diets. Subsequent STZ injection inflicts β-cell damage and frank hyperglycemia, without having a significant effect on the normal insulin levels in rodents [215]. In another report, 35 mg/kg STZ only induces diabetes in high fat fed animals and fail to do so in normal animals. This model was an attempt to mimic obesity as a causative factor of IR and T2DM [216]. Further, these animals responded normally to the actions of insulin, insulin sensitizing agents and insulinotropic drugs such as pioglitazone and gliclazide and therefore can be used to screen these drugs.
2.12.3.5. Multiple low dose Streptozotocin induced T2DM in mouse

In this model, STZ (50-60 mg/kg) is injected intraperitoneally to the 4-6 month old, 8-12 h fasted mouse for five consecutive days. STZ is prepared fresh every time and solubilized in citrate buffer (pH 4.5; 4-8°C). Single dose of STZ at this concentration does not produce diabetes in animals, however, multiple low doses is reported to induce diabetes, glucose intolerance, insulin signaling dysfunction, polyphagia and polydipsia in treated animals. This model produces stable long-standing diabetes and therefore is best suited to study long term complication of T2DM, especially central neurological complications which take a longer time to set in. In one such report, this model was used to evaluate T2DM induced memory dysfunction and depression in mice and for screening herbal antidiabetic drug for its potential to counter T2DM mediated neurological complications [41, 115, 217].

2.12.4. Animal models for stress

2.12.4.1. Early life maternal isolation stress

Exposing pups to maternal separation stress in the early stages of their life, induces a long-term alterations in their neuronal functioning and behavioral performances, which remains throughout the life and impairs quality of life [218]. In this model, 2 day old pups are separated from the mother for different time durations varying from 1 h to 24 h, depending on the severity of stress. Mother and its pups are placed in the different animal house facility such that no olfactory, visual or auditory connection occur. After the stipulated time, pups are replaced to their home cage with the mother [219]. Procedure may be repeated every 5-7 days to increase the intensity of stress. This model is used widely to investigate the consequence of neonatal stress on adult behavior including depression, addiction, anxiety etc. [220].

2.12.4.2. Low temperature-induced stress

Sudden drop or rise in the body temperature activates a stress response through HPA axis via activation of thermoregulatory receptors located in the brain [221]. Based on this physiology, an animal model to evaluate stress and anxiety has been developed by subjecting animals to sudden drop in the body temperature by placing them in the cold water or a cold chamber. Depending on the intensity of stress needed, temperature and duration of exposure can be adjusted. Most widely used protocol for this model consist of forcing animals to swim in a pool filled with cold water (16 ± 2°C) for 15-45 minutes or by keeping the home cage of the animals inside a cold room.
maintained at a temperature of 4°C for 15-30 min [222]. Stress can be repeated every day or frequently depending on the severity of stress needed. However, this model suffer from a drawback that with time exposure to same stress result in the development of resistance towards stressor and therefore after some time stressor may not be efficient to produce a desired effect.

2.12.4.3. Restrained and immobilization stress
Restraining movements of the rodents results in the severe stress and is one of the best model used to investigate stress and associated behavioral, biochemical and physiological changes [223]. Physiology of the development of the stress resembles human physiology via HPA hyper-activation and therefore this model has found a significant application in the research model to investigate acute stress. In this animal model, animals are placed inside a ventilated cylindrical tube such that all its body movements are restricted for a duration varying between 30 min to 6 h, depending on the severity of the stress needed. Further, to intensify the stress, some researchers use this model overnight or inside cold room [224]. In another model of immobilization, limbs of the animals are tied with the adhesive tape. Free movement of head is also restricted with the help of a metal loop. Duration of this model also depend upon the severity of stress needed [225]. This stress can be repeated multiple time to increase the severity of the stress. However, this model also suffer from the drawback that with time animals get adapted to the stressor and may not respond very well in the later stages, and therefore this model is also best suited for investigating acute stress [224].

2.12.4.4. Foot shock-induced stress
Giving repeated mild foot shock to the animals result in hyperactivation of the HPA axis and therefore, gives acute stress response. This model is a sub-type of fear conditioning. Animals are confined inside a chamber lined with grid floor capable of giving inescapable foot shock of 0.5-2 mA of 1-2 s duration, with 10-20 exposures every time. Animals are given repeated shock, and procedure is repeated on multiple occasions to increase the intensity of stress [226]. This model also suffer from adaptation drawback and thus can only be used for fear conditioning and acute stress studies.

2.12.4.5. Social defeat stress
Social defeat model is one of the best model to evaluate stress and depression mediated complications, including behavioral, physiological and biochemical alterations. This model was
initially developed by Klaus Miczec and in this model, an intruder animal (generally small in size) is introduced to the home cage of the resident dominant animal (aggressor) [227]. During the test, behavior of both the animals is recorded and animals may be separated if severe aggression is observed. Further, total time that intruder animal spent in the defeated posture (identified as immobility with all four paws on ground and head facing towards aggressor, crouching with all paws on ground with head facing away from the aggressor, escape from aggressor, and defeat upright stance with fore paws extended) is evaluated by blinded observer blind to the study [227].

2.12.4.6. *Chronic predicted and unpredictable stress*

These models are widely used and are the best models to investigate chronic and acute stress mediated behavioral, biochemical and physiological complications in the animals. Both of these models are of 21 days (or may be longer depending on the study). In chronic predicted stress model, animals are given same stress for 21 days at a particular time of the day by one researcher. This induces hyperactivation of HPA axis and induces stress response mimicking human physiology. However, due to same stress on same time of the day, animals get adapted to the situation and in later stages only acute effect is observed with not much alterations in the biochemical or physiological profile. Therefore, duration of the stress has to be increased up to 48 days or more to achieve desirable effect. To counter these drawbacks, an alternative animal model of chronic unpredicted stress of 21 day was developed. In this model, animals receive array of different randomly selected stress daily of 21 days at different time of the day by different researchers. Stressors include, mild foot shock of 0.2 mA (20 time at 2-10 sec interval), cold immersion (5-10 min at 4-15°C), forced swim in hot (25-30°C) or cold water (10-15°C), tilt cage, wet bedding, overnight illumination, restraining for 15-30 min, food and water deprivation etc. This model result in the development of chronic stress and induces severe biochemical and behavioral dysfunction. This model is a realistic stress model and represent closest physiological alterations to human stress, which is generally through environmental factors. This model has been validated multiple times with reproducible results and variety of drugs used to counter stress and depression such as (fluoxetine, desipramine, etc.) are also validated to be effective in this model [39, 114, 228].
2.13. Quercetin

Flavonoids are the very potent antioxidants occurring in the nature in almost all plant species. They are widely distributed in fruits, vegetables and beverages and have extraordinary ability to scavenge free radicals. Amongst all flavonoids, quercetin is the most widely studied molecule and is a part of flavonol sub-class. Quercetin and its glucosylated products compromises approximately 75% of total dietary flavonoid consumption [229]. Quercetin is a potent antioxidant and have an exceptional free radical scavenging ability. Based on its unique natural property, quercetin have been reported to be beneficial in the management of ailments such as cancer, atherosclerosis, and chronic inflammation [230, 231]. Structurally, flavonoids are having two phenyl groups interconnected by three carbon chain. Based on the chemical structure, flavonoids can be divided into two major categories, molecules with open three carbon bridge and those with closed heterocyclic three carbon bridge (Figure 2.9) [232]. Different structures of the flavonoids arise from the variations in the three-carbon chain and substitution on the phenyl rings [230]. Quercetin (3,5,7,3’ ,4’-penta-hydroxy flavone), (C_{15}H_{10}O_{7}; molecular weight 302.236) is a flavonol that belongs to a group of polyphenolic compounds and its structure of the quercetin is given in Figure 2.9. Antioxidant property of this molecule can be explained from the structure. When it interact with any free radical, it donates its proton and itself is converted to a free radical. Free electron thus generated, gets delocalized on the structure by the resonance phenomenon, rendering this free radical of quercetin to be too low in energy, and therefore non-reactive [233]. o-dihydroxyl groups, 2,3-alkene, 4-oxo group, and 3- and 5-hydroxyl groups present in the quercetin structure are essential for its antioxidant activity and helps in maintaining stability during reaction with the free radicals [230]. These functional groups helps to increase the number of resonating structures and aids in antioxidant potential and molecule stability [233].

![Open chain Flavonoid](image1.png) ![Closed chain Flavonoid](image2.png) ![Quercetin](image3.png)

**Figure 2.9:** Structure of flavonoids and quercetin
Apart from its property of hepatoprotection, cardioprotection, anti-cancer and renal protection, it is also known to improve diabetic complications by inhibiting bio-molecule oxidation, elevating insulin secretion, inhibition of NF-κB, preventing β-cell damage and anti-inflammatory property [234]. Several plants and plant preparation, such as Gingo biloba [63], sweetened dried and raw cranberries [64], green tea, grapes [65, 66], Dodonaea viscosa [67], Cecropia pachystachya [68], Vaccinium angustifolium [69], Helichrysum plicatum [70], etc. are having high quercetin content. These plants and plant preparations are previously reported as good antidiabetic preparations when screened through various animal models and in-vitro assays. Majority of these research work related to the antidiabetic potential of quercetin are only preliminary studies reporting reduction in blood glucose and insulin level, inflammatory stress and hypolipidemic and anti-oxidative activity [235-239]. Very few researchers had worked on the diabetic neuropathy using quercetin and reports depicts only preliminary findings. An in-vitro study conducted on isolated rat dorsal root ganglionic neuron, in which neuropathy was induced by supplying high glucose concentration, reports that quercetin efficiently prevent diabetic neuropathy by activating Nrf-2/HO-1 and by inhibiting NF-κB signaling [240]. Anjaneyulu and Chopra studied analgesic effect of quercetin in diabetic neuropathic pain (STZ induced) by using tail flick mouse model and reports elevation in pain threshold giving an indication of prevention of diabetic neuropathy [241]. Further, quercetin improved memory dysregulation in STZ-diabetic rats by evaluating memory performance inside Morris water maze apparatus [242]. Oral administration of quercetin showed dose dependent cell proliferation in mice hippocampus [243]. Their study was based on the level of cyclic-AMP response-element binding protein which enhances hippocampal cellular differentiation and neurogenesis/proliferation. However, no detailed mechanism or underlying molecular and genetic changes in the hippocampus region/neurogenesis was provided by them. PPAR-γ is a well-known target for anti-diabetic drugs. Quercetin analog, pentamethyl quercetin, had been reported to up-regulate PPAR-γ and improve glucose uptake and has the potential to compete with rosiglitazone for the binding site at PPAR-γ [244, 245]. In CNS, quercetin improves neuronal functioning and survival under challenging conditions [246]. Quercetin is reported to possess the good potential to alleviate stress, reduce blood cortisol levels [247] and is known to provide beneficial effect during the depression, anxiety and memory dysfunction [248, 249-251]. The mechanism underlying the CNS modulatory effect of quercetin is still unknown, and need to be explored further.
CHAPTER 3

OBJECTIVES
3. OBJECTIVES OF THE STUDY

- To screen small molecules of natural origin through *in-silico* docking studies and *in-vitro* assays for the management of diabetes and associated neurological complications.

- To evaluate the effect of quercetin on diabetes and associated neurological complications during STZ mediated diabetes in Swiss albino mice.

- To investigate the effect of quercetin on insulin resistance in an experimental model of chronic unpredicted stress.

- To elucidate the mechanism through which quercetin modulates glucose homeostasis.
CHAPTER 4

MATERIALS AND METHODS
4. MATERIALS AND METHODS

4.1. Materials

Unless otherwise specified, all the chemicals, reagents etc. used in the present study were of analytical grade and were procured from Sigma–Aldrich Co. (Bangalore, India) and Hi-Media (Mumbai, India).

4.2. Molecular docking

We identified 267 small molecules of natural origin from literature that are regularly consumed in form of fruits, vegetables, and beverages throughout the world. These molecules were screened for their potential to benefit diabetes through in-silico docking using AutoDock (4.2.6) by using insulin receptor (PDB: 1IR3), PPAR-γ (PDB: 2PRG) and DPP-4 (PDB: 4PNZ) as a potential target [252]. Crystal structure of target proteins (1IR3, 2PRG, and 4PNZ) were obtained from the protein data bank (www.rcsb.org). Phosphoaminophosphonic acid-adenylate ester, rosiglitazone, and omarigliptin were used as standard internal ligands for 1IR3, 2PRG and 4PNZ respectively. By using AutoDock software, polar hydrogen atom was added to the crystalline structure (pH 7.4) and the water of crystallization was removed from this complex. Further, pdbqt files of both, ligands and receptors, were prepared using AutoDock tool of the Molecular Graphic Laboratory (MGL). Each complex was individually assigned Gasteiger charges and grid boxes of 30 Å × 30 Å × 30 Å were located on each target protein, with following coordinates, 1IR3: x = -24.435, y = 37.828 and z = 37.828; 2PRG: x = 51.217, y = -17.389 and z = 24.528; 4PNZ: x = 40.239, y = 55.444 and z = 38.316.Docking analysis was initiated within these grid boxes using AutoDock tool and results were expressed in terms of free energy (kcal/mol; docking score) of ligand-protein interaction. Results of the docking studies predicted ascorbic acid, gallic acid, quercetin, caffeine, piperine, cinnamic acid and ellagic acid as the potential antidiabetic and therefore these molecules were selected for further screening through in-vitro assays.

4.3. In-vitro assays

We narrowed down the screening process through various in-vitro assays to identify a potential antidiabetic molecule that may prove beneficial in the management of neurological complications associated with diabetes. For this, we identified pathways that lead to the development and advancement of these neurological complications and concluded that primarily
oxidative stress, genotoxicity and impaired glucose homeostasis are involved in the development of these alterations. Potential of natural molecules (viz. ascorbic acid, gallic acid, quercetin, caffeine, piperine, cinnamic acid and ellagic acid) to interfere with these pathways was studied by mimicking these conditions in in-vitro assays. We further tested marketed drugs (viz. rosiglitazone, metformin and glimepiride) through these assays to get an idea whether or not marketed drugs are capable of interfering with the pathways that lead to the development of neurological complications. To get a better insight, we compared the results of marketed drugs with natural molecules and evaluated their comparative efficacy.

4.3.1. Antioxidant assay

Antioxidant potential of the test molecules was screened through (i) 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and (ii) lipid peroxidation inhibition assay. These assays are simple, reproducible, and widely used in-vitro tools to determine antioxidant potential of drugs, molecules, and plant preparations. A stock solution of the test compounds (100 mM) was prepared by dissolving required quantity of test compounds in 95% methanol, followed by dilution in double distilled water to get the desired working concentration of 0.625 - 6.25 mM for DPPH assay and 6.25 - 50 mM for inhibition of lipid peroxidation assay. Selection of the test concentrations was determined according to assay sensitivity by running successive experiments.

DPPH assay was performed as per the method of Sharma et. al. (2009), with slight modifications [253]. In the solution form, DPPH exists as free radical with purple coloration. This assay is based on neutralization of the DPPH free radical by the antioxidant molecules, resulting in discoloration, and thereby detecting scavenging of DPPH free radical or amount of discoloration produced by the spectrophotometric method. Briefly, 0.4 mM DPPH solution was prepared in 95% methanol. 3 ml of this solution was added to 10 ml of different concentrations of test molecules (0.625 - 6.25 mM) and mixed vigorously. The reaction mixture was kept at 37°C for 30 min and then the absorbance was recorded spectrophotometrically at 517 nm using UV spectrophotometer (Go Scan Microplate Reader, Thermo Scientific, USA). Blank reaction was run simultaneously under similar conditions, by taking equal volume of 95% methanol in place of drugs, to serve as a control. % DPPH radical scavenging activity was determined by using following equation:

\[
\% \text{ DPPH radical scavenging} = 1 - \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \times 100
\]
Inhibition of lipid peroxidation assay was performed in accordance with the method of Shah et. al. (2013) [254], after including some necessary modifications. Briefly, 10% solution of egg yolk was prepared in 1.15% potassium chloride solution. Mixture was homogenized properly and then subjected to 5 min ultrasonication. Reaction mixture consisted 100 µl test sample at different concentrations (6.25 - 50 mM), 400 µl distilled water and 500 µl of yolk homogenate. 1500 µl acetic acid (20 mM; pH 3.5) and thiobarbituric acid (0.8%; prepared in 1.1% sodium dodecyl sulphate) was added to the reaction. Reaction mixture was mixed vigorously and incubated for 90 minutes over a water bath maintained at 95ºC. Afterwards, reaction mixture was brought to 25ºC, 1000 µl n-butanol was added to it, vortexed and then subjected to centrifugation at 5000 rpm for 300 s. Supernatant was carefully seperated and corresponding absorbance was recorded at 532 nm using UV-spectrophotometer. Blank reaction was run simultaneously under similar conditions, by taking equal volume of 95% methanol in place of test molecules, to serve as a control reaction. Results were determined as percent anti lipid peroxidation activity, which was calculated by using following equation:

\[
\text{% inhibition of lipid peroxidation} = 1 - \left( \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \right) \times 100
\]

Entire experimentation was performed in triplicate. IC\textsubscript{50} values (µM) for each drug tested was determined and the results were determined as mean ± standard deviation (SD).

4.3.2. Genotoxicity assay

Comet assay is used to determine the genotoxicity or DNA fragmentation in both, in-vitro and in-vivo experimentations. We used this model to investigate whether or not test molecules possess potential to inhibit H\textsubscript{2}O\textsubscript{2} induced genotoxicity in human blood cells and compared them with marketed drugs. Briefly, 5 µl of whole human blood was drawn by pricking the fingertip with sterile needle. Blood sample was incubated with 100 µl of H\textsubscript{2}O\textsubscript{2} (100 µM prepared in normal saline) and 10 µM test compounds for 20 minutes at the temperature of 4ºC. Samples were centrifuged at 3000 rpm for 300 seconds and resulting pellet was washed twice with excess of normal saline. Pellet was mixed with 95 µl low melting agarose solution (0.75%) heated to 40 ± 2ºC. Mixture was quickly transferred to a glass slide that was previously coated with thin layer
of high-melting agarose (1% w/v) and a coverslip was placed over it to make a thin layer and uniform spreading of cell suspension. Slides were immersed in freshly prepared ice cold lysis buffer composing NaCl (2.5 M), Tris (10 mM), EDTA (100 mM), Triton X-100 (1%) and DMSO (10%) at pH 10.2 for 30 min at 4°C. Slides were gently removed and were subjected to electrophoresis in freshly prepared ice cold electrophoresis buffer consisting of NaOH (300 mM) and EDTA (1 mM) at 25 Volt and 300 mA for 20 minutes in a dark room. Slides were removed, neutralized by washing with 0.4 M Tris (pH 7.5), DNA was stained with propidium iodide and observed under fluorescent microscope (Nikon Eclipse-80i, Japan). Images were acquired at 200 X magnification and the olive tail movement was determined using CaspLab software [255].

4.3.3. Anti-diabetic assays

We screened small molecules of natural origin for their antidiabetic activity by evaluating their potential to inhibit of α-amylase activity, enhance neuronal glucose uptake and GLUT4 expression. Different test concentrations in the amylase assay were determined by running experiments at different concentrations. Through MTT assay we determined that at 10 µM concentration, none of the test molecules exhibited significant toxicity towards neuronal cells, and therefore this concentration was used in the glucose uptake and GLUT4 expression assay. All the samples for neuronal primary culture based assays were prepared in DMSO and diluted with serum free DMEM nutrient media such that concentration of 10 µM is achieved in each well of 96-well plate having 200 µl media and the concentration of DMSO remain below 0.04%.

4.3.3.1. α-Amylase inhibition assay

Potential of test compounds to inhibit α-amylase activity was performed in accordance with previously described method [256], with slight modifications. This assay was performed in a 96-well plate. For this, 30 µl of test drugs at different concentrations (50 - 500 µM) were incubated with 60 µl enzyme (1 U/ml prepared in phosphate buffer saline (PBS); pH 6.9) for 10 minutes at 37°C. Equal volume of distilled water was incubated with enzyme to serve as a control reaction. Reaction was terminated by adding 30 µl HCl (1M) and 120 µl IKI (5% w/v) and absorbance of each well was recorded at 630 nm using UV spectrophotometer. All procedures were performed in triplicate and the results are expressed as % α-amylase inhibition (mean ± SD) as per following equation:

\[
\% \text{ amylase inhibition} = 1 - \left( \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \right) \times 100
\]
4.3.3.2. Glucose uptake and neuronal membrane GLUT4 translocation

Glucose uptake and GLUT4 expression were evaluated in-vitro on primary hippocampal cultures prepared from E18 rat embryo as per previously described method [257]. Briefly, healthy female Wistar rat (200 - 250 g; 14-16 week age) with proven fertility was housed with male Wistar rat (200 - 250 g; 14-16 week age) on the day of the beginning of the estrous cycle. Vaginal smears were regularly taken to identify the sign of mating, and when the mating was observed, the male was separated from female cage and that day was considered as day 0.5 of the pregnancy. At E18 day, mother rat was anesthetized (5 mg/kg xylazine and 90 mg/kg ketamine) and embryos were desiccated out inside a cold room maintained at 4ºC. Embryonic hippocampi were quickly dissected out and 6-8 hippocampi were digested with 2 ml of 0.25 % trypsin (prepared in serum free DMEM) for 10 min at 37ºC. Neuronal cells were dissociated by gentle trituration using pasture pipette. Samples were centrifuged and the pellet was washed twice with an excess of DMEM having 10 % fetal bovine serum (FBS). Pellet was suspended in a complete medium, i.e. DMEM having 1X Penicillin-Streptomycin and 10% FBS, viable cells density was determined using trypan blue and seeded on to 96-well plate at a cell density of 1 × 10^6 cells per well. Culture plates were then incubated at 37ºC inside CO_2 incubator having 5% CO_2 for 14 days. On 15th day of the experimentation, regular nutrient media was replaced with low glucose media. Cells were starved for 12 h. Afterwards, media was again replaced with serum free DMEM having 10 µM test compounds and 5 g/l glucose and plates were incubated for 24 hours at 37ºC inside the atmosphere of 5% CO_2. Amount of glucose present in the media was determined by using glucose-oxidase method. Percent glucose uptake by primary neuronal cultures was determined for each treatment. Proceeding further, cells were fixed in 4% paraformaldehyde solution for 2 h. Fixed cultures were washed with PBS, permeabilized by incubating them with Triton-X 100 solution (0.1% prepared in PBS) for 10 min at 25ºC. Cultures were washed with an excess of PBS and blocked by incubating them for 45 min with 5 % bovine serum albumin (BSA) (prepared in PBS having 0.1% tween 20 (PBST)) at 37ºC. Cultures were incubated with primary GLUT4 antibody (1:250) (Glut4 (N-20); goat polyclonal IgG, SC-1606) (prepared in 5 % BSA having 0.1 % tween 20) for 12 h at 4ºC. Cultures were again washed with an excess of PBS and twice with an excess of PBST. Further, cells were incubated with corresponding secondary donkey anti-goat IgG-FITC antibody (1:1000; SC-2783) (prepared in 3% BSA having
0.1% tween-20) for 120 minutes in dark at 37ºC. After washing, cultures were stained with DAPI and then visualized under fluorescent microscope. Images were acquired at 200 X magnification and were later analyzed for total FITC fluorescence of GLUT4 using image-J software and total fluorescence was depicted as corrected total cell fluorescence (CTCF) [258].

CTCF = Integrated density – (Area of the selected cell x Mean background fluorescence)

**4.4. Animals**

Swiss albino mice (20-25 g and 12-16 week old) were used in the entire experimentation. Animals were obtained from the central animal facility of National Institute of Nutrition (NIN) (Hyderabad, India) and National Institute of Pharmaceutical Education and Research (NIPER) (Mohali, India). Animals were housed at the animal house of the Jaypee University of Information Technology (JUIT) (Waknaghat, Himachal Pradesh, India) under standard laboratory conditions of light-dark cycle of 12 h, temperature maintained at 24 ± 2ºC temperature and 62 ± 3% humidity. Animals were fed with standard laboratory rodent chow and had free access to drinking water. All the experiments were performed according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India after approval from the Institute Animal Ethics Committee (IAEC). All necessary efforts were made to minimize suffering to the animals under study.

**4.5. Experimental Design**

**4.5.1. Anti-diabetic study**

Swiss albino mice (20-25 g; 12-16 week age; both sex) were used in the entire study. Animals were kept in a group of four animals per cage and were divided into six groups (n ≥ 12):

Group 1:- Control + vehicle (CTRL); (received 0.3 % carboxymethyl cellulose as a vehicle)
Group 2:- Control + Quercetin (CTRL + Q); (received 15 mg/kg quercetin in vehicle)
Group 3:- Control + Rosiglitazone (CTRL + ROSI); (received 5 mg/kg rosiglitazone in vehicle)
Group 4:- Diabetes + vehicle (STZ); (received vehicle)
Group 5:- STZ + Quercetin (STZ + Q); (received 30 mg/kg quercetin in vehicle)
Group 6:- STZ + Rosiglitazone (STZ + ROSI); (received 5 mg/kg rosiglitazone in vehicle)

Animals in group 4, 5 and 6 were administered 50 mg/kg streptozotocin (STZ) (dissolved in ice cold citrate buffer; pH 4.5) through intraperitoneal injection for 5-consecutive days. Change in
the FBG levels were regularly monitored and animals showing FBG ≥ 200 mg/dl on day 7 were used in further experimentations and rest were excluded. All the treatments were administered orally, between 7 and 8 am daily for 8 weeks. Changes in food intake, body weight, and water consumption was monitored regularly. 8 h FBG level was monitored weekly. Behavioral studies were performed between day 57 and day 63, after which animals were sacrificed. Serum and brain samples were collected for biochemical, molecular and neuroanatomical studies. Experimental design of the antidiabetic study is depicted graphically in Figure 4.1.

![Figure 4.1: Streptozotocin (STZ) induced diabetes procedure and experimental design](image)

4.5.2. Chronic Unpredicted Stress (CUS) study

For CUS study, male Swiss albino mice (20-25 g; 12-16 week age) were used. Animals were kept in a group of four animals per cage and were divided into 4 experimental groups, having n ≥ 12 animals in each group.

Group 1:- Control + vehicle (CTRL); (received 0.3 % carboxymethyl cellulose as a vehicle)
Group 2:- Control + Quercetin (CTRL + Q); (received 30 mg/kg quercetin in vehicle)
Group 3:- Chronic Unpredicted Stress + vehicle (CUS); (received vehicle)
Group 4:- CUS + Quercetin (CUS + Q); (received 30 mg/kg quercetin in vehicle)

Animals in group 3 and group 4 were subjected to CUS for 21 days. Various stressors included in the study were tail pinch (5 min), food and water deprivation (8–18 h), foot shock (0.2 mA; 4 time in 2 min interval), cold swim (3 min at 10°C), overnight illumination, tilted cage (at 45° overnight) and warm swim (5 min 24°C). Stressors were selected randomly and were given to the animals between 8 am and 6 pm at a randomly selected time [39]. Animals subjected to CUS
were housed in an isolated room to avoid any sort of contact with the control animals. All the treatments were given orally (once daily) between 7 and 8 am for 21 days. Body weight, food intake and water intake was measured regularly. 8 h FBG level was monitored weekly. Behavioral evaluation was conducted between 22–28 days of the study, after which animals were sacrificed either by cervical dislocation or under anesthesia (90 mg/ kg ketamine + 5 mg/kg xylazine). Serum and brain samples were collected for biochemical, molecular and neuroanatomical studies. Experimental design of CUS study is depicted in Figure 4.2.

Figure 4.2: CUS procedure and experimental design. FWD: food and water deprivation (8–18 h); OL: overnight illumination; FS: foot shock (0.2 mA; 4 time in 2 min interval); tail pinch (5 min); cold swim: 3 min at 10°C; TC: tilted cage at 45° and warm swim: 5 min 24°C.

4.6. Behavioral studies

4.6.1. Locomotion and muscle coordination

4.6.1.1. Actophotometer

Actophotometer is a reliable and well-established method to evaluate locomotion in rodents [179]. We used this model to evaluate the effect of long standing diabetes/CUS and quercetin treatment on the locomotor performance in mice on day 57 in the STZ study and on day 22 in the CUS study. Actophotometer apparatus consisted of a rectangular chamber (36 × 36 × 10 cm; L × W × H), which was equipped with light emitting probes on two adjacent walls and photodetector on the other two walls. Every time light beam was interrupted by the moving animal, a digital meter recorded the reading. Locomotor activity of the animals was recorded in an experimental session of 10 min in terms of number of light beam interrupted/crossed (or simply as number of line crossing). Between each experimental session, Actophotometer was wiped with 70% ethanol to eliminate any sign of olfactory cues.
4.6.1.2. Rota-rod Test
Rota-rod model is a well-established model used to evaluate the muscle coordination and strength in rodents [179]. We used this model to investigate the effect of long standing diabetes/CUS and quercetin treatment on muscle coordination in Swiss albino mice on day 57 in the STZ study and on day 22 in the CUS study. For this, each mouse was individually placed on the rotating bar of Rota-rod apparatus (25 rpm). Time (seconds) in which mice fall from the rotating bar was recorded as a measure of muscle coordination/strength, with the maximum cut-off time duration of 180 sec and the animals which remained on the rotating bar for 180 sec were removed manually and were assigned time of 180 sec. Each animal was tested thrice and maximum time spent by it in three trials was used considered as a final time duration. To eliminate any interference of the olfactory cues between different experimental sessions, entire apparatus was cleaned thoroughly with 70% ethanol after every trial.

4.6.1.3. Open Field Test (OFT)
OFT is a reliable, well-established and commonly used model to evaluate the locomotor activity in experimental animals [259, 260]. We used this model to investigate the effect of various treatments on the locomotion during diabetes and stressed condition. OFT was performed on day 60 of diabetes study and day 25 of CUS between 4–7 pm. OFT apparatus consisted of a wooden box having dimensions of 50 cm (L) × 50 (W) × 25 cm (H). Apparatus was painted black and was placed inside a dark room illuminated with low-intensity red light. Animals were placed in the middle of the chamber during the experimental session and they were allowed to freely explore the arena for 10 min. Experimental session was video-recorded by overhead camera and was later analyzed for locomotor activity of the mice in terms of a total number of line crossings. Entire apparatus was thoroughly cleaned between every experimental session.

4.6.2. Anxiety
4.6.2.1. Elevated Plus Maze Test (EPM)
EPM is one of the best known model to evaluate anxiety behavior in rodents [259, 260]. In our study, we used this model to evaluate the effect of various treatments on diabetes and stress mediated anxiety in Swiss albino mice. EPM test was performed on the day 57 of the diabetic study and day 22 of the CUS study between 2 and 4 pm. EPM apparatus consisted of a black painted wooden plus maze elevated to the height of 50 cm from the ground, having two open
arms, 50 cm (L) × 50 cm (W), and two closed arms, 50 (L) × 50 (W) × 25 cm (H), originating perpendicularly from a central platform, 10 cm (L) × 10 cm (W). Apparatus was placed in a dark room illuminated with low intensity light. In an experimental session of 3 min, test animals were placed at the central platform of EPM such that their head faced toward the open arm. Animals were then allowed to freely explore the EPM apparatus, during which total time they spent in the open arm and the closed arm of the apparatus was recorded. Entire apparatus was thoroughly cleaned between every experimental session.

4.6.2.2. Open Field Test (OFT)
OFT is used to evaluate anxiety like behavior experimental animals [259, 260] and we evaluate the effect of various treatment on long standing diabetes and chronic stress induced anxiety by using OFT. OFT was performed as described in the section 4.6.1.3. Anxiety behavior in animals was determined in terms of total entries to the central region of OF and total time spent exploring the central region of the apparatus during an experimental session of 10 min. Entire apparatus was thoroughly cleaned between every experimental session.

4.6.3. Depression
4.6.3.1. Sucrose Preference Test (SPT)
We used SPT to determine the effect of various treatments on diabetes and chronic stress mediated depression in Swiss albino mice as per previously defined method [259, 260]. SPT was performed on day 59-60 of diabetes study and day 24-25 of the CUS study. In this test, animals were habituated with two water sources in their home cage for 24 h. After 24 h of habituation, water in one of the bottle was replaced with 10 % sucrose solution and a choice was provided to animals between regular drinking water and sweetened water for next 24 h. Position of bottles were interchanged every 6 h in order to eliminate any sort of side bias. Total sucrose intake and percent sucrose preference was recorded as a marker of depressive like behavior.

4.6.3.2. Tail Suspension Test (TST)
We used TST to establish the effect of various treatments on diabetes and chronic stress mediated depression in Swiss albino mice according to the previously described method [179]. In this test, the mouse was suspended 60 cm above the floor, from its tail by placing adhesive tape 5 cm below the base of its tail on to the edge of a beam. TST experimental session was of 6 min duration, during which frequency of upward turning and total immobility time was recorded as
the measure of depression-like behavior in mice. Animals were considered immobile when they remained hanging motionless and all their body activities except respiration ceased.

4.6.3.3. Forced Swim Test (FST)
FST is another well-established and extensively used model to evaluate depression-like behavior in rodents [179]. In this test, animals were forced to swim inside a glass cylindrical chamber (50 cm high and 25 cm radius), which was filled with water up to 25 cm at 23 ± 2°C. Experimental session was of 6 min duration during which total immobility time was recorded as a measure of depression in animals. Animals were considered immobile when they float motionless and all their body movements, except respiration and gentle movements to keep head above water surface, ceases.

4.6.4. Learning and Memory
4.6.4.1. Novel Object Recognition Test (NOR)
NOR is one of the best animal model for evaluating recognition memory dysfunction in rodents. In our study, we also used this model to demonstrate the effect of different treatments on memory dysfunction associated with long standing diabetes and chronic stress [41, 179]. This test was performed on day 57-58 of diabetes study and day 22-23 of CUS study inside a black wooden chamber, 50 cm (L) × 50 cm (W) × 25 cm (H), placed in a dark room illuminated by low-intensity light. Animals were placed at the center of the apparatus and were allowed to freely explore the arena for 300 seconds. Object A was then introduced in the arena and the animals were allowed 10 min more to explore the object and to get familiarize with object A. Animals were then returned to their respective cages and memory retention was investigated after 24 h. During memory retention trial was performed in the similar way, however, this time two diagonally placed objects were introduced to them in the arena, object A (familiar object) and object B (novel object), and animals were allowed to explore them for next 10 min. Experimental session was video recorded and was later analyzed for total time spent by the animals in exploring the novel and the familiar objects. Entire apparatus was thoroughly cleaned between every experimental session.

4.6.4.2. Morris Water Maze (MWM)
We used MWM test to investigate the effect of various treatments on learning & memory dysfunction associated with long standing diabetes and chronic stress as per previously defined
method [39, 179]. MWM was performed between day 59-63 of diabetes study and day 24-28 of stress study. MWM apparatus comprised a circular pool of 100 cm radius. The pool was painted black and was filled with water (23 ± 2°C) up to 50 cm height. Circular pool was divided into 4 hypothetical quadrants and an escape platform of 5 cm radius was placed 2 cm below the surface of water in a randomly selected quadrant, such that it remained invisible to the animals. Learning trial was given for 4 days, having 4 experimental sessions each day. During experimental session, animals were placed in quadrant such that their head faced the wall of the pool when released. Animals were allowed to find the hidden escape platform for next 60 sec. As soon as animals find the platform, the time taken to find it was recorded as transfer latency. Animals stayed on the platform for next 10 sec, before they were removed, dried and returned to their respective cages. In case the animal failed to find the platform in 60 sec, it was manually directed towards the escape platform, where it stayed for 10 sec, after which it was removed, dried and returned to the respective cage. Probe trial was performed 24 h after last learning trial to evaluate memory index. During this, the platform was removed from the pool and animals were released from the randomly selected quadrant. During the experimental session of 60 sec, number of platform crossing and total time spent by the animals in searching for escape platform in the platform quadrant was recorded as a measure of memory index.

4.6.4.2. Passive Avoidance Step through Test (PAST)

PAST test is commonly used model to evaluate the associative learning and memory performance in rodents [40, 179]. We used PAST to determine the effect of various treatments on long standing diabetes and chronic stress mediated memory dysfunction in Swiss albino mice. PAST apparatus consisted of a two compartment wooden chamber. Light chamber, 16 cm (L) × 20 cm (W) × 20 cm (H), was painted white and was brightly illuminated with 100 W bulb placed 25 cm above the compartment. Dark chamber (25 × 20 × 20 cm; L × W × H) was painted black and was covered by a wooden lid such that no light entered this chamber. Dark chamber was lined with wire grid that was capable of delivering an inescapable foot shock to the animals. Both compartments were interconnected by small guillotine door (5 × 5 cm) such that animals could freely move between the compartments. Learning trials were given on day 57 of diabetes study and day 22 of the CUS study, during which animals were placed at the center of the light chamber, such that their head faced towards the door of dark chamber and time taken by it to enter the dark chamber was recorded. Guillotine door was immediately closed when the animals
stepped into dark chamber and an inescapable foot shock (0.5 mA) was given to them. Animals were quickly removed and were returned to their respective cage. Short term and long-term memory retention was evaluated after 24 h and 120 h of the learning trial in the similar manner, however, foot shock was not given to the animals during retention studies. During retention trials, transfer latency to enter dark chamber was recorded with maximum cut-off duration of 180 s. Entire apparatus was thoroughly cleaned between every experimental session.

4.6.4.3. Passive Avoidance Step Down Test (PASD)

PASD paradigm is a commonly used model to evaluate memory dysfunction in rodents. We performed PASD according to the previously established method [179]. PASD apparatus consisted of a wooden chamber (50 × 50 × 50 cm; L × W × H) having a grid floor capable of delivering inescapable foot shock to the animals. During learning trials, a wooden platform (5 cm × 5 cm) was positioned in the center of the PASD apparatus and the test animal was placed over it. Time taken by the mouse to step-down from the wooden platform was recorded and as soon as all its four paws touched the floor, electric shock (0.2 mA) was delivered to it. Short term and long-term memory retention was evaluated after 24 h and 120 h of the learning trial in the similar manner, however, foot shock was not given to the animals during retention studies. Step-down latency was recorded with maximum cut-off duration of 180 s.

4.7. Symptomatic progression of diabetes

Body weight, food intake and water consumption were checked regularly throughout the study to determine the progression of diabetes. FBG levels (mg/dl) were estimated weekly by using commercially available Accu-check blood glucose monitoring system (Roche Diagnostics GmbH, Germany). Oral glucose tolerance test (OGGT) was performed at the end of the study. For this, 8 h fasted animals were orally administered 2 g/kg glucose solution and changes in the blood glucose levels (mg/dl) was monitored after 0 min, 30 min, 60 min, 90 min and 120 min time.

4.8. Animal sacrifice and sample collection

Animals were sacrificed after 24 hours of the last behavioral study. Six animals were selected from each group in random manner. Animals were sacrificed by cervical dislocation and their brains were quickly harvested over ice. Hippocampus of three brains were quickly removed over ice, weighed, homogenized in RIPA buffer having proteinase inhibitor cocktail (MP
Biomedicals, LLC, Cat # 158,837). All the samples were then centrifuged at 16,000 g for 30 minutes during which temperature was maintained at 4 ºC. Supernatant was separated and stored at -80ºC until used for western blot protein expression studies and biochemical studies. Other three brains were directly used for Golgi-cox staining. Blood samples of all the sacrificed animals was collected, serum was isolated and stored at -80ºC until used for biochemical studies. Remaining six animals were anesthetize using combination of ketamine (90 mg/kg) and xylazine (5 mg/kg). Brains tissue was fixed by double circulation by infusing 1 X PBS followed by 2% formalin and 2% glutaraldehyde solution through heart. After fixation, brains were isolated and subjected to cryotome/microtome sectioning to prepare 5 μm thick sections. Prepared sections were mounted on glass slides and were stored at 4°C until used for neuroanatomical investigation and immunofluorescence studies.

4.9. Biochemical studies

4.9.1. Estimation of serum Insulin levels
Serum insulin levels were quantified by commercially available kit (AccuLite CLIA Microwells, Monobind Inc., USA) using chemiluminescent immunoassay under fasting conditions and the results were expressed as mU/l [39].

4.9.2. Estimation of Insulin resistance (IR)
IR was determined in terms of homeostasis model assessment-estimated insulin resistance (HOMA-IR) index. Fasting insulin (mU/l) and FBG levels (mmol/l) were determined and HOMA-IR index was calculated by using following equation:

$$\text{HOMA index} = \frac{\text{Fasting insulin (mU/l)} \times \text{Fasting glucose (mmol/l)}}{22.5}$$

4.9.3. Estimation of serum corticosterone levels
Levels of serum corticosterone were estimated by using HPLC-UV system by taking dexamethasone as an internal standard. For this, known amount of dexamethasone and 500 μl of serum was extracted with 5000 μl dichloromethane. The prepared extract was dried and then dissolved in 0.1 ml mobile phase, which was composed of methanol : water (70:30). 20 μl of the prepared extract was injected into HPLC (flow rate 1.2 ml/min) and corticosterone was detected at 250 nm by injecting [261].
4.9.4. Estimation of thiobarbituric acid reactive substance (TBARS) levels in hippocampus

TBARS levels in the hippocampal homogenate were determined spectrophotometrically at 540 nm [262]. Reaction mixture consisted of 100 μl of hippocampal homogenate, 1 ml of acetic acid (20%; pH 3.5), 100 μl sodium dodecyl sulphate (8%), and 1000 μl thiobarbituric acid (0.67%). Reaction mixture was heated for one hour at 95°C over a water bath and then brought to room temperature. After cooling, 1000 μl water and 5000 μL n-butanol : pyridine (15:1, v/v) mixture were added to each tube. Samples were the centrifuged for 10 minutes at 5000 rpm, supernatant was collected and absorbance of organic layer was determined spectrophotometrically at 540 nm.

4.9.5. Estimation of total thiol (TTH) levels in the hippocampus

Hippocampal TTH levels were determined spectrophotometrically at 412 nm [263]. Briefly, 100 μl of hippocampal homogenate, 900 μl of disodium-EDTA (2 mM; prepared in 0.2 M Na₂HPO₄) and 20 μl DTNB (10 mM; prepared in 0.2 M disodium hydrogen phosphate) were mixed together and kept at 25°C for 5 min. Absorbance was then recorded spectrophotometrically at 412 nm as a measure of TTH levels.

4.9.6. Estimation of nitric oxide (NO) levels in the hippocampus

Levels of hippocampal NO in diabetic and stressed animals were determined by using Greiss reagent (as nitrite plus nitrate) according to previously defined method [40], with some modifications. NO is highly unstable and is immediately oxidized to nitrite and nitrate. Therefore, total nitrates present in 100 μl hippocampal homogenate were firstly reduced to nitrite by treating them with ammonium molybdate (2%) and ferrous ammonium sulphate (4%). Total NO on the given sample was then quantified spectrophotometrically at 450 nm by adding Greiss reagent (1% sulphanilamide and 0.1% naphthyl ethylenediamine di-hydrochloride) to the reaction mixture.

4.9.7. Estimation of catalase levels in the hippocampus

Hippocampal catalase levels were also determined spectrophotometrically according to previously established method [264], with slight modifications. Briefly, 100 μl hippocampal homogenate was added to the cuvette having 1900 μl phosphate buffer (50 mM; pH 7.2). Reaction was initiated by adding 1000 μl of freshly prepared H₂O₂ (30 mM) and the levels of
catalase were determined spectrophotometrically at 240 nm in terms of rate of H$_2$O$_2$ decomposition.

4.10. Immunoblot

Immunoblot was performed according to protocol standardized in our lab as per the method described previously [39, 41, 217]. Hippocampal samples for SDS-PAGE/western blot assay were prepared as per method described under section 4.8 above and the hippocampal expression levels of GAPDH, insulin, insulin receptor (InR), glucose transporter 4 (GLUT4), and doublecortin (DCX) were determined.

Amount of protein in the hippocampal homogenate was quantified using BSA standard curve through Bradford assay method. For this, 400 µl of known concentration of BSA (1–320 µg/ml) was added to 1600 µl Bradford reagent. The reaction mixture was incubated for 10 minutes at 25°C and the corresponding absorbance was recorded spectrophotometrically at 595 nm. A standard curve of the BSA was plotted and regression equation of straight line was derived. To quantify the amount of protein in the hippocampal homogenate, 3 µl sample was mixed with 397 µl distilled water and 1600 µl Bradford reagent. Reaction mixture was incubated for 10 min at 25°C, corresponding absorbance was recorded and the total amount of protein present in the sample was quantified using regression equation obtained from BSA standard curve.

Sample volume corresponding to the 30 µg protein was denatured with 6 µl Laemmli buffer by heating the mixture at 95 ± 1°C for 10 min, brought to room temperature and then resolved on 10% sodium dodecyl sulphate-polyacrylamide gel. Resolved proteins were electroblotted from the gel to the nitrocellulose membrane. Nitrocellulose membrane was then blocked with 3-5% BSA (prepared in PBST) for 60 min at 25°C, followed by overnight incubated at 4°C with respective primary antibodies (1:3000; prepared in PBST): GAPDH (GAPDH (FL-335); rabbit polyclonal IgG, SC-25778), insulin (Insulin antibody (H-86); rabbit polyclonal IgG, SC-9168), InR (Insulin Rβ (C-19); rabbit polyclonal IgG, SC-711), GLUT4 (Glut4 (N-20); goat polyclonal IgG, SC-1606), and DCX (Doublecortin antibody (C-18); goat polyclonal IgG, SC-8066). Blots were washed with PBS (3 × 5 min) and PBST (2 × 5 min). Nitrocellulose membrane was then incubated with respective HRP-conjugated secondary antibodies (1:5000), goat anti-rabbit IgG (SC-2030) and donkey anti-goat IgG (SC-2020), for 120 minutes at 25°C. Blots were again washed with PBS (5 × 5 min) and PBST (3 × 5 min) and then developed by using 10 ml 0.06 %
3,3’-diaminobenzidine tetrahydrochloride (DAB), 100 µl 0.025 % cobalt chloride and 10 µl 0.01 % H₂O₂. Images of the blots were captured and analyzed using ImageJ software. Results were expressed in terms of relative expression of GAPDH.

4.11. Hematoxylin and eosin staining

Effect of CUS and different treatments on the neuronal morphology in the hippocampus region of the brain was evaluated through hematoxylin and eosin staining method, as described previously [265], with some modifications. Animals were sacrificed and 5 µm sections were prepared as described under section 4.8. Sections were gradually rehydrated by treating them with xylene (6 min), xylene:ethanol (1:1) (6 min), 100% ethanol (120 sec), 90% ethanol (120 sec), 70% ethanol (120 sec), 50% ethanol (120 sec) and PBS (2 × 5 min). Hippocampal sections were then stained with hematoxylin (15 min), counterstained with 1% eosin (15 min), and then gradually dehydrated by treating the with 50% ethanol (120 sec), 70% ethanol (120 sec) 90% ethanol (120 sec), 100% ethanol (10 min), xylene: ethanol (1:1) (6 min) and xylene (6 min). CA1, CA2, and CA3 regions of the hippocampus were observed at 400X magnification under light microscope (Olympus BX51TF microscope with DP70 color camera). The number of neuronal cells per 0.1 mm² area were determined in these regions to evaluate neuronal damage.

4.12. Cresyl violet staining

Cresyl violet staining method is one of the best and widely used procedure to demonstrate the morphological changes in the neurons [266]. We used this procedure to access the integrity of hippocampal neurons during chronic stress. Animals were sacrificed and 5 µm sections were prepared as described under section 4.8. Sections were gradually rehydrated (as described above) and then stained with 2% cresyl violet for 10 min at room temperature and excessive stain was removed under tap water. Sections were then gradually dehydrated and CA1, CA2 and CA3 regions were observed under light microscope at 400X magnification and the neuronal damage was assessed visually.

4.13. Golgi-cox staining

Golgi-cox method is one of the best method for visualizing complete neuronal morphology and it allows us to evaluate neurodegeneration, integrity of neurons, synaptic connections, interneuronal networking and dendritic spine density. We used this method to understand the effect of various treatments on the long standing diabetes mediated alterations in neuronal
morphology according to method described previously [267]. Golgi-cox solution was prepared fresh for each experiment. 50 mg/ml stock solution of mercuric chloride (HgCl$_2$), potassium dichromate (K$_2$Cr$_2$O$_7$), and potassium chromate (K$_2$CrO$_4$) were individually prepared and stored in dark. Working solution of Golgi-cox was prepared by mixing 10.41 ml K$_2$Cr$_2$O$_7$, 10.41 ml HgCl$_2$, 8.33 ml K$_2$CrO$_4$ and 20.83 ml ddH$_2$O in a reagent bottle. This solution was protected from light and stored for 48 h before use. Animals were sacrificed under ketamine-xylazine anesthesia, their brains were isolated over ice, washed with chilled water and chilled Golgi-cox solution. A block, having entire hippocampus, was cut from the brain and incubated with Golgi-cox solution for 24 h at 37ºC. Block was removed, dried using tissue paper, 200 µm thick sections were prepared and fixed on glass slides. These sections were rinsed twice (5 min each) with ddH$_2$O, dehydrated with ethanol (50% ) and then immersed in 3:1 ammonia : water solution for 10 min in dark. Sections were washed with ddH$_2$O (2 × 5 min) and then immersed in sodium thiosulphate solution (5%) for 10 min in dark. Sections were again washed with ddH$_2$O (2 × 5 min) and were gradually dehydrated using 50%, 70%, 80%, 95% and 100% ethanol (5 min each). Sections were cleared with xylene and mounted with DPX on the slide. Neuronal morphology, interneuronal connections, neurodegeneration etc. in the different regions of the hippocampus were observed under light microscope at 200X and 400X magnification and dendritic spine density was visualized at 1000X magnification. Images were captured, processed and were later analyzed for various parameters of neuronal integrity.


Animals were sacrificed and sections were prepared for immunofluorescence studies as described under section 4.8. Slides were brought to room temperature and for microtome paraffin embedded sections, they were deparaffinized and rehydrated by treating them with xylene (6 min), xylene:ethanol (1:1) (6 min), 100% ethanol (10 min), 90% ethanol (6 min), 70% ethanol (6 min) and 50% ethanol (6 min). These sections (and cryotome sections) were then rehydrated by immersing them in PBS for 10 min (2 × 5 min). Sections were permeabilized with 0.1% Triton X-100 solution (10 minutes) at room temperature, washed with PBS (2 × 5 min) and then blocked with 3-5% BSA (prepared with PBST) for 45 min at 37ºC. Sections were then incubated for 12 h at 4ºC with InR (Insulin Rβ (C-19); rabbit polyclonal IgG, SC-711) primary antibodies (1:100). Sections were washed with PBS (3 × 5 min) and PBST (2 × 5 min) and then they were incubated for 60 minutes at 25ºC with corresponding FITC tagged goat anti-rabbit IgG antibody
(1:750) (Santa Cruz Biotechnology, Inc.; SC-2012) secondary antibody (1:750). Sections were washed with PBS (5 × 5 min) and PBST (3 × 5 min) and then incubated with GLUT4 (Glut4 (N-20); goat polyclonal IgG, SC-1606) primary antibody 12 h at 4°C. After washing with PBS and PBST, sections were incubated for 1 h at room temperature with corresponding TRITC tagged corresponding donkey antigoat IgG antibody (Santa Cruz Biotechnology, Inc.; SC-2783) secondary antibody, washed with PBS (5 × 5 min) and PBST (3 × 5 min), stained with DAPI and expression of InR and GLUT4 was observed under fluorescence microscope (Nikon eclipse Ti fluorescence microscope) at 100X and 400X magnification [267]. Results were demonstrated as total FITC (InR) and TRITC (GLUT4) fluorescence intensity in terms of CTCF calculated using image-J software with the following equation:

\[
CTCF = \text{Integrated density} - (\text{Area of selected cell} \times \text{Mean background fluorescence})
\]

Further, GLUT4:InR ratio was calculated to evaluate the role of quercetin in modulating hippocampal insulin signaling pathway.

4.15. Experiments to confirm mechanism of quercetin action

4.15.1. 2-NBDG uptake assay

2-NBDG is a fluorescent analogue of D-glucose. Like glucose, it is taken up by the cells through GLUTs, however, 2-NBDG cannot be metabolized within the cells and therefore it gets accumulated inside the cells. 2-NBDG generates a fluorescence, which is proportional to glucose uptake by the cells and thus, it can be used to measure glucose uptake [268]. We used 2-NBDG assay to confirm the potential of quercetin to enhance neuronal glucose uptake. Further, we wished to investigate whether quercetin is acting through insulin signaling pathway or not, and for this we used LY294002, which is a specific inhibitor of insulin signaling pathway [269]. Entire experimentation was performed on L6 rat skeletal muscle cell line (ATCC CRL-1458). L6 cells were seeded at \(2 \times 10^5\) cells per well in an adherent micro-well plate having DMEM media (with 10% FBS) 24 h before the start of experiments. Plates were incubated inside an incubator having 5% CO\(_2\) atmosphere at 37°C. After 24 h (or after 70% confluency is achieved) media was replaced with serum free and glucose free DMEM. Culture palates again incubated for 6 h in incubator. Afterwards, cells were incubated for 15 min with 10 \(\mu\)M LY294002 at 37°C (equal volume of PBS served as a control reaction). 10 \(\mu\)M quercetin and 1 \(\mu\)M insulin was then added
to respective wells for 20 min, after which 0.1 mM 2-NBDG was added and culture plates were incubated for 20 more minutes at 37°C inside a CO₂ incubator. Cultures were washed with chilled PBS (3 × 5 min) and fixed with 4% formalin solution. Images of the 2-NBDG uptake were acquired at 200 X magnification using fluorescent microscope and the fluorescence intensity was quantified in terms of CTCF using image-J software.

4.15.2. Docking studies to predict quercetin-insulin receptor (InR) interaction site
To predict whether quercetin is able to interact with InR and to predict the interaction site of ligand-protein interaction, we performed the docking studies. We used 1IR3 as the crystallographic structure of InR protein at a resolution of 1.9 Å, 306 A.a length and at a residue position of 1005-1310. Active site was predicted through Metapocket server. Docking between quercetin and InR was performed through Autodock for flexible docking between them, using the Genetic algorithm. Docking between insulin molecule (2VJZ) and InR protein (1IR3) was done through rigid docking using Z-dock. Interaction energy, RMSD, and ligplot analysis were performed to determine the interaction and binding site for quercetin and insulin on IR (1IR3).

4.16. Statistical analysis
Statistical significance of the data was determined by using GraphPad Prism 6 software. Data were expressed as mean ± SD and the statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple comparison post hoc test at a significance level of *P < 0.05, **P < 0.01, and ***P < 0.001). Two-way ANOVA with Bonferroni post hoc test was performed for all data to evaluate main effect and interaction at a confidence level of P < 0.05. Data of the MWM were analyzed through repeated measures ANOVA. Further, eta-squared effect size (η²) for ANOVA results and Cohen's d effect sizes estimates were performed.
CHAPTER 5

RESULTS
5. Results

5.1. Screening of natural molecules for their antidiabetic potential

5.1.1. Molecular docking

We performed docking study to screen small molecules of natural origin for their antidiabetic potential against InR (1IR3), PPAR-γ (2PRG) and DPP-IV (4PNZ) proteins. Results of the molecules predicted to be potential antidiabetic moieties are depicted below in Table 5.1 and Figure 5.1 represent their ligand-receptor binding images.

Table 5.1: Interaction energies (kcal/mol) of natural molecules with InR protein (1IR3), PPAR-γ protein (2PRG) and DPP-IV protein (4PNZ)

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Docking Score (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1IR3</td>
</tr>
<tr>
<td>Phosphoaminophosphonic acid-adenylate ester</td>
<td>-8.0</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>-</td>
</tr>
<tr>
<td>Omarigliptin</td>
<td>-</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>-4.8</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>-6.2</td>
</tr>
<tr>
<td>Quercetin</td>
<td>-8.9</td>
</tr>
<tr>
<td>Caffeine</td>
<td>-5.4</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>-7.2</td>
</tr>
<tr>
<td>Piperine</td>
<td>-7.6</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>-5.7</td>
</tr>
</tbody>
</table>

Our results demonstrated a good interaction of quercetin with 1IR3 (-8.9 kcal/mol) and 2PRG (-7.5 kcal/mol) proteins. Docking scores for these interactions were observed to be better than internal standard ligand of 1IR3 and 2PRG, viz. phosphoaminophosphonic acid-adenylate ester (ANP) (-8.0 kcal/mol) and rosiglitazone (-7.0 kcal/mol), respectively. Interaction of quercetin with 2PNZ protein (-7.3 kcal/mol) was comparable to omarigliptin (-7.9 kcal/mol), which was used as an internal standard ligand for 2PNZ. Further, ellagic acid also demonstrated good interaction with 2PRG (-7.8 kcal/mol) and 4PNZ (-8.1 kcal/mol) proteins and results were comparable to quercetin.
and respective internal ligands, besides, docking score of -7.2 kcal/mol was observed when it was docked with 1IR3 protein. Furthermore, ascorbic acid, piperine, cinnamic acid, gallic acid and caffeine also showed appreciable interaction with 1IR3, 2PRG and 2PNZ protein targets. These results predicted ascorbic acid, gallic acid, quercetin, caffeine, ellagic acid, piperine, and cinnamic acid as the potential antidiabetic moieties. Screening process was narrowed down by subjecting these molecules through in-vitro assays.
Figure 5.1: Docking interaction of herbal molecules with 1IR3, 2PRG and 4PNZ.

A - internal standard ligand; B - ascorbic acid; C - gallic acid; D - quercetin; E - caffeine; F - ellagic acid; G - piperine; H - cinnamic acid.
5.1.2. Antioxidant activity

In the present study, we investigated the *in-vitro* antioxidant potential of the natural molecules through DPPH radical scavenging assay and inhibition of lipid peroxidation assay, to evaluate whether or not natural molecules can prove beneficial during oxidative stress, and compared them with marketed antidiabetic drugs. Results of the DPPH radical scavenging assay are depicted below in Table 5.2. Ascorbic acid, gallic acid, quercetin and ellagic acid are well known antioxidants and here also they demonstrated a good potential to scavenge DPPH free radical. Maximum activities of 94.09 ± 0.54%, 94.80 ± 1.64%, 90.14 ± 0.82% and 88.71 ± 0.54% were observed at 6.25 µM concentration respectively. The IC$_{50}$ values were observed to be in the range of 0.006 – 0.03 µM. Further, caffeine, piperine and cinnamic acid did not showed promising results and their IC$_{50}$ values for DPPH radical scavenging activities were observed in the range of 12.90 – 16.67% at 6.25 µM concentration. Similarly, marketed drugs were observed to be poor antioxidant and their free radical scavenging activity was observed to be 2 – 3 fold lower than ascorbic acid, gallic acid, quercetin and ellagic acid.

Table 5.2: Percent DPPH radical scavenging activities of herbal molecules and commercially available antidiabetic drugs and their calculated IC$_{50}$ values (µM). Values are represented as mean ± SD (n = 4). Values followed by different letters in the same column are significantly different at p < 0.05 by Duncan’s multiple range test.

<table>
<thead>
<tr>
<th>Test compounds</th>
<th>% DPPH radicle scavenging activity</th>
<th>IC$_{50}$ value (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.625 µM</td>
<td>1.25 µM</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>83.69±1.89$^f$</td>
<td>87.45±1.35$^f$</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>84.77±0.82$^f$</td>
<td>87.45±0.82$^f$</td>
</tr>
<tr>
<td>Quercetin</td>
<td>83.33±1.07$^f$</td>
<td>85.30±0.82$^f$</td>
</tr>
<tr>
<td>Caffeine</td>
<td>8.79±3.41$^{cd}$</td>
<td>11.11±1.11$^c$</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>76.34±0.54$^e$</td>
<td>80.46±0.31$^e$</td>
</tr>
<tr>
<td>Piperine</td>
<td>1.97±1.35$^a$</td>
<td>6.45±1.94$^a$</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>6.45±1.94$^{bc}$</td>
<td>9.14±0.93$^{bc}$</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>10.75±0.93$^d$</td>
<td>18.99±0.62$^d$</td>
</tr>
<tr>
<td>Metformin</td>
<td>4.30±1.42$^{ab}$</td>
<td>6.99±3.27$^{ab}$</td>
</tr>
<tr>
<td>Glimepiride</td>
<td>2.15±1.42$^a$</td>
<td>6.45±1.07$^a$</td>
</tr>
</tbody>
</table>
Further, we observed a concentration dependent inhibition of lipid peroxidation in the order of quercetin > ellagic acid > piperine > ascorbic acid > gallic acid > caffeine > cinnamic acid > glimepiride > metformin > rosiglitazone (Table 5.3). Quercetin and ellagic acid were observed to be most effective moieties with maximum activity of 74.36 ± 3.47% and 84.36 ± 0.89% respectively at 50 µM concentration. IC₅₀ values were observed to be 0.92 µM and 1.07 µM respectively. Our results demonstrated that piperine, ascorbic acid and gallic acid possess a moderate potential to inhibit lipid peroxidation and their activity was observed to be in the range of 69.74 – 79.23% at 6.25 µM concentration (IC₅₀ value ranging between 7.03 – 8.47 µM). Herein, we demonstrated that natural molecules possess good potential to scavenge free radicals and prevent lipid peroxidation, especially quercetin, and thereby may prove to be beneficial in alleviating oxidative stress during diabetic condition, besides, marketed drugs failed as an antioxidant moieties.

Table 5.3: Percent inhibition of lipid peroxidation by natural molecules and commercially available antidiabetic drugs and their calculated IC₅₀ values (µM). Values are represented as mean ± SD (n = 4). Values followed by different letters in the same column are significantly different at p < 0.05 by Duncan’s multiple range test.

<table>
<thead>
<tr>
<th>Test compounds</th>
<th>% Inhibition of lipid peroxidation</th>
<th>IC₅₀ value (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.25 µM</td>
<td>12.5 µM</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>45.13±3.47ᵇ</td>
<td>64.36±4.24ᶜ</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>47.69±2.03ᵇ</td>
<td>53.08±3.08ᵇ</td>
</tr>
<tr>
<td>Quercetin</td>
<td>65.64±1.93ᶜᵈ</td>
<td>72.30±1.33ᵈ</td>
</tr>
<tr>
<td>Caffeine</td>
<td>32.56±1.17ᵃ</td>
<td>33.59±1.17ᵃ</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>68.46±2.03ᵈ</td>
<td>74.87±1.60ᵈ</td>
</tr>
<tr>
<td>Piperine</td>
<td>45.38±0.77ᵇ</td>
<td>61.79±1.93ᶜ</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>34.36±1.17ᵃ</td>
<td>36.15±2.03ᵃ</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>33.33±1.60ᵃ</td>
<td>33.59±1.17ᵃ</td>
</tr>
<tr>
<td>Metformin</td>
<td>32.05±0.44ᵃ</td>
<td>32.56±1.17ᵃ</td>
</tr>
<tr>
<td>Glimepiride</td>
<td>34.87±0.44ᵃ</td>
<td>36.15±0.77ᵃ</td>
</tr>
</tbody>
</table>
5.1.3. Genotoxicity

We evaluated the effect of test molecules on the H$_2$O$_2$ induced genotoxicity in whole human blood, since hyperglycemia is frequently associated with genotoxicity within the biological system which exaggerates the progression and severity of neurological complications associated with hyperglycemia. We further compared the results of natural molecules with the commonly used marketed drugs to understand the comparative activity of natural and marketed drugs. Results of the comet assay are depicted in Figure 5.2 and our results suggest that H$_2$O$_2$ induced genotoxicity, as indicated by the DNA fragmentation and significantly higher (p < 0.001) olive tail movement, when compared to control.

![Figure 5.2: Effect of test molecules on H$_2$O$_2$ induced genotoxicity. Values are represented as mean ± SD (n = 4) measured from comet assay. Significance is represented at *p < 0.05, **p < 0.01, ***p < 0.001; #p < 0.05, ##p < 0.01, ###p < 0.001. *Control vs treatments; #H$_2$O$_2$ vs treatments.]

Quercetin, gallic acid, caffeine, piperine and cinnamic acid were observed to be most effective natural molecules and a significant (p < 0.001) reduction in DNA fragmentation and olive tail movement was observed in the cells treated with these molecules, when compared to H$_2$O$_2$ treatment. Further, ellagic acid, ascorbic acid and marketed drugs failed to rescue cells from H$_2$O$_2$ mediated DNA fragmentation and the olive tail movement was observed to be significantly higher than control. These results suggest that natural molecules are better than marked drugs in countering genotoxicity, therefore can prove beneficial in managing hyperglycemia mediated complications.
5.1.4. Anti-diabetic activity

The comparative in-vitro antidiabetic activity of natural molecules and marketed drugs were evaluated through inhibition of α-amylase assay, neuronal glucose uptake assay and neuronal GLUT4 expression analysis. Results of the in-vitro inhibition of α-amylase activity are depicted in Table 5.4. Our results suggests that quercetin and ellagic acid possesses high potential to inhibit enzyme in concentration dependent manner, with the maximum inhibition of 85.83 ± 0.83% and 81.11 ± 1.27% respectively achieved at 500 µM concentration, with IC₅₀ values of 18.31 µM and 12.49 µM respectively. Ascorbic acid, caffeine and piperine showed a moderate potential to inhibit amylase activity with their percent inhibition of 72.22 ± 2.09%, 54.44 ± 1.73% and 51.94 ± 2.54% respectively at 500 µM concentration having IC₅₀ value of 72.4 ± 0.03 µM, 311.7 ± 0.04 µM and 464.2 ± 0.06 µM respectively. Further, marketed drugs were ineffective in inhibiting the actions of α-amylase enzyme and their IC₅₀ values observed to be >1000 µM. These results suggest that natural molecules can very well manage dietary inflow of glucose, which cannot be achieved with the tested marketed drugs.

Table 5.4. Percent α-amylase inhibition by test molecules and their IC₅₀ values (µM). Values are represented as mean ± SD (n = 4). Values followed by different letters in the same column are significantly different at p < 0.05 by Duncan’s multiple range test.

<table>
<thead>
<tr>
<th>Test compounds</th>
<th>% α-amylase inhibition</th>
<th>IC₅₀ value (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 µM</td>
<td>100 µM</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>47.22±1.27f</td>
<td>51.94±1.27f</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>40.28±1.73c</td>
<td>42.78±1.27c</td>
</tr>
<tr>
<td>Quercetin</td>
<td>63.05±0.96g</td>
<td>72.22±2.09h</td>
</tr>
<tr>
<td>Caffeine</td>
<td>37.78±0.96de</td>
<td>42.50±0.83de</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>62.78±1.27g</td>
<td>68.33±2.20g</td>
</tr>
<tr>
<td>Piperine</td>
<td>35.83±0.83cd</td>
<td>40.00±0.83d</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>32.78±0.48c</td>
<td>34.17±0.83c</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>18.05±4.19b</td>
<td>17.22±2.54ab</td>
</tr>
<tr>
<td>Metformin</td>
<td>7.22±1.92a</td>
<td>14.72±0.48a</td>
</tr>
<tr>
<td>Glimepiride</td>
<td>20.28±3.37b</td>
<td>18.61±1.27b</td>
</tr>
</tbody>
</table>
We further evaluated the effect of herbal molecules on the glucose uptake and GLUT4 expression in the primary E18 embryonic hippocampal neuronal cultures, and compared them with rosiglitazone, metformin and glimepiride. Each drug was tested at 10 µM concentration, as the cytotoxicity studies did not demonstrated any signs of neuronal toxicity at this concentration. Results of the *in-vitro* glucose uptake assay are depicted in Figure 5.3. Ascorbic acid, caffeine, gallic acid, quercetin, cinnamic acid and ellagic acid significantly enhanced neuronal glucose uptake (29.44% – 35.94%), when compared to control, whereas basal glucose uptake of 24.53% was observed. Enhanced glucose uptake could be attributed to the potential of natural molecules to upregulate neuronal GLUT4 expression (Figure 5.4 and 5.5). Rosiglitazone, ascorbic acid, quercetin, caffeine and ellagic acid resulted in enhanced expression of GLUT4 and thereby, a proportionally enhanced glucose uptake was observed in cultures treated with these molecules. Marketed drugs also enhanced neuronal glucose uptake and their activity was observed to be in the range of 28.05 – 33.62%, which was significantly higher than control cells, but differed insignificantly from ascorbic acid.

![Figure 5.3](image-url)

**Figure 5.3:** Effect of the herbal molecules and antidiabetic drugs on percent glucose uptake into primary E18 hippocampal neurons after 24 h incubation in DMEM media containing 5 g/L glucose. Values are represented as mean ± SD (n = 4). Significance is represented at *p < 0.05, **p < 0.01, ***p < 0.001. *Control vs treatments.
Figure 5.4: Effect of herbal molecules and antidiabetic drugs on GLUT4 expression in the E18 embryonic hippocampal primary neuronal culture. GLUT4 is depicted as green fluorescence (FITC), counterstained with DAPI blue fluorescence.
quercetin, caffeine and ellagic acid. A proportional increase in GLUT4 expression was observed in rosiglitazone treated cells, which may be responsible for higher glucose utilization in these cells. Comparatively, metformin and glimepiride showed lower GLUT4 expression than rosiglitazone, and therefore glucose uptake was also observed to be on the lower side for these drugs. These results suggest that, natural molecules, especially quercetin and caffeine, can prove to be beneficial in managing diabetes and IR by disposing excessive glucose from the blood into the cells during hyperglycemia and T2DM.

![Figure 5.5](image)

**Figure 5.5:** Corrected Total Cell Fluorescence (CTCF) of GLUT4 translocation. Values are represented as mean ± SD (n = 4). Significance is represented at *p < 0.05, **p < 0.01, ***p < 0.001. *Control vs treatments.

### 5.2. Antidiabetic study

#### 5.2.1. Effect of quercetin on symptomatic progression of diabetes

Progression of diabetes during 8 weeks of the study was evaluated in terms of symptomatic parameters which include blood glucose levels, polyphagia, polydipsia, and body weight. FBG was evaluated weekly during the study. Post hoc analysis of the FBG level revealed a significant STZ/quercetin interaction \[F (2,30) = 15.25, p < 0.001 \text{ and } \eta^2 = 0.15\]. We successfully induced diabetes in experimental animals and the effect of quercetin and rosiglitazone treatment on it was evaluated at the end of 8 week. Fasting glucose levels in STZ group were significantly (p < 0.001)
higher than CTRL animals and drug treatments significantly \( p < 0.001 \) lowered hyperglycemia (Figure 5.6A).

Subjecting animals to long standing diabetes revealed a nonsignificant interaction between diabetes and treatments \( [F (2,30) = 1.19, p > 0.05 \text{ and } \eta^2 = 0.21] \). These results revealed that diabetes induced a significant \( p < 0.01 \) reduction in the body weight of diabetic mice. Treating diabetic animals with quercetin for 8 weeks improved body weight, however, rosiglitazone treatment did not showed much effect on body weight (Figure 5.6B). Further, results of feed intake did not revealed any significant STZ/treatments interaction \( [F (2,30) = 1.67, p > 0.05 \text{ and } \eta^2 = 0.05] \).
Post hoc analysis revealed that diabetes induced a significant ($p < 0.05$) polyphagia in animals and by treating diabetic animals with quercetin efficiently ($p < 0.05$) improvement feed intake, which was not observed in rosiglitazone treated animals (Figure 5.6C). We further evaluated the effect of diabetes and drug treatment on polydipsia and observed a significant STZ/drug treatments interaction [$F(2,12) = 5.34$, $p < 0.05$ and $\eta^2 = 0.18$]. Diabetic animals showed significant ($p < 0.001$) polydipsia and treating these animal with quercetin and rosiglitazone significantly ($p < 0.05$) lowered the water consumption (Figure 5.6D). These results confirms the development of diabetes in animals after STZ injection and revealed that 8 week quercetin treatment provide a symptomatic improvement against STZ mediated diabetes in mice.

We further evaluated the progression of diabetes during the study by measuring FBG levels at the end of every second week (Figure 5.7A). Although glucose levels in the animals injected with STZ were significantly ($p < 0.001$) greater than CTRL, we did not observed any significant difference in blood glucose levels of STZ, STZ + Q and STZ + ROSI animals up to 2nd week of the study. Diabetes progressed steadily in STZ animals and the FBG levels remained higher than CTRL and our results showed a significant interaction between STZ and treatments for fasting glucose levels during week 4 [$F(2,30) = 14.23$, $p < 0.001$ and $\eta^2 = 0.06$], week 6 [$F(2,30) = 23.23$, $p < 0.001$ and $\eta^2 = 0.11$] and week 8 [$F(2,30) = 19.37$, $p < 0.001$ and $\eta^2 = 0.12$] of the study. Treating diabetic animals with quercetin and rosiglitazone significantly ($p < 0.05$) reduced FBG levels at week 4, 6 and 8, when compared to STZ. Interestingly, none of the treatments actually cured the disorder, but somehow managed to keep glucose levels almost constant, suggesting that they did not reversed the STZ mediated diabetes, but prevented it from worsening.
5.2.2. Effect of quercetin on diabetes mediated glucose intolerance

To get a conclusive evidence for the development of diabetes and glucose intolerance after STZ injection, we performed OGTT and our results demonstrated a significant levels of glucose intolerance in STZ animals. Blood glucose remained significantly ($p < 0.001$) higher in STZ group throughout OGTT study, when compared to CTRL. Treating diabetic animals with quercetin improved glucose tolerance, as blood glucose was found to be significantly ($p < 0.001$) lower than STZ at every time point of the study. Rosiglitazone treated animals also showed improvement in glucose tolerance and blood glucose remained significantly lower at 120 minutes ($p < 0.05$) and 180 minutes ($p < 0.001$) of OGTT study. Interestingly, blood glucose did not increased as per expectations in quercetin treated normal as well as diabetic animals, suggesting the strong potential of quercetin to dispose blood glucose Figure 5.7B.

5.2.3. Effect of quercetin treatment on locomotion and muscle coordination during diabetes

5.2.3.1. Open field test (OFT)

The effect of long standing diabetes on locomotion was evaluated through OFT in terms of number of line crossings. Our results demonstrated a significant diabetes/drug treatments interaction [$F(2,30) = 7.41$, $p < 0.01$ and $\eta^2 = 0.16$]. Long standing diabetes significantly ($p < 0.001$) lowered
the locomotion in animals. Quercetin and rosiglitazone treated animals were observed to freely explore the open arena and the locomotor activity was significantly higher \((p < 0.05)\), when compared to STZ Figure 5.8A.

### 5.2.3.2. Rota-rod and beam walk

We evaluated the effect of diabetes and quercetin treatment on muscle coordination and strength through Rota-rod and beam walk test. We did not observed any significant diabetes/treatment interaction in the Rota-rod test \([F (2,30) = 0.11, p > 0.05 \text{ and } \eta^2 = 0.006]\) and beam walk test \([F (2,30) = 0.65, p > 0.05 \text{ and } \eta^2 = 0.04]\). Diabetes did not impaired muscle coordination and muscle strength. We did not observed any significant difference in the time taken to fall from Rota-rod apparatus and time take to cross the beam, suggesting normal muscle activity in all the treated animals Figure 5.8 B-C.

\[\text{Figure 5.8: Effect of diabetes and quercetin treatment on locomotion (A), muscle strength and muscle coordination (B-C). Results are depicted as mean ± SD (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. \#p < 0.05; \##p < 0.01; ###p < 0.001 versus STZ.}\]
5.2.4. Effect of diabetes and quercetin treatment on anxiety behavior

5.2.4.1. Elevated Plus Maze (EPM)
EPM is most widely used model to evaluate the anxiety in rodents. We evaluated the effect of diabetes and quercetin treatment on anxiety through this model and observed a significant diabetes/treatment interaction \([F (2,30) = 12.21, p < 0.001\) and \(\eta^2 = 0.013\)]. Diabetic animals were highly anxious and spent significantly \((p < 0.001)\) lesser time in the open arm of the EPM. Quercetin showed marked anxiolytic effect and animals were observed to freely explore both the arms of EPM. A significant \((p < 0.001)\) improvement in anxiety was observed after treating animals with quercetin and rosiglitazone, however, quercetin was having better anxiolytic activity than rosiglitazone Figure 5.9A.

![Figure 5.9: Effect of diabetes and quercetin treatment on anxiety. Elevated plus maze (A), open field test number of center entries (B) and open field test time spent in the center (C). Results are depicted as mean ± SD (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. #p < 0.05; ##p < 0.01; ###p < 0.001 versus STZ.](image-url)
5.2.4.2. Open Field Test (OFT)
OFT anxiety was evaluated in terms of number of entries to the central region Figure 5.9B and amount of time spent there Figure 5.9C. Like EPM, in this test also diabetic animals were observed to highly anxious and a significant interaction between diabetes and drug treatment was observed for the number of entries to the central region \( F(2, 30) = 11.57, p < 0.001 \) and \( \eta^2 = 0.18 \) and for the time spent in the central region of OFT \( F(2, 30) = 14.72, p < 0.001 \) and \( \eta^2 = 0.23 \). Quercetin treatment significantly \( (p < 0.001) \) lowered the anxiety in diabetic animals and effect of quercetin was more pronounced than rosiglitazone. These results suggest that diabetes is associated with anxiety behavior in Swiss albino mice and quercetin possesses strong anxiolytic effect.

5.2.5. Effect of diabetes and quercetin treatment on depressive like behavior
5.2.5.1. Sucrose Preference Test (SPT)
Development of depressive like behavior in diabetic mice was evaluated in term of anhedonia through SPT Figure 5.10A. We observed a significant STZ/drug treatment interaction \( F(2, 18) = 8.95, p < 0.01 \) and \( \eta^2 = 0.27 \). Long standing diabetes induced a significant \( (p < 0.001) \) levels of anxiety in animals, as indicated by the results of SPT where animals showed almost equal preference towards normal and sweetened water, which is a prominent indicator of anhedonia. Both, quercetin and rosiglitazone, were observed to alleviate STZ mediated depression and treated animals showed significantly \( (p < 0.01) \) higher preference towards sweetened water, when compared to STZ.

5.2.5.2. Tail Suspension Test (TST)
TST is used to evaluate the despair behavior in rodents and we evaluated the effect of quercetin treatment on diabetes induced depression through this model. A significant STZ/treatment interaction was observed in TST \( F(2, 30) = 4.97, p < 0.05 \) and \( \eta^2 = 0.10 \). Diabetic animals were observed to spent significantly \( (p < 0.001) \) higher time in immobility, indicating the development of despair behavior in them. Quercetin and rosiglitazone treated animals showed higher struggling time in TST. A significantly lower immobility time was observed in diabetic animals treated with quercetin \( (p < 0.001) \) and rosiglitazone \( (p < 0.05) \) Figure 5.10B. These results suggest that quercetin efficiently alleviated diabetes mediated depressive like behavior in mice.
5.2.5.3. Forced Swim Test (FST)

Like TST, FST also measures the despair behavior in animals by evaluating the effort of animal to get out of a distressing condition. We observed a significant STZ/treatment interaction [$F (2,30) = 4.97, p < 0.05$ and $\eta^2 = 0.10$] in animals subjected to FST. Subjecting results to post hoc analysis suggest that diabetes induced a marked ($p < 0.001$) level of depression in animals, which was efficiently attenuated by quercetin ($p < 0.01$) and rosiglitazone ($p < 0.05$) treatment Figure 5.10C. Effect of quercetin on depressive like behavior was more pronounced than rosiglitazone. These results suggest that quercetin is having antidepressant activity and efficiently alleviate depression associated with long standing diabetes.

![Figure 5.10: Effect of diabetes and quercetin treatment on depression. Sucrose preference test (A), tail suspension test (B) and forced swim test (C). Results are depicted as mean ± SD (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. *p < 0.05; **p < 0.01; ***p < 0.001 versus STZ.](image-url)
5.2.6. Effect of diabetes and quercetin treatment on learning and memory dysfunction

5.2.6.1. Novel Object Recognition (NOR) Test

NOR was used to evaluate the effect quercetin treatment on diabetes mediated recognition memory dysfunction, in terms of discrimination index. A significant STZ/treatment interaction was observed [F (2,30) = 14.62, p < 0.001 and \( \eta^2 = 0.28 \)] in the animals. Diabetes significantly (p < 0.001) impaired the recognition memory function in the animals as they failed to discriminate novel object from the familiar one. Animals treated with quercetin and rosiglitazone showed significantly (p < 0.001) improved memory performance and these animals spent significantly (p < 0.001) greater time exploring the novel object, when compared to STZ Figure 5.11A. These results suggest that long standing diabetes induced by STZ inflicts marked memory dysfunction in Swiss albino mice and quercetin treatment improved memory in rodents.

5.2.6.2. Morris Water Maze (MWM)

Effect of diabetes and quercetin treatment on the learning and working memory was evaluated through MWM test. Our results did not demonstrate any significant interaction between diabetes and drug treatments on the first day of the learning trial [F (2,30) = 2.56, p > 0.05 and \( \eta^2 = 0.10 \)]. However, the impact of diabetes and drug treatments on learning became evident after second day of the learning trial. Significant STZ/treatment interaction was observed on second day [F (2,30) = 13.05, p < 0.001 and \( \eta^2 = 0.27 \)], third day [F (2,30) = 13.69, p < 0.001 and \( \eta^2 = 0.27 \)] and fourth day [F (2,30) = 12.28, p < 0.001 and \( \eta^2 = 0.33 \)] of the learning trial. These results suggest that diabetes impaired learning abilities of the animals and they struggled to find the hidden platform. Treating diabetic animals with quercetin and rosiglitazone significantly improved learning, and the animals found the hidden platform in significantly lesser time, when compared to STZ Figure 5.11B.

We further evaluated the memory consolidation through probe trial of MWM. We did not observed significant STZ/treatment interaction for number of platform crossing [F (2,30) = 2.56, p > 0.05 and \( \eta^2 = 0.10 \)], however a significant interaction was observed for time spent in the platform quadrant [F (2,30) = 9.19, p < 0.001 and \( \eta^2 = 0.17 \)]. Subjecting animals to STZ induced diabetes resulted in marked memory impairment, as suggested by the significantly lesser platform crossings.
and significantly lesser amount of the time spent in the platform quadrant during the probe trial of MWM. Quercetin and rosiglitazone treatment improved diabetes mediated memory dysfunction and drug treated animals spent significantly ($p < 0.001$) higher time in searching for the escape platform in the platform quadrant and showed significantly ($p < 0.05$) higher number of platform crossings. These results suggest that long standing diabetes induced memory dysfunction which can be efficiently attenuated by quercetin treatment Figure 5.11C-D.

**Figure 5.11:** Effect of diabetes and quercetin treatment on learning and memory functions. NOR test discrimination index (A), time taken to find hidden platform in MWM (B), number of platform crossings in MWM probe trial (C) and time spent in the platform quadrant in MWM (D). Results are depicted as mean ± SD (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control group. #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$ versus STZ.
5.2.6.3. Passive Avoidance Step Through (PAST)

Effect of diabetes and quercetin on associated memory dysfunction was evaluated through passive avoidance test in terms of animal's transfer latency to enter dark compartment, calculated as inflexion ratio. We observed a significant diabetes/treatment interaction on second day \( [F (2,30) = 9.19, p < 0.001 \text{ and } \eta^2 = 0.17] \) and fifth day \( [F (2,30) = 13.44, p < 0.001 \text{ and } \eta^2 = 0.40] \) of the PAST test. Long standing diabetes significantly impaired short-term as well as long-term memory in mice, as indicated by the significantly \((p < 0.001)\) lower inflexion ratio in diabetic animals on day 2 (IF1) and day 5 (IF2). Drug treatments improved memory function and animals retained the memory of foot shock they received during learning trial on day 1 of the test. Inflexion ratio in quercetin and rosiglitazone treated animals was significantly \((p < 0.05)\) higher than STZ, when evaluated on day 2 and day 5 of the test Figure 5.12A. Quercetin treated animals showed nonsignificantly higher inflexion ratio than STZ + ROSI, suggesting that quercetin is better than rosiglitazone in improving associated memory dysfunction.

A: Passive Avoidance Step Through Test

![Graph showing inflexion ratio in PAST test](image)

**Figure 5.12 (A):** Effect of diabetes and quercetin treatment on associative memory dysfunction. Inflexion ratio in PAST. **IF1:** inflexion ratio on day 2 of the study. **IF2:** inflexion ratio on day 5 of the study. Results are depicted as mean ± SD \((n = 6)\). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *\(p < 0.05\); **\(p < 0.01\); ***\(p < 0.001\) versus control group. #\(p < 0.05\); ##\(p < 0.01\); ###\(p < 0.001\) versus STZ.
5.2.6.4. Passive Avoidance Step Down (PASD)

PASD is a commonly used method to evaluate short-term and long-term memory performance in rodents. Our results demonstrated a significant interaction between diabetes and drug treatments for short-term [F (2,30) = 20.39, p < 0.001 and η² = 0.45] and long-term memory performance [F (2,30) = 13.71, p < 0.001 and η² = 0.43] evaluated on day 2 and day 5 of the PASD test. Diabetic animals showed significant (p < 0.001) impairment in the associative memory and were not able to remember the foot shock received during the learning trial. Treating animals with quercetin improved short-term as well as long-term memory performance and inflexion ratio was significantly (p < 0.001) higher than STZ on day 2 (IF1) and day 5 (IF2) of the test. Rosiglitazone treated animals showed higher latency to remember the foot shock received during learning trial and showed a significantly (P < 0.5) higher inflexion ratio on the day 2 (IF1) and day 5 (IF2) of the test Figure 5.12B.

**Figure 5.12 (B):** Effect of diabetes and quercetin treatment on associative memory dysfunction. Inflexion ratio in PASD. **IF1:** inflexion ratio on day 2 of the study. **IF2:** inflexion ratio on day 5 of the study. Results are depicted as mean ± SD (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. *p < 0.05; **p < 0.01; ***p < 0.001 versus STZ.
5.2.7. Effect of diabetes and quercetin treatment on biochemical parameters

5.2.7.1. Serum insulin levels and HOMA-IR index

Insulin levels were determined in the serum of the experimental animals. Our results did not demonstrate any significant diabetes/treatment interaction \([F (2,18) = 1.01, p > 0.05\) and \(\eta^2 = 0.05\)]. Subjecting animals to diabetes by injecting STZ induced significant \((p < 0.05)\) reduction in serum insulin levels. Neither of the drug treatments significantly altered the serum insulin levels when compared to STZ, which may be due to the permanent destruction of pancreatic beta cells by STZ treatment (Figure 5.13A).

We calculated the effect of STZ on the development of IR in terms of HOMA-IR index. Post hoc analysis of the HOMA-IR index demonstrated a significant diabetes/treatment interaction \([F (2,18) = 7.16, p < 0.01\) and \(\eta^2 = 0.23\)]. Results of the HOMA-IR index demonstrated the development of IR in animals, as the HOMA-IR index was significantly \((p < 0.001)\) higher in diabetic animals, when compared to CTRL. Quercetin and rosiglitazone treatments alleviated IR and significantly \((p < 0.05)\) lowered HOMA-IR index in treated animals. These results validated our model of T2DM by injecting 50 mg/kg STZ on five consecutive days, as diabetic animals were observed to be insulin resistant. Moreover, quercetin treatment efficiently attenuated IR in diabetic animals (Figure 5.13B).

![Figure 5.13](image_url): Effect of diabetes and quercetin treatment on serum insulin levels and insulin resistance. Serum insulin levels (A) and HOMA-IR index (B). Results are depicted as mean ± SD (n = 4). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. #p < 0.05; ##p < 0.01; ###p < 0.001 versus STZ.
5.2.7.2. Hippocampal oxidative stress

Hippocampal oxidative stress was evaluated in terms of the expression levels of antioxidants (TTH and catalase) and oxidative stress markers (TBARS and NO) in the hippocampus. Post hoc analysis of the results of the hippocampal TTH levels demonstrated a significant diabetes/drug treatment interaction \[F (2,18) = 21.39, p < 0.001 \text{ and } \eta^2 = 0.18\]. Long standing diabetes induced marked oxidative stress in the hippocampus, as indicated by the significantly (p < 0.001) lower level of the TTH, which acts as an antioxidant and protects neurons from oxidative damage. Further, a significant interaction between diabetes and drug treatment was also observed for hippocampal catalase expression \[F (2,18) = 21.99, p < 0.001 \text{ and } \eta^2 = 0.11\]. Along with depletion of hippocampal TTH, long standing diabetes significantly (p < 0.001) reduced hippocampal catalase levels. Treating diabetic animals with quercetin produced a strong antioxidant effect and the levels of TTH and catalase were significantly (p < 0.001) restored in the hippocampus. Rosiglitazone treatment also showed significant improvement in hippocampal TTH (p < 0.001) and catalase levels (p < 0.05), however, the effect of quercetin treatment was observed to be better than rosiglitazone (Figure 5.14A-B).

Further, hippocampal oxidative stress was evaluated in terms of the expression of oxidative stress markers (TBARS and NO). Our results demonstrated a nonsignificant diabetes/drug treatment interaction for hippocampal TBARS expression \[F (2,18) = 2.65, p > 0.05 \text{ and } \eta^2 = 0.06\] and a significant interaction for NO expression \[F (2,18) = 10.52, p < 0.001 \text{ and } \eta^2 = 0.08\] in the hippocampus. Post hoc analysis of the results demonstrated that long standing diabetes markedly (p < 0.001) increased the levels of oxidative stress markers in the hippocampus, when compared to CTRL. Quercetin and rosiglitazone treatment significantly (p < 0.001) alleviated oxidative stress by lowering the levels of TBARS and NO in the hippocampus (Figure 5.14C-D). These results suggest that diabetes is associated with the generation of oxidative stress markers and depletion of antioxidants in the hippocampus, which may have enhanced neuronal dysfunction and induced behavioral dysfunction in diabetic animals. Quercetin treatment efficiently restored hippocampal antioxidants and reduced the levels of oxidative stress markers, thereby may protect neurons from the oxidative damage.
5.2.8. Effect of diabetes and quercetin treatment on neuronal integrity in the hippocampus

5.2.8.1. Golgi-cox staining

Golgi-cox staining method was used to check the neuronal integrity and interneuronal connections in the CA3 region of the hippocampus. Golgi-cox stained images were acquired at 400X.

Figure 5.14: Effect of diabetes and quercetin treatment on hippocampal oxidative stress. Hippocampal TTH expression (A), catalase expression (B), TBARS expression (C) and NO expression (D). Results are depicted as mean ± SD (n = 4). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. #p < 0.05; ##p < 0.01; ###p < 0.001 versus STZ.
magnification to visually check signs of neurodegeneration and neuronal integrity, besides, number of primary branches arising from soma (Np), critical value (Rc), maximum number of intersections (Nm) and Schoenen ramification index (Nm/Np) was calculated through Sholl analysis using image-J software. Images were captured at 1000 X magnification to calculate the dendritic spine density. Diabetes inflicted marked neuronal damage in the hippocampus, especially in the CA3 region, where number of dendrites were few, neurons appeared brittle and degenerating, and the number of interneuronal connections were lower in the animals. Quercetin treatment rescued neurons from diabetes mediated damage and healthy neurons with significant dendritic arborization were observed. Improvement in neuronal morphology was also observed in rosiglitazone treated animals, however, neuroprotective effect of rosiglitazone was not as prominent as observed for quercetin (Figure 5.15A).

**Figure 5.15 A:** Golgi-cox staining. Images of the Golgi-cox stained neurons in the CA3 region of the hippocampus. Neuronal morphology and arborization was determined at 400X magnification (Left panel). Images showing dendritic spines at 1000X magnification (Right panel).
We did not observe any significant diabetes/treatment interaction for (Np) \[F (2,18) = 0.64, p > 0.05 \text{ and } \eta^2 = 0.06\] and Rc \[F (2,18) = 0.90, p > 0.05 \text{ and } \eta^2 = 0.07\] calculated through Sholl analysis. Np did not vary significantly between different groups suggesting that diabetes do not affect dendrites arising from the soma and probably because primary branching had occurred in neurons before diabetes was induced. Rc value was significantly (p < 0.001) reduced in diabetic animals and a significant improvement was observed after quercetin (p < 0.001) and rosiglitazone (p < 0.01) treatment (Figure 5.15B-C).

Further, post hoc analysis of Nm at 100 μm length revealed a significant diabetes/treatment interaction \[F (2,18) = 11.02, p < 0.001 \text{ and } \eta^2 = 0.47\]. Nm was observed to be significantly lower in diabetic animals despite of nonsignificant difference in Np, suggesting that diabetes induced degenerating effect on the dendrites and therefore shorter and lesser number of dendrites were observed in STZ group. Diabetic animals treated with quercetin and rosiglitazone significantly (p < 0.05) increased number of intersections, suggesting that they efficiently protected dendrites from diabetes mediated degeneration (Figure 5.15D).

To get a better insight, we calculated Schoenen ramification index (Nm/Np) which gives the measure of dendritic arborization. Post hoc analysis demonstrated a significant \[F (2,18) = 7.92, p < 0.01 \text{ and } \eta^2 = 0.40\] diabetes/treatment interaction. Ramification index was significantly reduced in diabetic animals, indicating lower dendritic arborization in these animals. Treating diabetic animals with quercetin and rosiglitazone significantly (p < 0.05) improved Nm/Np value and thereby suggested that neurons in treated animals were having good arborization, which was comparative to control animals (Figure 5.15E).

Furthermore, we calculated the dendritic spine density at 1000X magnification and post hoc analysis demonstrated a significant diabetes/treatment interaction \[F (2,30) = 5.26, p < 0.05 \text{ and } \eta^2 = 0.15\]. Our results suggest that long standing diabetes significantly (p < 0.001) reduced the dendritic spine density in dendrites of hippocampus, due to which lesser number of dendrites and interneuronal connections were observed in these animals. Reduced spine density may have resulted in reduction in the formation of new connections and thereby may have resulted in behavioral dysfunction. Quercetin and rosiglitazone treatment rescued neurons from diabetes mediated damage and significantly (p < 0.05) enhanced spine density, which may have contributed in behavior improvement after drug treatment (Figure 5.15F).
Figure 5.15 B-F: Effect of diabetes and quercetin treatment on neuronal integrity in the CA3 region of the hippocampus (Sholl analysis). Number of primary branches (Np) (B), critical value (Rc) (C), maximum number of intersection (Nm) (D), Schoenen ramification index (Nm/Np) (E) and dendritic spine density (F). Results are depicted as mean ± SD (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test) and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus STZ.
5.2.9. Effect of diabetes and quercetin treatment on hippocampal insulin signaling and neurogenesis (Immunoblot)

The effect of diabetes and quercetin treatment on hippocampal insulin signaling was evaluated in terms of hippocampal expression of insulin, InR, and GLUT4 proteins through SDS-PAGE-immunoblot analysis and the results are depicted in Figure 5.16A. Two way ANOVA of the hippocampal insulin expression levels demonstrated a significant diabetes/treatment interaction \[F (2,18) = 10.88, p < 0.001 \text{ and } \eta^2 = 0.41\]. Hippocampal expression levels of insulin were observed to be significantly \(p < 0.001\) lower than CTRL, which may be the result of pancreatic destruction caused by STZ injection. Treating diabetic animals with quercetin and rosiglitazone significantly \(p < 0.05\) upregulated hippocampal insulin expression, when compared to STZ. Interestingly expression of insulin was observed to be nonsignificantly lower in normal animals treated with quercetin and rosiglitazone, when compared to CTRL Figure 5.16B.

Post hoc analysis of the hippocampal expression of InR demonstrated a significant between diabetes/drug treatment interaction \[F (2,18) = 16.68, p < 0.001 \text{ and } \eta^2 = 0.29\]. Our results demonstrated a significant reduction \(p < 0.001\) in InR expression in the hippocampus of diabetic animals. Treating diabetic animals with quercetin and rosiglitazone improved InR expression and its levels were significantly \(p < 0.05\) higher than STZ. Interestingly, we observed a significant reduction in the InR expression in normal animals treated with quercetin \(p < 0.01\) and rosiglitazone \(p < 0.05\), when compared to CTRL Figure 5.16C.

We further evaluated the expression of GLUT4 in the hippocampus and the results of two way ANOVA demonstrated a nonsignificant diabetes/drug treatment interaction \[F (2,18) = 1.49, p > 0.05 \text{ and } \eta^2 = 0.01\]. Post hoc analysis revealed that subjecting animals to long standing diabetes significantly \(p < 0.001\) reduced hippocampal expression of GLUT4, which was efficiently upregulated by quercetin \(p < 0.001\) and rosiglitazone \(p < 0.01\) treatment. Interestingly, we observed a significant increase in the GLUT4 expression in the hippocampus of normal animals treated with quercetin \(p < 0.01\) and rosiglitazone \(P 0.05\), when compared to CTRL. Keeping in view the expression levels of insulin, InR and GLUT4 in the hippocampus, our results suggest that quercetin is having potential to upregulate insulin signaling in normal as well as diabetic animals Figure 5.16D.
Figure 5.16: Effect of diabetes and quercetin treatment on the expression of GAPDH, insulin, InR, GLUT4 and DCX in the hippocampus (A). Relative expression of insulin (B), InR (C), GLUT4 (D) and DCX (E). Results are depicted as mean ± SD (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001. #p < 0.05; ##p < 0.01; ###p < 0.001 versus STZ.
We evaluated the effect of diabetes and drug treatments on hippocampal neurogenesis by evaluating the expression of DCX and our results demonstrated a significant diabetes/treatment interaction \( [F (2,18) = 10.56, p < 0.001 \text{ and } \eta^2 = 0.09] \). Our results demonstrated a significantly (\( p < 0.001 \)) reduced hippocampal neurogenesis, as indicated by the DCX expression levels. Quercetin treatment showed market neurogenesis promoting activity and the expression of DCX was observed to be significantly higher in STZ + Q (\( p < 0.001 \)) and CTRL + Q (\( p < 0.001 \)), when compared with STZ and CTRL respectively. Further, rosiglitazone did not showed promising effect on neurogenesis and levels of DCX were significantly (\( p < 0.05 \)) higher in STZ + ROSI (compared to STZ) and were significantly (\( p < 0.05 \)) lower in CTRL + ROSI (compared to CTRL) Figure 5.16E.

5.2.10. Immunofluorescence

Effect of quercetin treatment on neuronal insulin signaling was evaluated in terms of the InR and GLUT4 expression in the hippocampal CA3 region, as this region showed maximum diabetes mediated damage in Golgi-cox studies. Our results did not demonstrated any significant diabetes/treatment interaction \( [F (2,18) = 2.66, p > 0.05 \text{ and } \eta^2 = 0.063] \) for neuronal InR expression in the CA3 region. Further we did not observed any significant diabetes/treatment interaction for GLUT4 expression \( [F (2,18) = 0.87, p > 0.05 \text{ and } \eta^2 = 0.01] \) and GLUT4:InR \( [F (2,18) = 2.29, p > 0.05 \text{ and } \eta^2 = 0.003] \). Post hoc analysis revealed that diabetic animals expressed significantly (\( p < 0.001 \)) higher InR levels in the CA3 region, when compared to CTRL. Treating diabetic animals with quercetin (\( p < 0.01 \)) and ROSI (\( p < 0.001 \)) significantly reduced neuronal expression levels of InR.

Results of GLUT4 expression demonstrated a significant (\( p < 0.001 \)) reduction in diabetic animals, when compared to CTRL. Treating diabetic animals with quercetin significantly (\( p < 0.001 \)) enhanced neuronal GLUT4 expression in diabetic animals, when compared to STZ. However, a we also observed a significant (\( p < 0.01 \)) increase in GLUT4 expression in normal animals treated with quercetin, when compared to CTRL. ROSI treated animals also demonstrated a significant (\( p < 0.05 \)) improvement in GLUT4 expression in diabetic animals, when compared to STZ.

Moreover, GLUT4:InR ratio was observed to be significantly (\( p < 0.001 \)) increased in normal animals treated with quercetin and significantly (\( p < 0.001 \)) reduced in STZ group, when compared to CTRL. Treating diabetic animals with quercetin and ROSI resulted in a significant (\( p < 0.05 \))
increase in GLUT4:InR. These results suggest the development of IR like state in the neurons of diabetic animals, as the levels of GLUT4 failed to increase despite having significantly higher InR levels. Quercetin treatment improved GLUT4 expression, and thereby attenuated neuronal IR like state, in normal as well as diabetic animals (Figure 5.17A-D).

**Figure 5.17:** Effect of diabetes and quercetin treatment on the expression of insulin receptor (InR) and glucose transporter 4 (GLUT4) in the CA3 region of the hippocampus. Immunofluorescence images depict CA3 region of the hippocampus at 400 X magnification. Immunofluorescence detection of DAPI (blue), FITC (green), and TRITC (red) in the CA3 region of the hippocampus represents nucleus, InR, and GLUT4 respectively (A). Graph showing expression of InR (B), GLUT4 (C) and GLUT4 : InR ratio (D). Results are depicted as mean ± SD (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001; ##p < 0.01; ###p < 0.001 versus STZ.
5.3. Chronic Unpredicted Stress (CUS) study

5.3.1. Effect of quercetin on changes in body weight, feed intake and water consumption during CUS

Body weight, feed intake and water consumption were measured regularly during the study. Results are depicted below in Figure 5.18. Subjecting animals to chronic stress revealed a significant CUS/quercetin interaction \([F (1,20) = 31.49, p < 0.001 \text{ and } \eta^2 = 0.19]\), CUS main effect \([F (1,20) = 89.62, p < 0.001, \eta^2 = 0.51 \text{ and } d = 6.5]\) and quercetin \([F (1,20) = 35.05, p < 0.001, \eta^2 = 0.2 \text{ and } d = 3.56]\) for the percent change in the body weight. Body weight in the CUS group dropped consistently throughout the study, however, a significant improvement was observed in quercetin treated animals, especially in during 14-21 days of the study.

![Figure 5.18: Effect of CUS and quercetin on percent change in body weight (A), feed intake per gram body weight (B) and water intake (C). Results are depicted as mean ± SD (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS.](image)
We observed insignificant interaction between CUS and quercetin treatment \([F (1,20) = 3.49, p > 0.05 \text{ and } \eta^2 = 0.06]\). However, a significant CUS main effect \([F (1,20) = 22.41, p < 0.001, \eta^2 = 0.36 \text{ and } d = 2.73]\) and quercetin \([F (1,20) = 15.52, p < 0.001, \eta^2 = 0.25 \text{ and } d = 2.44]\) was observed when data was analyzed for feed intake (calculated as per gram body weight). Subjecting animals to chronic stress resulted in reduced feed intake which was improved after quercetin treatment.

Further, results of the water intake demonstrated a non-significant interaction between CUS and quercetin \([F (1,8) = 1.09, p > 0.05 \text{ and } \eta^2 = 0.07]\) and quercetin main effect \([F (1,8) = 0.83, p > 0.05, \eta^2 = 0.05 \text{ and } d = 2.62]\), however, a significant CUS main effect \([F (1,8) = 5.38, p < 0.05, \eta^2 = 0.35 \text{ and } d = 2.03]\) was observed.

### 5.3.2. Effect of quercetin treatment on locomotion and muscle coordination during CUS

#### 5.3.2.1. Actophotometer

Effect of CUS and quercetin treatment on locomotor activity in Swiss albino mice was determined as the no. of line crossings in Actophotometer apparatus. Two way ANOVA suggested a significant interaction between CUS and quercetin \([F (1,20) = 22.15, p < 0.001 \text{ and } \eta^2 = 0.34]\), CUS main effect \([F (1,20) = 15.93, p < 0.001, \eta^2 = 0.25 \text{ and } d = 4.58]\) and main effect of quercetin treatment \([F (1,20) = 6.60, p < 0.05, \eta^2 = 0.10 \text{ and } d = 4.27]\). These results suggest that chronic stress induces hypo-locomotion in mice and quercetin treatment efficiently improved locomotor activity in stressed mice, as indicated by the significantly higher number of line crossings in the Actophotometer (Figure 5.19A).

#### 5.3.2.2. Open field test (OFT)

Locomotor activity in mice was further evaluated through OFT in terms of number of line crossings. Our results depicted a significant chronic stress/quercetin interaction \([F (1,20) = 9.59, p < 0.01 \text{ and } \eta^2 = 0.16]\). A significant main effect of quercetin treatment \([F (1,20) = 8.79, p < 0.01, \eta^2 = 0.15 \text{ and } d = 3.43]\) and CUS \([F (1,20) = 20.64, p < 0.001, \eta^2 = 0.35 \text{ and } d = 6.5]\) was also observed. Post hoc comparison of the data revealed that chronic stress markedly impaired locomotion in mice and quercetin treatment efficient improved it (Figure 5.19B).
5.3.2.3. Rota-rod test

We used Rota-rod apparatus to evaluate the muscle coordination and strength in animals subjected to 21 day chronic stress and quercetin treatment. Post hoc comparison of the data revealed a nonsignificant interaction between CUS and quercetin \[F (1,20) = 0.27, \ p > 0.05 \text{ and } \eta^2 = 0.01\], main effect of 21 day chronic stress \[F (1,20) = 0.18, \ p > 0.05, \eta^2 = 0.008 \text{ and } \ d = 0.48\] and quercetin treatment \[F (1,20) = 0.53, \ p > 0.05, \eta^2 = 0.03 \text{ and } \ d = 0.08\]. These findings suggest that CUS has no significant effect on the muscle coordination and strength, and thereby, reduced locomotion cannot be due to the muscle function impairments, rather may have resulted from the abnormal neuronal functioning during CUS Figure 5.19C).

![Graphs of Actophotometer, Open Field Test, and Rota-rod Test](image)

**Figure 5.19:** Effect of CUS and quercetin on locomotion and muscle coordination. Number of line crossings in Actophotometer (A), number of line crossings in OFT (B) and time take to fall from Rota-rod (C). Results are depicted as mean ± SD (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS.
5.3.3. Effect of quercetin treatment on anxiety behavior in stressed animals during CUS

5.3.3.1. Elevated Plus Maze (EPM)

We evaluated anxiety behavior in stressed animals through EPM test and investigated the effect of quercetin treatment on CUS mediated anxiety. Subjecting data through two way ANOVA analysis demonstrated a significant interaction between chronic stress and quercetin treatment \[ F (1,20) = 26.93, p < 0.001 \] and \[ \eta^2 = 0.29 \], main effect of 21-day CUS \[ F (1,20) = 38.16, p < 0.001, \eta^2 = 0.41 \text{ and } d = 4.05 \] and quercetin treatment \[ F (1,20) = 8.03, p < 0.05, \eta^2 = 0.08 \text{ and } d = 3.99 \]. Chronic stress induced a significant levels of anxiety in Swiss albino mice and 21 day quercetin treatment efficiently alleviated CUS mediated anxiety behavior, as quercetin treated animals were found to spent more time in exploring the open arm of EPM than stressed animals (Figure 5.20A).

![Figure 5.20: Effect of CUS and quercetin on anxiety behavior in Swiss albino mice. Total time spent exploring the open arm of EPM (A), number of entries to the central region of the OFT (B) and total time spent in the central region of the OFT (C). Results are depicted as mean ± SD (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS.](image-url)
5.3.3.2. Open Field Test (OFT)

We used OFT to investigate the effect of quercetin treatment and chronic stress on anxiety behavior in mice in terms of number of line crossings and total number of entries to the central region of the apparatus. Our results demonstrated a significant interaction between chronic stress and quercetin interaction \[F(1,20) = 10.06, p < 0.01 \text{ and } \eta^2 = 0.21\], 21-day CUS main effect \[F(1,20) = 14.06, p < 0.01 \text{ and } \eta^2 = 0.29 \text{ and } d = 2.34\], and quercetin main effect \[F(1,20) = 4.89, p < 0.05 \text{ and } \eta^2 = 0.1 \text{ and } d = 1.98\] for number of center entries and a significant chronic stress/quercetin interaction \[F(1,20) = 28.80, p < 0.001 \text{ and } \eta^2 = 0.42\], 21-day CUS main effect \[F(1,20) = 7.28, p < 0.05 \text{ and } \eta^2 = 0.1 \text{ and } d = 4.62\], and quercetin main effect \[F(1,20) = 12.20, p < 0.01 \text{ and } \eta^2 = 0.18 \text{ and } d = 3.11\] for total time spent in center. Post hoc analysis of the results revealed that chronic stress is associated with significant levels of anxiety in mice, as evident from the significantly lower number of entries to the central region of apparatus and significantly lesser time spent in this region. Further, 21 day quercetin treatment attenuated CUS mediated anxiety and animals were observed to freely explore the OFT arena (Figure 5.20B-C).

5.3.4. Effect of quercetin treatment on depressive like behavior in stressed animals during CUS

5.3.4.1. Sucrose Preference Test (SPT)

CUS mediated anhedonia in mice and the effect of quercetin treatment on it was evaluated through SPT. We observed a significant interaction between CUS and quercetin \[F(1,8) = 18.75, p < 0.01 \text{ and } \eta^2 = 0.36\], 21-day CUS main effect \[F(1,8) = 16.93, p < 0.01 \text{ and } \eta^2 = 0.32 \text{ and } d = 4.42\] and the main effect of quercetin \[F(1,8) = 8.37, p < 0.05 \text{ and } \eta^2 = 0.16 \text{ and } d = 4.19\], when tested for preferential sucrose consumption. 21 day chronic stress induce a significant depressive like behavior in mice and 21 day quercetin treatment efficiently alleviated CUS mediated depression (Figure 5.21A).

5.3.4.2. Tail Suspension Test (TST)

CUS mediated depression in mice was further evaluated through TST in terms of total immobility duration and frequency of upward turnings and the effect of quercetin treatment was investigated. Our results depicts a significant interaction between chronic stress and quercetin interaction \[F(1,20) = 4.95, p < 0.05 \text{ and } \eta^2 = 0.08\], 21-day CUS main effect \[F(1,20) = 4.46, p < 0.05 \text{ and } \eta^2 = 0.08\], and quercetin main effect \[F(1,20) = 4.20, p < 0.05 \text{ and } \eta^2 = 0.18 \text{ and } d = 3.11\], and 21-day CUS main effect \[F(1,20) = 7.28, p < 0.05 \text{ and } \eta^2 = 0.1 \text{ and } d = 4.62\], and quercetin main effect \[F(1,20) = 12.20, p < 0.01 \text{ and } \eta^2 = 0.18 \text{ and } d = 3.11\] for total time spent in center. Post hoc analysis of the results revealed that chronic stress is associated with significant levels of anxiety in mice, as evident from the significantly lower number of entries to the central region of apparatus and significantly lesser time spent in this region. Further, 21 day quercetin treatment attenuated CUS mediated anxiety and animals were observed to freely explore the OFT arena (Figure 5.20B-C).
0.07 and \(d = 2.91\) and main effect of quercetin treatment \([F (1,20) = 33.90, p < 0.001, \eta^2 = 0.54\) and \(d = 3.06\) for total immobility time. Further, results for frequency of upward tuning in TST revealed an insignificant interaction between CUS and quercetin \([F (1,20) = 3.65, p > 0.05\) and \(\eta^2 = 0.09\] however, a significant CUS main effect \([F (1,20) = 5.25, p < 0.05, \eta^2 = 0.13\) and \(d = 3.00\] and main effect of quercetin treatment \([F (1,20) = 10.13, p < 0.01, \eta^2 = 0.26\) and \(d = 2.63)\] was observed. Post-hoc analysis suggest that quercetin treatment efficiently alleviated CUS mediated depression, as indicated by the longer duration spent struggling to get out of adverse condition by quercetin treated animals (Figure 5.21B-C).

**Figure 5.21:** Effect of CUS and quercetin treatment on depressive like behavior in Swiss albino mice. Percent sucrose preference in SPT (A), total immobility time in TST (B), frequency of upward turnings (C) and total immobility time in FST (D). Results are depicted as mean ± SD (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *\(p < 0.05\); **\(p < 0.01\); ***\(p < 0.001\) versus control group. \(*p < 0.05\); \(##p < 0.01\); \(###p < 0.001\) versus CUS.
5.3.4.3. Forced Swim Test (FST)
CUS mediated depression in mice was evaluated through FST in terms of total immobility duration and the effect of quercetin treatment was investigated. Our results depicted a significant interaction between CUS and quercetin \([F (1,20) = 14.82, p < 0.001 \text{ and } \eta^2 = 0.14]\), 21-day CUS main effect \([F (1,20) = 58.87, p < 0.001, \eta^2 = 0.55 \text{ and } d = 4.43]\) and main effect of quercetin treatment \([F (1,20) = 13.75, p < 0.01, \eta^2 = 0.13 \text{ and } d = 2.83]\). Subjecting our results to post-hoc analysis suggested that chronic stress is associated with significant levels of depression in mice. Results of FST confirms the observations made in SPT and TST. Here also, quercetin treated animals kept struggling to get out of adverse condition for significantly longer duration and therefore, quercetin treatment efficient alleviated CUS mediated depressive like behavior in Swiss albino mice (Figure 5.21D).

5.3.5. Effect of quercetin treatment on learning and memory in stressed animals during CUS
5.3.5.1. Novel Object Recognition Test (NOR)
Effect of chronic stress and quercetin treatment on recognition memory was evaluated through NOR. Post hoc analysis of the recognition memory performance demonstrated a significant interaction between CUS and quercetin interaction \([F (1,20) = 6.88, p < 0.05, \text{ and } \eta^2 = 0.08]\), 21-dya CUS main effect \([F (1,20) = 35.71, p < 0.001, \eta^2 = 0.41, \text{ and } d = 2.90]\) and main effect of quercetin \([F (1,20) = 24.97, p < 0.001, \eta^2 = 0.28, \text{ and } d = 2.59]\). Stressed animals were not able to discriminate the novel object from the familiar one, thereby indicating the development of impaired recognition memory in these animals. Treating stressed animals with quercetin for 21-day showed marked improvement in memory performance, as indicated by the significantly higher discrimination index in quercetin treated animals (Figure 5.22A).

5.3.5.2. Morris Water Maze (MWM)
Effect of quercetin treatment on learning and memory performance during CUS was evaluated through MWM. A two-way ANOVA of learning ability in Morris Water Maze task revealed a nonsignificant interaction between CUS and quercetin \([F (1,20) = 0.03, p > 0.05, \eta^2 = 0.001]\), 21-day CUS main effect \([F (1,20) = 0.13, p > 0.05, \eta^2 = 0.005, \text{ and } d = 0.38]\), and the main effect of
quercetin treatment \[F (1,20) = 2.45, p > 0.05, \eta^2 = 0.11, \text{ and } d = 0.65\] on the first day of the learning trial.

We observed a significant interaction between 21-day chronic stress and quercetin treatment \[F (1,20) = 10.66, p < 0.01, \eta^2 = 0.22\], CUS main effect \[F (1,20) = 12.59, p < 0.01, \eta^2 = 0.26, \text{ and } d = 5.46\] and the main effect of quercetin treatment \[F (1,20) = 4.35, p < 0.05, \eta^2 = 0.09, \text{ and } d = 2.66\] on second day of learning trial. Similarly, two way ANOVA of 3rd day learning trial revealed a significant interaction between CUS and quercetin \[F (1,20) = 36.58, p < 0.001, \eta^2 = 0.36\], 21-day CUS main effect \[F (1,20) = 35.88, p < 0.001, \eta^2 = 0.35, \text{ and } d = 5.44\] and the main effect of quercetin treatment \[F (1,20) = 9.47, p < 0.01, \eta^2 = 0.09, \text{ and } d = 3.79\].

Two way ANOVA of learning trial on fourth day demonstrated a significant chronic stress/quercetin interaction \[F (1,20) = 41.93, p < 0.001, \eta^2 = 0.32\], 21-day CUS main effect \[F (1,20) = 33.35, p < 0.001, \eta^2 = 0.26, \text{ and } d = 4.09\] and the main effect of quercetin treatment \[F (1,20) = 32.99, p < 0.001, \eta^2 = 0.26, \text{ and } d = 4.00\]. Post hoc analysis of the learning ability in animals suggested a nonsignificant difference in the platform finding latencies of the animals belonging to different treatment groups on day 1. Further, we observed that escape latency of every animal decreased from day one to day four as a result of learning trials, however, escape latencies of stressed animals were observed to be significantly higher than CTRL animals. 21 day quercetin treatment improved learning in stressed animals and their escape latency was observed to be significantly lower than CUS animals on 2nd, 3rd, and 4th day of the trial period (Figure 5.22B).

Effect of CUS and quercetin treatment on memory consolidation and retrieval was performed after 24 h of the last learning trial. During probe trial, memory index was evaluated as no. of platform crossings and amount time spent exploring the target quadrant. Post-hoc analysis revealed a significant interaction between CUS and quercetin interaction \[F (1,20) = 8.19, p < 0.01, \eta^2 = 0.02\], a main effect of quercetin treatment \[F (1,20) = 13.02, p < 0.01, \eta^2 = 0.29, \text{ and } d = 3.04\] for total no. of platform crossings. Further, a nonsignificant main effect of chronic stress \[F (1,20) = 2.62, p < 0.05, \eta^2 = 0.06, \text{ and } d = 1.83\] was also observed.

Two-way ANOVA of the total time spent exploring platform quadrant in MWM test demonstrated a significant interaction between CUS and quercetin treatment \[F (1,20) = 17.97, p < 0.01, \eta^2 = 0.28\]. We also observed a significant main effect of 21-day chronic stress \[F (1,20) = 5.93, p <
Our results suggest that CUS is associated with marked impairment in memory consolidation and retrieval as suggested by the results of memory index. Stressed animals showed significantly lower platform crossings and spent significantly less amount of time inside platform quadrant, indicating the development of memory dysfunction. 21 day treatment with quercetin resulted in a significant improvement in the memory performance. Quercetin treatment improved memory functions in stressed animals and these animals not only spent greater time in the platform quadrant, but also showed higher platform crossings. (Figure 5.22C-D).

**Figure 5.22:** Effect of quercetin treatment on CUS mediated memory dysfunction in Swiss albino mice. Discrimination index in NOR (A), latency to find the hidden platform in MWM (B), number of platform crossings MWM (C) and total time spent in the platform quadrant MWM (D). Results are depicted as mean ± SD (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS.
5.3.5.3. Passive Avoidance Step Through (PAST)

Effect of CUS and quercetin treatment on short-term and long-term associative memory performance was evaluated through PAST in Swiss albino mice animals. A two-way ANOVA for the transfer latencies on day 1 of the test did not reveal any significant difference. Short-term memory was evaluated 24 h after the learning trial and a two-way ANOVA of transfer latencies revealed significant interactions between CUS and quercetin treatment [F (1,20) = 8.07, p < 0.001 and η² = 0.24], 21-day CUS main effect [F (1,20) = 10.19, p < 0.001, η² = 0.29 and d = 4.45] and the main effect of quercetin treatment [F (1,20) = 10.44, p < 0.001, η² = 0.3 and d = 3.85]. Further, two way ANOVA of the transfer latencies on day 5 (long-term memory retention) of this study also demonstrated a significant chronic stress/quercetin interaction [F (1,20) = 33.57, p < 0.001 and η² = 0.21], 21-day CUS main effect [F (1,20) = 57.00, p < 0.001, η² = 0.35 and d = 6.87] and the main effect of quercetin treatment [F (1,20) = 52.42, p < 0.001, η² = 0.32 and d = 6.63]. Our results suggest that subjecting animals to 21 day unpredictable stress impaired short-term and long-term memory performance in mice. Treating stressed animals with quercetin for 21 day produced beneficial effect on memory and the animals were observed to remember the foot shock they received in the dark chamber, and thereby showed significantly greater transfer latency (Figure 5.23A).

5.3.5.4. Passive Avoidance Step Down (PASD)

We evaluated the effect of CUS and quercetin treatment on the short-term and long-term memory through PASD task in Swiss albino mice. Two way ANOVA of the transfer latencies during learning trial (day 1) did not revealed any significant interaction between CUS and quercetin [F (1,20) = 0.41, p > 0.05 and η² = 0.02]. We also observed a significant main effect of 21-dya CUS [F (1,20) = 0.38, p > 0.05, η² = 0.02 and d = 0.009] and quercetin treatment [F (1,20) = 1.15, p > 0.05, η² = 0.05 and d = 0.54]. Animals of all the groups quickly stepped-down from the wooden platform and were given an inescapable foot shock.

Short-term memory retention was evaluated 24 h after the learning trial and our results revealed a significant interaction between CUS and quercetin [F (1,20) = 28.80, p < 0.001 and η² = 0.24]. Further, a significant main effect of chronic stress [F (1,20) = 45.90, p < 0.001, η² = 0.38 and d = 6.36] and quercetin treatment [F (1,20) = 26.50, p < 0.001, η² = 0.22 and d = 3.05] was also
observed. Our results suggest that stressed animals showed impaired memory of the foot shock they received on day 1, however, quercetin and ROSI treated animals demonstrated good memory.

**A: Passive Avoidance Step Through**

![A: Passive Avoidance Step Through](image)

**B: Passive Avoidance Step Down**

![B: Passive Avoidance Step Down](image)

**Figure 5.23:** Effect of quercetin treatment on CUS mediated associative-memory dysfunction in Swiss albino mice. Time take to enter the dark chamber/step-through in PAST (A) and time taken to step down from the wooden platform in PASD (B). Results are depicted as mean ± SD (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. 

α/*p < 0.05; αα/**p < 0.01; ααα/***p < 0.001 versus control group. β/#p < 0.05; ββ/##p < 0.01; βββ/###p < 0.001 versus CUS.

Effect of CUS and quercetin treatment on long-term memory performance in mice was evaluated on the day 5 of the PASD test. Two way ANOVA of the transfer latencies demonstrated a significant chronic stress/quercetin interaction [F (1,20) = 116.20, p < 0.001 and η² = 0.31]. We observed a significant main effect of 21-day chronic stress [F (1,20) = 74.38, p < 0.001, η² = 0.20 and d = 9.38] and quercetin treatment [F (1,20) = 168.42, p < 0.001, η² = 0.44 and d = 22.80]. Post
hoc analysis suggest that subjecting animals to 21 day unpredicted stress impaired short-term and long-term memory performance in mice, as indicated by the significantly lower transfer latencies of stressed animals when compared to CTRL. Treating stressed animals with quercetin for 21 day produced beneficial effect on memory and the animals were observed to remember the foot shock they received on day 1, and thereby showed significantly greater step-down latency (Figure 5.23B).

5.3.6. Oral Glucose Tolerance Test (OGTT)

The results of OGTT are depicted below in Figure 5.24. A two way ANOVA demonstrated a significant interaction between chronic stress and quercetin treatment at 0 min \([F (1,20) = 7.91, p < 0.05 \text{ and } \eta^2 = 0.18]\). We also observed a significant main effect of 21-day chronic stress \([F (1,20) = 7.23, p < 0.05, \eta^2 = 0.16 \text{ and } d = 4.32]\) and quercetin treatment \([F (1,20) = 9.36, p < 0.01, \eta^2 = 0.21 \text{ and } d = 7.80]\).

At 30 min of the OGTT we did not observed any significant interaction between CUS and quercetin treatment \([F (1,20) = 0.69, p > 0.05 \text{ and } \eta^2 = 0.004]\). However, a significant main effect of 21-day chronic stress \([F (1,20) = 96.58, p < 0.001, \eta^2 = 0.56 \text{ and } d = 5.18]\) and quercetin treatment \([F (1,20) = 55.03, p < 0.001, \eta^2 = 0.32 \text{ and } d = 11.21]\) was observed.

Two way analysis of OGTT at 60 min time demonstrated nonsignificant interaction between CUS and quercetin treatment \([F (1,20) = 0.107, p > 0.05 \text{ and } \eta^2 < 0.001]\). We further observed a significant main effect of 21-day CUS \([F (1,20) = 75.48, p < 0.001, \eta^2 = 0.65 \text{ and } d = 7.28]\) and quercetin treatment \([F (1,20) = 20.13, p < 0.001, \eta^2 = 0.17 \text{ and } d = 5.32]\).

At 90 min of the OGTT, a nonsignificant interaction between CUS and quercetin treatment \([F (1,20) = 0.01, p > 0.05 \text{ and } \eta^2 > 0.001]\) was observed. Further, our results suggest a significant 21-day CUS main effect \([F (1,20) = 59.23, p < 0.001, \eta^2 = 0.69 \text{ and } d = 3.91]\) and quercetin treatment \([F (1,20) = 6.26, p < 0.05, \eta^2 = 0.07 \text{ and } d = 6.51]\).

Two way analysis of the OGTT at 120 min revealed a significant interaction between CUS and quercetin treatment \([F (1,20) = 20.49, p < 0.001 \text{ and } \eta^2 = 0.18]\). Further, a significant main effect of 21-day CUS \([F (1,20) = 47.96, p < 0.001, \eta^2 = 0.43 \text{ and } d = 9.38]\) and quercetin treatment \([F (1,20) = 20.94, p < 0.001, \eta^2 = 0.19 \text{ and } d = 7.33]\) was also observed at 120 min of OGTT. Post hoc analysis revealed that 21 day chronic stress induced severe glucose intolerance in the animals. Blood glucose levels in the stressed animals were found to be significantly high throughout the study, when compared to CTRL. Further, treating stressed animals with quercetin for 21 days
alleviated CUS mediated glucose impairment, as indicated by the significantly lower glucose levels in OGTT study when compared to CUS. Interestingly, levels of the blood glucose in quercetin treated normal animals were also found to be lower in OGTT at different time interval, when compared to CUS or CTRL, thereby suggesting that quercetin may have direct role in controlling rise in blood glucose.

**Figure 5.24:** Effect of quercetin treatment on CUS mediated glucose intolerance in Swiss albino mice. Graph represents the blood glucose levels measured at 0, 30, 60, 90 and 120 min after administering 2 g/kg glucose to the animals during OGTT. Results are depicted as mean ± SD (n = 6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS.

### 5.3.7. Effect of CUS and 21 day quercetin treatment on biochemical parameters

#### 5.3.7.1. Fasting Blood Glucose (FBG) level

FBG was measured by the commercially available kit and the results are depicted in Figure 5.25A. Subjecting FBG levels to two-way ANOVA demonstrated a significant interaction between chronic stress and quercetin treatment [F (1,20) = 10.84, p < 0.01, and η² = 0.37]. We observed a significant main effect of 21-day CUS [F (1,20) = 220.12, p < 0.001, η² = 0.74, and d = 7.16] and quercetin treatment [F (1,20) = 45.25, p < 0.001, η² = 0.15, and d = 4.86]. Our results revealed that chronic stress is associated with a significantly increase in the fasting glucose levels, when
compared to CTRL, which suggest the development of diabetes-like state in these animals and these animals could be considered as pre-diabetic. 21 day quercetin treatment efficiently lowered the blood glucose levels in stressed animals.

5.3.7.2. Serum insulin level

Results of the serum insulin levels are depicted in Figure 5.25B. Post-hoc analysis of the insulin levels in serum demonstrated a significant interaction between CUS and quercetin \[ F (1,12) = 8.99, p < 0.05, \text{ and } \eta^2 = 0.73 \]. Further, a significant main effect of chronic stress \[ F (1,12) = 130.12, p < 0.001, \eta^2 = 0.73, \text{ and } d = 6.60 \], and quercetin treatment \[ F (1,12) = 27.99, p < 0.001, \eta^2 = 0.16, \text{ and } d = 3.18 \] was also observed. These results suggested that chronic stress significantly upregulated the serum insulin levels and treating animals with quercetin resulted in a significant lowering in serum insulin levels, when compared to CUS.

5.3.7.3. HOMA-IR index

Development of IR in the chronically stressed animals was evaluated in terms of HOMA-IR index. Two-way ANOVA of HOMA-IR index demonstrated a significant interaction between CUS and quercetin \[ F (1,12) = 19.15, p < 0.001, \text{ and } \eta^2 = 0.08 \], moreover, a significant main effect of 21-day CUS \[ F (1,12) = 180.0, p < 0.001, \eta^2 = 0.72, \text{ and } d = 6.88 \] and 21-day quercetin treatment \[ F (1,12) = 39.57, p < 0.001, \eta^2 = 0.16, \text{ and } d = 4.00 \] was also observed. Post hoc comparison of the results of HOMA index suggest that CUS is associated with the development of significant IR, when compared to CTRL. Treating stressed animals with quercetin for 21 days significantly lowered the HOMA-IR index when compared to CUS, suggesting that quercetin can efficiently improve insulin sensitivity in stressed animals (Figure 5.25C).

5.3.7.4. Serum corticosterol level

Hypercortisolemia is the outcome of stress and we evaluated the effect of CUS and quercetin treatment on serum corticosterol levels. Results of the serum cortisol levels depicted a significant interaction between CUS and quercetin treatment \[ F (1,12) = 14.84, p < 0.01, \text{ and } \eta^2 = 0.08 \]. Further, we observed a significant main effect of chronic stress \[ F (1,12) = 144.91, p < 0.001, \eta^2 = 0.79, \text{ and } d = 8.08 \], and 21-day quercetin treatment \[ F (1,12) = 12.20, p < 0.01, \eta^2 = 0.06, \text{ and } d = 3.11 \]. Post hoc analysis revealed that chronic stress significantly elevated serum corticosterol
levels when, compared to CTRL. Quercetin efficiently lowered the stress levels and the serum corticosterol levels were found to be significantly lower than CUS (Figure 5.25D).

![Figure 5.25: Effect of CUS and quercetin treatment on fasting blood glucose levels (A), serum insulin levels (B), HOMA-IR index (C) and serum corticosterone levels (D). Results are depicted as mean ± SD (n = 4-6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS.]

### 5.3.7.5. Oxidative stress

Effect of CUS and quercetin treatment on the hippocampal oxidative stress was evaluated in terms of hippocampal TBARS, TTH, catalase and NO expression levels. Subjecting hippocampal TBARS levels to two way ANOVA analysis revealed a significant interaction between CUS and quercetin [F (1,20) = 12.97, p < 0.01 and $\eta^2 = 0.14$], 21-day CUS main effect [F (1,20) = 48.30, p
< 0.001, \eta^2 = 0.51 \text{ and } d = 4.45\] and the main effect of quercetin treatment \[F (1,20) = 14.36, p < 0.01, \eta^2 = 0.15 \text{ and } d = 2.5\] (Figure 5.26A).

Hippocampal TTH levels demonstrated a significant interaction between CUS and quercetin \[F (1,20) = 9.53, p < 0.01 \text{ and } \eta^2 = 0.08\]. Further, a significant main effect of chronic stress \[F (1,20) = 75.39, p < 0.001, \eta^2 = 0.62 \text{ and } d = 6.42\] and 21-day quercetin treatment \[F (1,20) = 17.01, p < 0.001, \eta^2 = 0.14 \text{ and } d = 2.81\] was also observed (Figure 5.26B).

Further, post-hoc analysis of the hippocampal catalase levels demonstrated a significant interaction between CUS and quercetin treatment \[F (1,20) = 10.51, p < 0.01, \eta^2 = 5.84\]. Further, a significant main effect of 21-day CUS \[F (1,20) = 97.14, p < 0.001, \eta^2 = 0.64 \text{ and } d = 7.99\] and 21-day quercetin treatment was also observed \[F (1,20) = 22.83, p < 0.001, \eta^2 = 2.96 \text{ and } d = 3.16\] (Figure 5.26C). Similarly, NO levels also revealed a significant interaction between CUS and quercetin \[F (1,20) = 10.34, p < 0.01 \text{ and } \eta^2 = 0.12\], besides, a significant 21-day CUS main effect \[F (1,20) = 49.96, p < 0.001, \eta^2 = 0.57 \text{ and } d = 4.66\] and the main effect of quercetin treatment was also observed \[F (1,20) = 6.89, p < 0.05, \eta^2 = 0.08 \text{ and } d = 2.16\] (Figure 5.26D). Post hoc analysis demonstrated that CUS inflicts marked neuronal oxidative stress in the hippocampus, as indicated by the significantly reduced hippocampal antioxidant levels (TTH and catalase) and significantly elevated oxidative stress markers (TBARS and NO). Treating stressed animals with quercetin for 21 days restored neuronal antioxidant levels and lowered the oxidative stress markers, thereby alleviated CUS mediated hippocampal oxidative stress.
5.3.8. Effect of CUS and quercetin treatment on hippocampal insulin signaling and neurogenesis (Immunoblot)

The effect of CUS and quercetin treatment on hippocampal insulin signaling was evaluated in terms of hippocampal expression of insulin, InR, and GLUT4 proteins through SDS-PAGE-immunoblot analysis and the results are depicted in Figure 5.27A. Two way ANOVA of the hippocampal insulin expression levels demonstrated a significant CUS/quercetin interaction \[ F(1,8) = 8.81, p < 0.05 \text{ and } \eta^2 = 0.21 \] and a main effect of quercetin \[ F(1,8) = 22.91, p < 0.01, \eta^2 = 0.56 \text{ and } d = 4.75 \]. These results suggest that 21-day chronic stress upregulated hippocampal insulin levels, and quercetin efficiently reduced insulin levels, when compared to CTRL (Figure 5.27B).

Further, results of hippocampal InR expression demonstrated a significant interaction between chronic stress and quercetin treatment \[ F(1,8) = 9.69, p < 0.05 \text{ and } \eta^2 = 0.22 \]. Further, we observed a significant main effect of 21-day CUS \[ F(1,8) = 5.36, p < 0.05, \eta^2 = 0.12 \text{ and } d = 2.83 \] and 21 day quercetin treatment \[ F(1,8) = 20.83, p < 0.01, \eta^2 = 0.47 \text{ and } d = 3.80 \]. These results indicate that chronic stress is associated with significantly elevated InR expression levels in the
hippocampus, when compared to CTRL. Treating stressed animals with quercetin for 21 days significantly reduced InR expression, when compared to CUS. Further, we also observed a non-significant lowering of hippocampal InR expression in normal animals treated with quercetin for 21 days, suggesting that quercetin is capable of lowering hippocampal InR expression in stressed as well as normal animals (Figure 5.27C).

Post-hoc analysis of the hippocampal GLUT4 expression also demonstrated a significant interaction between CUS and quercetin \( [F (1,8) = 6.26, p < 0.05 \text{ and } \eta^2 = 0.16] \). We observed a significant main effect of 21-day CUS \( [F (1,8) = 7.38, p < 0.05, \eta^2 = 0.19 \text{ and } d = 0.19] \) and quercetin treatment \( [F (1,8) = 18.13, p < 0.01, \eta^2 = 0.46 \text{ and } d = 6.44] \). Post hoc analysis demonstrated a significant reduction in hippocampal GLUT4 expression, when compared to CTRL. Further, treating stressed animals with quercetin for 21 day quercetin significantly elevated GLUT4 expression in the hippocampus, when compared to CUS. Moreover, a slight non-significant increase in the hippocampal GLUT4 was also observed in normal animals treated with quercetin (Figure 5.27D). These results suggest that quercetin treatment is capable of upregulating GLUT4 expression in the normal as well as stressed conditions.

Dentate gyrus region of the hippocampus is actively involved in the neurogenesis in adult brain. Hippocampal expression of DCX is a marker of neurogenesis and we evaluated the effect of CUS and quercetin treatment on hippocampal DCX expression through immunoblot analysis. Two way ANOVA of the hippocampal DCX expression revealed a significant interaction between CUS and quercetin treatment \( [F (1,8) = 11.50, p < 0.01 \text{ and } \eta^2 = 0.26] \). Further, our results depicts a significant main effect of chronic stress \( [F (1,8) = 7.84, p < 0.01, \eta^2 = 0.17 \text{ and } d = 3.69] \) and 21 day quercetin treatment \( [F (1,8) = 11.502, p < 0.01, \eta^2 = 0.38 \text{ and } d = 4.23] \). Post hoc analysis of DCX expression demonstrated that chronic stress is associated with a marked reduction in hippocampal DCX expression levels, which were observed to be significantly elevated after 21 day quercetin treatment. These results suggest that quercetin may have a beneficial role in promoting neurogenesis in the hippocampus during CUS (Figure 5.27E).
Figure 5.27: Effect of CUS and quercetin treatment on the expression levels of insulin, InR, GLUT4, DCX, and GAPDH in the hippocampus. Images of the immunoblot obtained after western-blot analysis of protein expression (A), relative expression of insulin (B), InR (C), GLUT4 (D) and DCX (E). Values are represented mean ± SD (n = 4). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS.
5.3.9. Effect of CUS and quercetin treatment on neuronal integrity in the hippocampus

5.3.9.1. Hematoxylin and eosin staining

Hematoxylin and eosin staining is one of the best method to evaluate morphological alterations in the cells. In our study we used this method to determine the effect of quercetin treatment on integrity of hippocampal neurons during CUS. Histopathological examination demonstrated that CUS inflicts severe neuronal damage in the CA2 and CA3 regions of hippocampus.

Figure 5.28: Effect of CUS and quercetin treatment on neuronal morphology. Values are represented mean ± SD (n = 4). Images depict hematoxylin and eosin-stained sections (5 μm) of the hippocampus and the effect of quercetin treatment on neuronal integrity in the CA1, CA2, and CA3 region of the hippocampus of stressed animals (damaged neurons are depicted by red arrows) (A), number of neuronal cells/mm$^2$ in the CA1 region of the hippocampus (B), number of neuronal cells/mm$^2$ in the CA2 region of the hippocampus (C), number of neuronal cells/mm$^2$ in CA3 region of the hippocampus (D). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS.
Treating stressed animals with quercetin for 21 days rescued neurons from CUS mediated damage and the neurons in quercetin treated animals were observed to resemble CTRL animals in morphology (Figure 5.28A). Two way ANOVA of the number of viable neurons in the CA1 region did not revealed any significant different amongst different treatment groups (Figure 5.28B). Our results demonstrated a nonsignificant interaction between CUS and quercetin \( [F (1,20) = 2.62, p > 0.05, \eta^2 = 0.08] \). Further, a significant main effect of 21-day CUS \( [F (1,20) = 6.47, p < 0.05, \eta^2 = 0.19, \text{and } d = 1.52] \) and quercetin treatment was observed \( [F (1,20) = 5.35, p < 0.05, \eta^2 = 0.15, \text{and } d = 1.53] \) when evaluated for number of viable neurons in the CA2 region of the hippocampus (Figure 5.28C). Two way ANOVA of the neuronal integrity in the CA3 region demonstrated a significant interaction between CUS and quercetin \( [F (1,20) = 4.58, p < 0.05, \eta^2 = 0.08] \). A significant main effect of chronic stress \( [F (1,20) = 24.06, p < 0.001, \eta^2 = 0.43, \text{and } d = 3.37] \) and quercetin treatment \( [F (1,20) = 6.69, p < 0.05, \eta^2 = 0.12, \text{and } d = 2.64] \) was also observed (Figure 5.28D). Post hoc analysis demonstrated that subjecting animals to 21 day chronic stress severely damages the neurons, especially in the CA3 region of the hippocampus and quercetin protected neurons from stress-mediated damage.

**5.3.9.2. Cresyl violet staining**

Cresyl violet staining is specifically used for accessing the integrity of neurons and we used this method to evaluate the neuroprotective effect quercetin on the CUS mediated alterations in neuronal morphology (Figure 5.29). We compared the morphology of neurons in the different regions of the hippocampus of CTRL and CUS groups and observed that CUS induces marked neuronal damage in the hippocampus. CA3 region was most venerable to stress mediated damage, followed by CA2 and CA1 region. Further, morphology of the neurons in the quercetin treated stressed and normal animals resembled with CTRL animals, suggesting that quercetin did not have any damaging effect of itself and efficiently rescue hippocampal neurons from the CUS mediated damage.

**5.3.10. Immunofluorescence**

**5.3.10.1. Expression of the InR and GLUT4 in the CA3 region of the hippocampus**

InR and GLUT4 expression in the hippocampal CA3 region was evaluated through immunofluorescence studies on 5 µm thick hippocampal sections and total FITC (InR) and TRITC (GLUT4) fluorescence was determined in acquired images using image-J software (Figure 5.30).
Figure 5.29: Effect of CUS and quercetin treatment on the integrity of hippocampal neurons. Images represent cresyl violet stained hippocampal CA1, CA2 and CA3 region at 400 X magnification and yellow arrows depicts the damaged neurons.

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**Hippocampus**

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Results of the InR expression in the CA3 region of the hippocampus demonstrated a significant interaction between CUS and quercetin \([F (1,12) = 289.29, p < 0.001, \text{and } \eta^2 = 0.65]\). We observed a significant main effect of 21 day unpredicted stress \([F (1,12) = 42.64, p < 0.001, \eta^2 = 0.23, \text{and } d = 6.34]\) and 21 day quercetin treatment \([F (1,12) = 101.69, p < 0.001, \eta^2 = 0.23, \text{and } d = 12.04]\). Likewise, GLUT4 expression in the CA3 region was having a significant chronic stress/quercetin treatment interaction \([F (1,12) = 98.44, p < 0.001, \text{and } \eta^2 = 0.27]\). Further, a significant main effect of 21-day CUS \([F (1,12) = 32.36, p < 0.001, \eta^2 = 0.09, \text{and } d = 6.93]\) and quercetin treatment \([F (1,12) = 216.77, p < 0.001, \eta^2 = 0.60, \text{and } d = 9.21]\) was also observed for GLUT4 expression.

Post hoc analysis of the obtained results revealed that subjecting animals to 21 day chronic stress resulted in a significant reduction in InR and GLUT4 expression in hippocampal CA3 region and treating the stressed animals with quercetin significantly upregulate the expression of these proteins. Interestingly, two way ANOVA further suggest that quercetin treatment in normal animals significantly lowers the expression of InR and elevates the expression of GLUT4 in the CA3 region, when compared to normal animals.

Since GLUT4 is the end product of InR activation, we calculated the ratio of the expression of GLUT4 to InR (GLUT4:InR) and subjected them to two way ANOVA analysis which did not reveal any significant interaction between CUS and quercetin \([F (1,12) = 0.07, p > 0.05, \text{and } \eta^2 = 0.0003]\) and the main effect of there week chronic stress \([F (1,12) = 3.08, p > 0.05, \eta^2 = 0.02, \text{and } d = 1.05]\), however, a significant main effect of quercetin \([F (1,12) = 179.43, p < 0.001, \eta^2 = 0.92, \text{and } d = 5.57]\) was observed. Post-hoc analysis of GLUT4:InR suggest a significant difference between quercetin treated animals (CTRL + Q and CUS + Q) and vehicle treated animals (CTRL and CUS).
**Figure 5.30 (A-C):** Effect of CUS and quercetin treatment on the expression of InR and GLUT4 in the entire hippocampal CA3 region. CA3 expression of InR (A), GLUT4 (B) and the ratio of GLUT4:InR expression (C). Values are represented as mean ± SD (n = 4) of total cell fluorescence calculated from ImageJ software. Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. &p < 0.05; &p < 0.01; &p < 0.001 versus CUS.

**Figure 5.30 (D):** Immunofluorescence images depict CA3 region of the hippocampus at 100 X magnification. Immunofluorescence detection of DAPI (blue), FITC (green), and TRITC (red) in the CA3 region of the hippocampus represents nucleus, InR, and GLUT4 respectively.
5.3.10.2. Effect of CUS and quercetin treatment on per cell InR and GLUT4 expression in the hippocampal neurons (Immunofluorescence)

Expression of InR and GLUT4 in the hippocampal CA3 neurons was calculated as total cell fluorescence (TCF) and the results are depicted in Figure 5.31(A–D). Two way ANOVA of the InR expression of CA3 neurons did not showed any significant interaction between CUS and quercetin \([F (1,16) = 0.85, p > 0.05, \text{ and } \eta^2 = 0.02]\). We observed a nonsignificant main effect of 21 day chronic stress \([F (1,16) = 1.75, p > 0.05, \eta^2 = 0.03, \text{ and } d = 0.13]\), and a significant main effect of 21-day quercetin treatment \([F (1,16) = 37.18, p < 0.001, \eta^2 = 0.67, \text{ and } d = 3.23]\). Two way ANOVA of the neuronal GLUT4 expression revealed a significant interaction between 21-day chronic stress and quercetin treatment \([F (1,16) = 13.26, p < 0.01, \text{ and } \eta^2 = 0.11]\) and quercetin treatment main effect \([F (1,16) = 86.02, p < 0.001, \eta^2 = 0.72, \text{ and } d = 3.92]\), besides, a non-significant main effect of chronic stress was also observed \([F (1,16) = 3.36, p > 0.05, \eta^2 = 0.03, \text{ and } d = 0.98]\). Post hoc analysis suggested a non-significant difference in the expression of InR between CTRL and CUS groups. Further, quercetin-treated groups showed significantly lower InR expression levels, when compared with CTRL and CUS animals. Compared to CTRL or CUS, we observed a significantly higher expression of GLUT4 in the CA3 neurons of quercetin treated animals. We further calculate the GLUT4:InR ratio and the results resembled that observed for the entire CA3 region expression levels. Two way ANOVA revealed a non-significant interaction between three weak chronic stress and quercetin treatment \([F (1,16) = 0.26, p > 0.05, \text{ and } \eta^2 = 0.002]\). Further, we observed an insignificant main effect of CUS \([F (1,16) = 0.009, p > 0.05, \eta^2 > 0.001, \text{ and } d = 1.09]\), and a significant main effect of quercetin treatment \([F (1,16) = 91.92, p < 0.001, \eta^2 = 0.85, \text{ and } d = 3.15]\). These results suggest that quercetin is capable of upregulating hippocampal GLUT4 expression in normal and stressed animals, independent of insulin and InR.
Figure 5.31 (A-C): Effect of CUS and quercetin treatment on the expression of InR and GLUT4 in hippocampal neurons. Expression of InR (A), GLUT4 (B) and the ratio of GLUT4:InR expression (C). Values are represented as mean ± SD (n = 4) of total cell fluorescence calculated from image-J software. Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus control group. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS.

(D) CA3 region of Hippocampus (400X magnification)

Figure 5.31 (D): Immunofluorescence images depict CA3 region of the hippocampus at 400 X magnification. Immunofluorescence detection of DAPI (blue), FITC (green), and TRITC (red) in the CA3 region of the hippocampus represents nucleus, InR, and GLUT4 respectively.
5.4. Mechanism of quercetin action

5.4.1. 2NBDG uptake assay

To confirm whether quercetin is acting by utilizing insulin signaling pathway or not, we performed 2NBDG uptake assay in the presence and absence of insulin signaling inhibitor (LY294002). Our results did not demonstrated any significant interaction between 21 day chronic stress and quercetin treatment [F (2,12) = 3.35, p > 0.05 and η² = 0.03]. Post hoc analysis revealed that quercetin (p < 0.05) and insulin (p < 0.01) treatment significantly enhanced 2NBDG uptake in L6 cells, when compared to CTRL. Treating cells with LY294002 significantly (p < 0.001) reduced 2NBDG uptake into the L6 cells. 2NBDG fluorescence did not varied significantly in LY294002 + quercetin and LY294002 + insulin treated wells, indicating that both quercetin and insulin failed to enhance 2NBDG uptake in these cells after LY294002 treatment. These results suggests that intact insulin signaling pathway is essential for the quercetin mediated glucose uptake Figure 5.32A-B).

Figure 5.32: 2NBDG uptake into L6 cells in the presence and absence of insulin signaling inhibitor (LY294002). Images show 2NBDG uptake in terms of green fluorescence (A) and graph represents calculated fluorescence intensity depicting 2NBDG uptake (B). Values in (B) are represented as mean ± SD (n = 4) of. Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test and two-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001 versus CTRL group. #p < 0.05; ##p < 0.01; ###p < 0.001 versus LY294002.
5.4.2. Predicting interaction site of quercetin on the insulin receptor (InR)

Docking study revealed that quercetin is having strong interaction with InR and we observed a receptor-ligand binding energy of -6.42 kcal/mol. Further, our results demonstrated that quercetin interacts with InR at a site which is entirely different from that utilized by insulin. Ligplot analysis revealed that quercetin form hydrogen bonds with Thr293, Lys1165, Gly1169, Glu314, Arg212, Leu1171, Arg1136, Asp1132 residues and hydrophobic interaction with Ala291, Leu5, Arg20, Glu294, Asn1215, Tyr312, Val1173, Lys1168, Ala315, Ser4, Ile6, Trp1175 residues of the InR protein. Moreover, insulin forms hydrogen bonds with Asp1132, Arg1136, Gly1169, Lys1165, Met11, Met13, Tyr10, Asp9, Gly8, Leu15, Ala14 residues and hydrophobic interaction with Gly20, Leu6, Ile2, Val12, Tyr26, Glu1004 residues of InR. Images of the ligplot analysis and ligand-receptor interactions are demonstrated in Figure 5.33.

Figure 5.33: Interaction of quercetin and insulin with insulin receptor (InR). Quercetin demonstrated 8 hydrogen bonds and 12 hydrophobic interactions with InR (A). Insulin demonstrated 11 hydrogen bonds and 6 hydrophobic interactions with InR (B).
CHAPTER 6

DISCUSSION
6. DISCUSSION

Molecular docking studies have emerged as a highly effective and reliable method for screening molecules for their potential to interact with target proteins, such as receptors, enzymes etc. Docking studies predicts the possible ligand-protein interactions and thereby gives an idea about the therapeutic potential drug candidates [270]. In our study, we screened 267 small molecules of natural origin and studied their docking interactions with crystalline structures of InR (1IR3), PPAR-γ (2PRG) and DPP-4 (4PNZ) proteins [271-273] to predict which of these molecules possesses potential anti-diabetic activity. Our results suggested that quercetin had good interaction with all three proteins targeted, therefore might be useful in managing diabetes and associated. Moreover, molecular docking predicted quercetin, ellagic acid, ascorbic acid, piperine, cinnamic acid, gallic acid and caffeine as an antidiabetic moieties and therefore these molecules were further subjected to in-vitro screening to refine and narrow down the screening process, where activities of these molecules were comparing with the standard marketed antidiabetic drugs, rosiglitazone, metformin and glimepiride.

Antioxidant potential of ascorbic acid, gallic acid, quercetin, caffeine, ellagic acid, piperine and cinnamic acid was evaluated through DPPH radical scavenging and lipid peroxidation inhibition assay and compared them with rosiglitazone, metformin and glimepiride (Table 5.2, 5.3). It is a simple, rapid, reproducible and highly sensitive method and is widely used for evaluating antioxidant potential of both natural as well as synthetic molecules [253]. DPPH exist as a free radical in methanol solution and gives a strong absorbance at 517 nm. DPPH is reduced to more stable form in the presence of antioxidants as per the equation given below, which results in the discoloration of DPPH solution. Stronger the antioxidant molecule, more DPPH will be reduced and greater discoloration will be observed. This discoloration is measured spectrophotometrically at 517 nm as a measure of antioxidant activity of the test molecules [253].

\[
\text{DPPH}^* \ (\text{colored}) + \text{antioxidant-H} \rightarrow \text{DPPH-H} (\text{discolored}) + \text{antioxidant}^* 
\]

Ascorbic acid, gallic acid, quercetin and ellagic acid were observed as a potent antioxidant molecules Table 5 and marketed drugs showed poor antioxidant activity.
In a biological system, oxidative stress is associated with the peroxidation of phospholipid cell membrane, which generates large number of highly reactive and unstable lipid hydroperoxide. These hydroperoxides degrades to malondialdehyde, which serves as an important biomarker and is associated with the destruction of biological membrane and cell [274]. In *in-vitro* testing, lipid peroxidation is measured spectrophotometrically at 532 nm by measuring TBARS generated as result of reaction between malondialdehyde and thiobarbituric acid at 95°C [254]. It is highly reliable, rapid and widely used method to screen molecules for their potential to inhibit lipid peroxidation. Hyperglycemia is associated with lipid peroxidation and excessive production of free radicals, which contributes in the development and progression of secondary complications associated with diabetes, such as central neuropathy [6]. Several reports suggest that, reduced oxidative stress in the biological system improves neurological functioning during cognitive dysfunction, depression, anxiety, memory impairments etc. [41, 95, 115, 217]. Herein we demonstrated that natural molecules, especially quercetin, possess good potential to scavenge free radicals and prevent lipid peroxidation, and thereby might prove to be beneficial in alleviating oxidative stress during diabetic condition. Considering the role of oxidative stress during neurological complications, failure of prescribed drugs to alleviate oxidative stress can be a possible reason for continuous progression of neurological complications during diabetes, despite of regular therapeutics. Therefore, supplementing currently used antidiabetic medications with antioxidants may aid in controlling oxidative stress and may slow down the rate of progression of diabetes associated neuropathy.

Hyperglycemia frequently leads to the development of genotoxicity in the biological system. Genotoxicity is known to augment diabetic complications, besides being a maleficent condition in itself [275, 276]. Therapeutic interventions which are capable of checking the development and progression of genotoxicity in the biological system can very well benefit in controlling diabetic complications. We evaluated the potential of test molecules against H$_2$O$_2$ induced genotoxicity through comet assay Figure 5.2. Our results we demonstrated that H$_2$O$_2$ induces severe genotoxicity in whole human blood. Quercetin, gallic acid, caffeine, piperine and cinnamic acid efficiently prevented H$_2$O$_2$ mediated DNA fragmentation and these molecules were observed to be most effective natural molecules. Here also marketed drugs failed to impart protection against H$_2$O$_2$ mediated genotoxicity, suggesting that marketed drugs are not capable of interfering with genotoxicity and therefore may prove to be ineffective in preventing neuronal
genotoxicity, hence, the diabetic neuropathy. Previously, genotoxicity has been associated with several neurological complications, such as cognitive dysfunction, and the natural molecules which reversed genotoxicity, have been reported to improve memory performance in the experimental animals [255, 277, 278]. Our findings suggest that natural molecules can be exploited as therapeutics for diabetic complications.

Small molecules of natural origin and marketed drugs were screened and compared for their antidiabetic potential through \textit{in-vitro} $\alpha$-amylase inhibitory assay and neuronal glucose uptake assay. $\alpha$-amylase is an important enzyme involved in the digestive process. It increases blood glucose levels through by degrading non-absorbable dietary polysaccharides into absorbable monomeric unit (D-glucose), which gets freely absorbed from the gastro intestinal track [6, 71]. Previously, quercetin [279] and ellagic acid [280] have been demonstrated to possess moderate $\alpha$-amylase inhibitory activity, however, our results suggests that both of these molecules possesses high potential to inhibit the enzyme in a concentration dependent manner (IC$_{50}$ values of 18.31 and 12.49 $\mu$M, respectively) (Table 5.4). Ascorbic acid, caffeine and piperine showed a moderate potential to inhibit amylase activity and their activity was observed to be significantly lower than quercetin and ellagic acid. Rosiglitazone, metformin and glimepiride demonstrated insignificant effect on the $\alpha$-amylase activity, suggesting that they cannot control dietary inflow of glucose, which is one of the prime reason for the development of hyperglycemia. Quercetin, ellagic acid and ascorbic acid showed good potential to inhibit enzyme activity and therefore may prove beneficial in managing hyperglycemia by lowering dietary inflow of glucose.

To get a better insight into the antidiabetic potential of natural molecules, we screened their potential towards cellular glucose uptake through \textit{in-vitro} glucose uptake and GLUT4 expression assay on E18 hippocampal primary neuron cultures. Diabetes is characterized by persistent hyperglycemia and the drugs which are capable of disposing excessive glucose from the blood will eventually reduce circulating glucose levels and therefore can be considered as an efficient antidiabetic agents [71, 281]. Ascorbic acid, gallic acid, ellagic acid, caffeine, quercetin, and cinnamic acid efficiently disposed the glucose into the neuronal cells (Figure 5.3), and interestingly, their results were comparable to rosiglitazone and were significantly higher than basal glucose uptake of 24.53 %. Further, we also observed that these molecules upregulated the neuronal GLUT4 expression (Figure 5.4). Enhanced glucose uptake could be attributed to the
potential of ascorbic acid, caffeine, quercetin, ellagic acid and rosiglitazone to upregulate neuronal GLUT4 expression. Our results for quercetin contradicts the reports of Wang and Yang, 2014 [282], where they demonstrated quercetin to lower GLUT4 translocation by inhibiting InR on cancer cells, probably because they conducted studies on cell lines, where just one type of cells are present. GLUT4 expression was observed to be lower than rosiglitazone in cells treated with metformin and glimepiride, and therefore glucose uptake was also observed to be on the lower side. Recent reports demonstrated that neuronal GLUT4 expression is essential for normal neuronal functioning and its downregulation was associated with impaired neurological performance in rodents during diabetes [40, 41]. Our results suggest that natural molecules, especially quercetin, ascorbic acid, ellagic acid and caffeine are efficient in disposing glucose into the cells, which may be attributed to their potential to enhance GLUT4 expression, and therefore may be beneficial in managing hyperglycemia and associated complications.

After careful evaluation of the results of the in-vitro screening, we concluded that quercetin was the most promising molecule amongst all the tested small molecules of natural origin. It showed good interaction with InR, PPAR-γ and DPP-4 in the docking study, demonstrated good antioxidant activity and efficiently prevent H₂O₂ induced DNA fragmentation, besides, showing potential antidiabetic activity through inhibition of α-amylase, disposing glucose into neuronal cells and by upregulating GLUT4 expression in the neuronal cells. Therefore, we selected quercetin for further evaluation of its potential to modulate type II diabetes and associated neurological complications in Swiss albino mice.

In an animal model of experimental diabetes, we investigated the T2DM mediated neurological complications and their association with neuronal insulin signaling pathway. We also evaluated the effect of quercetin on the development of IR, glucose intolerance and insulin signaling cascade in the hippocampus and its associated behavioral dysfunction and molecular mechanisms. We induced hyperglycemia in Swiss albino mice by injecting 50 mg/kg STZ for five consecutive days and confirmed the development of type II diabetes by calculating HOMA-IR index. Our results demonstrated that, during 8 weeks of study hyperglycemia progressed steadily and fasting blood glucose levels were found to be significantly higher in STZ animals, when compared to CTRL. Treating animals with quercetin and rosiglitazone improved diabetes, blood glucose levels remained almost similar throughout the study and were significantly lower
than STZ treated animals. Previously, where we have validated this model to induce long standing diabetes in Swiss albino mice [41, 115, 217]. Symptomatically, T2DM is characterized by polydipsia, polyphagia and weight loss in experimental animals [217] as well as in clinical patients [1, 71, 281]. In our study, we demonstrated the development of weight loss, polydipsia, polyphagia and glucose intolerance in STZ treated animals and quercetin treatment provided symptomatic improvement. Further, a reduction in insulin levels was observed in the serum of diabetic animals, which may be the effect of pancreatic damage induced by STZ injection. We further demonstrated the development of IR state in diabetic animals through HOMA-IR index, which, along with symptoms of T2DM in diabetic animals, validates our model to represent type II diabetes. Quercetin and rosiglitazone treatment resulted in significant improvement in insulin levels and attenuated IR. Quercetin treatment alleviated diabetes mediated glucose intolerance in OGTT and interestingly, glucose levels in CTRL + Q group were found to be lower than CTRL animals, suggesting that quercetin is having potential to dispose circulating glucose in normal as well as in diabetic animals. These effects of quercetin treatment may be the result of enhanced glucose uptake mediated through GLUT4 expression, as demonstrated previously in in-vitro studies, however, further validation is necessary.

Subjecting Swiss albino mice to long standing diabetes resulted in the development of marked behavioral complications like impaired locomotion, anxiety, depression and learning & memory dysfunction. Diabetic animals demonstrated reduced locomotion in both, actophotometer and OFT (Figure 5.8). These animals were highly anxious, as suggested by the results of EPM and OFT where they preferred closed arm of EPM and hesitated to enter the central region of open field arena (Figure 5.9). STZ mediated diabetes induced marked depression in mice as suggested by the results of SPT, TST and FST (Figure 5.10). Diabetes induced a significant levels of anhedonia in animals, as suggested by comparatively lower preference towards sweetened water in SPT. Further, diabetes induced despair behavior in animals and these animals demonstrated significantly increased immobilization time in TST and FST, suggesting the development of despair behavior, which is a characteristic of clinical depression. Further, long standing diabetes impaired learning & memory functions in mice. STZ treated animals showed learning impairment in MWM task as they struggled to find the hidden platform during the learning trials. These animals showed impaired memory during probe trial of MWM (Figure 5.11), impaired recognition memory during NOR test (Figure 5.11), and impaired associative memory in PAST.
and PASD tests (Figure 5.12). These results are in agreement with our previous reports where we have demonstrated long standing diabetes to induce behavioral dysfunction in mice by impairing hippocampal insulin signaling, cholinergic signaling and by exaggerating neuronal oxidative stress [41, 115, 217]. Moreover, we did not observed any significant effect of hyperglycemia on muscle coordination and muscle strength in Rota-rod test and beam walk (Figure 5.8). These results suggest that reduced locomotor activity in the actophotometer and OFT may be a result of anxiety and depression induced by long standing diabetes and therefore lowers the possibility of the interference from impaired motor coordination in the results of MWM, FST and TST, where animal struggle and need proper muscle coordination and strength. These results are in agreement with the past literature, which demonstrates that hyperglycemia is associated with behavioral dysfunction in experimental animals by impairing normal functioning of insulin signaling cascade, IR [283, 284], and autophagy pathway [285], apoptosis [286] and by exaggerating oxidative and inflammatory stress [287] in the brain.

Hippocampus regulates several behavioral functions and hippocampal damage results in memory and mood dysfunctions, which include depression and anxiety in both, experimental animals [39, 111] as well as in human subjects [288-290]. Neurons are highly vulnerable towards the damaging effects of hyperglycemia. Brain need continuous and uninterrupted glucose supply for maintaining regular functioning. Unlike peripheral system, glucose uptake into the neurons depends upon the extracellular glucose concentration, which makes neurons prone to damage after persistent episodes of hyperglycemia [291]. Hyperglycemia inflicts damage in the hippocampus by exaggerating neuronal oxidative stress, apoptosis and mitochondrial dysfunction [292-294], by disrupting synaptic plasticity [295, 296], inflicting glucocorticoid toxicity [13] and reducing hippocampal volume [297], thereby impairing hippocampal based behavioral functions.

Earlier, we have reported that Urtica dioica leaf extract efficiently is capable of attenuating diabetes mediated behavioral dysfunctions [41, 115, 217]. Phytochemical screening demonstrated high amount of quercetin in the hydroalcoholic U. dioica extract [41, 217], which might be responsible for the neuromodulatory effect of the plant extract. Herein, we administered quercetin to the diabetic animals orally for 8 weeks and evaluated its effect on long standing diabetes mediated behavioral dysfunction. Quercetin treated mice were healthy and did not show any signs of toxicity during experimentation. Quercetin treatment improved locomotion in
Actophotometer and OFT. Quercetin treatment attenuated diabetes mediated anxiety, as evident from the greater time spent exploring open arm of EPM, higher entries to the central region of the OFT and significantly greater time spent there, when compared to STZ. Quercetin alleviated STZ mediated anhedonia and depressive like behavior. Animals preferred sweetened water and immobility was significantly lower than diabetic animals during TST and FST. Similarly, quercetin treatment improved learning & memory functions in hyperglycemic mice, these animals efficiently discriminated the novel object from the familiar one during NOR test, showed lower latency to find the hidden platform in MWM, demonstrated good memory index during probe trial of MWM and efficiently remembered the foot shock received during PAST and PASD. Our results are consistent with the previous findings where quercetin is known to attenuate diabetes and improve behavioral dysfunction in rodents [298-300]. Further, previous reports suggest that quercetin is having neuromodulatory potential and is known to improve neuronal survival, physiology and functioning by alleviating neuronal oxidative, nitritative and inflammatory stress, by improving mitochondrial dysfunction and by attenuating apoptotic pathway [246, 301-306].

Brain works continuously during which it metabolizes approximately 60-70% of total body glucose for maintaining normal physiological functions [19-22]. Brain is having its own antioxidant defense in the form of enzymes and thiols, which protects it from the oxidative damage. However, the antioxidant defense system of the brain is limited and saturable, which makes it highly susceptible towards the oxidative stress [307], especially during conditions such as hyperglycemia. Neuronal oxidative stress is characterized by either elevated levels of oxidation products (TBARS, ROS and RNS) or by reduced level of antioxidants (catalase, SOD, thiols etc.) [6]. These highly reactive ROS and RNS interacts with various biomolecules, such as proteins, enzymes, DNA, RNA etc., and adversely affect their normal physiology and functioning, leading to the development and progression of variety of degenerative processes in the brain [6, 308]. Prolonged hyperglycemia is known to induce neurodegeneration by elevating neuronal mitochondrial dysfunction and oxidative stress [309, 310]. Evidence suggest that neuronal oxidative stress is associated with the development of several neurological abnormalities, which includes autism, anxiety, memory impairment, depression, Parkinsonism, Alzheimer’s disease, etc. [311-313]. Further, these findings are consolidated by the observations where attenuating oxidative stress has resulted in marked behavioral improvements in animals.
and clinical patients [39, 41, 217, 314]. We observed a significantly higher levels of oxidative stress markers (TBARS and NO) in the hippocampus of diabetic animals, which were complimented with reduced antioxidants (catalase and TTH) (Figure 5.14). Quercetin is a natural antioxidant and is known to protect neurons and improve behavior [315, 316]. Treating diabetic animals with quercetin and rosiglitazone reduced the levels of TBARS and NO, which may have in turn prevented the depletion of catalase and TTH levels in the hippocampus. Interestingly, we observed a more pronounced effect in quercetin treated animals than rosiglitazone. Reduction of oxidative stress observed in STZ + ROSI may be because of the significant reduction in blood glucose and rosiglitazone may not have any direct action on the oxidative stress in the hippocampus, as indicated in our in-vitro results. Further, in our previous reports we have demonstrated the association between reduced oxidative stress and behavioral improvement in rodents [39].

Intact neuroanatomical and neurophysiological integrity in the certain parts of the brain, which includes hippocampus, is essential for the normal animal behavior and activity. Accumulated evidence from the past suggest that hyperglycemia initiates a neurodegenerative process in the hippocampus, and compromises mood functions [317, 318]. We evaluated the effect of long standing diabetes on neuronal integrity in the hippocampus region of brain through Golgi-cox analysis. Neurons were stained using Golgi-cox solution, visualized under 400 X and 1000 X magnification (Figure 5.15). Subjecting animals to diabetes mediated damage for 8 week resulted in a marked neuronal destruction in the hippocampus. Diabetic neurons appeared degenerated, especially in the CA3 region. Although, results of number of primary branches arising from soma differed insignificantly in different treatment groups, we observed a significantly lower number of dendrites were observed to reach 100 µm length, suggesting the degenerating effect of hyperglycemia. Further, good dendritic spine density reflects the healthy state of neuron and is associated with the possibility of number of new connection a neuron can make. Diabetic animals showed reduced dendritic spine density, which may be a reason for lower arborization and behavioral dysfunction in diabetic animals. Previous reports suggest that reduced neuronal arborization and dendritic spine density has been associated with the development of behavioral dysfunctions in rodents [319, 320]. Further, integrity of the neurons in the hippocampal CA3 region is necessary for the intact behavioral functions in animals, and neuronal damage in this region is associated with several behavioral dysfunctions, which includes impaired cognition and
memory performance [321, 322]. Quercetin and rosiglitazone treatment efficiently rescued neurons from diabetes mediated damage and hippocampal neurons appeared healthy with significantly higher arborization and dendritic spine density. Interestingly, neurons in the CTRL + ROSI group appeared to be distorted, and an insignificant reduction in arborization was also observed. Comparatively, quercetin showed better neuroprotective effect than rosiglitazone as evaluated from Golgi-cox method. These findings are in agreement to the previous reports which depicts that intact neuronal integrity is essential for regulating normal behavioral functions [323, 324] and quercetin is demonstrated to improve neuronal survival and integrity, which can be primarily attributed to its potential to alleviate neuronal oxidative stress [246].

We further explored the neuronal insulin signaling pathway during long standing diabetes and evaluated the effect of quercetin. We observed that hyperglycemia was associated with low serum insulin level and development of insulin resistant state in these animals and quercetin treatment alleviated hyperglycemia and IR. Role of the insulin signaling pathway in the regulation of neuronal functions and maintaining neuronal physiology is a conflicting debate. There are several evidence which suggest that working of brain is independent of the insulin actions [16, 17]. These reports are contradicted by different set of reports which suggest that intact central insulin signaling pathway is essential for the normal physiology and functioning of neurons [15, 18, 23]. Intact insulin signaling in the CNS is well documented for its neuromodulatory effects and is critical for normal behavioral functioning, neuronal survival, maintaining neuronal integrity, neurogenesis, energy homeostasis and synaptic plasticity in the hippocampus [56, 58, 325-328]. Argument regarding effect of quercetin on glucose homeostasis still prevails, since there are reports which suggest that quercetin is having an inhibitory effect on InR and thereby downregulates insulin signaling [282, 329], however, it is also well documented that quercetin has the potential to alleviate IR [330, 331] and upregulate insulin signaling pathway [332]. Moreover, upregulating the expression of GLUT4 in the hippocampus is associated with improved neuronal survival and functioning, besides, improving behavioral functions [33, 39, 41]. To answer this debate and to explore the association of neuronal insulin signaling in the development of diabetes mediated behavioral impairements, we investigated the effect of diabetes and quercetin treatment on neuronal insulin signaling pathway through protein expression studies using western blot and immunofluorescence studies.
We observed a significant reduction in insulin, InR and GLUT4 expression in the hippocampus of diabetic animals (Figure 5.16). Insulin-InR interaction activates the downstream cascade which ultimately leads to the enhanced expression of GLUT4 in the cells, and therefore, significantly lower insulin and InR levels compliments lower GLUT4 expression levels in the STZ group. Treating diabetic animals with quercetin and rosiglitazone significantly upregulated hippocampal insulin and InR expression, and thereby a significant increase in the expression of GLUT4 was also observed. Interestingly, levels of insulin and InR did not have much difference in STZ + Q and STZ + ROSI groups, however, GLUT4 expression in quercetin treated animals was observed to be on the higher side. This can be explained on the basis of mechanism of action of rosiglitazone, which is thorough insulin sensitization rather than by increasing GLUT4 directly. Moreover, GLUT4 level in CTRL + Q animals was significantly higher than CTRL animals, suggesting that quercetin may have directly upregulated GLUT expression in the normal as well as diabetic animals.

Neurogenesis is essential for maintaining adequate synaptic plasticity and normal mammalian behaviors [333, 334], and therefore, it is now being considered as a promising new therapeutic strategy for the management of neuronal disorders like anxiety and depression [335]. Herein we evaluate the effect of long standing diabetes and quercetin treatment on adult neurogenesis and demonstrated a significant reduction in the neurogenesis during diabetes, as suggested by the significantly lower DCX expression levels. Quercetin treatment enhanced hippocampal neurogenesis and the levels of DCX were observed to be significantly higher than STZ as well as STZ + ROSI (Figure 5.16). Moreover, DCX expression was significantly higher in control animals treated with quercetin when compared to CTRL. Further, DCX expression in quercetin treated animals suggest that quercetin is having neurogenesis promoting effect, which rosiglitazone lacks. These findings are in agreement with previous findings where hyperglycemia is reported to adversely affect neurogenesis and induce behavioral dysfunction in rodents [309, 310, 336, 337].

To get a better insight whether the alteration in the neuronal insulin signaling in hippocampus during diabetes is involved in the development and progression of neurological dysfunction and to generate a conclusive evidence whether or not quercetin is having a potential to modulate hippocampal insulin signaling, we evaluated the hippocampal expression of InR and GLUT4
immunofluorescence studies (Figure 5.17). Through Golg-cox staining it became evident that diabetes inflicts maximum damage in the CA3 region of the hippocampus and therefore, immunofluorescence studies were focused in this region. We observed a significantly higher InR expression and significantly lower GLUT4 expression in STZ treated animals, suggesting the development of IR state since GLUT4 failed to increase despite having higher InR expression. Treating diabetic animals with quercetin and rosiglitazone lowered InR expression and the levels of GLUT4 were upregulated. Despite having lower InR expression in normal animals treated with quercetin (CTRL + Q), a significant increase in GLUT4 expression was observed. These results suggest that quercetin can upregulate neuronal insulin signaling and attenuate the development of neuronal insulin resistance like state. To verify this possibility, we calculated the ratio of GLUT4:InR, since GLUT4 is directly upregulated by the activation of InR. We observed a significant reduction in GLUT4:InR ratio in STZ group, suggesting the development of insulin resistant state. GLUT4:InR ratio was significantly improved by quercetin and rosiglitazone treatment. Interestingly, GLUT4:InR was significantly higher in CTRL + Q, when compared to CTRL, suggesting that quercetin is capable of upregulating insulin signaling and GLUT4 expression in hippocampus in both, normal as well as diabetic animals. Thereby it can be concluded that quercetin might have improved long standing diabetes mediated neurological alterations by directly modulating neuronal insulin signaling and by alleviating central IR. Previously, central IR had been associated with the development of T2DM-like state, dysregulation of GLUT expression in the brain and induction of several neurological disorders [134]. Further, accumulated evidence from the clinical and preclinical studies demonstrates the involvement of defective InR mediated signaling in the development of neuropsychological abnormalities [338].

From the second objective it became evident that quercetin attenuate diabetes mediated neurological alterations by alleviating central insulin signaling dysfunction. We now wished to explore the mechanism of quercetin action for its antidiabetic and neuromodulatory potential, and therefore we used CUS to induce IR in Swiss albino mice and evaluated the neuromodulatory effects of quercetin during IR state with intact pancreatic functions.

CUS model in rodents is used to induce physiological stress and associated neurobehavioral and neuroanatomical alterations [39, 114]. By using this model, we determined the effect of quercetin
on behavioral alterations, insulin signaling dysfunction and biochemical alterations during IR state. Stressed animals showed significant reduction in the body weight, feed intake and water intake. Quercetin treated stressed animals lost some weight during first two weeks of the test, however, it recovered between 14-21 days of the study. Further, these animals showed improvement in feed and water intake, when compared to CUS.

Subjecting Swiss albino mice to 21 day chronic stress resulted in a marked behavioral alterations, which included impaired locomotion, anxiety, depression and learning and memory dysfunction. Stressed animals showed impaired locomotion in both, Actophotometer and OFT (Figure 5.19). Animals were observed to be highly anxious, as suggested by the results of EPM and OFT, where animals were observed to prefer closed arm of EPM and hesitated to enter the central region of the open field (Figure 5.20). Stressed animals showed severe signs of depression in SPT, TST and FST (Figure 5.21). CUS induced a significant levels of anhedonia in these animals, as suggested by comparatively lower preference towards sweetened water in SPT. Further, stressed animals showed significantly lower struggling time in TST and FST. Animals spent significantly higher time in immobility and remained motionless during these tests, suggesting the development of despair behavior, which is a characteristic symptoms of clinical depression. Further, CUS impaired learning and memory functions in mice. Stressed animals struggled to find the escape platform during the learning trials of MWM. These animals showed impaired working memory during probe trial of MMM (Figure 5.22), impaired recognition memory during NOR test (Figure 5.22), and impaired long term and short-term associative memory in PAST and PASD (Figure 5.23). Moreover, we did not observed any significant effect of CUS on muscle coordination in Rota-rod test and beam walk test (Figure 5.19) and animals were observed to have good muscle coordination and strength. These results suggest that reduced locomotor activity in the Actophotometer and OFT may be a result of anxiety and chronic stress induced CUS paradigm. Therefore, any possibility of interference from impaired muscle strength can be considered non-significant, especially in the MWM, FST and TST. These results are consistent with the previous reports, where chronic stress is reported to induce behavioral dysfunction in experimental animals by impairing normal functioning of insulin signaling cascade [39], neuronal Smo-Gli pathway [114], and autophagy pathway [339], exaggerating oxidative and inflammatory stress [39, 117, 124-126], etc. in the brain. Hippocampus region of the brain regulates several behavioral functions and hippocampal damage results in memory and
mood dysfunctions, which includes depression and anxiety in both, experimental animals [39, 111] as well as in human subjects [288-290]. Further, chronic stress results in the prolonged activation of HPA axis and thereby induces hypercorticosteronemia. Hippocampal neurons are highly susceptible towards the damaging effects of glucocorticoids. Excessive circulating glucocorticoids induces neuronal toxicity and damages hippocampal neurons through neuronal remodeling [111], by disrupting synaptic plasticity [340, 341], reducing hippocampal volume [108] and impairing neuronal homeostasis [39], thereby impairing hippocampal based behavioral functions.

In the present study we administered quercetin to the animals orally for 21 days along with CUS and evaluated its effect on CUS mediated behavioral dysfunction. Quercetin treatment improved locomotion in Actophotometer and OFT. Quercetin treatment attenuated CUS mediated anxiety and animals spent greater time exploring open arm of EPM, frequently entered the central region of the OFT and spent significantly greater time there, when compared to CUS. Quercetin alleviated CUS mediated anhedonia and depressive like behavior. Animals were observed to prefer sweetened water and spent significantly lower time in immobility during TST and FST. Similarly, quercetin treatment improved learning and memory functions in chronically stressed mice. Quercetin treated stressed animals efficiently discriminated the novel object from the familiar object during NOR test, showed lower latency to find hidden platform in MWM, demonstrated good memory index during probe trial of MWM and were able to remember the foot shock they received during PAST and PASD. Previously, quercetin have been reported to alleviate stress and associated behavioral dysfunction in experimental animals [242, 247, 248, 315, 316, 342].

Previous reports suggest that quercetin is having a neuroprotective potential and it improve neuronal survival and functioning by alleviating neuronal oxidative stress, modulating neuronal mitochondrial signaling, apoptotic pathway and by protecting neurons from oxidative/inflammatory stress mediated degeneration [246, 301-306]. We observed similar results in the STZ model, as depicted above. Brain works continuously during which it consumes large amount of oxygen for maintaining normal physiological functions. Brain is having its own antioxidant defense in form of enzymes and thiols, which protects it from the oxidative damage. However, the antioxidant defense system of the brain is limited and saturable, which makes it
highly susceptible towards the oxidative damage [307]. As discussed earlier, oxidative stress adversely affects the functioning of various biomolecules and is associated with the progression of variety of degenerative processes in the brain [6, 308]. Hypercorticosteronemia in known to induce neurodegeneration by elevating neuronal oxidative stress [110, 308]. Accumulated evidence suggest the strong association between oxidative stress and the development and progression of autism, anxiety, memory impairment, depression, Parkinsonism, Alzheimer’s disease, etc. [311-313], which could be alleviated by lowering oxidative stress through pharmacological interventions [39, 41, 217, 314]. In our study, we demonstrated that CUS induces significant neuronal oxidative stress in mice by elevating TBARS and NO levels and lowering the levels of antioxidants (catalase and TTH) in the hippocampus (Figure 5.26), which might have assisted the development of CUS mediated behavioral dysfunction. Quercetin is a well-known natural antioxidant and treating stressed animals with it efficiently lowered the hippocampal oxidative stress, as demonstrated above during diabetes. Quercetin treatment lowered TBARS and NO levels and restored hippocampal antioxidant levels in the form of catalase and TTH. These results are consistent with the previous reports where quercetin has been reported to improve behavioral impairments in mice by alleviating oxidative stress [315, 316]. Further, in our previous reports we have demonstrated that attenuating oxidative stress in rodents result in improved memory functions and alleviated depressive like behavior [39].

Normal behavioral functions depends on the neuroanatomical and neurophysiological integrity in the certain regions of the brain, which includes hippocampus. Accumulated evidence from the past suggests that hippocampus is highly susceptible towards CUS mediated damage and high levels of circulating GCs adversely affect hippocampal neuronal functioning and initiates a neurodegenerative process [323, 324, 343], and thereby compromising cognition, mood and other neurological functioning. We evaluated the effect of CUS on neuronal integrity in the hippocampus region of brain. Neurons were stained with hematoxylin-eosin (Figure 5.28) and cresyl violet stain (Figure 5.29). Subjecting animals to 21 day unpredicted stress paradigm resulted in marked neuronal damage in hippocampus, especially in the CA3 region. Neurons appeared degenerated with altered morphology in both, hematoxylin-eosin and cresyl violet staining. Number of viable cells in CA2 and CA3 regions were significantly lower in CUS animals, when compare to CTRL. Accumulated evidence suggest that integrity of CA3 region is necessary for the intact behavioral functions in animals, and neuronal damage in this region is
associated with several behavioral dysfunctions, which includes impaired cognition and memory performance [321, 322]. Quercetin treatment efficiently rescued neurons from CUS mediated damage. Hippocampal neurons in the quercetin treated animals appeared healthy and number of functional neurons were significantly higher the CUS in these animals, suggesting a strong neuroprotective role of quercetin during chronic stress. These findings are consistant with previous reports, which suggest that intact neuronal integrity is essential for normal behavioral functions [323, 324] and quercetin is demonstrated to improve neuronal survival and integrity during stressful conditions, which can be primarily attributed to its potential to alleviate neuronal oxidative stress [246].

We further explored whether or not behavioral dysfunctions mediated through CUS are associated with the impaired insulin functioning and investigated the role of quercetin in modulating insulin actions. For this we measured FBG level, serum insulin and corticosterone levels and determined the development of IR by calculating HOMA-IR index. Subjecting animals to 21 day CUS resulted in significantly elevated fasting glucose levels and animals were observed to be pre-diabetic. Further, stressed animals were having significantly higher serum insulin and corticosterone levels, when compared to CTRL. Chronic stress is known to induce IR state, impair insulin signaling pathway, induce glucose intolerance and inflict type II diabetes like state in experimental animals [36-39, 41], which were further associated with the development of behavioral dysfunctions in these animals [39, 41]. Treating stressed animals with quercetin for 21 days alleviated pre-diabetic state, as indicated by significantly reduced FBG levels, and significantly attenuated IR and serum corticosterone levels. These findings suggest that impaired insulin signaling during chronic stress may play a crucial role in the development of behavioral dysfunction in mice, besides, oxidative stress and CUS mediated neurodegeneration being the additional factors. These findings can be justified by the observed improvement in the behavioral functions after attenuating IR and improving insulin signaling through quercetin treatment. Further, the argument regarding effect of quercetin on glucose homeostasis still prevails, since there are reports which suggest that quercetin is having an inhibitory effect on InR and thereby downregulates insulin signaling [129, 329], however, it is also well documented that quercetin has a potential to alleviate IR [330, 331] and upregulate insulin signaling pathway [332].
Role of the insulin and insulin signaling pathway maintaining neuronal physiology and functioning is not clearly understood. There are several evidence which suggest that working of brain is independent of insulin actions [16, 17], while other suggest that intact insulin and insulin signaling in the CNS is essential for the normal physiology and functioning of neurons [15, 18, 23]. Intact insulin signaling in the CNS is well documented for its neuromodulatory effects and is critical for normal behavioral functioning, neuronal survival, maintaining neuronal integrity, neurogenesis, intact energy homeostasis and synaptic plasticity in the hippocampus [56, 58, 325-328]. We have demonstrated that CUS induces IR and there are reports which agrees with our findings and suggest that stress mediated IR is associated with depression and cognitive dysfunctions in rodents [344]. Moreover, upregulating the expression of GLUT4 in the hippocampus is associated with improved neuronal survival and functioning, besides, improving behavioral functions [33]. Therefore, to get a better insight and to understand the interlink between CUS, IR and hippocampal insulin signaling during CUS mediated behavioral alterations and to understand the role of quercetin in this scenario, we evaluated hippocampal insulin signaling pathway in terms of the expression of insulin, InR, GLUT4 and proteins involved in the neurogenesis through western blot and immunofluorescence studies. Through western blot analysis, we demonstrated a significant increase in hippocampal insulin and InR expression in chronically stressed mice, whereas, GLUT4 expression was observed to be significantly lower when compared to normal animals (Figure 5.27). Insulin-InR interaction activates the downstream cascade which ultimately leads to the enhanced expression of GLUT4 in cells. Interestingly, results of protein expression in stressed animals suggest that GLUT4 levels fails to increase in the hippocampus despite of significantly higher insulin and InR levels, suggesting that CUS markedly impairs the insulin signaling pathway in hippocampus. Quercetin treatment enhanced the expression of GLUT4 in the hippocampus of both, stressed and normal animals, besides, expression of insulin and InR in stressed animals were significantly lowered. Quercetin treated normal animals further demonstrated an insignificant reduction in hippocampal insulin and InR levels, suggesting that quercetin may have potential to upregulate GLUT4 expression directly and thereby upregulate neuronal insulin signaling independent of insulin and InR.

Neurogenesis is essential for maintaining adequate synaptic plasticity and normal mammalian behaviors [333, 334], and therefore, it is now being considered as a promising new therapeutics strategy for the management of psychiatric disorders [335]. Herein we evaluate the effect of CUS
and quercetin treatment on adult neurogenesis and demonstrated that chronic stress significantly reduces neurogenesis, as indicated by the significantly lower expression of DCX. Quercetin treatment enhanced hippocampal neurogenesis and the levels of DCX were observed to be significantly higher than CUS (Figure 5.16). In earlier reports, chronic stress is reported to adversely affect neurogenesis and induce behavioral dysfunction in rodents [333, 345].

Proteomic studies in the immunoblot analysis gives us an idea about the alterations in the insulin signaling during inulin resistant state in the entire hippocampus region of the brain. Through histopathological examination we have clearly demonstrated that CUS inflicts marked damage in the CA3 region, whereas, CA1 and dentate gyrus region are comparatively less affected. To understand the role of hippocampal insulin signaling during neurological dysfunction and to investigate the effect of quercetin treatment in this scenario, we evaluated InR and GLUT4 expression in the hippocampus. Our results demonstrated that expression levels of both, InR and GLUT4 is reduced in the CA3 region of the hippocampus during CUS. Consideration higher serum levels of insulin and higher hippocampal insulin expression in stressed animals, these findings suggest the development of neuronal IR in the hippocampus, as a consequence of which, GLUT4 expression was reduced in this region despite of high serum and hippocampal insulin levels. Further, quercetin treated normal animals showed significantly lower InR and enhanced GLUT4 expression (Figure 5.30). These findings, along with lower serum and hippocampal insulin levels in quercetin treated animals, suggest that quercetin may have a capability to upregulate GLUT4 expression independent of InR expression. To verify this possibility, we calculated the GLUT4:InR and demonstrated that GLUT4:InR was significantly higher in normal animals treated with quercetin, when compared to CTRL, and therefore confirming that quercetin can directly upregulate GLUT4 expression. Moreover, treating stressed animals with quercetin for 21 days significantly upregulated InR and GLUT4 levels in the CA3 region and GLUT4:IR ratio was also significantly higher than CUS (Figure 5.30), suggesting the beneficial effect of quercetin in improving hippocampal insulin signaling during insulin resistant state. Interestingly, there were no significant difference in the GLUT4:InR ratio when quercetin treated normal and stressed animals were compared with each other, however, this ratio was significantly higher than CTRL and CUS groups. Taking into consideration the observed serum and hippocampal insulin levels, these results suggest that quercetin is capable of enhancing GLUT4 expression in the CA3 region, irrespective of InR levels in both, stressed and normal conditions, and thereby it
can be concluded that quercetin might have improved CUS mediated neurological alterations by directly modulating neuronal insulin signaling and by alleviating central IR. Previously, central IR has been associated with the development of T2DM, dysregulation of GLUT expression in the brain and induction of several neurological disorders [346]. Further, accumulated evidence from previous studies demonstrates comorbid existence of defective InR mediated signaling and HPA axis dysfunction, which results in the development of neuropsychological abnormalities [338]. Chronic stress is a degenerative process and is associated with reduced hippocampal volume and lower number of viable neurons [347]. It is worthy to mention that CA3 region of the hippocampus of stressed animals have significantly lower number of functional neurons (Figure 5.29). Therefore, to reach an evidence based decisive conclusion for insulin signaling and quercetin interaction, we evaluated InR and GLUT4 expression in an individual viable neuronal cell in terms of TCF. We observed some interesting results which are demonstrated in Figure 5.31. Quercetin treated animals (both normal and stressed) showed significantly lower InR expression, when compared to CTRL or CUS animals. We further observed that GLUT4 expression in the quercetin treated animals (CTRL + Q and CUS + Q) was significantly higher than control or stressed animals. These findings confirms that quercetin has the potential to upregulate GLUT4 expression in the neurons of normal as well as insulin resistant animals. Neuronal InR expression in quercetin treated animals was observed to be significantly lower than CTRL and CUS, which might be a result of feed-back inhibition. We further calculated the GLUT4:InR expression ratio for individual neuron and the results clearly suggest that quercetin has directly elevated GLUT4 expression, in both stressed and control animals, and this action was independent of InR expression levels and probably insulin too.

To confirm the mechanism of quercetin action, we performed an \textit{in-vitro} 2NBDG uptake assay on L6 rat muscle cell line in the presence and absence of LY294002, which is an inhibitor of insulin signaling [348, 349]. Our results demonstrated that quercetin and insulin enhanced 2NBDG uptake in L6 muscle cells in the absence of inhibitor, however, LY294002 treatment resulted in a significant reduction in 2NBDG uptake in both quercetin and insulin treated cells. These results are consistent with the earlier literature reports where LY294002 is known to inhibit insulin signaling pathway [348, 349]. These results suggest that quercetin exploits insulin signaling pathway to exert its beneficial effects. Moreover, docking studies demonstrated that quercetin is having strong interaction with InR (PDB: 1IR3) and the interaction site was different
than insulin. These findings suggest that quercetin is having potential to activate insulin signaling by directly activating InR and downstream signaling cascade, which could explain the enhanced GLUT4 expression and reduced InR expression in normal, stressed and diabetic animals.
CHAPTER 7

CONCLUSION
7. CONCLUSION

In the present study, we screened natural molecules for their antidiabetic potential through *in-silico* docking studies and demonstrated a good interaction of ascorbic acid, gallic acid, quercetin, ellagic acid, caffeine, piperine and cinnamic acid with the insulin receptor, PPAR-γ and DPP-IV. Screening process was narrowed down to quercetin through *in-vitro* assays where quercetin demonstrated excellent potential to inhibit oxidative stress, genotoxicity, α-amylase activity and upregulated neuronal GLUT4 expression and glucose uptake. Quercetin was then evaluated for its antidiabetic and neuromodulatory potential in the animal model of experimental diabetes. 8-week quercetin treatment alleviated hyperglycemia and development of insulin resistance in Swiss albino mice. Quercetin treatment attenuated diabetes mediated behavioral dysfunctions, which was attributed to its potential to alleviate neuronal oxidative stress, neurodegeneration and insulin signaling dysfunction in the hippocampus region of the brain. The mechanism through which quercetin demonstrated antidiabetic and neuromodulatory effects seems to be through modulation of central insulin resistance and insulin signaling. To confirm this mechanism, we induced insulin resistance in Swiss albino mice through experimental model of chronic stress and evaluated the neuromodulatory effects of quercetin. Chronic stress induced insulin resistance and pre-diabetic state in animals and was accompanied by behavior dysfunction, neurodegeneration, and impaired neuronal insulin signaling pathway. Quercetin treatment attenuated stress mediated insulin resistance, hyperglycemic state, behavioral dysfunction and rescued neurons from degeneration. Quercetin directly upregulated neuronal insulin signaling pathway, as it enhanced neuronal expression of GLUT4 despite of significantly lower insulin and insulin receptor levels in the hippocampus, in normal as well as insulin resistant state. This mechanism was confirmed thorough cell line based assay where quercetin treatment enhanced 2NBDG uptake into muscle cells, but failed to so in the presence of an insulin signaling inhibitor (LY294002). Through docking studies we confirmed that quercetin is having strongly interaction with insulin receptor and the binding site was predicted to be different form that used by insulin. Therefore, we concluded that quercetin is having potential to alleviate type II diabetes and associated neurological complications by directly modulating neuronal insulin signaling pathway, which seems to be independent of the hippocampal insulin levels.
Chapter 8

References
8. REFERENCES


155


166


CHAPTER 9

APPENDICES
<table>
<thead>
<tr>
<th>Name of the Compounds</th>
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*Remaining molecules were the isomers (α/β or +/- forms) of the compounds listed above*
9. APPENDICES

List of Publications


List of Conferences Attended


4. Vineet Mehta, Ashwani Kumar, Tiratha Raj Singh, Udayabanu Malairaman. Screening of herbal molecules against Type 2 Diabetes mediated memory dysfunction. Poster presentation delivered at 33rd Annual Conference of Indian Academy of Neuroscience “Neuroscience Research from Mechanisms to Applications” organized at Panjab University, Chandigarh, India. 31st October-02nd November 2015.

List of Workshops Attended

1. Attended 16-day IBRO-APRC School of Neuroscience on “Epigenetic Regulation of Neuronal Gene Expression”, organized at Banaras Hindu University, Varanasi, India. April 1-16, 2017.


Awards

1. Received International Brain Research Organization grant for attending “IBRO-APRC advanced school on functional brain mapping with advanced neurotechnologies” to be organized at Daegu, South Korea. October 19-25, 2017

2. Received International Brain Research Organization grant for attending 16-day workshop on “Epigenetic regulation of neuronal gene expression” during IBRO-APRC School of Neuroscience organized at Banaras Hindu University, Varanasi, India. April 1-16, 2017.

3. Received Frank A. Beach Award for my publication entitled “Quercetin ameliorates chronic unpredicted stress-mediated memory dysfunction in male Swiss albino mice by attenuating insulin resistance and elevating hippocampal GLUT4 levels independent of insulin receptor expression” published in Hormones and Behavior. 2017.
Quercetin ameliorates chronic unpredicted stress-induced behavioral dysfunction in male Swiss albino mice by modulating hippocampal insulin signaling pathway

Vineet Mehta\textsuperscript{a}, Tiratha Raj Singh\textsuperscript{a}, Malairaman Udayabanu\textsuperscript{a}\textsuperscript{b}\textsuperscript{**}

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Abstract

Chronic stress is associated with impaired neurogenesis, neurodegeneration and behavioral dysfunction, whereas the mechanism underlying stress-mediated neurological complications is still not clear. In the present study, we aimed to investigate whether chronic unpredicted stress (CUS) mediated neurological alterations are associated with impaired hippocampal insulin signaling or not, and studied the effect of quercetin in this scenario. Male Swiss albino mice were subjected to 21 day CUS, during which 30 mg/kg quercetin treatment was given orally. After 21 days, behavioral functions were evaluated in terms of locomotor activity (Actophotometer), muscle coordination (Rotarod), depression (Tail Suspension Test (TST), Forced Swim Test (FST)) and memory performance (Passive-avoidance step-down task (PASD)). Further, hippocampal insulin signaling was evaluated in terms of protein expression of insulin, insulin receptor (IR) and glucose transporter 4 (GLUT-4) and neurogenesis was evaluated in terms of doublecortin (DCX) expression. 21 day CUS significantly impaired locomotion and had no effect on muscle coordination. Stressed animals were depressed and showed markedly impaired memory functions. Quercetin treatment significantly improved stress-mediated behavior dysfunction as indicated by improved locomotion, lesser immobility time and greater frequency of upward turning in TST and FST and increased transfer latency on the day 2 (short-term memory) and day 5 (long-term memory) in PASD test. We observed significantly higher IR expression and significantly lower GLUT-4 expression in the hippocampus of stressed animals, despite of nonsignificant difference in insulin levels. Further, chronic stress impaired hippocampal neurogenesis, as indicated by the significantly reduced levels of hippocampal DCX expression. Quercetin treatment significantly lowered insulin and IR expression and significantly enhanced GLUT-4 and DCX expression in the hippocampus, when compared to CUS. In conclusion, quercetin treatment efficiently alleviated stress mediated behavioral dysfunction by modulating hippocampal insulin signaling and neurogenesis.

1. Introduction

Stress response is an essential defense mechanism of our body, which enables us to deal with the daily life stressors \cite{1,2}. However, chronic stress is highly deleterious process, which initiates several degenerative processes throughout the body, especially in the brain, which is highly vulnerable to stress mediated damage. In the CNS, chronic stress is associated with impaired synaptic plasticity \cite{3,4}, reduced hippocampal volume \cite{5} and reduced dendritic complexity \cite{6}, besides being associated with neurodegeneration \cite{7}, depression and impaired learning and memory functioning \cite{8,9}, which compromises healthy state of living.

Mechanisms responsible for the development and progression of chronic stress-mediated neurological complications are poorly understood. Previous studies suggest that chronic stress is associated with enhanced oxidative and inflammatory stress \cite{8}, neurodegeneration, glucose intolerance, insulin resistance and impaired insulin signaling in the brain, which plays a part in the development of these complications \cite{9,10}. Brain utilizes large proportion of the body's glucose and regulates the energy homeostasis throughout the body, and therefore, a continuous and uninterrupted glucose supply is essential for the proper functioning of neurons. Unlike peripheral system, role of insulin and insulin signaling in the brain energy homeostasis is debatable. Evidence suggest that insulin signaling is essential for glucose utilization and metabolism in the brain \cite{11,12}, while it is also reported that this process is independent of the insulin actions \cite{13,14}. Interestingly,
Quercetin prevents chronic unpredictable stress induced behavioral dysfunction in mice by alleviating hippocampal oxidative and inflammatory stress

Vineet Mehta, Arun Parashar, Malairaman Udayabanu *

Department of Biotechnology, Bioinformatics and Pharmacy, Jaypee University of Information Technology, Waknaghat, India

HIGHLIGHTS

• Chronic unpredictable stress induced anxiety, depression and cognitive deficit in mice.
• Stress elevated oxidative and inflammatory stress markers in hippocampus.
• Chronic quercetin treatment reversed behavioral dysfunction.
• Quercetin attenuated hippocampal oxidative stress and lowered pro-inflammatory cytokine expression.
• Quercetin rescued hippocampal neurons from stress induced damage.

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Stress
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ABSTRACT

It is now evident that chronic stress is associated with anxiety, depression and cognitive dysfunction and very few studies have focused on identifying possible methods to prevent these stress-induced disorders. Previously, we identified abundance of quercetin in Urtica dioica extract, which efficiently attenuated stress related complications. Therefore, current study was designed to investigate the effect of quercetin on chronic unpredictable stress (CUS) induced behavioral dysfunction, oxidative stress and neuroinflammation in the mouse hippocampus. Animals were subjected to unpredictable stress for 21 days, during which 30 mg/kg quercetin was orally administered to them. Effect of CUS and quercetin treatment on animal behavior was assessed between day 22-28. Afterward, the hippocampus was processed to evaluate neuronal damage, oxidative and inflammatory stress. Results revealed that stressed animals were highly anxious (Elevated Plus Maze and Open Field), showed depressive-like behavior ( sucrose preference task), performed poorly in short-term and long-term associative memory task (passive avoidance step-through task) and displayed reduced locomotion (open field). Quercetin alleviated behavioral dysfunction in chronically stressed animals. Compared to CUS, quercetin treatment significantly reduced anxiety, attenuated depression, improved cognitive dysfunction and normalized locomotor activity. Further, CUS elevated the levels of oxidative stress markers (TBARS, nitric oxide), lowered antioxidants (total thiol, catalase), enhanced expression of pro-inflammatory cytokines (IL-6, TNF-α, IL-1β and COX-2) in the hippocampus and damaged hippocampal neurons. Quercetin treatment significantly lowered oxidative and inflammatory stress and prevented neural damage. In conclusion, quercetin can efficiently prevent stress induced neurological complications by rescuing brain from oxidative and inflammatory stress.

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1. Introduction

Acute stress is an essential defense mechanism which prepares an individual, physically and mentally, to deal with the variety of stressful situations faced regularly, however, chronic stress initiates several deleterious processes which compromise healthy living and induces a variety of complications [1,2], amongst which etiology of neurological complications is least known. Prolonged activation of hypothalamic-pituitary-adrenal (HPA) axis chronically elevates the levels of
Frank A. Beach Award Paper

Quercetin ameliorates chronic unpredicted stress-mediated memory dysfunction in male Swiss albino mice by attenuating insulin resistance and elevating hippocampal GLUT4 levels independent of insulin receptor expression

Vineet Mehta, Arun Parashar, Arun Sharma, Tiratha Raj Singh, Malairaman Udayabanan *

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Keywords:
Chronic unpredicted stress
Insulin resistance
Quercetin
Cognition
Hippocampus
GLUT4
Immunofluorescence
Insulin
Learning and memory

A B S T R A C T

Chronic stress is associated with impaired neuronal functioning, altered insulin signaling, and behavioral dysfunction. Quercetin has shown neuroprotective and antidiabetic effects, besides modulating cognition and insulin signaling. Therefore, in the present study, we explored whether or not quercetin ameliorates stress-mediated cognitive dysfunction and explored the underlying mechanism. Swiss albino male mice were subjected to an array of unpredicted stressors for 21 days, during which 30 mg/kg quercetin treatment was given orally. The effect of chronic unpredicted stress (CUS) and quercetin treatment on cognition were evaluated using novel object recognition (NOR) and Morris water maze (MWM) tests. Hippocampal neuronal integrity was observed by histopathological examination. Blood glucose, serum corticosterone, and insulin levels were measured by commercial kits and insulin resistance was evaluated in terms of HOMA-IR index. Hippocampal insulin signaling was determined by immunofluorescence staining. CUS induced significant cognitive dysfunction (NOR and MWM) and severely damaged hippocampal neurons, especially in the CA3 region. Quercetin treatment alleviated memory dysfunction and rescued neurons from CUS-mediated damage. Fasting blood glucose, serum corticosterone, and serum insulin were significantly elevated in stressed animals, besides, having significantly higher HOMA-IR index, suggesting the development of insulin resistance. Quercetin treatment alleviated insulin resistance and attenuated altered biochemical parameters. CUS markedly down-regulated insulin signaling in CA3 region and quercetin treatment improved neuronal GLUT4 expression, which seemed to be independent of insulin and insulin receptor levels. These results suggest that intact insulin functioning in the hippocampus is essential for cognitive functions and quercetin improves CUS-mediated cognitive dysfunction by modulating hippocampal insulin signaling.

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1. Introduction

Chronic stress is associated with prolonged activation of hypothalamic–pituitary–adrenal (HPA) axis which is known to impair hippocampal neuronal plasticity and induce hypercorticosteremia, neurodegeneration, depression, and cognitive dysfunction (Bondi et al., 2008; Pioli et al., 2007; Rossetti et al., 2014; Sousa et al., 2008). Corticosterones easily cross blood–brain barrier and interact with the high density of corticosterone receptors present in the hippocampus. Hippocampal neurons are highly susceptible to glucocorticoid toxicity, leading to hippocampus-dependent cognitive impairment during chronic stress (Lupien and Lepage, 2001; McEwen, 2000). Prolonged activation of hypothalamic–pituitary–adrenal (HPA) axis chronically elevates blood corticosterone level which impairs glucose homeostasis and induces type-2 diabetes-like state by lowering insulin secretion from pancreatic β-cells, reducing glucose uptake/utilization, and interfering insulin resistance (Andrews and Walker, 1999; Ghaisas et al., 2009; Jatwa et al., 2007; Patel et al., 2016c; Patel and Udayabanan, 2014). Interestingly, both chronic stress and type 2 diabetes are well-known risk factors for the development of learning and memory impairment, cognitive decline, and Alzheimer's disease (Patel et al., 2016a; Patel et al., 2016c; Patel and Udayabanan, 2014; Riedinger et al., 2011; Rothman and Mattson, 2010). However, the exact mechanism and cellular targets which are involved in the development and progression of chronic stress-mediated CNS complications are not completely understood.

Continuous and uninterrupted supply of glucose is essential for maintaining normal neuronal functions, and glucose level in the brain plays a critical role in regulating energy metabolism throughout the
Original Article

Quercetin, ascorbic acid, caffeine and ellagic acid are more efficient than rosiglitazone, metformin and glimepiride in interfering with pathways leading to the development of neurological complications associated with diabetes: A comparative in-vitro study

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1. Introduction

Diabetes mellitus (DM) is a metabolic disorder which has become epidemic in recent years, in both developed and developing nations. Despite of enormous advancement in healthcare science, prevalence of DM is increasing at alarming rate and is expected to increase by 54.6% by the year 2040 [1]. Mortality and morbidity associated with DM are primarily attributed to hyperglycemia induced secondary complications such as neuropathy, nephropathy, cardiovascular complications, retinopathy etc. Diabetic neuropathy is least understood and most distressing complication associated with hyperglycemia and it continues to grow uncontrolled despite of regular anti-diabetic therapy. DM is associated with several neurological complications such as memory dysfunction, depression, dementia, Alzheimer’s disease, phobias etc. [2–5] and pre-diabetic patients are at higher risk to develop these complications [6]. Diabetic neuropathy is primarily attributed to enhanced oxidative stress and downregulated neuronal energy homeostasis, resulting in neurodegeneration, reduction in membrane glucose transporters (GLUTs) and neuronal starvation [3,7].

During diabetes, excessive circulating glucose generate highly reactive oxygen/nitrogen species through polyol, hexosamine, pro-
Chapter 13
Flavonoids: Prospective Strategy for the Management of Diabetes and Its Associated Complications

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ABSTRACT
Diabetes Mellitus is one of the major healthcare problems faced by the society today and has become alarmingly epidemic in many parts of the world. Despite enormous knowledge and technology advancement, available diabetes therapeutics only provide symptomatic relief by reducing blood glucose level, thereby, just slows down development and progression of diabetes and its associated complications. Thus, the need of the day is to develop alternate strategies that can not only prevent the progression but also reverse already “set-in” diabetic complications. Many flavonoids are reported, traditionally as well as experimentally, to be beneficial in averting diabetes and lowering risk of its accompanying complications. In the present chapter we have convened different flavonoids beneficial in diabetes and comorbid complications and discussed their mechanisms of action. Further, we conclude that coupling current therapeutics with flavonoids might provide exceptional advantage in the management of diabetes and its complications.

INTRODUCTION
Diabetes mellitus (DM) is a complex metabolic disorder arising from variety of factors, genetic or environmental, resulting in hyperglycemia. Hyperglycemia leads to acute and chronic metabolic abnormalities, amongst which neuropathy, nephropathy, retinopathy, learning and memory impairment and cardiovascular complications are the major causes of morbidity (Goodman, 2011). Hyperglycemia is a consequence of either pancreatic β-cells destruction, thereby decreasing the level of insulin secretion (Type-1 Diabetes Mellitus; T1DM) or due to decreased respon