Prevention of Diabetes-Associated Cognitive Impairment through the Administration of Herbal Products

Author
Hibba Riaz

Registration Number

318293

Supervisor

Dr. Aneeqa Noor

DEPARTMENT BIOMEDICAL ENGINEERING AND SCIENCES

SCHOOL OF MECHANICAL & MANUFACTURING ENGINEERING

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY

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Author

Hibba Riaz

Registration Number

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Thesis Supervisor:

Dr. Aneeqa Noor

Thesis Supervisor's Signature: _____

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NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY

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We hereby recommend that the dissertation prepared under our supervision by: Hibba Riaz (00000318293)
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Examination Committee Members

1.	Name: Saima Zafar	Signature:
2.	Name: Adeeb Shehzad	Signature:
3.	Name: Muhammad Asim Waris	Signature:
4.	Name: Aneeqa Noor	Signature:

Supervisor: Aneeqa Noor Signature:

Date: 09 - Aug - 2023

Head of Department 09 - Aug - 2023
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Signature:

Name (Supervisor): Aneeqa Noor

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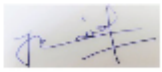
Signature (DEAN): 

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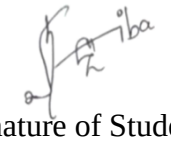

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Abstract

The condition of long-term high blood glucose concentration i.e., chronic hyperglycaemia resulting from deficiency of insulin leads to Diabetes Mellitus. This deficiency leads to metabolic disturbances notably in carbohydrate, protein and lipid metabolism. The number of diabetic patients has significantly increased in the past few years. An association between Type 1 Diabetes Mellitus (TIDM) and cognitive impairment has been previously reported and is extremely dominant in ageing patients. We confirmed that TIDM mice induced by streptozotocin (STZ) injections presented impairments in working and spatial memory. Long-term potentiation (LTP) induction defects and synaptic loss were also observed in these mice. Modified levels of synaptic proteins i.e. N-methyl-D-aspartic acid receptor (NMDAR) subunit NR2A were found in the cortical region. We administered natural herbs including Nigella sativa and Cassia angustifolia post-STZ injections in diabetic mice and observed improvements in memory, neuronal loss, and synaptic proteins even with the retention of hyperglycaemia. Incorporation of these herbs in the diet of diabetic and pre-diabetic patients may improve debilitating cognitive deficits associated with TIDM.

Keywords: Diabetes Mellitus, cognitive impairment, STZ, NR2A

Table of Contents

Declaration ..... **i**

Proposed Certificate for Plagiarism ..... **ii**

Copyright Statement ..... **iii**

Acknowledgements ..... **iv**

Abstract ..... **vi**

Table of Contents ..... **vii**

List of Figures ..... **xi**

List of Tables ..... **x**

List of Abbreviations ..... **xi**

CHAPTER 1: INTRODUCTION ..... **i**

1.1 Diabetes mellitus (DM) and Cognitive Impairments 2

1.1.1 Insulin resistance and deficiency 3

1.1.1.1 Role of Insulin 5

1.1.1.2 Role of GSK-3 5

1.1.1.3 Role of IDE in eradicating insulin and amyloid beta in brain 6

1.1.2 Hyperglycemia 7

1.1.2.1 Increased levels of AGEs 7

1.1.2.2 Increased levels of PKC 7

1.1.3 Vascular risk factors 8

1.1.4 Neurotransmitters 9

1.1.4.1 Impact of glutamate receptors 10

1.1.4.2 Elevation of glucocorticoids levels 10

1.2 Traditional medicines' healing effects on diabetes-related cognitive impaiment 11

1.2.1 AGE attenuation using herbs 11

1.2.2 Anti-oxidation and Anti-inflammation 12

1.2.3 Decreasing the Aβ levels in the brain 13

1.3 Effect of nigella sativa on DM 13

1.3.1 Anti-oxidant properties 14

1.3.2 Anti-inflammatory properties 15

1.3.3 Regulation of lipid profiles 16

1.4 Effect of Cassia angustifolia on DM 16

1.4.1 Anti-oxidant properties 17

1.4.2 Anti-inflammatory properties 17
1.5 Aims and Objectives 18
CHAPTER 2: MATERIALS AND METHODOLOGY 19
2.1 Animals 19
2.2 Preparation of the plant extract 19
2.2.1 Nigella sativa extract 19
2.2.2 Cassia angustifolia extract protocol 19
2.3 Experimental timeline 19
2.4 Behavioral tests 20
2.4.1 Object location test 20
2.4.2 Y-Maze Test 21
2.4.3 Morris Water Maze Test 21
2.4.4 Open Field Test 21
2.5 Histopathological Aessessment 22
2.6 Molecular docking 22
2.6.1 Protein-ligand interaction 23
2.6.2 Drug screening 23
2.7 Plymerase chain Reaction 23
2.7.1 Primer Selection 23
2.7.2 RNA extraction 24
2.7.3 Quality of RNA 25
2.7.4 cDNA Synthesis 25
2.7.5 Gene Expression Analysis by Quantitative real time Polymerase Chain Reaction 26
2.8 Statistical Analysis 26

CHAPTER 3: RESULTS 27
3.1 Object location test 27
3.2 Y-Maze Test 27
3.3 Morris Water Maze Test 28
3.4 Open Field Test 30
3.5 Histopathology results 31
3.5.1 Cell Count Graph 32
3.6 Molecular docking analysis 32
3.6.1 Binding Energy Graph 33
3.7 Molecular interaction analysis 34

3.7.1 Compounds of Cassia angustifolia and NR2a....34
3.7.2 Compounds of Nigella sativa and NR2a....35
3.8 PCR optimization results....36
3.8.1 qPCR Based Evaluation of NR2a Expression....38

CHAPTER 4: DISCUSSIONS 40

List of Figures

Figure 1: Pathological events leading to T1DM and T2DM 2
Figure 2: Physiological and Behavioral response of hyperglycemia 4
Figure 3: Timeline followed during research 20
Figure 4: BLAST technique showing match for forward primer 24
Figure 5: BLAST technique showing match for reverse primer 24
Figure 6: Exploration time in object location test 27
Figure 7: Number of entries in novel and other arm in Y-maze test 28
Figure 8: Number of entries in the target quadrant in MWM 29
Figure 9: Number of platform crossings in MWM 29
Figure 10: Time spent in target quadrant in MWM 30
Figure 11: Number of entries in the central area in OFT 30
Figure 12: Time spent in peripheral and central areas in OFT 31
Figure 13: Brain tissue stained with Hematoxylin and Eosin 31
Figure 14: Cell counts after histopathology analysis 32
Figure 15: Binding energy graph of NR2a and compounds of CA and NGS 34
Figure 16: 2D analysis of NR2a with compounds of Cassia angustifolia 35
Figure 17: 2D analysis of NR2a with compounds of Nigella sativa 36
Figure 18: BLAST technique showing match for forward primer 37
Figure 18: BLAST technique showing match for reverse primer 37
Figure 20: PCR optimization results 38
Figure 21: Standard Curve 39
Figure 22: Relative expression of NR2a evaluated by qPCR 39

List of Tables

Table 1: NR2a forward and reverse primer sequences. 23
Table 2: Ligands with NR2a and their binding energies..... 33

List of Abbreviation

DM	Diabetes mellitus
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
Mt	Mitochondrial
CSF	Cerebrospinal Fluid
GSK-3	Glycogen Synthase Kinase 3
IDE	Insulin Degrading Enzyme
RNS	Reactive Nitrogen Oxidative Species
ROS	Reactive Oxidative Species
AGEs	Advanced Glycated end Products
RAGE	Receptor for Advanced Glycated end Products
DAG	Di acyl glycerol
PKC	Protein Kinase C
IL-6	Interleukins-6
TNF	Tumor Necrosis Factor-alpha
ChE	Choline Esterase
NMDA	N-methyl-D-aspartate
WHO	World Health Organization
NIDDM	Non-Insulin-Dependent Diabetes mellitus
SOD	Superoxide Dismutase
STZ	Streptozotocin
CA	Cassia angustifolia
NGS	Nigella sativa
MWM	Morris Water Maze
cDNA	Complementary DNA
qPCR	Quantitative Real Time Polymerase Chain Reaction
SAS	Statistical Analysis System
LTP	Long Term Potentiation
LTD	Long Time Depression
AD	Alzheimer's Disease

CHAPTER 01
INTRODUCTION

Diabetes mellitus (DM), a multifaceted ailment, has been found to be associated with cognitive impairment and is characterized by persistent hyperglycemia. The global population of individuals with DM is estimated to be approximately 463 million. DM can impact various bodily organs such as the skin, nerves, arteries, kidneys, and retina. Given its significant deleterious impact on the human body resulting in retinopathy, neuropathy, and arteriosclerosis, DM has garnered international attention. Recently conducted studies have brought attention to the cognitive complications of diabetes (Feinkohl et al., 2015). Research has shown that Type 2 diabetes mellitus (T2DM) is particularly linked to heightened threat of Alzheimer's disease and vascular dementia (Ing et al., 2009 and Biessels GJ's).

There exist numerous risk factors that may contribute to the onset of DM and can ultimately influence the optimal functioning of the brain. These risk factors encompass a wide spectrum of diabetes-specific risk factors, which include insulin resistance, insulin deficiency, and hyperglycemia, DM-related vascular risk factors such as obesity, hyperlipidemia, and high blood pressure, and microvessel risk factors, such as stroke. According to a study conducted in 2002, these risk factors significantly impact cognitive function (Schoenle et al., 2002). While the exact mechanism linking diabetes to cognitive impairment remains unclear, certain theories hypothesize that oxidative stress and insulin receptor-mediated signal transduction may play a vital role.

DM can be classified into two distinct types i.e., Type 1 and Type 2 diabetes. Type 1 diabetes (T1DM), also known as insulin-dependent diabetes, has historically been referred to as juvenile-onset diabetes due to its propensity to develop during childhood. T1DM, an autoimmune disorder, is characterized by the targeting of the pancreas by antibodies within the body, resulting in a disability to produce insulin due to organ injury. The onset of this diabetes subtype can be attributed to genetic factors, as well as complications with insulin-producing cells within the pancreas (Eisenbarth et al., 2000). Diabetes, in general, has the potential to inflict damage upon the small blood vessels found in the eyes (diabetic retinopathy), nerves (diabetic neuropathy), and kidneys (diabetic nephropathy), which may consequently lead to plenty of health complications. Furthermore, the risk of heart disease and stroke is amplified in individuals who have T1DM.

Type 2 diabetes (T2DM), previously called non-insulin-dependent diabetes, has experienced a surge in prevalence among children and adolescents in recent years, partially attributable to the growing population of overweight or obese young individuals. Approximately 90% of diabetics fall under the category of Type 2. In most cases of T2DM, the pancreas produces some insulin, though in insufficient quantities or with an inability to be properly utilized by the body. Insulin resistance, characterized by the unresponsiveness of cells to insulin, typically affects adipose tissue, liver, and skeletal muscle cells. Although T2DM is often more manageable than T1DM, it still poses a significant threat to one's health, particularly in terms of the fragility of the blood vessels in the nerves, eyes and kidneys. Besides, T2DM increases one's risk of heart disease and stroke as well.

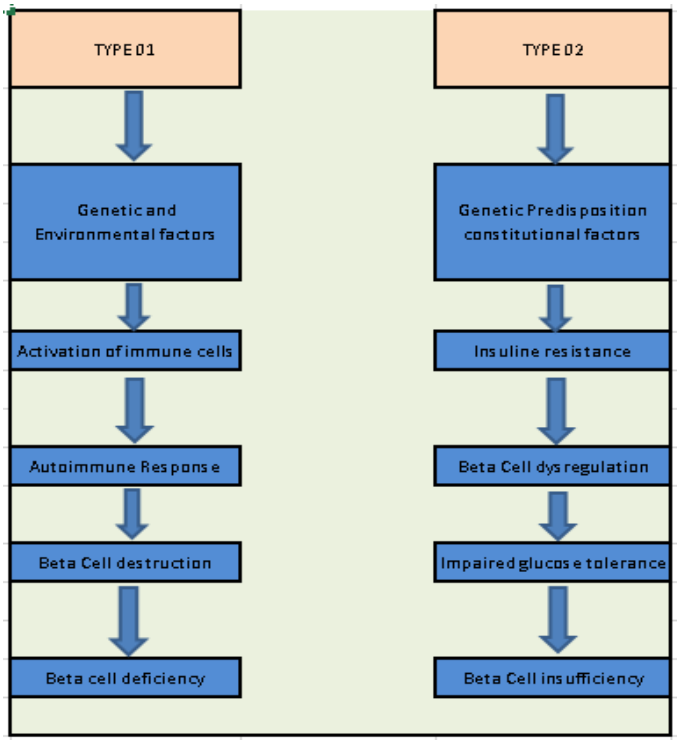


Figure 1: Pathological events leading to T1DM and T2DM. The diagram depicts that both hereditary and environmenta; factors contribute to the development of T1DM. Genetic predisposition and constitutional variables contribute to the development of T2DM.

1.1 Diabetes Mellitus (DM) and Cognitive Impairments

DM has been linked to decreased performance across a number of cognitive function categories. Diabetes can cause early-stage cognitive issues, and metabolic syndrome impairs them. The duration of diabetes and glycemic control may have an impact on the kind and severity of cognitive

impairment. Deficiencies in mitochondrial (Mt) metabolism, autonomic function, neuroinflammatory pathways, and insulin signalling are all part of the pathogenesis. There is mounting evidence that diabetes causes dementia-causing cognitive deterioration. The information as a whole indicates a stronger correlation between dementia and diabetes.

1.1.1 Insulin resistance and deficiency

Insufficient production of insulin and resistance to insulin are the key pathological factors behind T1DM and T2DM, respectively. The involvement of insulin resistance and deficiency in the onset of diabetes-related cognitive impairment has become a significant concern for the healthcare system. It is noteworthy that the brain also produces insulin, which is capable of passing through the blood-brain barrier (Banks et al., 2004). In fact, insulin is ubiquitously distributed throughout the brain, with the highest concentrations being found in the cortex, hippocampus, and hypothalamus. In addition to regulating food intake and its homeostasis, insulin also exerts a substantial neurotrophic effect (Gerozissis et al., 2004). According to a study, any disruption in the production of insulin or the activation of its receptors results in a deficiency. In the realm of cognitive development and pedagogy, considerable attention has been devoted to the impact of insulin on memory. Clinical investigations have evinced that individuals suffering from Alzheimer's disease exhibit diminished levels of insulin in their cerebrospinal fluid (CSF) (Zhao and Alkon et al., 2001). Remarkably, the administration of intravenous insulin to these patients has been demonstrated to significantly lessen cognitive impairments, reliant upon the maintenance of fasting blood sugar levels (Steen et al., 2005).

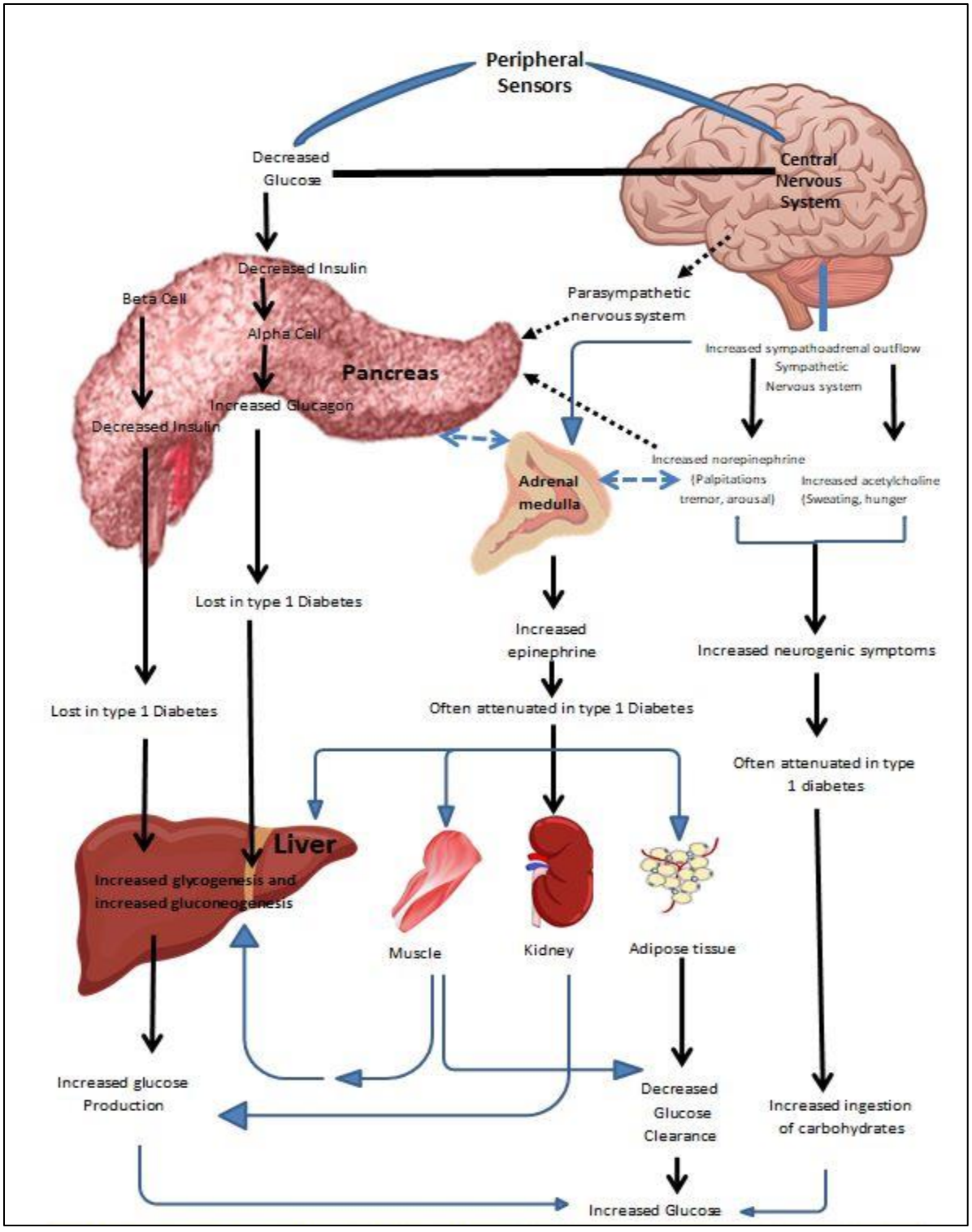


Figure 2: Physiological and Behavioral response of hyperglycemia. Hyperglycemia, which exhibits itself in various ways, is the root cause of metabolic dysfunctions affecting proteins, carbohydrates and fats.

1.1.1.1 Role of Insulin

Undoubtedly, insulin, a peptide hormone that is produced by the pancreas, plays a fundamental role as an important neurotrophic factor in the human body. However, it should be noted that individuals afflicted with DM should not have high levels of insulin. Although insulin does, in fact, operate as a neurotropic agent at a moderate level, it is crucial to acknowledge that it also leads to several consequences during hyperinsulinism in diabetes. A plethora of studies have found significant links between the aforementioned condition and several adverse effects, including but not limited to cardiovascular diseases, metabolic disorders, and even cancer. Therefore, it is imperative that healthcare providers and researchers alike continue to investigate the intricate relationship between insulin and diabetes in order to develop effective treatment strategies and improve overall patient outcomes. The risk factor of insulin resistance has been linked to cognitive decline and memory development, as well as having an impact on education. This correlation was originally demonstrated in a clinical study conducted, where it was found that patients suffering from Alzheimer's disease had lower insulin levels in their cerebrospinal fluid (CSF) (Zhao and Alkon et al., 2001). Interestingly, when insulin was administered intravenously to these patients and their blood sugar levels were kept at a fasting state, their cognitive impairment was significantly reduced (Steen et al., 2005). Undoubtedly, the significance of insulin as a neurotrophic factor cannot be overstated. However, individuals who suffer from diabetes should not be subjected to high levels of this crucial hormone. While insulin does, indeed, function as a neurotropic agent at a moderate level, it is important to note that hyperinsulinism in diabetes can result in numerous adverse outcomes. In fact, extensive research has established a correlation between insulin resistance, a major risk factor, and cognitive decline, as well as decline and dementia in diabetes patients (Young et al., 2006). When the signaling transduction system that is governed by the insulin receptor undergoes compromise, a physiological state of insulin resistance ensues within the body.

1.1.1.2 Role of GSK-3

Glycogen synthase kinase 3 (GSK-3), a fundamental enzyme, is typically phosphorylated during signal transduction mediated by the insulin receptor. Such phosphorylation ultimately leads to its deactivation, and hence, blocks insulin action. However, under circumstances of insulin resistance, the dephosphorylation of glycogen synthase kinase 3 (GSK3) results in its activation, which then

leads to tau hyperphosphorylation and an ensuing imbalance in the insulin receptor-mediated signal transduction pathway.

1.1.1.3 Role of IDE in eradicating insulin and Amyloid beta in the brain

It is additionally noteworthy that the protein known as amyloid beta peptide is created in substantial amounts (Phiel et al., 2003). In fact, the primary causes of the plaques that are present in the brain and serve as the hallmark of Alzheimer's disease are both amyloid β and tau protein.

The process of eliminating amyloid beta is considerably and significantly diminished when there is a substantial and notable presence of insulin within the brain, which serves as a definitive and decisive indication of both diabetes and cognitive impairment. This phenomenon occurs due to the competitive nature between insulin and amyloid-beta, as they both compete for the crucial and fundamental enzyme which is recognized and identified as the insulin-degrading enzyme (IDE). IDE, being a metalloproteinase by nature, is responsible for the eradication and obliteration of amyloid beta and insulin within the brain. It is a genuine and undeniable fact that insulin has a markedly higher inclination and predisposition to attach and connect to IDE as compared to amyloid beta (Phiel et al., 2003). This correlation and interconnection between DM and cognitive impairment is due to the fact that there is a notable decrease in the breakdown of amyloid β and an increase in the accumulation of amyloid β in the brain. It is indisputable and irrefutable that cognitive impairment is induced and caused by hyperglycemia, a condition that is commonly and frequently shared by both T1DM and T2DM.

Several studies of high repute have provided evidence that an elevation in the level of blood sugar inevitably leads to a deterioration in cognitive ability. One such study, focusing on preschoolers suffering from T1DM, was conducted and its findings proved to be intriguing. Children plagued by subpar glycemic management and higher levels of glycosylated hemoglobin exhibited lower motor function, poorer cognitive function, and worse receptive language scores (Patio-Fernández et al., 2010). A study was conducted on hyperglycemic rodents to investigate link between diabetes and cognitive function. The findings revealed that rats dealing with persistent hyperglycemia experienced a significant reduction in cognitive function and synaptic plasticity (Biessels et al., 1996). In fact, hyperglycemia can lower the threshold for dementia, even if it does not directly cause the condition. The process used to establish the connection between hyperglycemia and a decreased threshold for dementia is as follows.

1.1.2 Hyperglycemia

The function of oxidative stress in the progression of DM, as well as its progression and associated consequences, is widely believed to be significant. Hyperglycemia leads to elevation in the formation of reactive nitrogen oxidative species (RNS), which contribute to oxidative stress. Reactive oxygen species (ROS), which include peroxides, superoxide, lipid peroxidation, and protein peroxidation, are commonly used as indicators of oxidative stress (Comin et al., 2010). Studies have shown that this phenomenon is accompanied by a reduction in antioxidant levels, which is ultimately responsible for the cognitive deterioration observed in diabetic rats (Kuhad and Chopra, 2008). In order to alleviate these cognitive impairments and oxidative stress, antioxidants are utilized to lower glutathione levels. The aldose reductase enzyme, whose activation is stimulated by an excess of glucose in the bloodstream known as hyperglycemia, engages in the biochemical process of converting glucose present within individual cells into sorbitol, a sugar alcohol, in significant quantities. Sorbitol, due to its heightened polarity, exhibits a propensity for remaining indoors at the cellular level rather than diffusing outward into the surrounding environment. This tendency for accumulation within the cell indicates that the brains of rats suffering from diabetes display an excess of sorbitol, and this overabundance of sorbitol leads to a decline in spatial memory over the long term when compared to euglycaemic rats (Srivastava et al., 2005, Malone et al., 2007) have corroborated these earlier studies.

1.1.2.1 Increased level of AGEs

It is noteworthy to state that hyperglycemia, the condition of having high blood sugar levels, has been found to cause a significant increase in the quantity of advanced glycated end products (AGEs). The accumulation of these AGEs is observed to trigger an upsurge in the development of intracellular reactive oxygen species (ROS), which are extremely reactive molecules that can cause destruction of cells and tissues. It was revealed that rats with elevated levels of AGEs and RAGE (receptor for AGEs) in their brain's neurons exhibited cognitive abnormalities, thus providing concrete evidence of the connection between RAGE and the emergence of cerebral dysfunction (E. Wright et al., 2006).

1.1.2.2 Increased level of PKC

It is also essential to mention that DAG (diacylglycerol) and PKC (protein kinase C) are a pair of protein kinases that are involved in various cellular processes. In states of hyperglycemia, the PKC

pathway is activated, leading to the activation of different PKC isoforms. As a result of the overproduction of these PKC isoforms under hyperglycemic conditions, the excessive creation of ROS in the cells occurs, making the environment more conducive to oxidative stress. It was demonstrated that oxidative stress induced by diabetes can damage neurons and impair cognitive function (Toth et al., 2006).

To sum up, the damaging effects of oxidative stress brought on by diabetes can have detrimental effects on neurons, leading to impaired cognitive function. Therefore, it is of utmost importance to take preventive measures to manage blood sugar levels and minimize the risk of developing diabetes and its associated complications.

1.1.3 Vascular risk factors

DM, a metabolic disorder that affects glucose metabolism, is frequently accompanied by vascular conditions that have a damaging impact on cognitive and brain function. These vascular conditions include high blood cholesterol, hypertension, and obesity. Hypertension, in particular, has been identified as a significant risk factor for patients with T1DM who display impaired cognitive function, according to a study conducted by Framingham. Additionally, another study revealed that T2DM patients with chronic hypertension are more likely to experience cognitive decline (Elias et al., 1997).

Besides, it is noteworthy that a thorough retroscopic examination of a substantial number of 380,000 diabetic patients in a hospital setting has brought to light that the risk factor for hypertension has a significant impact on the likelihood of developing dementia. Furthermore, it has been established that patients with higher baseline blood pressure after a course of eight years are at a greater risk of developing Alzheimer's disease (Johnson et al., 2012). Additionally, it is pertinent to note that obesity and other vascular risk factors such as dyslipidemia have a direct correlation with cognitive performance. The research suggests that individuals with high plasma triglycerides exhibit considerably poor cognitive performance. Similarly, it has been observed that middle-aged obese diabetics exhibit worse verbal declarative memory (Bruehl et al., 2009). Admittedly, the extent of influence that vascular factors may have on cognitive health has not been fully explored. Nonetheless, it has been well-established that these vascular factors notably raise the probability of developing diabetes mellitus. There is an evidence indicating that individuals with diabetes often experience a range of cognitive difficulties, which may be due in part to

reduced blood flow in the cerebral cortex resulting from vascular illness. Such vascular illness may not only exacerbate inflammation but also lead to decreased blood flow, thereby increasing the threat of stroke and other brain-related problems. Indeed, studies have shown that people with diabetes are far more likely to have a stroke than people without diabetes mellitus, with the risk varying from 2 to 6 times higher (Luchsinger et al., 2002).

Likewise, stroke can have a significant impact on cognitive function, further intensifying the negative effects of diabetes-related complications. For instance, chronic inflammation, which is often observed in individuals with diabetes, can result in the elevation of various inflammatory markers like IL-6 and TNF-α. These markers have been associated with cognitive decline and other negative health outcomes, further highlighting the complex interplay between diabetes, vascular risk factors, and cognitive function.

Despite these findings, many questions remain regarding the precise mechanisms by which vascular risk factors contribute to cognitive decline and the emergence of cognitive deficits. Nevertheless, the evidence presented herein underscores the importance of addressing diabetes and its related complications, particularly in terms of its potential impact on cognitive function and overall health and well-being.

1.1.4 Neurotransmitters

The investigation of neurotransmitter function in rodent models with diabetes has revealed alterations that have been connected with cognitive impairment. To further explore this relationship, a study was conducted that utilized proton magnetic resonance spectroscopy to examine the concentration of neurotransmitters in the frontal brain region of patients with T1DM, specifically in relation to improved glucose levels (Lyoo et al., 2009). The findings of this study revealed the pathophysiological condition of the patients. However, it was observed that young adults with T1DM and underlying conditions showed abnormal levels of choline, myoinositol, and N-acetyl aspartate. Interestingly, patients over the age of 60 with T2DM did not exhibit any abnormality in neurotransmitter concentration. In a separate study, it was found that cholinergic neurotransmission is a critical factor in evaluating an individual's memory, learning, and cognitive function (Northam et al., 2009). It was also highlighted that choline esterase (ChE) plays an indispensable role in carrying out precise cholinergic activities. A study demonstrated that the activity of the crucial enzyme, choline esterase, witnessed a notable increase in the brains of rat

models suffering from diabetes, and this was found to be closely associated with cognitive impairment (Schmatz et al., 2009). Also, it was observed that a similar outcome emerged in another study wherein a rise in acetylcholinesterase activity in specific brain regions led to cognitive impairment in diabetic rats.

1.1.4.1 Impact of Glutamate Receptors

In addition to this, a separate study conducted on STZ-induced diabetic mice revealed that the deteriorating cognitive ability and function were a direct result of the frontal brain's defective NMDA receptor pathway. Additionally, when the glutamate receptor turnover increased in the brain of diabetic-induced rat models, it resulted in hippocampal synaptic plasticity. However, the administration of insulin could effectively counteract this condition of decreased hippocampus plasticity (Salceda, 2000).

1.1.4.2 Elevation of Glucocorticoids Levels

The interactions between the pituitary gland, hypothalamus, adrenal, and adrenal glands are collectively mentioned to as the hypothalamic pituitary adrenal axis. It is worth noting that chronic stress may obstruct cognitive function since it leads to the elevation of glucocorticoid levels. Numerous factors have been identified as having an impact on learning and memory formation when an individual is experiencing acute stress. Despite the existence of research on glucocorticoids and their effects on cognitive impairment in rodent models, a significant investigation to evaluate the impact of glucocorticoids on cognitive impairment in humans has yet to be conducted. Studies have demonstrated that elevated glucocorticoid levels or malfunction of the hypothalamic-pituitary-adrenal axis can be responsible for cognitive dysfunction and impairment (Reagan et al., 2008). A recent research study has revealed that in rat models, heightened levels of glucocorticoid corticosterone can have a negative impact on cognitive function. Notably, in rats with cognitive impairment due to diabetes, the hypothalamic pituitary adrenal axis is observed to be persistently activated. However, when glucocorticoid antagonists are administered to these rats, their cognitive losses are treated and reduced. It is worth noting that the excess amounts of corticosterone that lead to cognitive impairment also have an impact on synaptic plasticity (R. L. Wright et al., 2006).

1.2 Traditional medicines' healing effects on diabetes-related cognitive impairment

Herbal remedies, which have been utilized for centuries, are currently receiving a significant amount of attention globally. Moreover, there has been a surge in public financing for global research on traditional herbal therapy. The World Health Organization (WHO) has also been promoting the safe and efficient utilization of herbal medicines. Over an extended period of time, herbal remedies have been a dependable, satisfying, and popular choice of therapy. The market worth of herbal medicines is progressively rising each day, thereby leading to a rise in investors funding the development and study of these drugs. In traditional folk medicine, several herbal extracts have been employed for treating DM. Although some herbs possess hypoglycemic characteristics, the majority of them have been shown to have little to no effect on glycemic control in experimental trials (Xiaoham Xu et al., 2013). Chinese herbs have significantly contributed to the development of diabetes medications in recent years. Currently, there is an abundance of research literature that has been made available, which has undertaken the collection of pertinent data that pertains to the impact of herbaceous components on cognitive impairments that are related to diabetes in rat models. A plethora of studies have been published that have pointed towards the therapeutic worth of herbal constituents in mitigating the cognitive impairments that are associated with diabetes.

1.2.1 AGE attenuation using herbs

Danshensu, a bioactive compound derived from the roots of *Salvia miltiorrhiza*, has been extensively utilized in various Asian nations, including Japan, China, and Korea, for the treatment of cerebrovascular diseases owing to its remarkable pharmacological properties. In a previous investigation, sodium Danshensu, a salt form of Danshensu, was administered to diabetic rodents for a period of 84 days following the initial injection of streptozotocin. After 12 weeks of treatment, an extensive investigation was conducted on these rats, which revealed that the administration of Danshensu partially suppressed the expression of several inflammatory markers, including COX-2, RAGE, pr-38, and NF-kb (Wang et al., 2012). Moreover, the compound also demonstrated its efficacy in inhibiting the increased levels of pro-inflammatory cytokines like prostaglandin E2, IL-6, and TNF-α in the hippocampus of diabetic rats. Importantly, behavioral analysis of these diabetic rats demonstrated that the administration of Danshensu effectively lengthened their time in the water maze, indicating a decrease in the mean escape latency. Thus, it can be concluded that the administration of the bioactive compound, Danshensu, has a likely therapeutic potential for the

cure of cerebrovascular diseases. The administration of the Danshensu compound has been observed to significantly augment the memory and cognitive capabilities of rats that have been inflicted with diabetes-induced learning and memory impairment. These findings indicate that Danshensu may be a promising and viable alternative treatment option for the prevention of the impairments and cognitive dysfunction that are conventionally associated with diabetes.

1.2.2 Anti-oxidation and anti-inflammation

A multitude of research studies that exist in the literature demonstrates that inflammation and oxidative stress are the culprits behind late diabetic complications. When hyperglycemia is present, the generation of free radicals increases while the levels of antioxidants fall. Moreover, having high blood sugar levels is also correlated with an amplified inflammatory response. It was found that inflammation is the cause of both diabetes and cognitive impairment (Fukuhara et al., 2007). A theory was established that the onset of diabetes and cognitive impairment can be attributed to the presence of inflammation (Kuhad and Chopra, 2008). In this regard, ginsenoside Re, a triterpenoid saponin that is derived from Panax ginseng, is a substance with the potential to combat diabetes. The substance was found to improve the amount of time spent in a labyrinth, decrease escape latency, and exhibit a significant anti-diabetic effect. The introduction of the RE therapy to diabetic rodents developed in a noticeable decrease to the levels of glucose in fasting blood. Furthermore, inflammation and oxidative stress were significantly reduced, and the deficiencies and impairments in cognition caused by diabetes were also significantly reduced (Liu, Zhu, Li, et al., 2012).

Berberine, an alkaloid that is extracted from *Coptis chinensis*, has been found to be a highly efficacious substance in the treatment of hyperglycemia, dyslipidemia, and cognitive dysfunction in rat models with diabetes. As evidenced by a study, it has the ability to enhance the cognitive function of diabetic rats by reducing inflammation and oxidative stress. Interestingly, another substance that shares similar properties with berberine is derived from *Winthania somnifera* has been shown to effectively lower blood glucose levels and reduce inflammation, oxidative stress, motor dysfunction, and memory deficits in both diabetic rat and mouse models. In a study, it was demonstrated that this substance has the potential to be an effective therapeutic agent for the treatment of various diabetes-related complications (Bhutan et al., 2011).

1.2.3 Decreasing the Aβ levels in the brain

The present study has demonstrated that administering the renowned Chinese medicine Rhizoma anemarrhemae, an extract of *Anemarrhemae asphodeloides*, to diabetic rats caused in a noteworthy decrease in the intensities of $Aß_{40}$ and $Aß_{42}$ in the cerebral region. Upon the application of this chemical, the blood glucose levels were examined and were observed to be lower than their previous levels. These results have indicated that Rhizoma anemarrhemae has therapeutic potential in regulating blood glucose levels and justifying an accumulation in the brain, thereby implying its usefulness in managing diabetes-related cognitive impairment (Liu, Zhu, Lu, et al., 2012).

1.3 Influence of *Nigella sativa* on DM

The plant species *Nigella sativa L*., sometimes known as black seed, belongs to the Ranunculaceae genus. Over 2000 years have passed since it was first used as natural treatment in states surrounding in Mediterranean Sea. The components of black seed exhibit a wide range of natural, immunological, and pharmacological responses, but not limited to bronchodilatory, anti-inflammatory, antibacterial, hypoglycemic, and immunopotentiating activities.

The empirical evidence has evinced that the extract from *Nigella sativa* exhibits exceptional immunopotentiating, antioxidant, anti-tumor, and anti-diabetic effects. In rodents, the oil from N. sativa has been found to have exceptional analgesic and anti-inflammatory activities. The majority of the characteristics here have been primarily ascribed to the quinone components of N. sativa, among these thymoquinone is the principal active component of the essential oil derived from the black seeds. Notably, it has been shown that thymoquinone has potent antioxidant activities and to hinder the production of inducible NO synthase (Samad Alimohammadi et al., 2013).

Therefore, owing to its unique combination of bioactive compounds, N. sativa holds great promise in the treatment of diabetic animals. Numerous investigations have been conducted to examine the efficacy of N. sativa in the treatment of diabetes among diabetic animal models. In addition, it has been discovered that the crude aqueous extract of N. sativa helps to restore glucose homeostasis. Likewise, the petroleum ether extract of N. sativa has been proven to significantly reduce fasting plasma levels, while also restoring HDL cholesterol. N. sativa was shown in a study to increase the sensitivity of liver cells to insulin, which is a key factor in the emergence of non-insulin-dependent diabetes mellitus (NIDDM). (Le and colleagues) Given the fact that N. sativa possesses hypoglycemic properties, it has been traditionally used as an accepted therapy for diabetes in

various Middle Eastern countries. Therefore, owing to its unique combination of bioactive compounds, N. sativa holds great promise in the treatment of diabetic animals.

The research has been conducted with the intention of exploring the potential implications of *Nigella sativa*'s efficacy in managing diabetes. However, it is crucial to bear in mind that the establishment of *Nigella sativa*'s involvement in treating diabetes requires more comprehensive clinical trials, irrespective of the fact that some research has indicated its potential beneficial effects. The key aspects to consider in this regard include its ability to regulate blood sugar levels, its anti-inflammatory and antioxidant properties, its ability to enhance the lipid profile, as well as the level of high-density lipoprotein cholesterol.

It has been posited that the plant known as *Nigella sativa* possesses notable hypoglycemic properties, which could potentially serve to lessen elevated blood sugar levels. Research conducted on animals has suggested that extracts derived from nigella sativa, or its various active constituents, including thymoquinone, may serve to enhance insulin sensitivity and improve glucose tolerance. For individuals grappling with the burdens of diabetes, these salutary effects may prove to be quite advantageous. As is well-known, the onset and progression of diabetes are heavily influenced by oxidative stress and inflammation. However, nigella sativa has been demonstrated to have substantial anti-inflammatory effects, furthermore, is replete with a variety of potent antioxidants. Taken together, these various beneficial characteristics stand poised to mitigate the inflammation and oxidative damage that are so closely associated with diabetes.

1.3.1 Anti-oxidant properties

The existence of free radicals in the human body has the potential to generate oxidative stress, a condition that poses a threat to the well-being of various cells and consequently may result in numerous ailments. However, the utilization of antioxidants can aid in the safeguarding of the body from such stress. Based on various research studies, it has been established that the active constituents present in *Nigella sativa* and its derivatives exhibit potent antioxidant characteristics that can efficiently eliminate free radicals and mitigate oxidative damage. Therefore, the consumption of *Nigella sativa* may potentially prevent oxidative damage to cells and tissues by restricting oxidative stress and lowering the production of reactive oxygen species (ROS). This defense mechanism is applicable to several vital organs, including but not limited to the liver, kidney, heart, and brain. It has also been observed that endogenous antioxidant enzymes tend to

function more effectively when *Nigella sativa* is consumed. In the present context, it is noteworthy that the vital enzymes, namely catalase, glutathione peroxidase, and superoxide dismutase (SOD), play an indispensable role in the human body's persistent combat against the scourge of oxidative stress. It is imperative to emphasize that the aforementioned enzymes are not only crucial but also quintessential for the body's homeostasis and overall well-being.

1.3.2 Anti-inflammatory properties

The commencement and progression of numerous conditions are indissolubly linked with the prevalence of chronic inflammation. The anti-inflammatory potential of *Nigella sativa* has been extensively researched due to its efficacy in adapting inflammation. Conversely, chronic inflammation has been identified as a potential cause of diverse ailments, including but not limited to diabetes, cardiovascular complications, and certain types of cancer. *Nigella sativa* has demonstrated its anti-inflammatory properties by hindering the synthesis of cytokines, prostaglandins, and leukotrienes, which are inflammatory chemicals and enzymes. The ability of *Nigella sativa* extracts to alleviate inflammation in cell- and animal-based models has been demonstrated in several studies.

A multitude of pro-inflammatory mediators, comprising cytokines (which encompass TNF-alpha and interleukins) and inflammatory enzymes (such as cyclooxygenase-2 and inducible nitric oxide synthase), in addition to their discharge, have been discovered to be repressed by the plant nigella sativa. *Nigella sativa* could potentially be efficacious in mitigating inflammation by obstructing the initiation of these inflammatory chemicals. The immunomodulatory characteristics of *Nigella sativa* have been substantiated, with its influence on the equilibrium and operation of immune cells. It can potentially regulate the immunological responses and inhibit excessive inflammation, which is a beneficial attribute in autoimmune disorders and chronic inflammatory ailments. Moreover, the anti-inflammatory properties of *Nigella sativa* may also provide fortification to the tissues and organs against inflammation-induced damage. Diverse studies, inclusive of those that concentrate on conditions like colitis, arthritis, and asthma, have indicated evidence of this preventative impact.

1.3.3 Regulation of lipid profiles

Dyslipidemia, a condition marked by abnormal lipid levels, is a commonly observed phenomenon in individuals with DM. Pertaining to certain research studies, *Nigella sativa* has been known to

exert an ameliorative influence on lipid profiles by reducing the range of total cholesterol, triglycerides, and LDL cholesterol however simultaneously elevating levels of HDL cholesterol. This could potentially prove beneficial for individuals with DM, who are at a higher risk of developing cardiovascular complications.

Despite the promising preliminary findings, it is imperative to exercise caution while interpreting these results. Human clinical trials are scarce and largely underdeveloped, given that the majority of the research conducted so far has been confined to animal or in vitro studies. Additional research is required to regulate the optimal dosage, duration and long-term safety of *Nigella sativa* as a viable treatment option for DM.

1.4 Effect of *Cassia angustifolia* on DM

There have been numerous investigations conducted in the past aimed at discovering novel herbal remedies to alleviate diabetes, yet a more efficacious and potent herbal cure for diabetes is still in demand. *Cassia angustifolia*, commonly referred to as Senna or Indian senna, is a plant that has been historically employed in treating various medical conditions, including diabetes. However, the efficiency and safety of *Cassia angustifolia* in managing diabetes are still under investigation by scientists, and further research is imperative to ascertain its role in treating diabetes. The impact of *Cassia angustifolia* on diabetes management is a subject of ongoing scientific inquiry.

Several researchers have stated that Senna leaves, scientifically referred to as *Cassia angustifolia* Vahl., exhibit antioxidant, antihyperlipidemic, antihyperglycemic, and glucosidase inhibitory activities. It follows that the plant's properties make it competent in reducing diabetes and its complications (Deepti Kaushal Kumar Jani et al., 2019). In light of the existing gap in knowledge, the main purpose of study is to evaluate the anti-diabetic activity of *Cassia angustifolia* leaf extracts in a high-fat diet and low-dose streptozotocin-induced diabetes mellitus.

Multiple scientific investigations have indicated that *Cassia angustifolia* may possess potential anti-diabetic qualities. The plant has been found to contain anthraquinones and other substances that have hypoglycemic (blood sugar-lowering) effects. Therefore, it is plausible that *Cassia angustifolia* leaf extracts could be a viable treatment option for diabetes, although further research is needed to confirm its effectiveness.

The substances contained within *Cassia angustifolia* may potentially facilitate the release of additional insulin, enhance cellular absorption of glucose, or promote better insulin sensitivity. Additional research is necessary to determine the precise mechanisms of action exhibited *by Cassia angustifolia* and the overall effectiveness of the plant in treating diabetes.

1.4.1 Anti-oxidant properties

Cassia angustifolia is a type of plant that contains various substances with antioxidant properties. These substances include flavonoids, phenolic acids, and anthraquinones (Ahmed S.I. et al., 2016). Antioxidants are known to aid in the body's defence against harmful free radicals, which in turn reduces oxidative stress and the likelihood of cellular and tissue damage. Several studies have demonstrated the antioxidant activity of *Cassia angustifolia* extracts, which could potentially contribute to the plant's health benefits.

1.4.2 Anti-inflammatory properties

Inflammation is a natural immune response, but when it persists or becomes severe, it can contribute to the development of various disorders. To thoroughly grasp the underlying mechanisms of action and the therapeutic potential of *Cassia angustifolia* in human subjects, an extensive array of research must be conducted. It should be noted that the efficacy of *Cassia angustifolia* may be influenced by variations in dosage, formulation, and administration employed across different research studies. The efficacy of *Cassia angustifolia* may also be influenced by the variances in dosage, formulation, and administration employed across different research studies. It is noteworthy that researchers have directed their attention towards *Cassia angustifolia* owing to its potential to alleviate inflammation. Multiple investigations have documented the successful reduction of inflammatory markers synthesis and alleviation of inflammation in animal models through the use of *Cassia angustifolia* extracts. Anti-inflammatory characteristics of *Cassia angustifolia* could be recognized to presence of bioactive constituents such as flavonoids and anthraquinones.

1.5 Aims and Objectives

The present study aims to administer natural herbs, namely *Nigella sativa* and *Cassia angustifolia*, subsequent to STZ injections, and to observe enhancements in memory, neuronal loss, and synaptic proteins even in the presence of hyperglycemia. It is suggested that the inclusion of these

botanical constituents in the diabetic diet may ameliorate cognitive impairments. The aim of the project is to:

1. Examine the potential of herbal remedies such as *Nigella sativa* and *Cassia angustifolia* in mitigating diabetes-induced cognitive dysfunction.
2. Assess the expressions of molecular biomarker i.e. NR2a associated with cognitive impairment.

CHAPTER 02
MATERIALS AND METHODOLOGY

2.1 Animals

Thirty healthy male albino mice of 8 to 12 weeks were used weighing 200 to 250g each. These mice were bought from a local vendor and were put in separate cages before the start of our procedure. All animals received human care and were provided with food and water. Prior to the start of the experiment, animals were observed for two weeks and then all procedures were performed according to Ethics committee of the National University of Sciences and Technology.

2.2 Formation of the plant extract

2.2.1 *Nigella Sativa* extract

Nigella sativa oil extracted from its seed was bought from a local vendor. Using intragastric intubation, the oil was administered once daily orally in a dosage of (400 mg/kg body weight) for up to 12 weeks.

2.2.2 *Cassia Angustifolia* extract protocol

The leaves of *Cassia Angustifolia* were minced into fine powder using a grinding device. Subsequently, the 100 g of the herb's powder was subjected to extraction in 400 ml distilled boiled water for a duration of 30 minutes. Following this process, the resulting complex was filtered through Whatman No1 filter paper (1442 125, Cytiva, UK) and subsequently transmitted from the strainer centrifuge with 3500 rpm for 20 minutes. Post the filtration process, the extracts were concentrated in a hot water bath at 80°C for a period of 5 hours. The filtered extract was then stored at a refrigerated temperature of 5°C until it was utilized. Using intragastric intubation for up to 12 weeks, 150 mg/kg body weight dosage of Cassia Angustifolia aqueous extract was given.

2.3 Experimental Timeline

Animals were taken from the animal house NUST and achromatized for one week. After one week their body weight was measured. Animals were IP injected with STZ for 5 consecutive days. On 9^{th} day after the last injection of STZ, the blood glucose level of mice was measured. The animals were given herbal doses of NS and CA for 12 weeks. On 5^{th} week of dosage, the body weight of mice was again measured. After the completion of the time period of doses, weight of the body

and sugar level were measured. On 13th week, behavioral tests were performed. After all the tests conduction, animals were dissected for further histopathological testing and PCR.

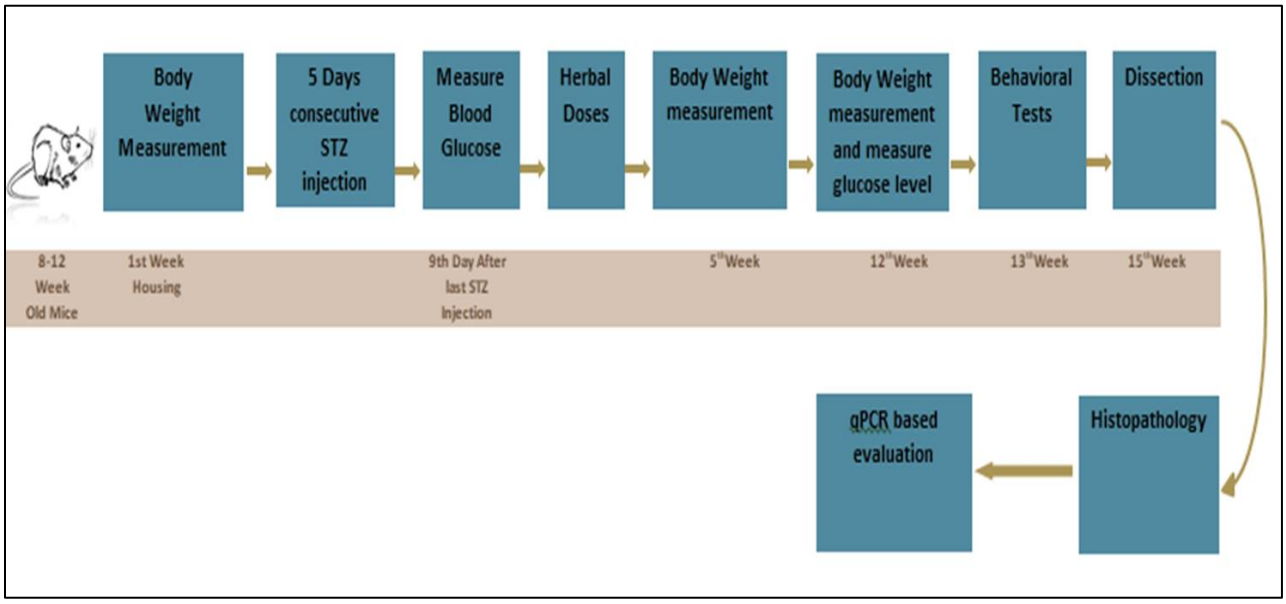


Figure 3: Timeline followed during research. The figure depicts the timeline of study. 12-week mice were injected with STZ and after the induction of diabetes were given herbal doses.

The induction of diabetes in the diabetic model was through dosage of STZ at 40 mg per kg. Before injecting streptozotocin, fasting blood sugar level of mice was observed and recorded. Thirty mice were distributed into five groups of six animals each. One of them considered as a reference group. The rest of the four groups were administered streptozotocin intraperitoneally for a period of five days. Among these four groups, one group was given *Nigella sativa* over a time period of thirty days. The other diseased group was given aqueous extracts of *Cassia angustifolia* for thirty days as well. The second last group containing diabetic mice was administered a mixture of extracts of both *Nigella sativa* and *Cassia angustifolia* for a month. The last group was left untreated as a disease control group.

2.4 Behavioral Tests

2.4.1 Object Location Test

The Object-Location Memory task serves as an assessment of cognition in mouse models of CNS diseases, with particular emphasis on spatial memory and discrimination. This evaluation is predicated on the innate preference of rodents to devote more attention to unfamiliar objects relative to those that are familiar, as well as their ability to detect when an object has been

displaced. The commencement of video recording signaled the start of the test, during which each mouse was situated in the release corner with its back against the walls. An allotted timeframe of five minutes was provided for the mice to explore the objects in question (Sophie L. Dix et al., 1999).

2.4.2 Y Maze Test

Rodents' tendency to explore novel habitats is evaluated using the Y Maze behavioral test. Mice often prefer to explore a fresh arm of the maze rather than going back to one they have already explored. Three opaque plastic arms in a Y-shaped maze with a 120° angle between them are used for testing. The three arms of the maze will be labelled as start, other and novel arm. The trial test will be recorded using a video camera and the video will be analyzed to record the number of times the animal entered the novel and other arm along with the duration of time spent in them. After each animal and between the trials, the maze will be thoroughly wiped with 70% ethanol to prevent any odour cues (Ann-Katrin Kraeuter et al., 2019).

2.4.3 Morris Water Maze Test

The Morris water maze (MWM) is a mouse latitudinal learning test that entails moving from starting points along the edge of an open swimming arena to an immersed escape stage using distal cues. The animal will undergo five days of training in a row to help it discover the tank's concealed platform. An only probe trial will be accomplished on the 6^{th} day, where the stage will be removed and the animals will be allowed to swim in the tank, trying to search the safety platform for 90 sec. Using a video camera, the trial will be recorded for video analysis, later. Using the videos, the animals' number of entries into the target quadrant, their stay there, and the number of platform crossings will all be recorded (Vorhees et al., 2006).

2.4.4 Open Field Test (OFT)

The OFT is a widely used method to evaluate an animal's ability to recognize items or stimuli, which in turn can serve as a measure of memory. In the context of rodent models of CNS diseases, The OFT is a simple sensorimotor test designed to measure general levels of activity, gross locomotor activity, and exploratory preferences. The test utilizes a square configuration for assessment purposes (Paul C. Guest et al., 2018). The test subjects, mice in this case, are placed in one of the corners of the square, and their behaviour is observed for a period of five minutes. The

number of squares filled in, as well as the animal's exploration of the outer squares near the wall and the inner squares, are counted separately.

2.5 Histopathological Assessment

Histopathological analysis was thought necessary to look at alterations in cellular activity and implement the impact of extracts on diabetic-related cognitive impairment. Brain tissue samples were taken from each mouse's cortical area and used for the analysis. Ten millilitres of neutral buffered formalin were used to fix these samples (10ml of formalin per cm3 of tissue). Following fixation, specimens were sized using a knife so they could fit into tissue cassettes with correct labelling. Using a paraffin block, tissues were processed into thin microscopic slices. In order to remove the water and formalin from the tissue, samples were dehydrated with increasing alcohol concentrations. An organic solvent, like xylene, was utilized to displace alcohol to make room for the infiltration of paraffin wax. To make the tissue visible, wax was scraped off the block's outside. Before sectioning, blocks were allowed to cool for ten minutes on a chilled plate or ice tray. Five um thick sections of tissue fragments were cut into. After being sliced, the tissue ribbons were carefully transferred to a warm water bath. Tissue segment was left intact while extra paraffin wax on the slides was gently melted by drying them upright at 37℃ for a few hours. Histochemical stains i.e., hematoxylin and eosin were used. To evaluate the slides and check the effect, light microscopy was done, and pictures were taken.

2.6 Molecular docking

In order to appraise the binding energies, binding conformations, and binding affinities of noncovalent interaction between a protein and a ligand, a methodology commonly referred to as molecular docking is employed. Using PyRx's integrated Vina Wizard module to dock ligands along with macromolecules, outcomes were found for the proteins and ligands listed in table. The accurateness and particularity of the binding molecules are increased by AutoDock Vina using multithreading on multiple core processors (Olson, 2010).

2.6.1 Interactions of Protein and Ligand

The bonding of the complex of ligands and protein were investigated by obtaining the complex using PyRx in the form of PDB and visualising it in BIOVIA Discovery Studio for 2D picture illustration.

2.6.2 Drug screening

Quercimeritrin, Rutin, and Scutellarein, three major compounds from *Cassia angustifolia*, and Thymoquinone, Carvacrol, t-anethole, and 4-terpinole, four major compounds from *Nigella sativa*, were chosen, and the structures were taken via PubChem in 2D.sdf format. A 2D picture of the bonding between the complexes was then obtained after the herb complexes were docked with the protein structures NR2a and using PyRx's Autodocking Vina Wizard. After that, discovery studio was used to visualize the drug-protein complex.

2.7 Polymerase Chain Reaction (PCR)

2.7.1 Primer Selection

NR2a protein was chosen due to its relevance to cognitive dysfunction. It constitutes the subunit of the glutamate receptor, namely the N-methyl-D-aspartic acid receptor (NMDAR). According to the literature, STZ-induced T1DM mice display reduced levels of synaptic proteins, such as NR2a, in the cortical area.

Name	Primer Sequence	Length (bp)	Temperature (°C)
NR2a Forward	**GAG ACG GTC TTG GGA TCT TAAC**	**22**	**49.9**
NR2a Reverse	**CCC ATT CCC GGT CCT TAT TC**	**20**	

Table 1: NR2a forward and reverse primer sequences. The forward primer sequence is having the length of 22bp while the reverse primer is having the length of 20bp and their annealing temperature is 49.9℃.

The sequence of primer was selected and checked using the BLAST technique (Table 1; Figure 4,5). The primer sequence was run against Mus musculus (taxonomy:10090). The mRNA showing a hundred percent specificity and similarity was selected in Nucleotide BLAST.

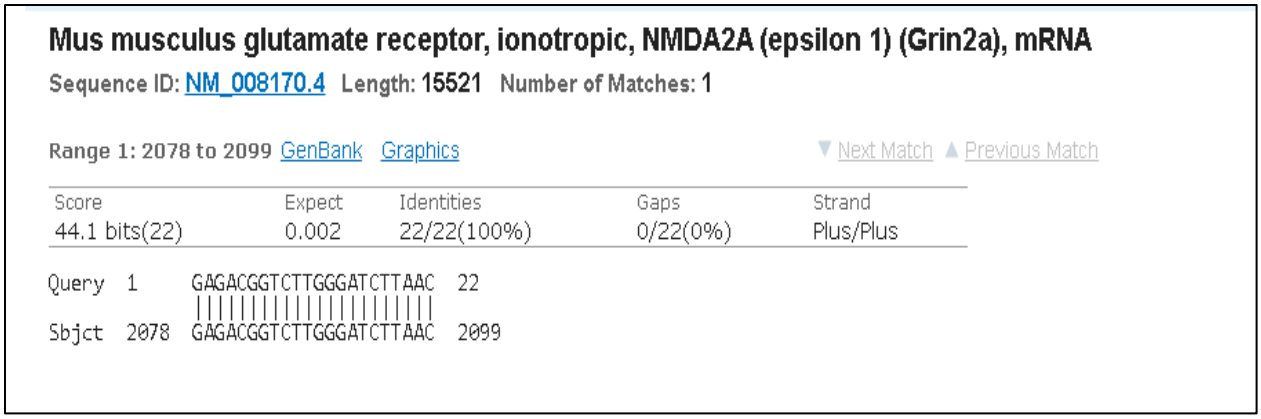


Figure 4: BLAST technique showing match for forward primer. Individual alignment of forward primer with Mus musculus genome are represented together with BLAST scores.

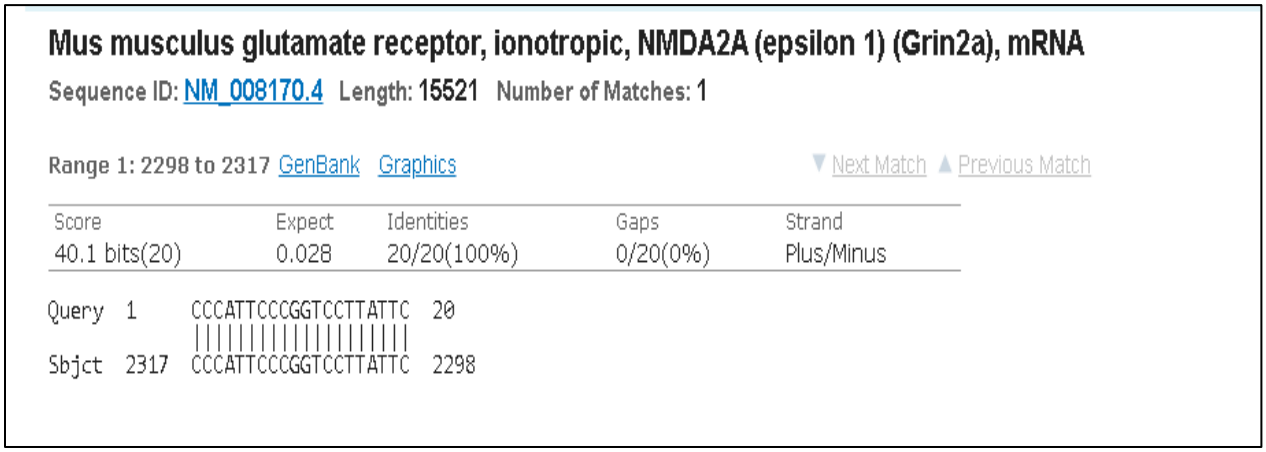


Figure 5: BLAST technique showing match for reverse primer. Individual alignment of reverse primer with Mus musculus genome are represented together with BLAST scores.

2.7.2 RNA extraction

The process of RNA extraction is carried out to ensure the preservation of RNA's structural integrity. A segment of the cerebral cortex was taken and subsequently subjected to the addition of 1000ul of Trizol solution. The process of cell lysis necessitates the homogenization of the sample, which was then left to incubate at room temperature for an interval of five minutes. Following this, the sample experienced centrifugation at a force of 12000 rpm for a time of 10 min at a temperature 4 ℃, after which the resultant supernatant was cautiously shifted into a fresh container. In order to facilitate the phase separation process, 200ul of chloroform was introduced into the solution, which was then subjected to 30 seconds of vigorous shaking prior to undergoing a second round of centrifugation under identical conditions. The extranatant was subsequently

eliminated, and the nucleotides and RNA were isolated through the utilization of 500ul of isopropanol.

The specimen underwent an incubation process lasting 10 min, at room temperature. Subsequently, samples were subjected to another centrifugation, under the same conditions. Following the elimination of the supernatant, the sediment was resuspended, and undergo a wash of 100µl with 75% ethanol. Vortexing the sample for a minute was then carried out. The sample was again subjected to centrifugation, this time for only two minutes under identical circumstances. The pellet was air-dried after discarding the supernatant, taking between 5-10 minutes. To prevent damage to the enzyme, 50µl of nuclease-free water was added, and the sample was stored at a temperature of -80°C until further processing.

2.7.3 Quality of RNA

Prior to the synthesis of complementary DNA (cDNA), all RNA samples underwent centrifugation at a rate of 12000 revolutions per minute for a duration of 5 minutes. Following centrifugation, supernatant was carefully removed, resulting pellet was re-suspended in 30 microliters of PCR water. To ensure the reliability of the RNA samples, all specimens were subjected to electrophoresis on a 2% agarose gel. Visualization of the gel was performed using a Benchtop 2UV Transilluminator (LM-20 | P/N 95044902, UVP Co., USA).

2.7.4 cDNA synthesis

The RNA that was extracted endured quantification using the Nanodrop 2000 instrument (Thermoscientific, USA). An equivalent amount of RNA (2ug) was transcribed into cDNA. To do so, 2ul of RNA, 4.5ul of 10mM dNTPs, and 4.5ul of 5 mM oligodts were utilized. The mixture was then incubated at 55℃ for a duration of 5 minutes. The next step entailed adding 12ul of RT buffer, 6ul of DTT, and 3ul of RT enzyme, along with 14.5ul of nuclease-free water. The temperature conditions were set to 37℃ for 10 minutes, 42℃ for 1 hour, and 95℃ for an additional 10 minutes.

2.7.5 Gene expression analysis by quantitative real-time polymerase chain reaction (qPCR)

The qPCR reaction was executed utilizing the ABI Prism 7300 Sequence Detection System (Applied Biosystems, 7300). Following the preparation of a reaction mixture comprising 4ul of

WizPure^{TM} qPCR Master (SYBR), 1ul of specific forward and reverse primers (Table), and 1ul of cDNA template, the volume was increased to 20ul using DNase-free water. The thermocycling settings comprised of two min at 50°C, ten min at 95°C, 40 cycles of 30s at 95°C, one min at 60°C, one min at 72°C, and a final dissociation step. In order to assess the quality of PCR effects, amplification curves and agarose gel electrophoresis were employed. The values obtained from these trials were analyzed in relation to gene expression using their ΔC_t values after all values were normalized to those obtained for ß-actin.

2.8 Statistical analysis

The results are shown as the mean and standard deviation. The Graph Pad was used to analyze the data. ANOVA variance analysis with Tukey's multiple comparison test was used to determine the significance of the comparison between the control and treatment groups using mean SEM. $p < 0.05$ was used to explain statistical significance for differences.

CHAPTER 03

RESULTS

3.1 Object Location Test

The results showed that diabetic mice spent the least time in exploration when compared to normal mice. No significant difference was observed in the exploration time of mice treated with *Nigella sativa* and extracts of both herbs. The mice treated with *Cassia angustifolia* showed significant differences as compared to diseased mice (Figure 6).

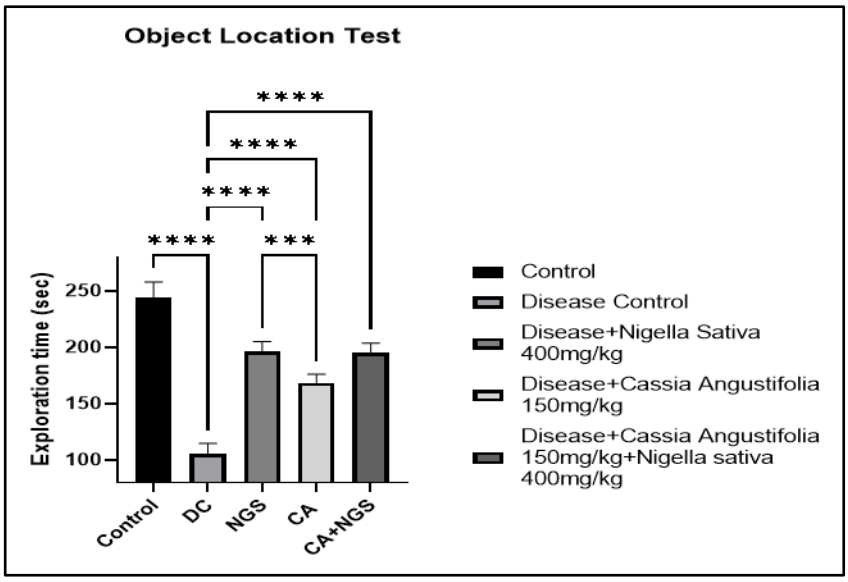


Figure 6: Exploration time in Object Location Test. This graph depicts that diseased mice spent least time in exploring the novel object. For statistical analysis, one-way ANOVA was employed followed by Tukey's multiple comparison test. SEM is displayed using error bars (***p<0.001, ****p<0.0001).

3.2 Y maze test

The findings of the Y maze test clearly demonstrated that the number of diabetic mice entering the novel arm as opposed to the other arm did not differ significantly from the other arm. While the treated mice accessed the novel arm more frequently than the alternate arm. The mice given both extracts entered both arms, but the novel arm saw the most movement (Figure 7).

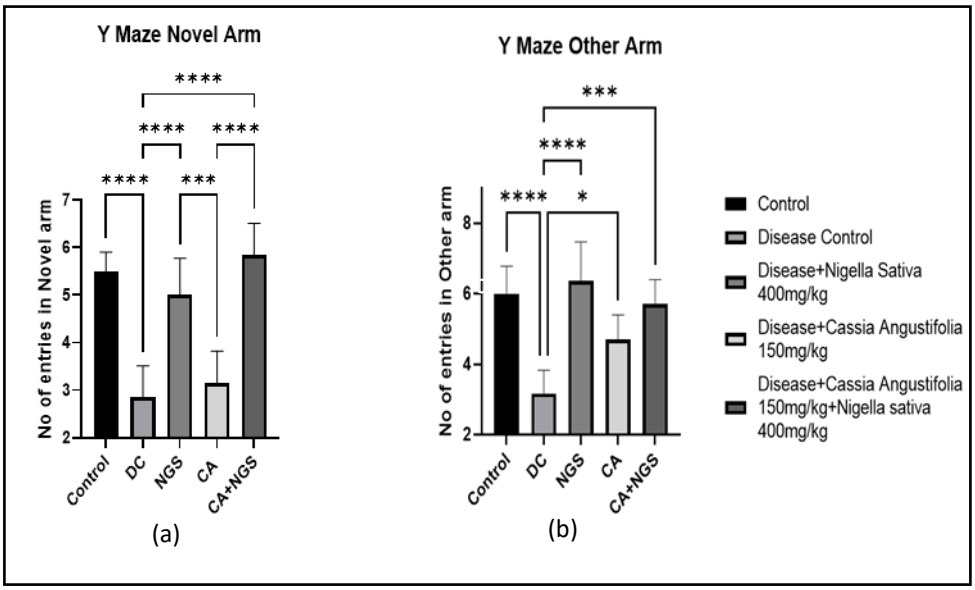


Figure 7: Number of entries in novel and other arm in Y Maze Test. These graphs depict that the number of entries of mice treated with both the extracts in novel arm is significantly more among all groups For statistical analysis, one-way ANOVA was employed followed by Tukey's multiple comparison test. SEM is displayed using error bars (*p<0.05, ***p<0.001, ****p<0.0001).

3.3 Morris Water Maze Test

During the probe test on Day 6, STZ-injected mice showed less number of entries and crossing time in target quadrant than normal mice. Diabetic mice also showed less number of platform crossings as compared to normal mice. While the treated mice were not having any significant difference in time spent or platform crossings as compared to normal mice. The mice treated with both extracts showed the maximum number of entries. NGS group didn't show any significant difference as compared to control. The number of entries of CA treated mice were in between disease and control groups (Figure 8, 9, 10).

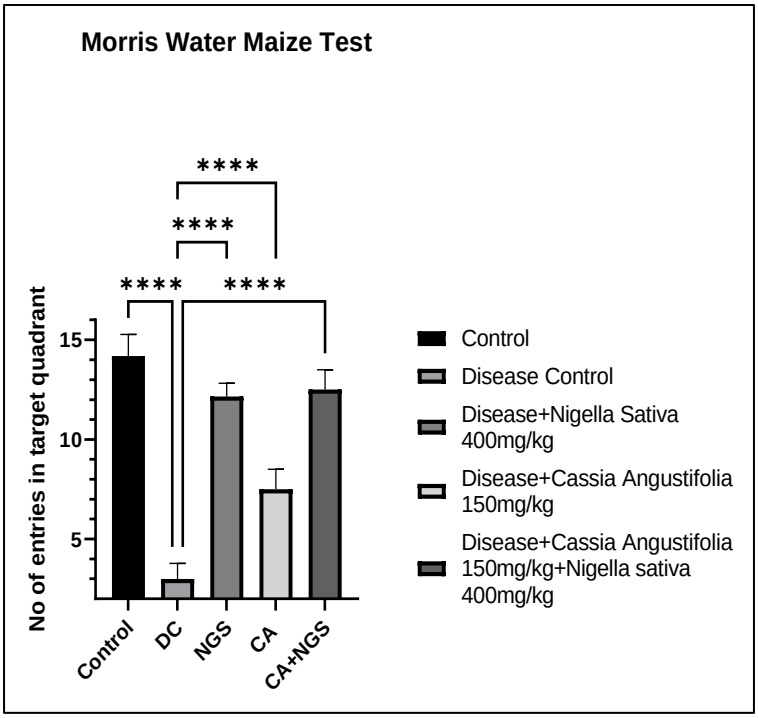


Figure 08: Number of entries in target quadrant in MWM. This graph depicts that the mice treated with NGS and CA collectively made the most number of entries in the target quadrant. For statistical analysis, one-way ANOVA was employed followed by Tukey's multiple comparison test. SEM is displayed using error bars (****p<0.0001).

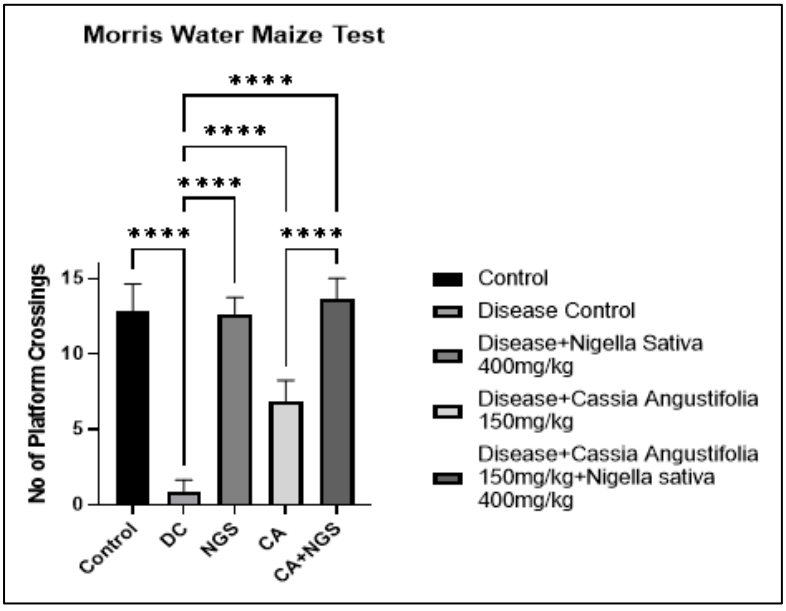


Figure 09: Number of platform crossings in MWM. The mice treated with both the extracts showed maximum number of platform crossings. For statistical analysis, one-way ANOVA was employed followed by Tukey's multiple comparison test. SEM is displayed using error bars (***p<0.001, ****p<0.0001).

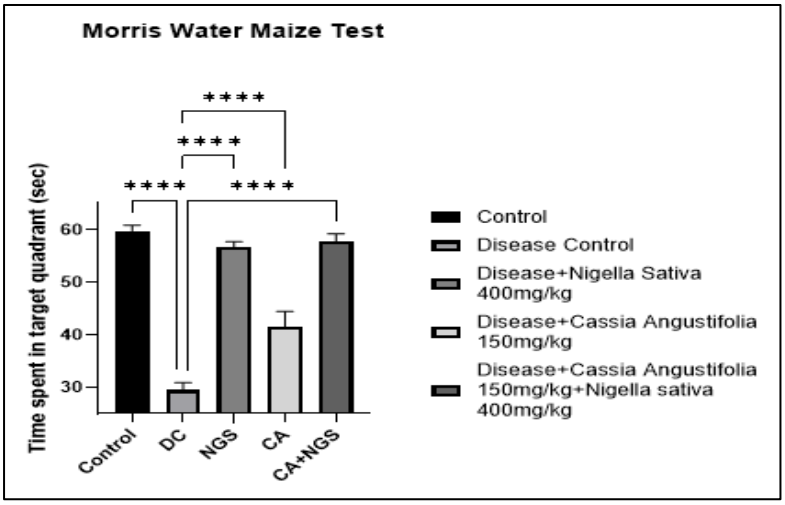


Figure 10: Time spent in the target quadrant in MWM. The graph depicts that the group treated with both the extracts spent most time in the target quadrant. For statistical analysis, one-way ANOVA was employed followed by Tukey's multiple comparison test. SEM is displayed using error bars (***p<0.001, ****p<0.0001).

3.4 Open Field Test

The results clearly show that diabetic mice were restricted to the peripheral area and their entry numbers in the center is significantly less. While the NS and CA-treated mice were likely to explore the central area. The group treated with both extracts spent maximum time in the center (Figure 11, 12).

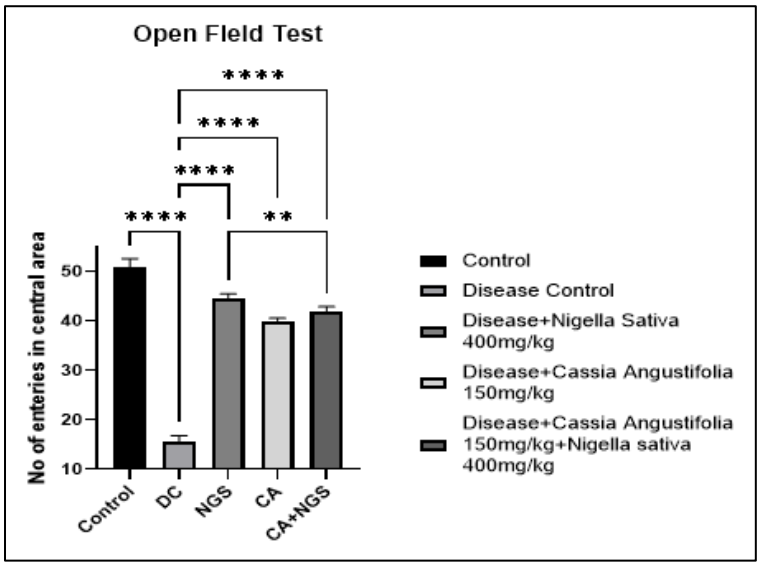
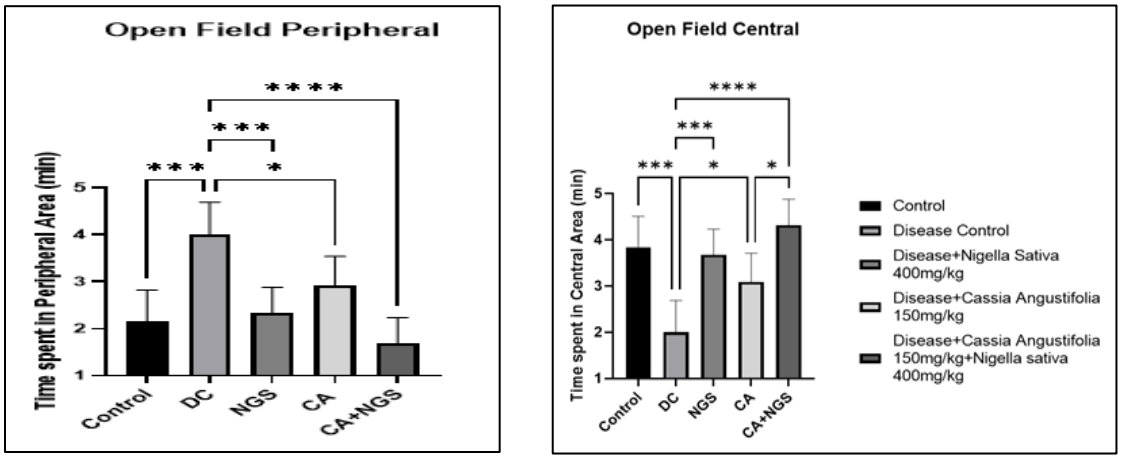


Figure 11: Number of entries in the central area in OFT. The graph showed that the group treated with NGS entered the central area the maximum number of times. For statistical analysis, one-way ANOVA was employed followed by Tukey's multiple comparison test. SEM is displayed using error bars (*p<0.05, **p<0.01, ****p<0.0001).



(a) (b)

Figure 12: Time spent in peripheral and central areas in OFT. The comparison of both graphs clearly depicts that the maximum time spent in the central area was by the mice treated with both herbs collectively. For statistical analysis, one-way ANOVA was employed followed by Tukey's multiple comparison test. SEM is displayed using error bars (*p<0.05, ***p<0.001, ****p<0.0001).

3.5 Histopathology Results

The number of cells in histopathology slides indicated the few neuronal cells in diabetic mice as compared to normal mice indicating the most neuronal loss. The mice treated with herbal extracts were not having a significant difference in the number of cell counts which indicates that our extracts can be used for the prevention of diabetes-associated cognitive impairment.

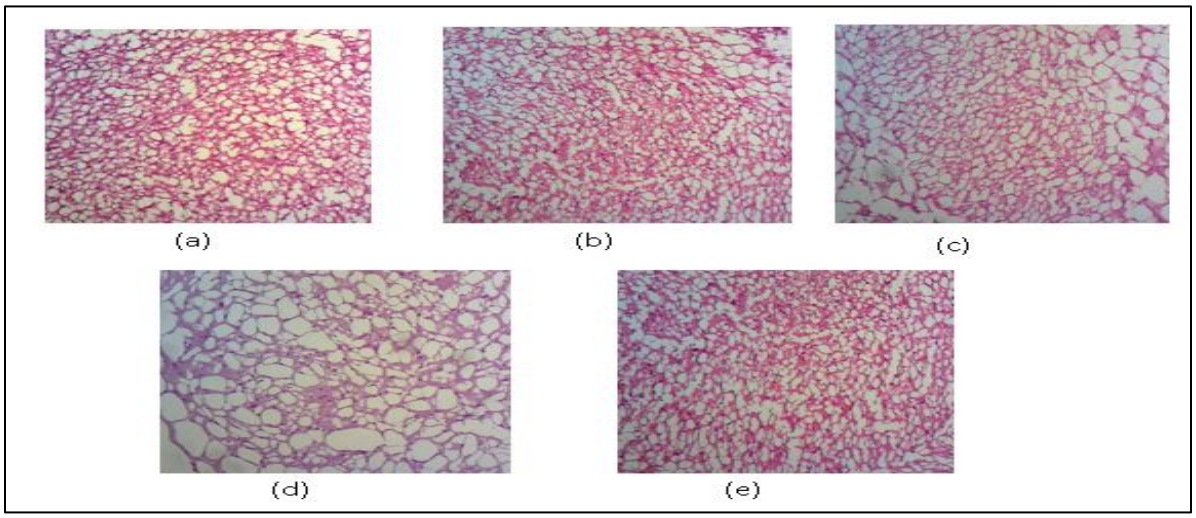


Figure 13: Brain tissues stained with Hematoxylin and Eosin. (a) Brain tissues of diabetic mice. (b) Brain tissues of mice of the control group. (c) Brain tissues of mice treated with NGS. (d) Brain tissues of mice treated with CA. (e) Brain tissues of mice treated with both extracts.

Cell nuclei stained blue-purple were observed. Brain tissues of diabetic mice showed the least number of cell counts indicating the most neuronal loss. The images were observed in a light microscope and pictures were taken from Pixel Pro software. The number of cells was counted by using Image J software (Figure 13).

3.5.1 Cell Count Graph

The results clearly show that the number of neurons is significantly less in diseased rodents in comparison with the control. The rodents given doses of both extracts are not having significant differences. The number of neurons in mice given the doses of *Nigella sativa* is close to control. The number of neurons in mice given doses of *Cassia angustifolia* is a significant difference from diseased mice but from control. The most significant difference from diseased mice is shown by mice given both extracts (Figure 14).

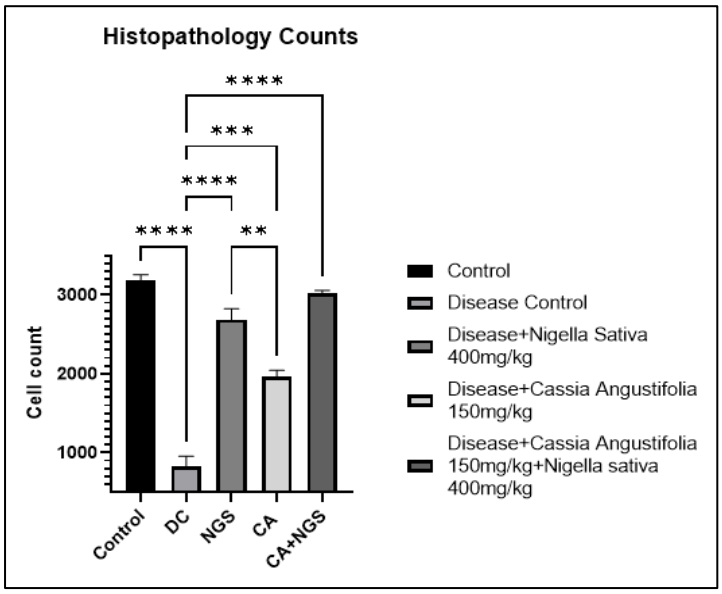


Figure 14: Cell counts after histopathology analysis. This graph depicts the maximum neuronal loss in diseased mice. For statistical analysis, one-way ANOVA was employed followed by Tukey's multiple comparison test. SEM is displayed using error bars (**p<0.01, ****p<0.0001).

3.6 Molecular Docking Analysis

The target protein was selected, and the compounds were selected as ligands. Molecular docking was done on PyRx and binding energies were attained listed in Table 2.

Ligand	Target	Binding Energy Kcal/mol
Cassia angustifolia		
(a) Quercimeritrin	**NR2a**	**-8.4**
(b) Rutin	NR2a	-7.5
(c) Scutellarein	NR2a	-7.2
Nigella sativa		
(a) 4-terpinole	NR2a	-4.9
(b) Carvacrol	**NR2a**	**-5.3**
(c) t-anethole	NR2a	-4.9
(d) Thymoquinone	**NR2a**	**-5.3**

Table 2: Ligands with NR2a and their binding energies. Compounds of Cassia angustifolia and Nigella sativa were docked with NR2a, and binding energies are listed with highest binding energies highlighted in bold.

PyRx Vina shows that Quercimeritrin among the compounds of CA displays the maximum binding energy with the target protein. In the case of NGS, Carvacrol and Thymoquinone show the highest binding energy with NR2a.

3.6.1 Binding Energy Graph

According to the comparison between the target protein and ligands, all of the ligands had greater binding affinities to the protein molecule (Figure 15). Quercimeritrin shows the highest binding energy of -8.4. 4-terpinole and t-anethole both show a binding energy of -4.9. Carvacrol and Thymoquinone show binding energy of -5.3. Rutin shows binding energy of -7.5. Scutellarein shows binding energy of -7.2. 4-terpinole and t-anethole show the lowest binding energy of -4.9 with NR2a among all.

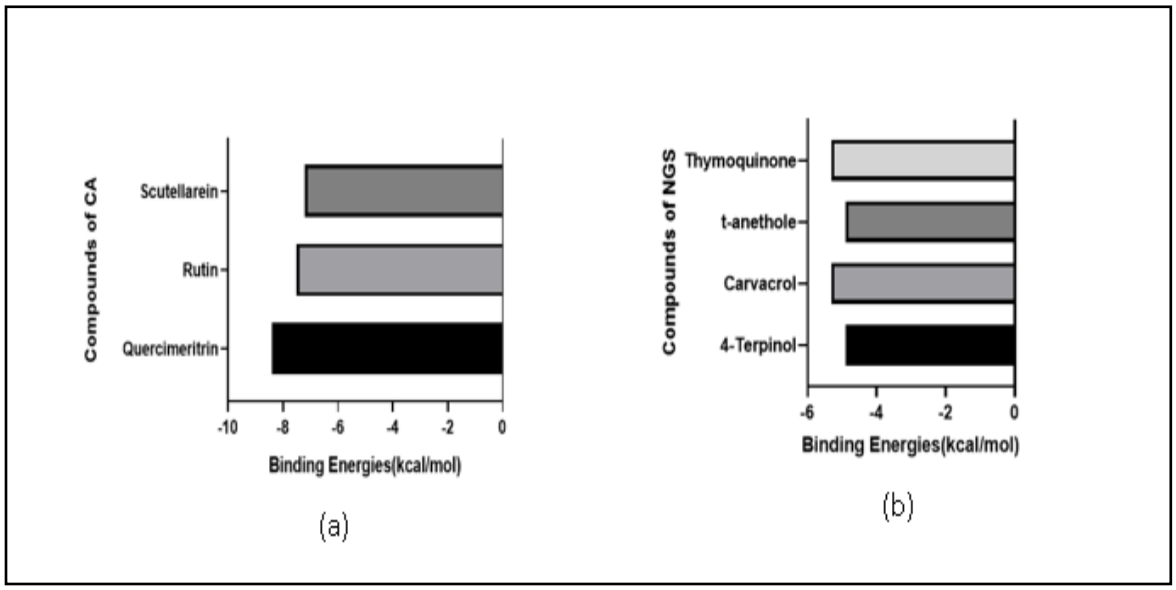


Figure 15: Binding Energy graph of NR2a and compounds of CA and NGS. (a) Binding energy graph of NR2a with compounds of CA. (b) Binding energy graph of NR2a with compounds of NGS.

The graph displays binding energies as determined using PyRx. The lowest binding energies for 4-terpinole and t-anethole are demonstrated to be -4.9 kcal/mol and -8.4 kcal/mol, that is, for Quercimeritrin.

3.7 Molecular interaction analysis

The complicated 2D diagrams showing the interactions between the protein and the ligand for the protein-ligand complex were made by Discovery Studio. The protein-ligand complex for all the compounds after being docked in Vina Wizard in PyRx was analyzed and visualized in Discovery Studio to obtain a 2D image of interactions and bonding.

3.7.1 Compounds of *Cassia angustifolia* and NR2a

The highest binding affinity with NR2a was shown by Quercimeritrin which was -8.4. Rutin and Scutellarein showed a binding affinity of -7.5 and -7.2. The protein-ligand complex for all three compounds after being docked in Vina Wizard in PyRx was visualized in Discovery Studio to obtain a 2D image of interactions and connecting to eachother. Along with Van der Waals and the unfavourable acceptor-acceptor contact, hydrogen bonds are the most noticeable interactions that exist in both carbon and conventional forms (Figure 16).

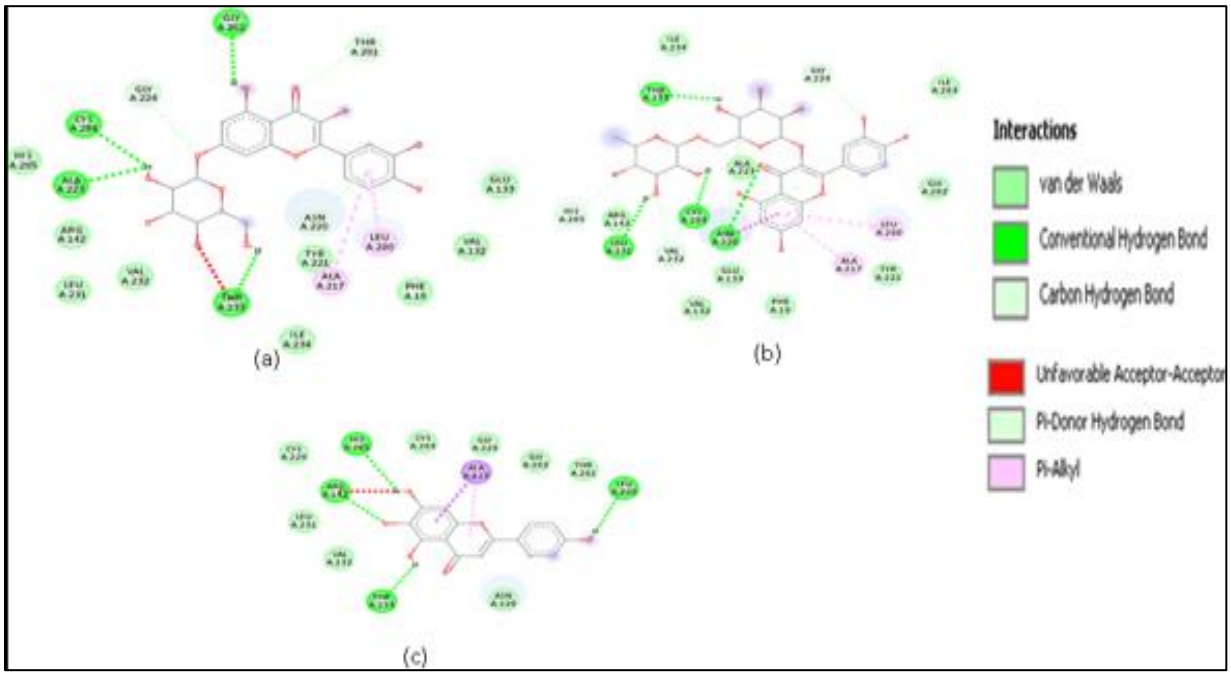


Figure 16: 2D Analysis of NR2a with compounds of *Cassia angustifolia*. (a) Quercimeritin showing interaction with protein NR2a. (b) Rutin showing interaction with protein NR2a. (c) Scutellarein showing interaction with protein NR2a.

In a 2D visualisation created by Discovery Studio, the bonds that hold the amino acids together are depicted as Van der Waals (shown in light green), hydrogen bonds (shown in dark green), carbon-hydrogen bonds (shown in blue), unfavourable acceptor-donor bonds and pi-alkyl bonds (shown in red).

3.7.2 Compounds of *Nigella sativa* and NR2a

The highest binding affinity with NR2a was shown by Carvacrol and Thymoquinone which was -5.3 for both. 4-terpinole and t-anethole, both showed the binding affinity of -4.9.

After docking in Vina Wizard in PyRx, the protein-ligand complex for each of the four compounds was visualised in Discovery Studio to create a 2D representation of interactions. The most prominent interactions in both carbon and conventional forms are hydrogen Waals, and unfavourable donor-donor interactions (Figure 17).

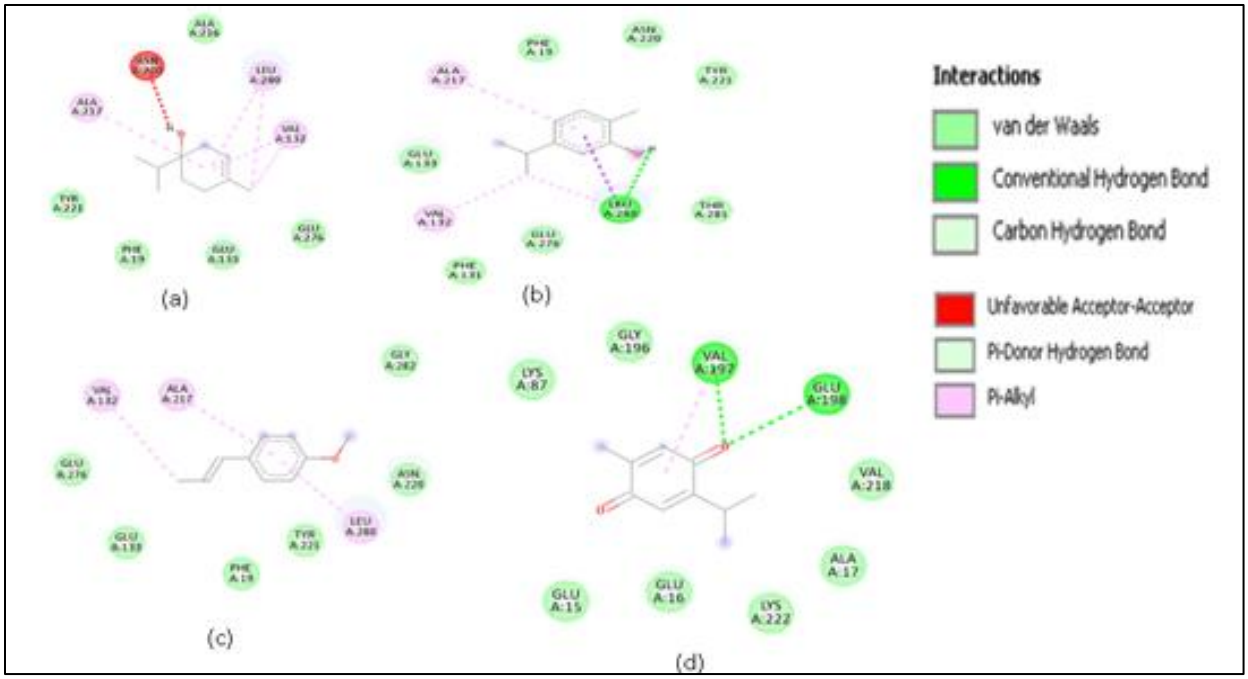


Figure 17: 2D Analysis of NR2a with compounds of Nigella sativa. (a) 4-terpinole showing interaction with NR2a. (b) Carvacrol showing interaction with NR2a. (c) t-anethole showing interaction with NR2a. (d) Thymoquinone showing interaction with NR2a.

2D visualization in Discovery Studio showing bonding amongst the amino acids including Van der Waals (shown in light green), hydrogen bond (shown in dark green), alkyl bond (shown in light pink) and Unfavorable donor-donor bond (shown in red).

3.8 PCR Optimization Results

After RNA extraction, gel electrophoresis was done to check the quality of the sample. The figure shows the results of optimization. At 239 base pairs, the bands were observed. The amplicon size was measured by studying the literature. The range of the forward and reverse primers were taken from the BLAST technique. The first range of forward primer was subtracted from the last range of reverse primer (Figure 18 and 19).

Last range of reverse primer = 2317

First range of forward primer= 2078

Amplicon size= 2327-2078= 239 bp

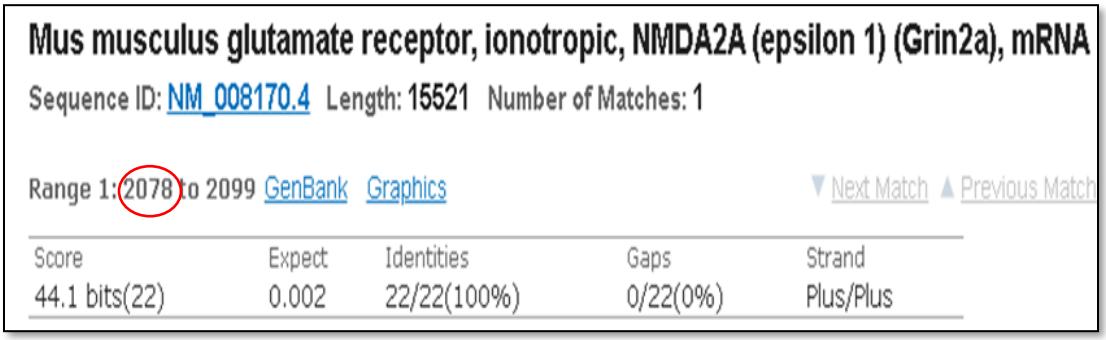


Figure 18: BLAST technique showing match for forward primer. Individual alignment of forward primer with Mus musculus genome is represented together with BLAST scores. The red mark shows the first range of forward primer.

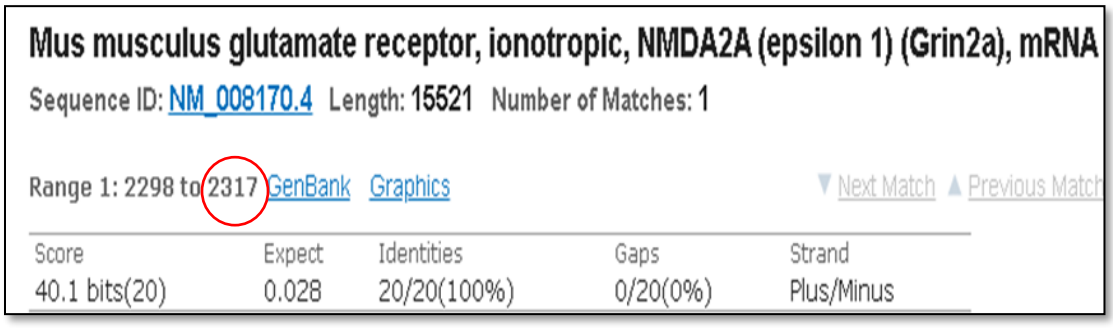


Figure 19: BLAST technique showing match for reverse primer. Individual alignment of reverse primer with Mus musculus genome is represented together with BLAST scores. The red mark shows the second range of reverse primer.

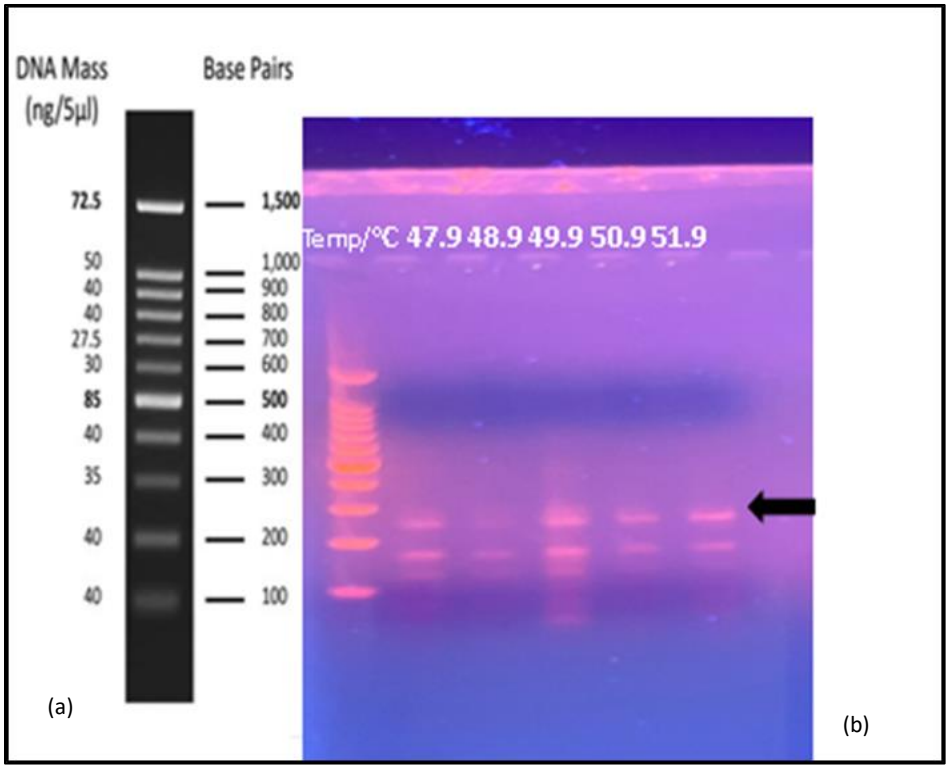


Figure 20: PCR optimization results. (a) Reference values for base pairs. (b) PCR optimization view. Five different temperatures were used to scrutinize the best annealing temperature for NR2a and bands were observed best at 49.9°C. The black mark shows the bands for annealed primer NR2a having amplicon size 239bp.

3.8.1 qPCR Based Evaluation of NR2a Expression

In order to make meaningful inferences regarding gene levels, the quantitative polymerase chain reaction (qPCR) experiment's data must first be examined. ΔCt values compare the expression of the housekeeping gene β-actin to that of the gene of interest, NR2a. The amplification curves represent the relative expression of NR2a evaluated by qPCR (Figure 21).

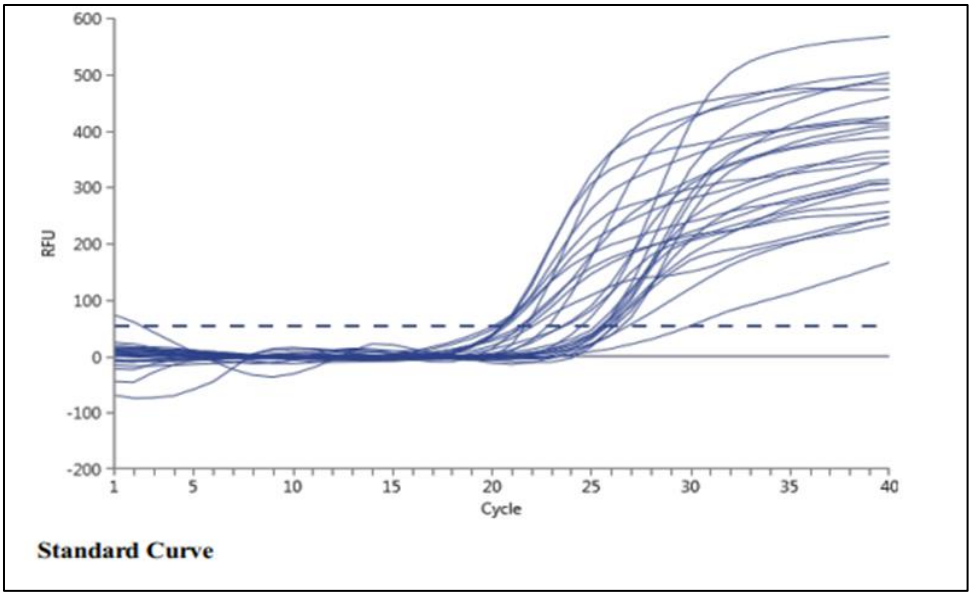


Figure 21: Standard curve. The representative amplification curves for NR2a. The graph depicts the relative expression of NR2a evaluated by qPCR.

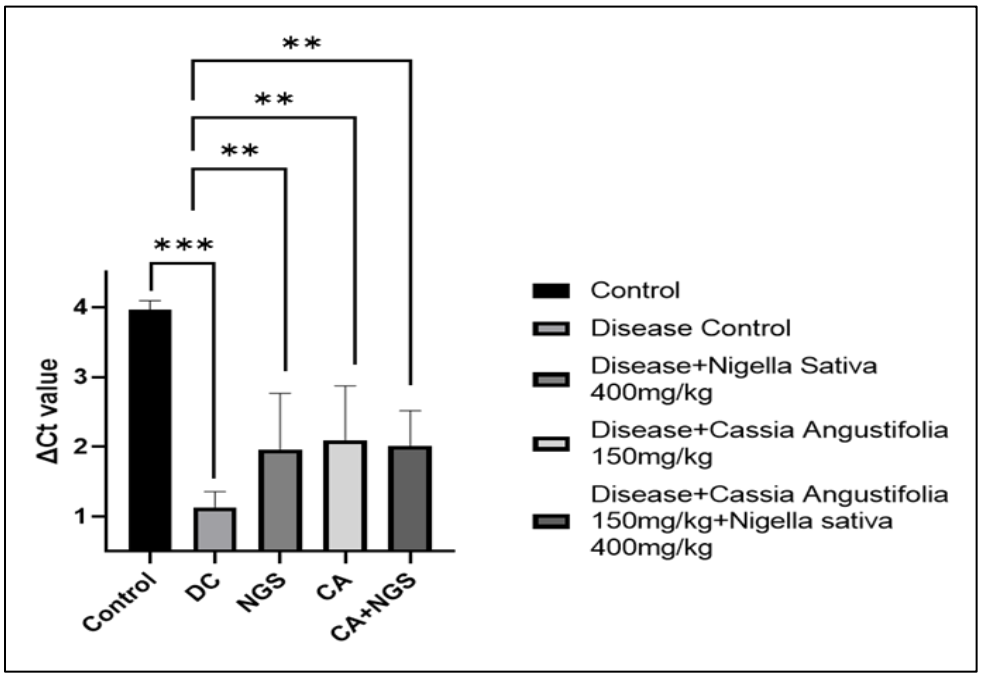


Figure 22: Relative expression of NR2a evaluated by qPCR. The graph depicts ΔCt values of all the groups after qPCR analysis. There is significant difference in NR2a protein expression of control and diabetic mice. Treated groups have shown improvement in protein expression. For statistical analysis, one-way ANOVA was employed followed by Tukey's multiple comparison test. SEM is displayed using error bars (**p<0.01, ***p<0.001).

CHAPTER 04
DISCUSSION

Diabetes is a persistent metabolic disorder that affects roughly 3 % of the global population. The mitigation of hyperglycemia over an prolonged period of time will reduce likelihood of preventing onset of microvascular diseases and reducing their possible side effects (Gaster and Hirsch, 1998). Extensive evidence indicates that diabetes elevates the likelihood of moderate cognitive impairment and accelerates the progression to dementia, hence cognitive impairment warrants classification as one of the chronic consequences of diabetes. Several risk factors have been identified as being associated with cognitive decline in diabetes, such as depression, altered neurotransmitters, hyperglycemia, hypoglycemia, insulin deficit, and insulin resistance. It is unlikely that any single cause of cognitive abnormalities among diabetic individuals is exclusively culpable, rather these factors may act in concert to disturb neuronal homeostasis, heighten neuronal susceptibility, and ultimately culminate in cognitive deterioration. Despite this, only a fraction of the pathophysiology underpinning cognitive impairment in diabetes has been elucidated (Xu et al., 2013).

The conventional pharmaceutical treatments for diabetes have a number of drawbacks, including adverse effects and high rates of subsequent failure. The efficiency of medicinal herbs is expected to be equivalent to that of traditional medication therapy but without unfavorable side effects. Thus, the aim of this investigation was to evaluate the anti-diabetic impact of Nigella extract and Senna leaves using models for T1D. The classical chemical agent known as STZ has the ability to selectively damage β-cells, which consequently results in diabetic rodent models. Due to its longer half-life, STZ is considered to be more stable and suitable for long-term research, as supported by previous studies (Rossini et al., 1977). In the present study, a T1DM model was established using continuous low-dose intraperitoneal STZ injections. As evidenced by our findings, STZ-induced mice displayed anxiety-like behavior, alongside decreased working and spatial memory. These results align with previous studies that have also reported memory loss in the T1DM model (Rom et al., 2019). Interestingly, even a little period after STZ injection, DM mice exhibited declined short-term and spatial memory, which was confirmed by our data to persist long after the initial injection. In the intervening, whilst engaged in the feeding process, we have ascertained that the rodents exhibit elevated levels of blood glucose subsequent to 14 weeks of STZ injection. This

finding implies the potential for long-standing hyperglycemia, indelible memory impairment, and irrevocable synaptic link loss. Our investigation has served to supplement the outcomes related to the enduring consequences of the injection of STZ. Cognitive and synaptic impairment display a strong correlation with T1DM. Prior research has noted a reduction in synaptic plasticity, notably long-term potentiation (LTP) and long-term depression (LTD), in young adult-onset STZ mice (Hamada et al., 2012). In this study, we assessed the protein expression levels of synapse-related proteins. NMDARs are serious for the commencement and conservation of LTP. The development of diabetic and AD neuropathy has been associated with the expression of NMDA receptors.

In the current study, it was observed that the N-methyl-D-aspartate (NMDA) receptor subunit, NR2a, was downregulated in mice induced with streptozotocin (STZ). Our findings revealed that T1DM mice exhibited impaired learning and memory, along with weakened synaptic strength, which was in line with the pathological features of Alzheimer's disease (AD) mouse models. These findings demonstrate the possibility of addressing synaptic and memory deficiencies in T1DM. The impact of both the extracts on body weight and food intake is insignificant. However, our findings indicate a significant improvement in cognitive functioning in albino mice after 12 weeks of treatment. In the current investigation, it was noted that the application of extracts manifested a noteworthy anti-hyperglycemic impact in albino mice, notwithstanding their non-representative nature with respect to human affliction. Nonetheless, these findings exhibit considerable potential, and if confirmed in forthcoming clinical trials, these extracts may emerge as a viable alternative for the management of T1D. Molecular analysis has demonstrated the existence of diverse bioactive phytoconstituents in the CA and NS extract. The extract encompasses flavonoids, namely Rutin in the CA extract and thymoquinone in NS. It has been extensively reported that flavonoids exhibit antihyperglycemic, antiobesity, and antioxidant properties (Singh R. et al., 2013). Rutin has been identified to possess anti-diabetic properties as well (Hossain M.K. et al., 2016). According to the literature, glycosides are recommended for use in antihyperglycemic activity (Singh R. et al., 2013). The discovery of these constituents within the extracts derived from the plants under study provides compelling evidence of their collaborative function in the therapeutic management of diabetes. The groups treated with extracts exhibited a significant improvement in cognitive functioning. According to research, glycosides, such as sennoside A and B, inhibit carbohydrate digestion, which partially accounts for the glucose-lowering activity of CA extracts (Choi et al., 2006). In models of diabetes, it has been demonstrated that the antihyperglycemic

properties of CA and NS extracts are efficacious, indicating that the inherent constituents may have acted independently to elicit the hypoglycemic response. Flavonoids contains the capability to restore cellular structures, while saponins enhance the signaling of insulin, prevent the formation of glucose, and impede the activity of glucosidase (Gaikwad S.B. et al., 2014). Rutin-fed diabetic animals exhibited noteworthy low level in glucose and high insulin levels (Hossain et al., 2007).

In the present research, it was observed that mice with diabetes induced by STZ exhibited a robust hypoglycemic response to the NS extract. Moreover, the NS extract's anti-hyperglycemic effect is liable on time, as per the data. This outcome is in alignment with the outcomes of research which revealed that the hypoglycemic results of black seed oil on blood glucose were similar to those of earlier studies (Fararh et al., 2002). A handful of investigations have been conducted on the bioactives and the mechanisms underpinning their anti-hyperglycemic activity. In an experimental analysis, it was determined that the enhanced insulin insensitivity in diabetic mice was attributable to black seed oil's ability to lower blood glucose levels (Alsaif et al., 2008). According to an alternative investigation, the hypoglycemic effects of black seed oil can be attributed to greater extrapancreatic impacts of insulin, as opposed to accelerated insulin production (Dakhakhny et al., 2002). Furthermore, it was suggested that the anti-hyperglycemic activity of its oil and its active compound, thymoquinone, could be a result of diminished oxidative stress (Abdelmeguid et al., 2010). The maintenance of pancreatic beta cell integrity leads to an elevation in insulin intensities. In addition, black seed oil possess various bioactive constituents, such as thymoquinone, p-cymene, pinene, dithymoquinone, and thymohydroquinone (Geng et al., 2009). The escalation in glycogen levels might be due to thess anti-diabetic properties of NS (Pari et al., 2009). The presence of free radicals has been known to exacerbate metabolic dysfunctions in individuals with diabetes (Seyedan A. et al., 2015). As such, the utilization of herbal medicines possessing antioxidant properties has been shown to diminish the metabolic consequences associated with this condition. This is supported by previous research indicating that both extracts possess significant antioxidant activity, thereby further augmenting their potential to lessen metabolic dysfunctions in diabetes (Gupta B.P. et al., 2018). The histopathological results of the brain revealed that the extracts exhibited the potential to restore the damage caused by introduction to STZ. In contrast, the groups administered with NS and CA demonstrated properties that were analogous to the control group with regard to the recuperation of brain impairment induced by STZ. Future research should investigate the downstream molecular pathways of the compounds of

Nigella sativa and Cassia angustifolia. Administration of these extracts is a natural approach with fewer side effects and more benefits. Further studies to understand its effects on DM pathology can establish them as potential preventive and therapeutic agents.

4.1 Conclusions

An association between T1DM and cognitive impairment has been previously reported and is extremely dominant in aging patients. It was confirmed that T1DM mice induced by STZ injections presented impairments in working and spatial memory. LTP induction defects and synaptic loss were also observed in these mice. Synaptic protein i.e. NR2a were found in the cortical region. Natural herbs like Nigella sativa and Cassia angustifolia were administered after STZ injections in diabetic mice and observed improvements in memory, neuronal loss and synaptic proteins even with the retention of hyperglycemia. Incorporation of these herbs in the diet of diabetic and pre-diabetic patients may improve debilitating cognitive deficits associated with T1DM.

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