Potential role of ***Rosmarinus officinalis*** on brain cognition in mice model for Type 2 Diabetes Mellitus

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Dedicated to

My loving Family

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Tahzeen Fatima

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS

DAB	3,3′ diaminobenzidine
AGEs	Advanced Glycation End Products
AD	Alzheimer’s Disease
APP	Amyloid precursor protein
HFD	High Fat Diet
Hr	Hour
IgG	Immunoglobulin G
Kg	Kilogram
Ug	Microgram
Ul	Microliter
Mmol/l	Mili moles per liter
Mg	Milligram
Min	Minutes
IDE	Insulin degrading enzyme
SVZ	Subventricular zone
SGZ	Subgranular zone
DG	Dentatae gyrus
NSCs	Neural stem cells
OB	Olfactory bulb
FGF2	Fibroblast growth factor 2
TACs	Transit amplifying cells
TIPS	Transit intermediate cells
PD	Parkinson disease
HD	Huntington’s disease
BDNF	Brain derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
PSANCAM	Polysialyted embryonic form of neural cell adhesion molecule
IGF-1	Insulin like growth factor 1

GLP-1	Glucagon like peptide 1
NTS	Nucleus tractus solitaries
RE	Rosemary extract
L	Liter
M	Molar
MWM	Morris water maze test
PBS	Phosphate buffer saline
Sec	Seconds
STZ	Streptozotocin
T2DM	Type 2 Diabetes Mellitus
GABA	γ-Aminobutyric acid

ABSTARCT

Several studies had revealed that Type 2 diabetes mellitus (T2DM) causes impairment of learning and memory and leads to dementia. T2DM negatively affects the hippocampal integrity and neurogenesis as insulin supports the fibroblast growth factor which regulates the neurogenesis process. The impaired insulin activity and secretion contributes in impairment of learning and memory. The present study elucidated the potential effects of *Rosmarinus officinalis*, commonly known as rosemary on impaired adult hippocampal neurogenesis in T2DM mice model using behavioral tests histological and immunohistochemical analysis. The male Balb/c mice were administered high fat diet and streptozotocin (STZ) (100 mg/kg i.p.) dose at 6^{th} and 9^{th} week of age. Following this, the 50 mg/kg dose of rosemary leaves extract was administered orally for 21 days starting 36 hr after the second STZ injection at 9 weeks of age. The results revealed the impairment of learning and memory associated with a decline in neurogenesis in T2DM mice. Interestingly after receiving *R. officinalis* extract diabetic mice reflect significant improvement in learning and memory and induction of neurogenesis process in hippocampus. The results are assertive of the notion that neurogenesis induced by the administration of *R. officinalis* extract lead to an improvement in learning and memory in diabetic mice. In conclusion it can be suggested that *R. officinalis* can be a potential drug for improvement of learning and memory in diabetes, however further studies are also required to explore the active constituents of *R. officinalis* that will further strengthen the preliminary finding of the present study.

CHAPTER 1

INTRODUCTION

Diabetes is a metabolic syndrome that can be characterized by hyperglycemia. Diabetes can be mainly classified into two types Type 1 Diabetes (T1D) and Type 2 Diabetes (T2D). In T1D production of insulin is not sufficient to fulfill body demand and in T2D body is not able to utilize insulin properly. Diabetes effect 422 million adults according to WHO report 2016(Global report on Diabetes 2016). Diabetes is a syndrome that affects multiple major organs such as eyes, kidney, brain and heart (Ho et al., 2013). Diabetes and its risks effecting patients, their families and health care system economically (Global report on Diabetes 2016).

Major cause of Type 2 Diabetes Mellitus is life style and genetic factors. Life style includes inactive life style, consumption of alcohol and smoking. About 55% of T2DM cases are due to obesity. Other then these factors environmental toxins can be the can also contribute towards development of T2DM (Olokoba et al., 2012).

Diabetes is controlled by number of factors that disturb function of beta cells and insulin sensitivity in tissues such as muscle, liver, adipose tissue and pancreas. Relation of beta cell dysfunction and insulin sensitivity can be explained by multi factors such as deposition of triglycerides in tissues and secretion of various inflammatory cytokines. Hyperglycemia major complication is its effect on central nervous system by which it produces a worst effect on quality of life. Insulin signaling impairment in diabetes may lead towards cognitive deficits and mental dysfunction. Optimal glycemic control and prophylactic approach is needed to prevent cognitive dysfunction in diabetes (Saedi et al, 2016).

Dementia is fifth leading cause of death. About 50-100% of diabetic patients are at higher risk to suffer dementia than non diabetic person (Mayeda et al, 2015). Dementia and some other neuropathological features can be regulated by some metabolic disturbances linked with diabetes (Ramos-Rodriguez et al., 2014). Overlaps of a lot of factors are responsible for co morbidity of diabetes and dementia (Ismail, 2010). Inactive lifestyle, diet, substance abuse are non physiological factors that can be responsible for diabetes and dementia co morbidity. Physiological factors responsible for co morbidity can be insulin resistance in the brain.

Relation between diabetes and cognitive deficits can be elaborated by the process of neuroplasticity and structure remodeling of brain. Neuroplastic changes take place in response to stress in stress sensitive region of brains such as hippocampus (McEwen, 2006). In hippocampus reduction in expression of dentric spines and synaptic protein and increase of apoptosis markers are observed due to stress (McIntyre et al., 2010;Eker and Gonul, 2010; Tata and Anderson, 2010).

Hippocampus play major role in learning and memory due to its involvement in process of neurogenesis. Neurogenesis takes place in dentate gyrus of hippocampus. Different forms of learning and memory such as pattern separation and spatial memory depends on neurogenesis process (Goodman et al., 2010; Snyder et al., 2005).

In diabetes reduction in neurogenesis can cause cognitive decline and depression. Specific treatment for diabetes related cognitive impairment and depression is not known currently. Treatments are available for depressed mood but their effectiveness in co morbidity of diabetes and depression is not clear. Although aims of clinical treatments are to maintain normal blood glucose levels to avoid the cognitive decline (Biessels et al., 2007).

Drug related side effects are major issue of pharmacological treatment. Complementary or alternative medicines such as herbal medicines may be promising source of treatment that are more beneficial, effective and produce less harmful effects than the conventional pharmacological treatments. Herbal compounds can be good source of treatment with fewer side effects to treat diabetes and related issues (Runtuwene et al., 2016).

Rosmarinus officinalis is a herb that is widely used in Brazilian folk medicine. This herb is used as sedative, antispasmodic, cardio tonic, intestinal, for treating inflammatory diseases (Nissen et al., 2000). *R. officinalis* extract have pharmacological activity as antioxidant (Inani et al., 1983), diuretic (Haloui et al., 2000), antinociceptive (González et al., 2007) and antiulcerogenic (Dias et al., 2000). *R. officinalis* extract produces noticeable anti depressant effect in animal model. *R. officinalis* interaction with neurotransmitters system is responsible for its antidepressant effects (Machado et al., 2009). Olfactory properties of *R. officinalis* can improve cognitive performance and memory quality according to some findings (Zanella et al., 2012).

Hippocampus has a significant role in memory formation. In adult brain neurogenesis occurs in dentate gyrus of hippocampus. Neurons that are formed newly have a deep impact on memory and learning. *R. officinalis* extract increase neurogenesis and in return also improves learning and memory significantly (Rasoolijazi1 et al., 2015).

In diabetes neurogenesis is decreased and can result cognitive decline (Biessels et al., 2007). Reduction of neurogenesis in diabetes is one the major factor that is responsible for cognitive decline. Agents that will increase the hippocampal neurogenesis can be the source of treatment memory and learning impairment in diabetes. *R. officinalis* extract improves learning and memory significantly by increasing neurogenesis (Rasoolijazi1 et al., 2015). By administration of *R. officinalis* extract to mice model of Type 2 Diabetes Mellitus followed by histopathological and immunohistochemical analysis will help us to understand the effect of potential role *of R. officinalis* on learning and memory in diabetes.

The present study intends to

- Identify the effects of *R. officinalis* on learning and memory in mice model of Type 2 Diabetes Mellitus through behavioral studies.
- Study the effects of *R. officinalis* on neurogenesis by histochemical and immunohistochemical analysis.

CHAPTER 2

LITERATURE REVIEW

Diabetes is characterized by unusually high plasma glucose levels and affecting multiple organs of body (Ho et al., 2013, Saedi et al 2016). Variety of secondary complication can be result of diabetes which can affect eyes, kidney, heart, and brain. Major brain complications in response to diabetes are cognitive decline and depression (Dejgaard et al., 1991).

Diabetes can be categorized mainly into two types: diabetes mellitus type 1 (T1D) and diabetes mellitus type 2 (T2D). This classification of diabetes based on body response to insulin. In T1D insulin production decreases due to damage of insulin producing pancreatic beta cells. T1D can be critical if insulin replacement therapy is not provided typically during childhood and early adolescence. In T2D insulin sensitivity is reduced in peripheral tissues and cause disturbance in insulin secretion. This deviation is generally connected with other metabolic disturbances such as hypercholesterolemia, hypertension, and obesity (Ho et al., 2013).

Type 2 DM insulin insensitivity occurs due to many factors such as of insulin resistance, decrease of insulin production, and at last pancreatic beta-cell dysfunction (Kahn, C.R et al., 1994; Robertson R.P., 1995). Hyperglycemia can be result of reduction in insulin production and increased insulin resistances (Olokoba et al., 2012).

Major energy source for brain is glucose but chronic hyperglycemia cause damage to the macrovasculature and microvasculature throughout the body. This damage includes ischemic stroke, which can lead to dementia (Gorelick et al., 2011). Chronic hyperglycemia damages microvascular tissue is by increasing reactive oxygen species, resulting in oxidative stress (Ceriello et al., 2002). Oxidative damage has also been implicated in the accumulation of beta-amyloid and neurofibrillary tangles(Nunomura et al ., 2001; Galasko et al ., 2010).

Hyperglycemia facilitates the formation of AGEs (advance glycation end products). AGEs are compounds formed by reactions between proteins, lipids, or nucleic acids and reducing sugars such as glucose that accumulate during aging (Goldin et al., 2006). In dementia AGEs are found

in amyloid plaques and neurofibrillary tangles suggesting that AGEs contribute to dementia pathogenesis (Srikanth et al., 2011; Yaffe et al ., 2011).

2.1 Insulin role in brain function:

In brain in addition to cerebral glucose utilization insulin has various function that support neuronal function including synaptogenesis, synaptic remodeling and modulating neurotransmitter levels (Craft et al., 2013). Insulin dysfunction may disturb the beta-amyloid clearance in the brain and lead to plaque formation by regulating expression of and competing for insulin-degrading enzyme (IDE), which degrades beta-amyloid in addition to insulin (Craft et al ., 2010).

Insulin has considerable effects on growth of nervous tissues. Insulin reaches central nervous system by crossing blood brain barrier by transport mechanism mediated by insulin receptors. Insulin receptors are localized at brain areas which play an important role in memory and learning such as hippocampus, entorhinal cortex and frontal areas. In DM2 a chronic hyperinsulinemia may cause down regulation of insulin receptors and cause reduction of insulin transport in brain.

Amyloid beta degradation can be reduced in hyperinsulinemia due to common degrading mechanism for insulin and beta amyloid “Insulin-Degrading Enzyme” (IDE). IDE is involved in degradation of insulin and amyloid beta. In case of DM2 hyperinsulinemia increase competition of insulin and amyloid beta for IDE and results in increased concentration of amyloid beta (Qiuand Folstein., 2006). Because IDE is more specific for insulin so hyperinsulinemia state can elevate the levels of amyloid beta and lead to accumulation of amyloid beta in brain. In result of this accumulation neurotoxic effects occurs (Craft and Watson., 2004).

Additional mechanisms through which insulin dysregulation may lead to brain dysfunction include the effects of insulin resistance and hyperinsulinemia on cerebral glucose metabolism, hyperphosphorylation of tau, vascular dysfunction, lipid metabolism, oxidative stress, and inflammation (Craft et al., 2013). Inflammation has role in development of T2D as it contributes to insulin resistance and insulin dysregulation and chronic hyperglycemia may in turn also promote inflammation (Dandona et al., 2004).

2.2 Diabetes and neurogenesis:

Neurogenesis occurs in two areas of dentate gyrus (DG) in hippocampus that are subventricular zone (SVZ) of the lateral ventricles and the sub-granular zone (SGZ) (van Praag, 2002). Neural stem cells (NSCs) migrate to olfactory bulb and differentiate into multiple types of local interneurons. NSCs are multipotent in nature and can differentiate into neurons, astrocytes, or oligodendrocytes (Palmer et al., 1999; Kukekov et al., 1999).

Insulin plays an important role in differentiation of NSCs by supporting fibroblast growth factor (FGF2). FGF2 play an important role in differentiation of NSCs (Arsenijevic et al., 2001; Hsieh et al., 2004). Newly formed neurons are integrated into OB and DG networks and indicating the considerable impact of adult neurogenesis on brain functions such as learning, memory processing, and odor discrimination (Van Praag et al., 2002; Gheusi et al., 2000).

In diabetes there are defects in insulin secretion and activity. As insulin has a significant effect on process of neurogenesis and in diabetes insulin dysfunction occurs due to which neurogenesis process impaired (Wakabayashi et al., 2016). Hippocampal morphological integrity is negatively affected by diabetes and in result volume of hippocampus reduces. As a result cognitive deficits appear in diabetes (Ho et al., 2013).

2.3 Neurogenesis process overview

In adults neurogenesis occurs in two brain areas of hippocampus: the subventricular zone (SVZ) and the sub granular zone (SGZ) of the hippocampus. The process of neurogenesis in brain is impaired in dementia and cause impairment of learning and memory (Fuster-Matanzoet al., 2013).

In adulthood mammals do not produce new neurons from precursor cells. Neurogenesis occurs in limited regions of the hippocampal dentate gyrus (DG) (Givre et al., 2003; Ernstet al., 2014). Newly formed neurons in adults function and incorporate into the brain networks (van praag et al., 2002).

Neurogenesis play a significant role in physiological functions such as learning, emotions, and memory such as pattern separation, temporal separation, high-resolution memory, fear conditioning, and synaptic plasticity (Braun et al., 2014; Aimone et al 2014).

Neurogenesis impairment can result various brain diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), stroke, epilepsy, and depression (Braun et al ., 2014; Cameron et al ., 2015).

Quiescent neural stem cells are slow growing self renewing and multipotent in nature. Divison of NSCs starts after they become activated. NSCs divide asymmetrically and produce transit amplifying cells (TACs) in the SVZ and transient intermediate progenitors (TIPs) in the SGZ. These cells after limited number of cell divisions give rise to neuroblasts. Neuroblast differentiates into newborn neurons then these neurons will integrate into brain circuit (Hsieh et al., 2012; Zaidi et al., 2009).

Variety of molecules play critical role in affecting process of neurogenesis such as morphogens, growth factors, neurotransmitters, transcription factors, and epigenetic factors (Liu and Song 2016).

Brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), and fibroblast growth factor 2 (FGF-2) are growth factors that play critical role in process of neurogenesis. Over expression of BDNF resulted in increased neurogenesis in adult DG (Scharfman et al ., 2005) and SVZ. (Zigova et al., 1998; Benraiss et al., 2001). TrkB is a membrane receptor of BDNF and its damage or loss can produce negative effects on adult hippocampal neurogenesis (Li et al., 2008).

IGF-1 have a significant effect on various neurogenesis processes including progenitor cell proliferation, neuronal differentiation, and maturation (Yaun et al 2015; Lichtenwalner et al 2001; Brooker et al 2000; Aberg et al 2003; Aberg et al 2000). In adult hippocampus IGF-1 increase cell and production of new neurons (Aberg et al 2000).

Activated FGF receptor over expression results in increase formation of new neurons and deletion of mentioned receptors cause reduction in neuronal number in mice. Interestingly, increase in activated FGF receptor in older mice produces a positive effects on neurogenesis

which is normally decreased (Kang et al 2015). In the same way FGF-2 infusion improved the production of new dentate granule cells and their dendritic growth in the hippocampus in middle-aged rats (Rai et al ., 2007). Gamma-aminobutyric acid (GABA), dopamine, glutamate, and serotonin are neurotransmitters that affect the adult neurogenesis (Faigle et al., 2013).

GABA, an inhibitory neurotransmitter is secreted from neuroblast and has role activation and proliferation of quiescent adult NSCs, granule cell maturation, and migration. $GABA_A$ ion channel is present on NSCs and their progeny. GABA inhibits NSC proliferation, forming a negative-feedback mechanism in maintaining neurogenesis homeostasis (Liu et al., 2005; Giachino et al., 2014).

2.4 Markers of neurogenesis:

Markers of neurogenesis can give different result at different steps of neurogenesis because expression of markers varies according to steps of neurogenesis (Kempermann et al., 2004; Ming and Song 2005). Nestin and glial fibrillary acidic protein (GFAP) is expressed by the newly generated cells during the proliferating phase (Fukuda et al., 2003; Filippovet al., 2003). Ki-67 is also used as proliferation marker (Scholzen and Gerdes 2000).

Precursors residing in hippocampus are progenitors having proliferative capacity and multipotent but have not self renewing ability thus unable to proliferate indefinitely. Stem cells involve in neurogenesis in hippocampus produce progenitors resides outside of hippocampus. Progenitors migrate into SGZ of DG and proliferate into new neurons (Bull and Bartlett 2005). The nestin/GFAP positive cells in proliferative stage represent progenitors. Stem cells show presence only for short time to create progenitor pool. As the dividing progenitor cells in DG are limited. The steep decline in neurogenesis with ageing could be attributed to the exhaustion of this progenitor pool.

In the SGZ, the immature neurons are formed by the differentiation of amplifying cells during differentiation and migration phase. During early differentiation phase cells are nestin-positive but GFAP-negative and highly proliferative (Kronenberg et al., 2003). At later points cells stops the production of nestin and starts the expression of doublecortin along with the polysialylated embryonic form of the neural cell adhesion molecule (PSA-NCAM) (Kronenberg et al., 2003;

Fukuda et al., 2003). Both PSANCAM and doublecortin are expressed by the immature neurons in the migratory stage in which they migrate a short distance into the granule cell layer of the DG. During axonal and dentritic targeting immature neurons send their dendrites toward the DG molecular layer and extend their axonal projections toward the hippocampal CA3 pyramidal cell layer. Neurons generated become post mitotic during this phase. Doublecortin and PSANCAM are expressed by the immature neurons (Kempermann et al., 2004; Ming and Song 2005).

Neuronal marker NeuN (neuron-specific nuclear protein) (Brandt et al., 2003) and the calcium-binding protein calretinin (Brandt et al., 2003; Llorens-Martin et al., 2006) are expressed by the post mitotic neurons. Synaptic contacts are established by the granule neurons for receiving inputs from the entorhinal cortex and for the transmission of outputs to the CA3 and hilus regions. After two to three weeks newly formed neurons become post mitotic and calretinin seems to be exchanged for calbindinin mature granule cells (Brandt et al., 2003; Kempermann et al., 2004). Calbindin is present in all mature granule cells (Rami et al., 1987; Baimbridge 1992), and neurons expressing calbindin become functionally integrated in the hippocampus (van Praag et al., 2002). NeuN is also expressed by these neurons (Kuhn et al., 1996).

2.5 Treatments that prevent deficits in neurogenesis in diabetes:

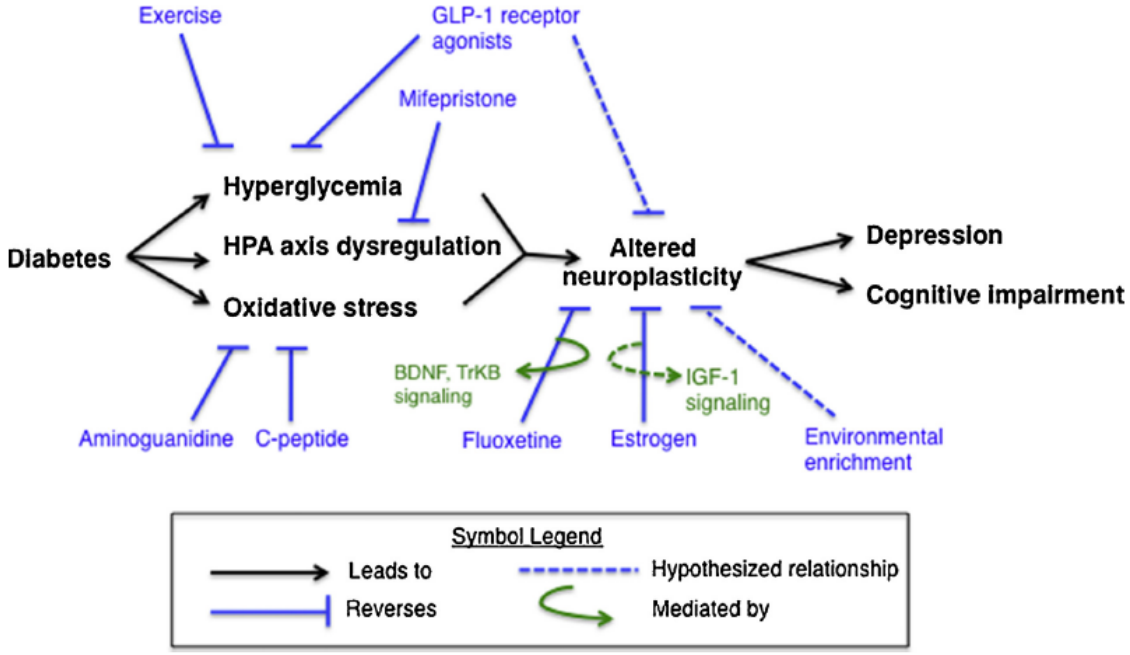

Figure 2.1 In diabetes hyperglycemia, HPA axis dysregulation and oxidative stress can lead to altered neuroplasticity in brain and result the symptoms like depression and cognitive impairment. Treating the hyperglycemia, HPA dysregulation or oxidative stress can improve the symptoms like depression and cognitive impairment. Hypothesized relationships are described with a dotted line, while solid lines support relationships that have been demonstrated by scientific evidence (adapted from Ho et al., 2013).

In diabetes fluoxetine depicts the complete reversal of cell proliferation reduction (Beauquis et al., 2006). Antidepressants increase hippocampal neurogenesis through mediation of BDNF and its receptor TrkB (Castren and Rantamaki, 2010; Pinnock et al., 2010). In diabetes fluoxetine ability to increase neurogenesis is potentiated by circulating CORT and exogenously administered glucocorticoids (Bilsland et al., 2006; Huangand Herbert, 2006). Activation of insulin-like growth factor-1 brought about by the activation of estrogen receptors on stem cells in DG promotes neurogenesis (Garcia-Segura et al., 2010).

Secretion of adrenal steroids is regulated by HPA axis. CORT low dose for maintenance of normal level of CORT after adrenalectomy prevent altered neurogenesis (Stranahan et al., 2008). According to recent study CORT act on glucorticoid receptors that result changes in hippocampal neurogenesis (Revsin et al., 2009). Glucocoticoid antagonist mifepristone also prevent hippocampal neurogenesis changes.

Environmental enrichment causes an increase in hippocampal neurogenesis and also reverses the structure and functional indications induced by diabetes (Beauquis et al., 2010; Piazza et al., 2011).

Exercise can control the hyperglycemia that can in turn increase neurogenesis. Exercise also lower blood pressure, improve brain perfusion and increasing growth factors like BDNF, insulin-like growth factor-1, and vascular endothelial growth factor (van Praag, 2008; Yi et al., 2009).

Glucagon-like peptide-1 (GLP-1) is a hormone which is involved in the modulation of glucose concentrations through the stimulation of insulin secretion and promotion of beta cell proliferation (Drucker and Nauck, 2006; Hayes, 2012). This hormone majorily produced by L-cells from the distal intestine and neurons in the nucleus tractus solitaries (NTS) of the caudal brain stem. GLP-1 is produced in the NTS, its receptor is distributed throughout the brain, including the hippocampus (Drucker and Nauck, 2006). GLP-1 analog effects include neuroprotective effects, cell survival improvement, reduce apoptosis, and increase hippocampal neurogenesis.

2.6 Pharmacological treatment vs Traditional medicine:

World Health Organization (WHO) recently defined traditional medicine as therapeutic practices (Kashaw et al., 2011). Pharmacological treatment can lead many drug-related side effects and problems. Drug related problems are observed frequently in patients receiving multiple drugs to for treatment of their medical conditions. Alternative or complementary medicines such as herbal medicine can be used for safe treatment. Herbal medicines can be a promising source of more effective and less harmful compounds that can be a part of new remedies for treating diseases (Runtuwene et al ., 2016).

2.7 *Rosmarinus officinalis:*

Rosemary (*Rosmarinus officinalis* L. family Lamiaceae) is a spice and medicinal herb. It was cultivated in Mediterranean first. Now cultivated and use all around the world (Zeng et al., 2001; Peng et al., 2007). Among the spices of genus Rosmarinus, *R. officinalis* L. is cost-effective.

Leaves of *R.officinalis* are used for food flavoring and food preservation because of its antioxidant properties. **(**Albuquerque et al., 2007). *Rosmarinus officinalis* is used as sedative, antispasmodic, cardiotonic, intestinal, for treating inflammatory diseases (Nissen et al., 2000). *R. officinalis* extract is known be pharmacologically active as an antioxidant (Inatani et al., 1983),antinociceptive (González et al., 2007), diuretic (Haloui et al., 2000), and antiulcerogenic (Dias et al., 2000). *R. officinalis* extract produces noticeable anti-depressant effect in animal model which could be attributed to its interaction with serotonergic, dopaminergic and noradrenergic systems (Machado et al., 2009). Olfactory properties of *R. officinalis* can improve cognitive performance and memory quality according to some findings. (Zanella et al., 2012). *R. officinalis* component rosmarinic acid has also reported hypoglycemic effects and has ability to treat diabetes and diabetes associated complications (Runtuwene et al., 2016).

Variety of chemical compounds that are known as plant secondary metabolites are responsible for medicinal an fragrance uses of *R. officinalis*. These metabolites include essential oils, polyphenolic compounds including flavanoids and phenolic acid derivatives. Essential oils basic constituents are 1,8-cineole, α-pinene, camphene, αterpineol, and borneol and have pharmacological effects such as antioxidant and antimicrobial and anticarcinogenic activities.

Polyphenolic compounds include flavonoids e.g., homoplantaginin, cirsimaritin, genkwanin, gallocatechin, nepetrin, hesperidin, and luteolin derivatives and phenolic acid derivatives e.g., rosmarinic acid (Habtemariam 2016). Phenolic diterpines include carnosic acid and its derivatives are main compounds responsible for *R. officinalis* extract efficacy (Rasoolijazi et al., 2015).

2.8 Effect of rosemary extract on memory:

The administration of RE improve the memory by inhibiting acetylcholinesterase activity and it can be a purposed medication for treatment of dementia (Ozarowski et al., 2013).

The phenolic diterpenes such as carnosic acid CA and carnosol are important component of RE and these components improve memory. CA is a lipophilic antioxidant ability to prevent lipid per oxidation and save biological membrane from disruption by scavenging free radicals. Hippocampus is highly sensitive to oxidative damage. As hippocampus is very important for brain cognition so normal redox state maintenance is very important for prevention of cognitive decline.

Newly formed neurons have deep impact on learning and memory. Evident study showed that animals receiving RE depict better memory and learning as compared to normal group. The improvement in memory is due to induction of neurogenesis by RE in hippocampus. There is quantitative relationship between neurogenesis and memory according to study conducted by **Drapeau et al.**

RE improves memory by increasing the activities of antioxidant enzymes at specified doses. Anti oxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) play noticeable roles in the management of oxidative stress within the cell and their activity increases in response to RE treatment (Rasoolijazi et al ., 2015).

Rosmarinic acid is another important an efficacious constituent of RE. Rosmarinic acid has multiple biological activities such as anti allergic, anti inflammatory, anti appoptic, hypoglycemic and neuroprotective effects (Ito et al., 2008; Runtuwene et al., 2016).

Rosmarinic acid effect on nervous system is that it can produce antidepressant effect by inducing cell proliferation in dentate gyrus of hippocampus (Ito et al., 2008). So it can be concluded that rosemary extract and its ingredients has reported effects on memory improvement.

CHAPTER 3

MATERIALS AND METHODS

3.1 Ethics Statement:

All experiments were performed in compliance with the rulings of the Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health, USA (Guide for the Care and Use of Laboratory Animals: Eighth Edition, 2011). All protocols employed were approved by the Internal Review Board (IRB), Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST).

3.2 Chemicals and Reagents:

Streptozotocin (STZ) was purchased from Calbiochem, Merck (Catalogue# 572201). *Rosmarinus Officinalis* was obtained from local market. Antibodies were purchased from Abcam. All other chemicals used in this study were gained from Sigma-Aldrich (USA) unless indicated otherwise.

3.3 Animals:

Male Balb/c mice (n=40) were used in this study. All animals were housed in the animal house facility of ASAB, NUST. Four mice were housed per cage and kept at constant temperature (25±2 ºC) under natural light-dark cycle (12-12 hr).

3.4 Development of Mouse Model of Type 2 Diabetes Mellitus:

Mouse model of T2DM was generated using a combination of High Fat Diet (HFD) (Table 3.1) and low doses of STZ.

Figure 3.1: High fat diet used for development of T2DM mouse model.

The controls were fed with a diet consisting of (%): crude protein 30, crude fat 9, crude fiber 4 and moisture 10.4. All animals used in this study were given distilled water.

Component	**Quantity (%)**
Basic Mice Feed	59
Sugar	20
Animal Fat	18
Egg Yolk	3

Table 3.1: Composition of High Fat Diet (Dong et al., 2013).

Animals used for the generation of T2DM were switched to HFD after weaning and rendered 100 mg/kg STZ at 6 and 9 weeks of age in 0.1 M citrate buffer (pH 4.5) after overnight fasting through intraperitoneal injections. The controls (n=10) were injected with citrate buffer only. FBG levels of animals were assessed after eight days of Streptozotocin injection using On-Call® EZ II Blood Glucose Monitoring System (Blood ACON International, USA). Mice with FBG levels higher than 7.8 mmol/L were considered diabetic (Dong et al., 2013; Li et al., 2011).

Figure 3.2: Monitoring of fasting blood glucose levels using On-Call® EZ II Blood Glucose Monitoring System (Blood ACON International, USA).

3.5 Study Design:

A 65 days long plan was formulated to generate a Type 2 Diabetes Mellitus mouse model by injecting STZ 100mg/kg at 6 and 9 week of age and giving HFD. Afterward investigate the effect of *R. officinalis* extract on neurogenesis in this mouse model. Behavioral tests were performed in the last five days, following which the animals were decapitated for histological studies

3.5.1 Animals groups for study:

Animals were randomly divided into groups. Each group had a total of 10 animals of 18-21 days of age. Details of all the groups are provided

Control group	**Diabetic group**	**Control+RE**	**Diabetic+RE**
Normal mice feed	High fat diet	Normal mice feed	High fat diet
Distilled water	Distilled water	Distilled water	Distilled water
Citrate buffer(PH 4.5)ip at 6 and 9 week of age	100mg/kg STZ in 0.1M citrate buffer (PH 4.5)ip at 6 and 9 week of age	Citrate buffer(PH 4.5)ip at 6 and 9 week of age	100mg/kg STZ in 0.1M citrate buffer (PH 4.5)ip at 6 and 9 week of age
Fasting blood glucose level monitoring	Fasting blood glucose level monitoring	Fasting blood glucose level monitoring	Fasting blood glucose level monitoring
		Rosemary extract is given through diet after 36 hours of citrate buffer injection (21 days)	Rosemary extract is given through diet after 36 hours of STZ injection (21 days)

Table 3.2: Strategy of experimental groups used in study.

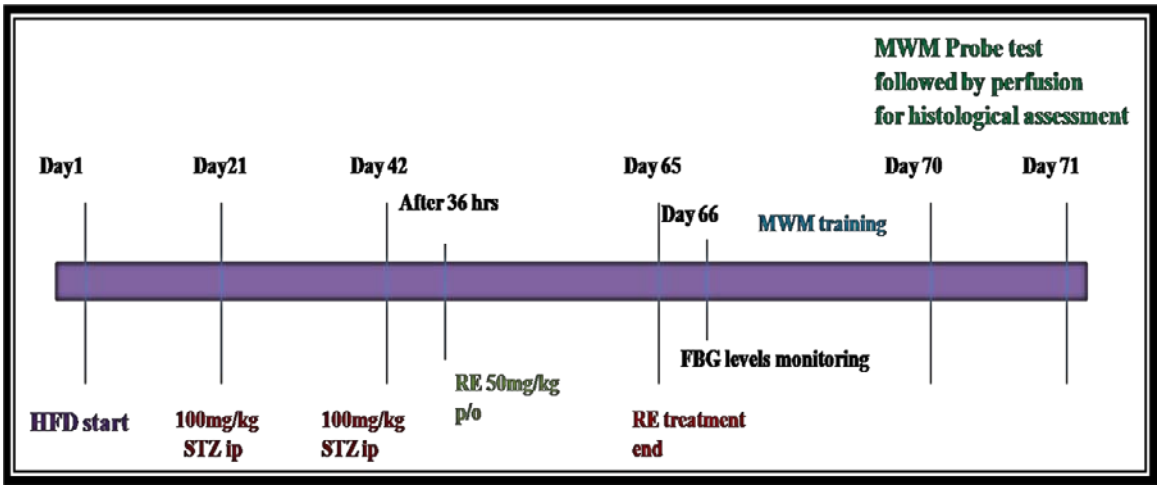

Figure 3.3: Balb/c male mice switched to HFD soon after weaning and given intraperitoneal STZ 100mg/kg at 6 and 9 week of age. After 36 hours of last STZ injection mice were switched to RE 50mg//kg orally through feed for 21 days. On day 66, animals were pre-trained in the MWM (five trials of 90 seconds each, inter trail interval of 10 minutes) with the platform protruding from the water surface. Between days 66 and 70 mice were trained to locate the hidden platform in the MWM. On day 71 they received a probe test in the MWM by placing them in the water without the platform for 90 seconds assessing the amount of time they spent in the target quadrant (where the platform used to be located) and number of entries in target quadrant.. Mice were sacrificed then transcardially perfused and their brains were processed for staining and immunohistochemistry.

3.6 Preparation of *Romarinus officinalis* extract:

Dried leaves of *R. officinalis,* which are commonly available, were procured during fall 2016 from local spice market of Rawalpindi, Pakistan and were verified by an experienced botanist. The extract was prepared using *R. officinalis leaves* (500 g) grinded into fine powder and sieved with 80 mesh sieve. The fine powder (10 g) was loaded into the main chamber of the Soxhlet extractor with distillation flasks containing extraction solvent (100% ethanol). After 24 h, the filtrate was collected and concentrated in rotary evaporator (R200 rotavapour Buchii) with reduced pressure at 68°C to afford a crude extract. The rem aining solvent extract if any, was removed by incubation at 37˚C and later stored at 4˚C until further use (Rahbardar et al., 2016).

3.7 Behavior Studies:

Morris Water Maize Test

Slight modification was done described by (Biessels et al., 1998) to conduct Morris Water Maze test. A circular water pool consists of four quadrants with 33cm depth of water (20ºC ± 2ºC). Blue dye was added to pool water to make it opaque and in North-West quadrant transparent platform was placed. Five acquisition trials per day with ten min time interval were given to each mouse for five consecutive days. Mice were released in water from different starting locations to find out platform for 90s. Mice that were successful to find out platform within 90s were allowed to sit there for 5s but those who were fail to find out platform guided towards the platform and left there for 20s. For each trial during five days average escape latency was calculated. On sixth day platform was removed to conduct probe trial test and for 90s mice were allowed to swim. Time spent in target quadrant and Number of platform crossings was calculated for each experimental group.

No. of Days	Direction of Release				
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
1	West	South	North	East	South
2	North	West	East	West	South
3	North	East	West	South	North
4	East	South	West	East	North
5	West	South	North	East	South
6	Single trial without platform, direction of release West.				

Table 3.3: Starting positions for MWM test training and spatial probe trial test**.**

3.8 Histological Examination of Brain Regional Tissues:

3.8.1 Tissue Perfusion/Fixation for Histological Assessment:

Heart perfusion was performed in accordance with the protocol of (Gage *et al*., 2012). The excised brain tissue was then placed in 4% paraformaldehyde for 24 h at 4 °C before being processed further for paraffin processing and embedding. After 24 h in 4% paraformaldehyde, the brain tissue was dehydrated through a series of alcohols (isopropanol), 70% (1h), 95% (1h), and 100% (1h) before paraffin infiltration. The brain tissues were then placed in xylene (4 h) and paraffin embedding was performed by keeping the tissue in molten paraffin (4 h at 60°C and left to solidify (4^{o}C) in mould (block formation) prior to cutting.

3.8.2 Haematoxylin and Eosin Staining:

Standard haematoxylin–eosin staining was performed on 5 µ tissue section. Tissue was deparaffinized and incubated for 8 min in Mayer's haematoxylin solution and washed in warm water for 10 min. Sections were dipped in 95% ethanol and counterstained with eosin for 30 s.

3.8.3 Congo red staining:

Congo red staining purpose is to stain amyloid protein. 1% congo red stain is prepared by mixing 1gm congo red powder and 100 ml D/W and then filter the solution. Then tissue was dewax and rehydrated in ethanol and D/W. Apply congo red stain for 25mins after this rinse several times

with D/W. In next step counter haematoxylin and eosin staining was done for 2 min. Rinse it with D/W. Dry and incubate for 20-25 min and in last step clear it in xylene solution.

3.9 Quantitative Analysis of Neurodegeneration:

Quantitative analysis of the cell number was carried out in dentate gyrus of hippocampus. The analysis was performed in an area of 10,000μm^2 from three randomly selected sites. Later,the average of values from all three sites were taken and plotted. **(**Farhat et al., 2017)

3.10 Brain Dissection & Hippocampus Isolation:

Mouse was anesthetized using chloroform. The head was gently but assertively stretched forward and a cut, posterior to the ears, was made using surgical scissors. A small incision, starting from caudal point was made followed by making a firm cut through the anterior part of the skull. Using curved narrow pattern forceps the parietal bone of both sides was tilted and broken off. The curved narrow pattern forceps was then slid under the anterior part of the brain which was gently lifted out of the skull. The removed brain was immediately transferred to a Petri dish containing pre chilled Phosphate Buffer Saline (PBS) such that the ventral side of the brain was facing the plate.

Once the brain was transferred to pre chilled PBS the first step was to remove olfactory bulb and cerebellum using scalpel. Small curved forceps was then held in a closed position between the cerebral halves. The forceps was gently opened revealing the opening of cortical halves. Once sufficient opening along the middle line was obtained, the closed forceps, at an angle of 30-40°, was directed counterclockwise and clockwise to separate the left and right hippocampus from the cortex respectively. The dissected hippocampus was transferred in a pre-chilled eppendorf and stored at -80° C till further use.

3.11 Immunohistochemistry:

5 µ sagital sections were mounted on Poly Lysine coated adhesive slides. Following rehydration (graded concentrations of ethanol), heat mediated antigen retrieval was performed by incubating sections for 35 minutes in sodium citrate (pH: 6) containing pressure cooker. The sections were subsequently washed and endogenous H_2O_2 was quenched by incubation sections in 35% H_2O_2. To minimize non specific labeling the section were incubated for 10 minutes in 5% bovine serum albumin in PBS. The tissue sections were then incubated overnight at 4°C in 0.1% bovine serum albumin in PBS containing: rabbit monoclonal antibody for NeuN (1:100; ab177487).The following day, for NeuN labeling, sections were washed and placed in 1:100 dilution of HRP conjugated anti-rabbit IgG (ab97051) for 1h at room tempertaure. The peroxidase reaction product was visualized by incubation in a solution containing 0.025% of 3,3diaminobenzidine (DAB, ab50185) for 10 minutes. Following haematoxylin counter staining, cover slips were mounted and sections were visualized by microscope (optika, Italy) at 4X, 10X and 40X resolutions. (Same procedure is repeated for Ki-67 antibody labeling).

CHAPTER 4

RESULTS

4.1 Development of mouse model for Type 2 Diabetes Mellitus:

Animals used for the generation of T2DM were switched to HFD after weaning and rendered 100 mg/kg STZ at 6 and 9 weeks of age in 0.1 M citrate buffer (pH 4.5) after overnight fasting through intraperitoneal injections. The controls (n=10) were injected with citrate buffer only. Mice with FBG levels higher than 7.8 mmol/L were considered diabetic (Dong et al., 2013; Li et al., 2011). Control mice show average fasting blood glucose levels (mmol/L) less than 7.8 mmol/L and mice fed with HFD and receiving STZ doses show average fasting blood glucose levels (mmol/L) more than 7.8 mmol/L so they can be used as Type 2 diabetic model for further studies (Figure 4.1).

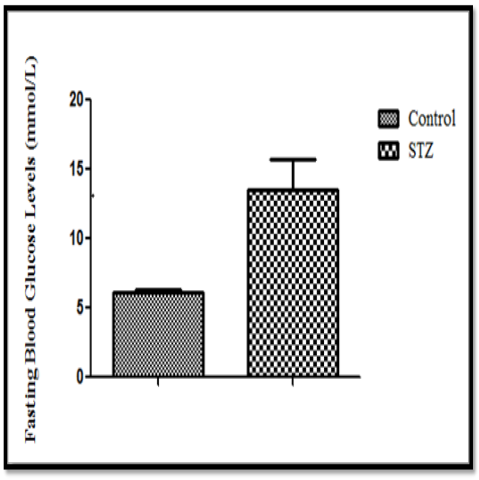

Figure 4.1:Average Fasting Blood Glucose levels (mmol/L) in control mice and mice fed with HFD from age of 3 to 10 weeks of age and rendered two doses of 100mg/kg STZ ip at 6 and 9 weeks of age

4.2 Effect of *R. officinalis* extract effect on hyperglycmia in diabetic mice:

R.officinalis (commonly known as rosemary) leaves extract treatment is administered to diabetic mice orally through mice feed. RE (rosemary extract) is given 50mg/kg for 21 days. The fasting blood glucose levels of diabetic mice receiving normal mice feed will increase from day0 to day22. FBG levels of diabetic mice receiving mice feed + RE remain almost same from day0 to day22. RE at dose of 50mg/kg helps to maintain the FBG levels of diabetic mice. Control mice receiving RE show non-significant changes in FBG levels from day0 to day22.

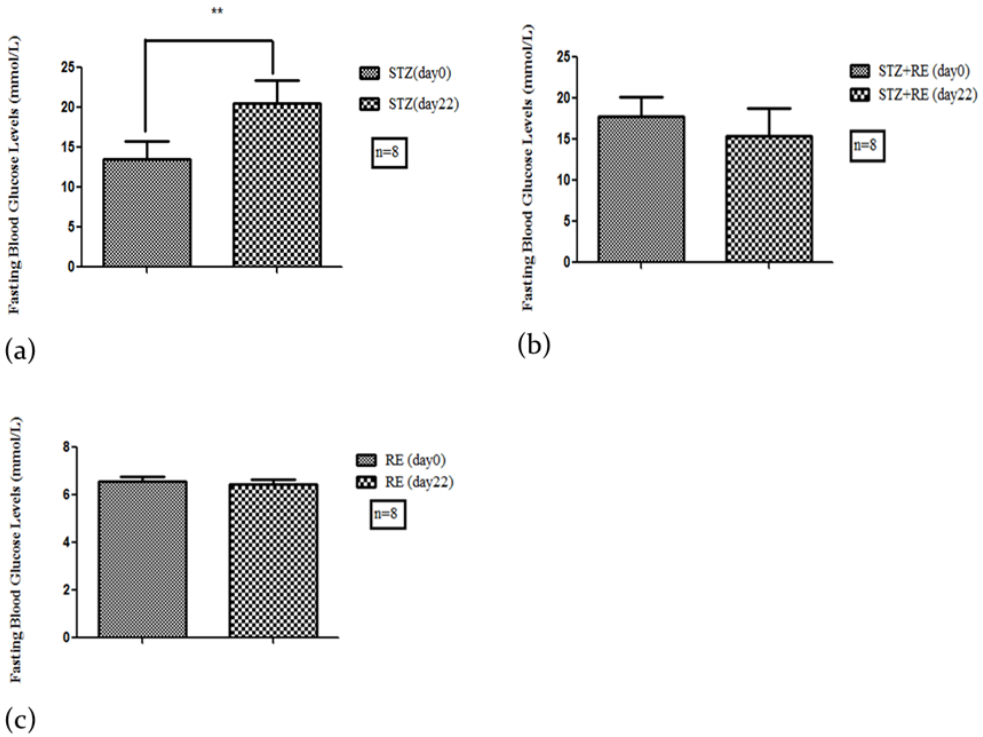

Figure 4.2: Effect of Rosemary Extract (RE) Treatment on Fasting Blood Glucose (FBG) levels: (a) The graph depicts the FBG levels mmol/L in Diabetic group. There was a significant increase in glucose levels from Day 0 to Day 22. **(b)**The graph depicts the FBG levels mmol/L in diabetic group receiving RE treatment (50mg/kg orally for 21 days) did not decrease significantly from day 0 to day 22. **(c)** The graph depicts that FBG levels did not change significantly in control mice receiving RE treatment. The data was analyzed using Student t-test. The data is shown as mean ± SEM, (p<0.05).

4.3 Behavorial analysis:

Impairment of cognitive function due to hyperglycemia:

Learning Phase

Morris water maze (MWM) test used to evaluate the cognitive function. MWM test determine the effect of diabetes and rosemary extract in diabetes on spatial learning and reference memory. Average escape latency of mice is time to reach platform is a direct reflection of spatial memory. In diabetic mice escape latency is higher than control group representing the decrease of spatial learning in diabetic mice. Average escape latency of diabetic mice receiving the RE 50mg/kg for 21 days reflect improvement in spatial learning as compared to diabetic mice. The escape latency of control mice receiving RE is less than control group representing improvement of spatial learning in control mice receiving RE.

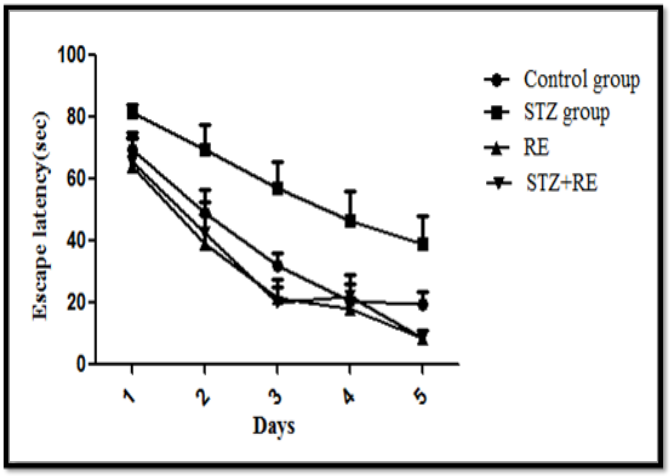

Figure 4.3 Escape latency(sec) recorded over period of five days, reflected a significant decrease in spatial learning in diabetic mice as compared to controls p<***0.001. Diabetic mice receiving treatment of RE 50mg/kg orally for 21 days show significant decrease of escape latency as compared to diabetic mice. Diabetic group depict lesser retention of spatial memory as compared to diabetic group receiving treatment of RE as diabetic mice find platform later than diabetic mice receiving treatment p<***0.001. Escape Latency of control mice and control mice receiving RE 50mg/kg for 21 days depicts that control mice receiving treatment find platform more quickly as compared to control mice *p<0.05 .Data is shown as Mean+SEM

Escape latency on Day 5:

Escape latency of Diabetic mice reflects significant decrease in learning in comparison to control on day 5. Significant decrease of escape latency is observed in diabetic mice receiving RE treatment as compared to diabetic mice. Non-significant difference in escape latency is shown between controls and controls receiving RE treatment.

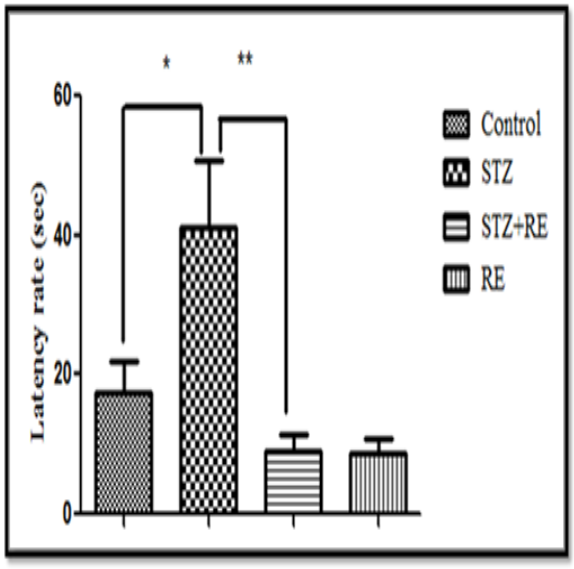

Figure 4.4 In comparison to controls diabetic mice showed a significant increase of escape Latency (sec) reflecting decline of spatial memory and learning *p<0.05. Diabetic mice receiving RE 50mg/kg for 21 days depicts the significant decrease in escape latency reflecting the improvement in learning and memory **p<0.01. Difference of escape latency (sec) in control and control receiving RE treatment is non-significant p>0.05 ns.

Reference Memory:

Probe trial was performed for assessing reference memory. Time spent in the target quadrant was recorded to check the exploration time for the previously placed hidden platform. More time spent in quadrant in which platform was previously placed reflect the improvement in memory and vice versa (Figure 4.5). Number of crossing from the point where platform was previously

placed is directly proportional to improved memory. More platform point crossings show better memory (Figure 4.6).

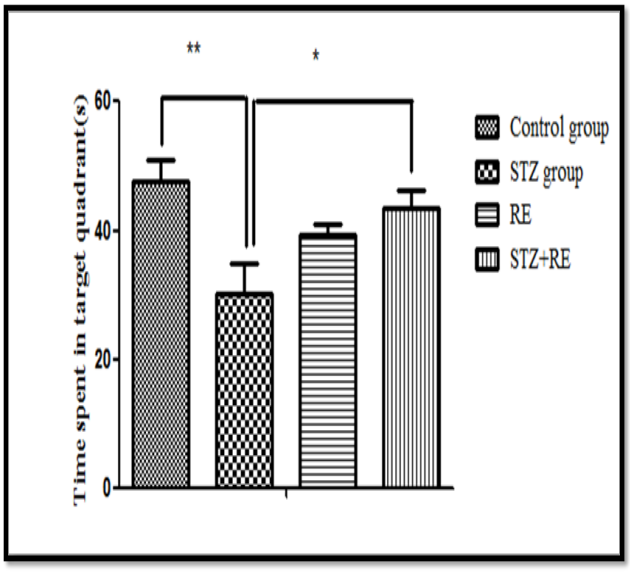

Figure 4.5 This graph reflect the impairment of learning and memory in diabetic mice as they spent less time in quadrant in which platform is previously located while control mice spent more time in quadrant in which platform is previously located. Data is shown as Mean+SEM, $^{**}p<0.01$. Improvement in memory and learning is observed in diabetic mice receiving treatment (RE 50mg/kg orally for 21 days) as compared to diabetic mice as diabetic mice spent more time in quadrant where platform is previously located $^{*}p<0.05$. Non-significant difference is observed in learning and memory of control mice and control mice receiving treatment (RE 50mg/kg orally for 21 days) as time spent in target quadrant in which platform is previously located is almost same. Data is represented as Mean+SEM, $p>0.05$ ns.

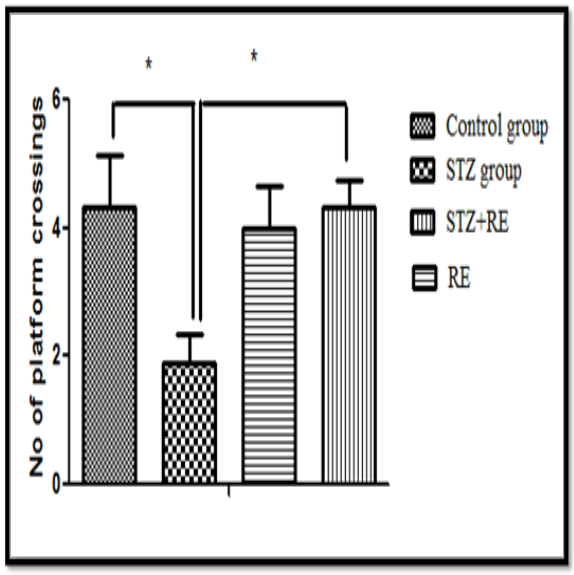

Figure 4.6 The graph indicates that control mice crossed the previously located platform point several times while diabetic mice cross platform in lesser time indicating the significant impairment in learning memory. Data is represented as Mean+SEM, **p*<0.01. Difference of no of previously located platform crossing in diabetic mice is significantly less as compared to diabetic mice receiving treatment (RE 50mg/kg orally for 21 days) reflecting the deficit in learning and memory. Data is shown as Mean+SEM, **p*<0.01. Control mice and controls receiving treatment (RE 50mg/kg orally for 21 days) shown non-significant difference of no of previously located platform crossings reflecting non-significant improvement of learning and memory in response of treatment in healthy animals.

4.4 Histological assessment:

4.4.1 Haematoxylin and eosin staining:

A comparison of the H&E stained brain sections of diabetic and control mice revealed a decrease in neuronal density accompanied by the classical appearance of shrunk neurons in diabetic brains. Improvement in neuronal density is observed in diabetic mice receiving RE treatment. Neuronal density difference is non-significant in control mice brain and control mice receiving RE treatment. Pattern and density of neurons in hippocampus is shown in Figure 4.7 Original magnification is 4X.

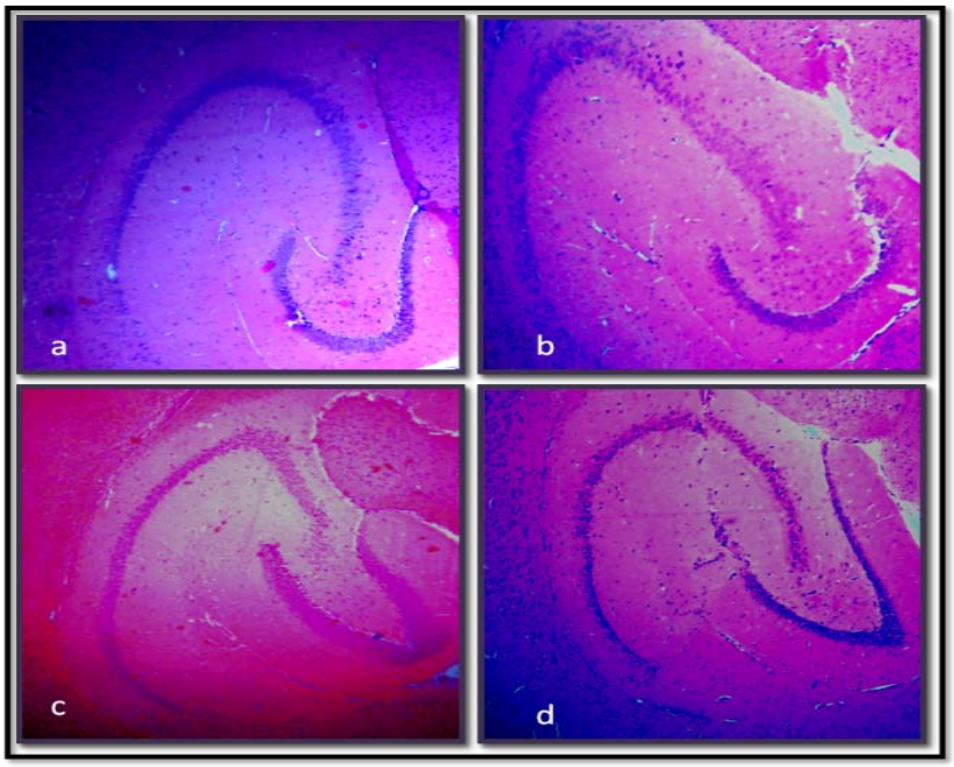

Figure 4.7 H&E stained sections of C- shaped hippocampus: (a) Pattern of dark neurons within hippocampus of control group. (b) Diabetic Group. (c) Diabetic group receiving RE treatment. (d) Control mice receiving RE treatment. Original magnification 4X.

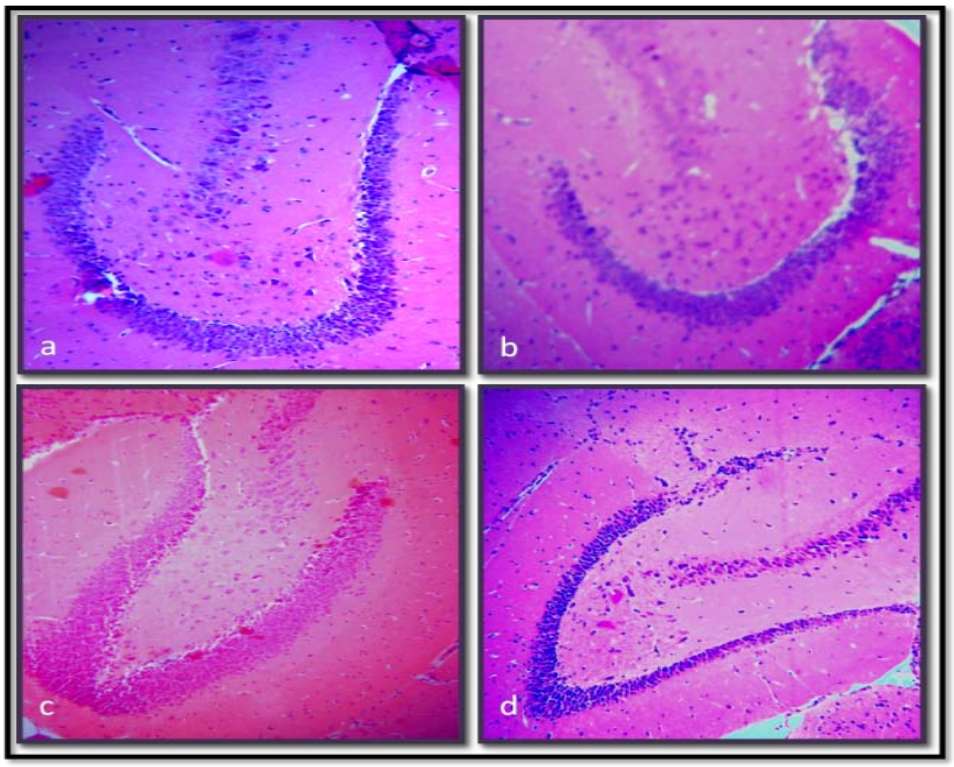

Figure 4.8 H&E stained sections of C- shaped hippocampus: (a) Pattern of dark neurons within hippocampus of control group. (b) Diabetic Group. (c) Diabetic group receiving RE treatment. (d) Control mice receiving RE treatment. Original magnification 10X.

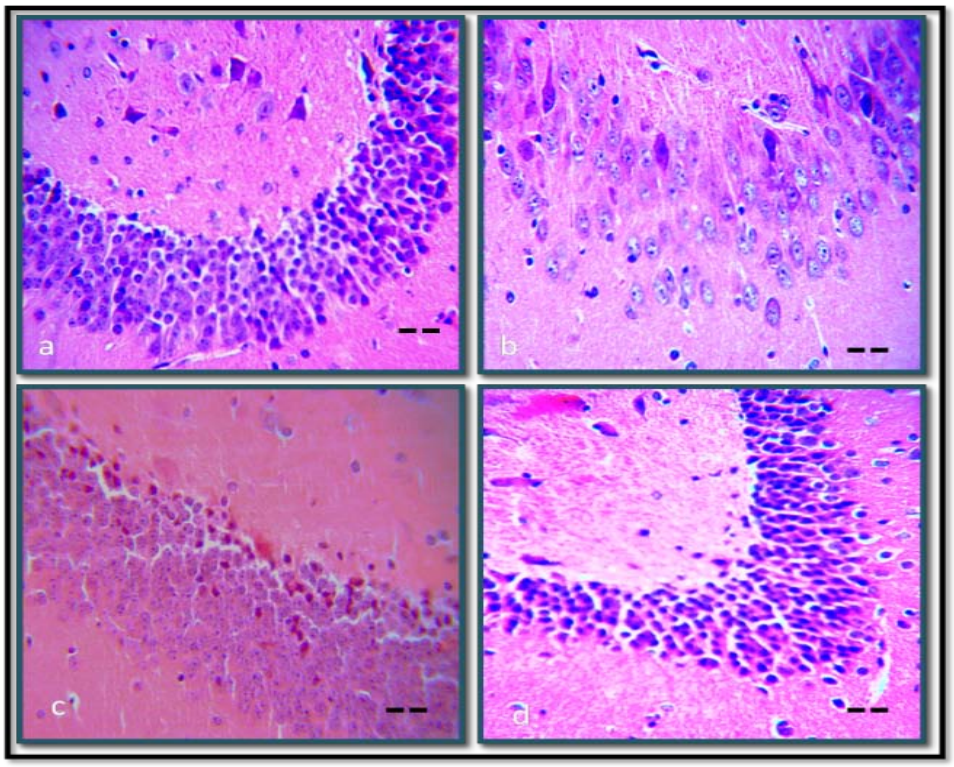

Figure 4.9 H&E stained sections of hippocampus (a) Neuron density with in dentate gyrus of hippocampus of control mice. (b) Diabetic group. (c) Diabetic mice receiving RE treatment. (d) Control mice receiving RE treatment. Original magnification 40X.

Cell count in dentate gyrus of hippocampus:

Neuron density decreases in diabetic mice in comparison to controls. Diabetic mice treated with RE show increase in neurons count as compared to diabetic mice. Control mice receiving RE treatment does not show any significant change in neuronal density in comparison to control mice

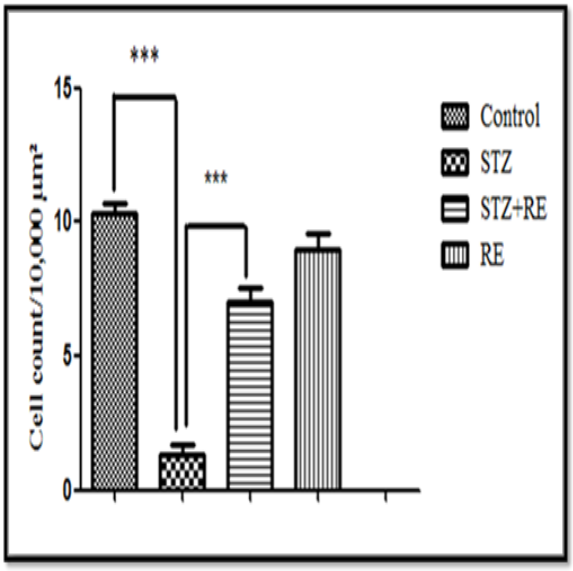

Figure 4.10 Neuronal count in dentate gyrus of diabetic mice is significantly less than control mice ****p*<0.001. Neuronal count in diabetic mice receiving RE treatment significantly higher as compared to diabetic mice ****p*<0.001. Control mice receiving RE treatment does not show any significant difference in neuron density as compared to controls.

4.4.2 Congo red staining:

Congo red staining shows the presence of amyloid plaques. Plaques represent the process of neurodegeneration in brain regions. Control mice do not show the presence of plaques whereas the Diabetic mice show the presence of plaques in dentate gyrus of hippocampus. Diabetic mice receiving RE treatment does not show any improvement in plaques presence. This means there is no effect of RE given dose (50mg/kg) on plaques presence. Control mice receiving RE treatment does not show any plaques same like control mice (Figure 4.10 & 4.11).

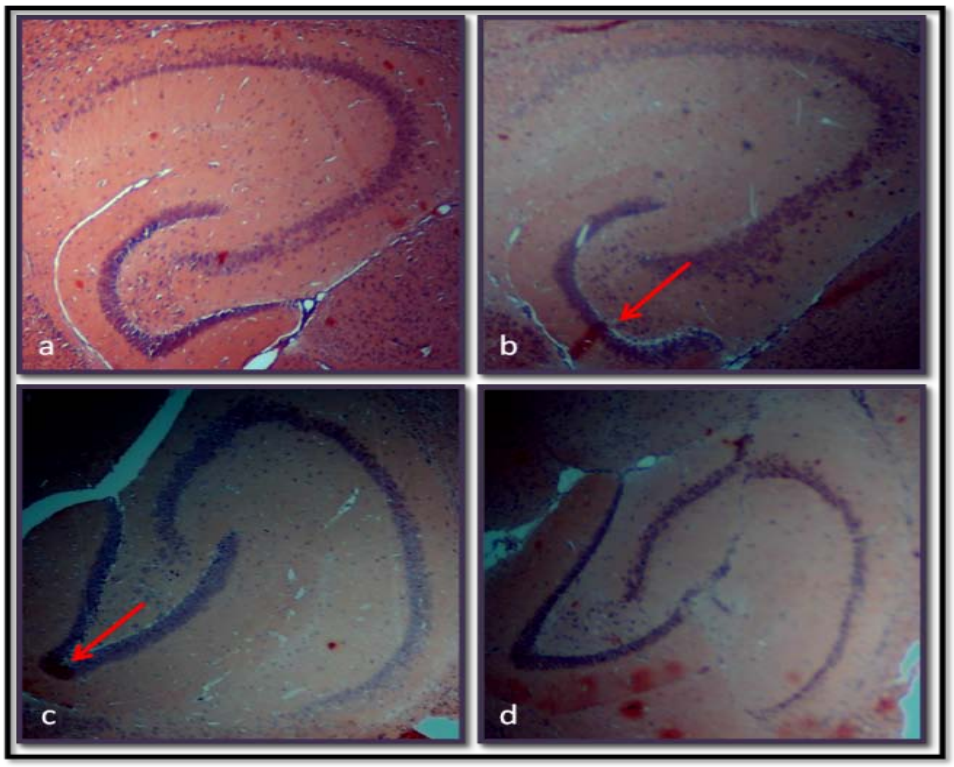

Figure 4.11 Congo red staining of hippocampus (a) Control mice.(b) Diabetic group shows the plaque in dentate gyrus of hippocampus. (c) Diabetic group receiving RE treatment also shows plaque in dentate gyrus . (d) Control group recieving RE treatment does not show any plaque in hippocampus regions. Original magnification 4X.

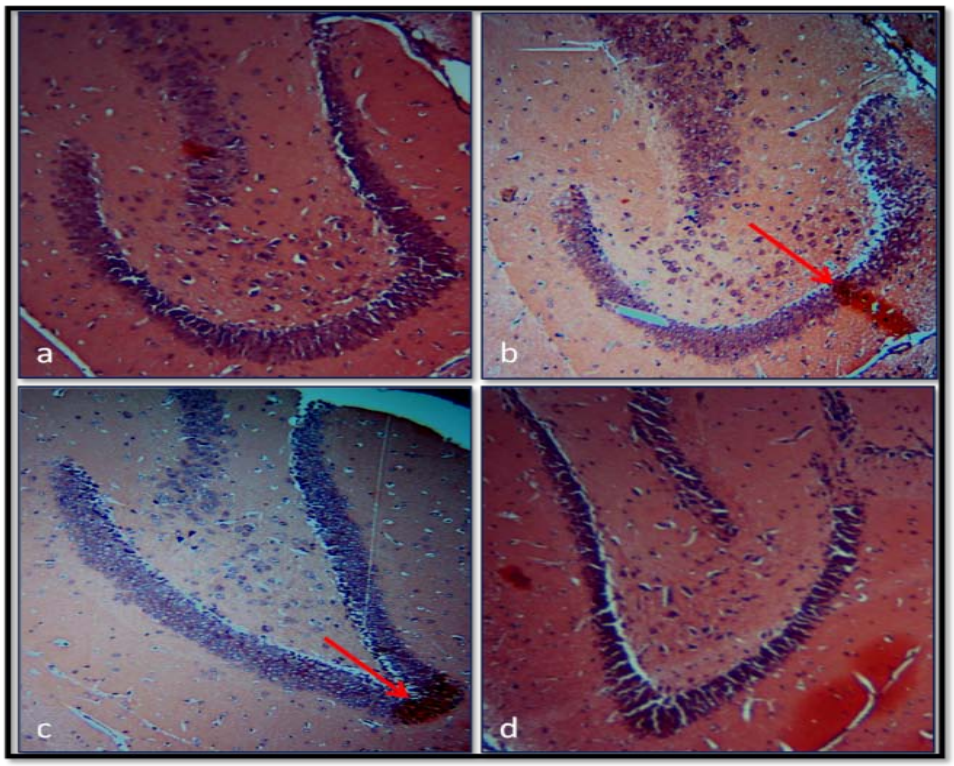

Figure 4.12 Congo red staining of hippocampus (a) Control mice.(b) Diabetic group shows the plaque in dentate gyrus of hippocampus. (c) Diabetic group receiving RE treatment also shows plaque in dentate gyrus . (d) Control group recieving RE treatment does not show any plaque in hippocampus regions. Original magnification 10X.

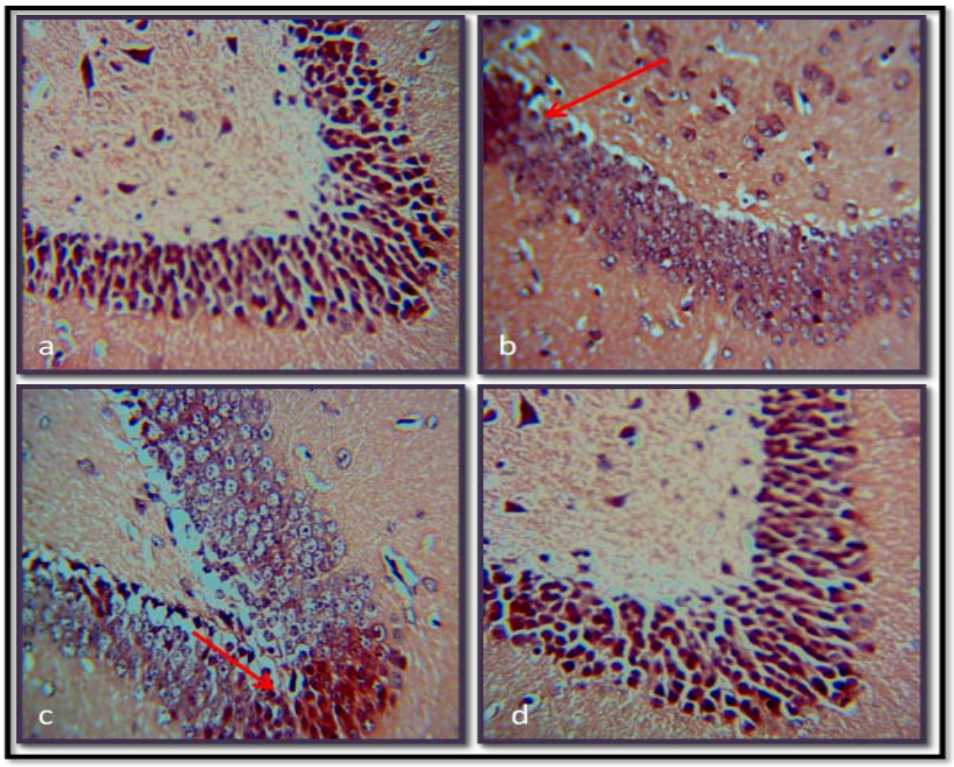

Figure 4.13 Congo red staining of hippocampus (a) Control mice. (b) Diabetic group shows the plaque in dentate gyrus of hippocampus. (c) Diabetic group receiving RE treatment also shows plaque in dentate gyrus . (d) Control group receiving RE treatment does not show any plaque in hippocampus regions. Original magnification 40X.

4.5 Immunohistochemical Evidence for Increased Neurogenesis in Hippocampus:

4.5.1 Ki-67 immunoreactive neurons in dentate gyrus:

Ki-67 is a proliferation marker protein as it is present in proliferating cells. During process of neurogenesis the ki67 immunoreactive Cells reflect the cells in proliferating phase.

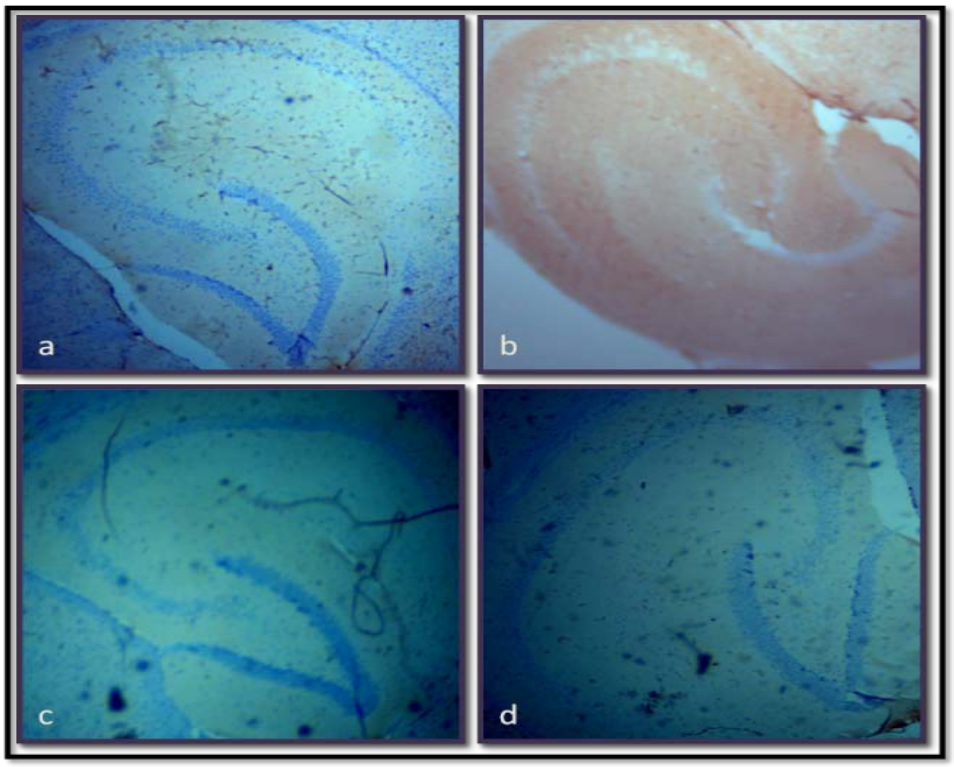

Figure 4.14 Tissue processed with proliferating marker Ki-67 (a) Control mice (b) Diabetic mice (c) Diabetic mice receiving RE (d) Control mice receiving RE .Original magnification 4X

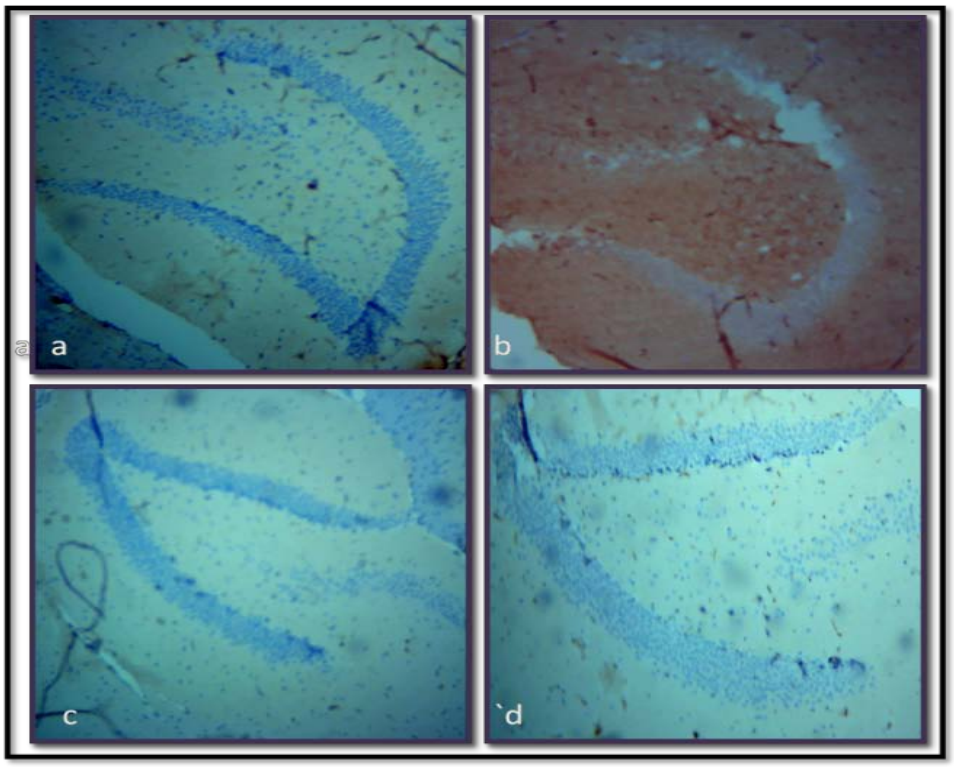

Figure 4.15 Tissue processed with proliferating marker Ki-67 (a) Control mice (b) Diabetic mice (c) Diabetic mice receiving RE (d) Control mice receiving RE .Original magnification 10X.

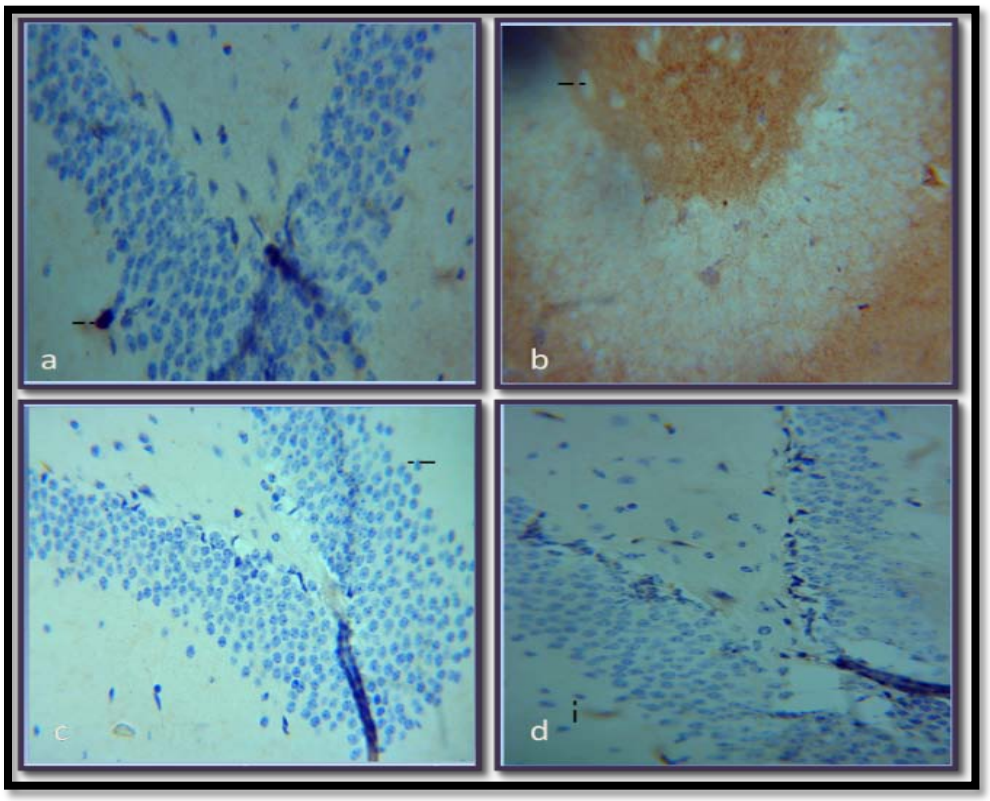

Figure 4.16Tissue processed with proliferating marker Ki-67 (a) Control mice (b) Diabetic mice (c) Diabetic mice receiving RE (d) Control mice receiving RE .Original magnification 40X.

Ki-67 immunoreactive neuronal count in dentate gyrus:

As Ki-67 is certainly linked with cell proliferation, its antigen can be detected exclusively within the nucleus during the interphase, whereas it gets relocated to the chromosomal surface in mitosis.

Ki-67 antibody shows reactivity with a nuclear structure which manifests exclusively in proliferating cells. It is expressed during the G1, S, and G2 phases of the cell division cycle and the mitotic phase in the cell nuclei. However, cells in the resting or G0 phase do not express the antigen. Its presence in the nuclei of all proliferating cells makes it an excellent proliferation marker which can also be used as an operational marker for the determination of the growth fraction of a given cell population.

In our study we use Ki-67 antibody to determine the neuronal cells that are in proliferation stage. In control mice the number of Ki-67 immunoreactive neurons are significant higher than in diabetic mice reflecting decreased number of cells that are in pro;iferation phase in turn reflecting decreased neurogenesis in diabetic mice.

Diabetic mice receiving RE treatment show significant increase in neurogenesis by showing increase number of Ki-67 immunoreactive neurons as compared to diabetic mice. Control mice receiving RE treatment does not reflect any difference in neurogenesis as compared to control mice as the number of Ki-67 immunoreactive neurons are not significantly different.

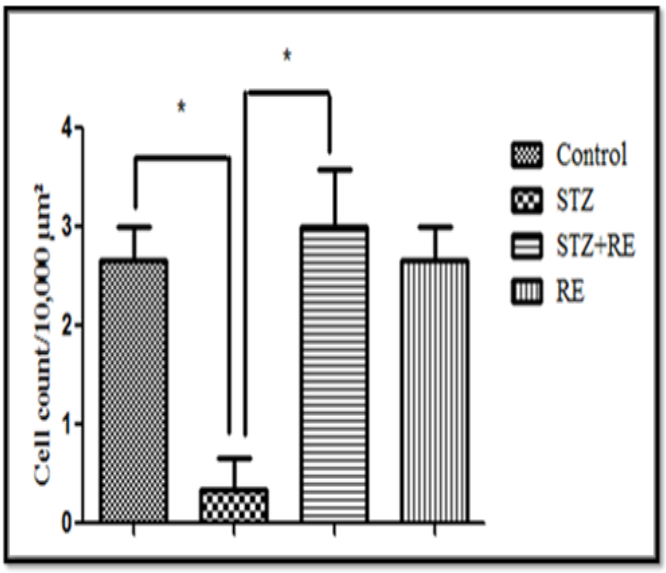

Figure 4.17 Ki-67 immunoreactive neuronal count in dentate gyrus of diabetic mice is significantly less than control mice *p<0.05. Diabetic mice receiving RE treatment show significantly higher Ki-67 immunoreactive neuronal count as compared to diabetic mice *p<0.05. Control mice receiving RE treatment does not show any significant difference in Ki-67 immunoreactive neuronal count as compared to controls.

4.5.2 NeuN immunoreactive neurons in dentate gyrus:

To study the consequences of STZ induced Type 2 Diabetes Mellitus on neurogenesis and putative rescue by RE administration, tissue sections were incubated with antibody NeuN. NeuN immunoreactive neurons were detected in dentate gyrus.

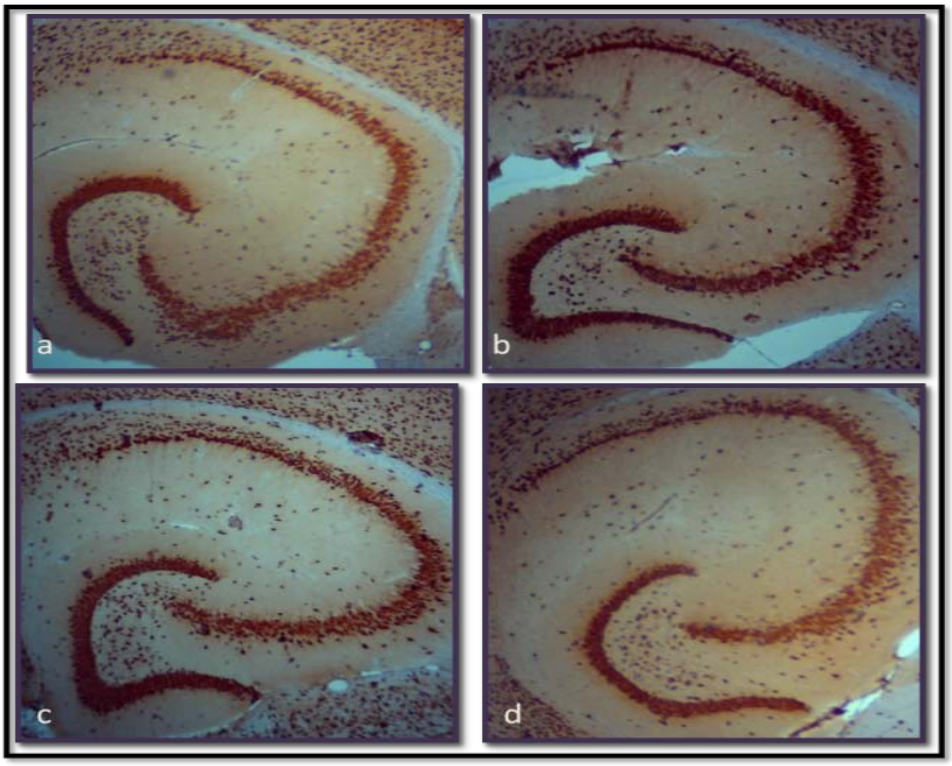

Figure 4.18 Tissue processed with neuron-specific antibody NeuN (a) Control mice (b) Diabetic mice (c) Diabetic mice receiving RE (d) Control mice receiving RE .Original magnification 4X.

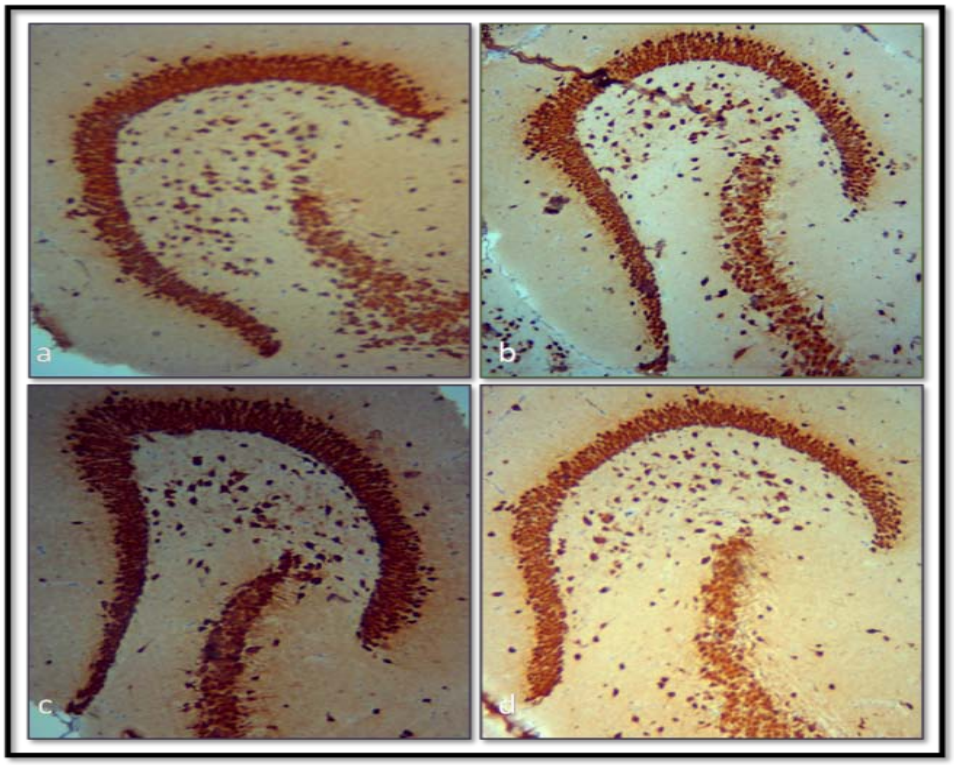

Figure 4.19 Tissue processed with neuron-specific antibody NeuN (a) Control mice (b) Diabetic mice (c) Diabetic mice receiving RE (d) Control mice receiving RE .Original magnification 10X.

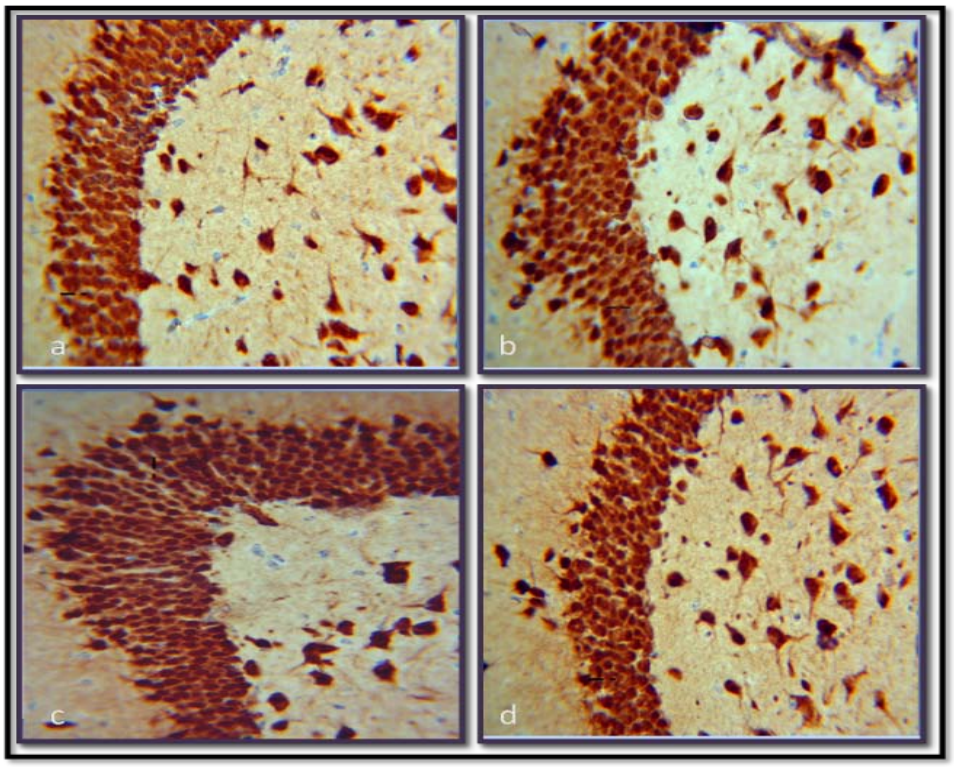

Figure 4.20 Tissue processed with neuron-specific antibody NeuN (a) Control mice (b) Diabetic mice (c) Diabetic mice receiving RE (d) Control mice receiving RE .Original magnification 40X.

NeuN immunoreactive neurons count in dentate gyrus:

NeuN is a soluble nuclear protein expressed in the nervous system in most neuronal cell types. It is localized to the postmitotic neurons where it can be found in the nucleus and the neuronal cytoplasm. Thereby, it marks the normal and the newly generated postmitotic cells in the hippocampus. In control mice the numbers of NeuN immunoreactive neurons are significantly higher than in diabetic mice reflecting decreased neurogenesis in diabetic mice.

Diabetic mice receiving RE treatment show significant increase in neurogenesis by showing increase number of NeuN immunoreactive neurons as compared to diabetic mice. Control mice receiving RE treatment does not reflect any difference in neurogenesis as compared to control mice due to similarity in the number of NeuN immunoreactive neurons.

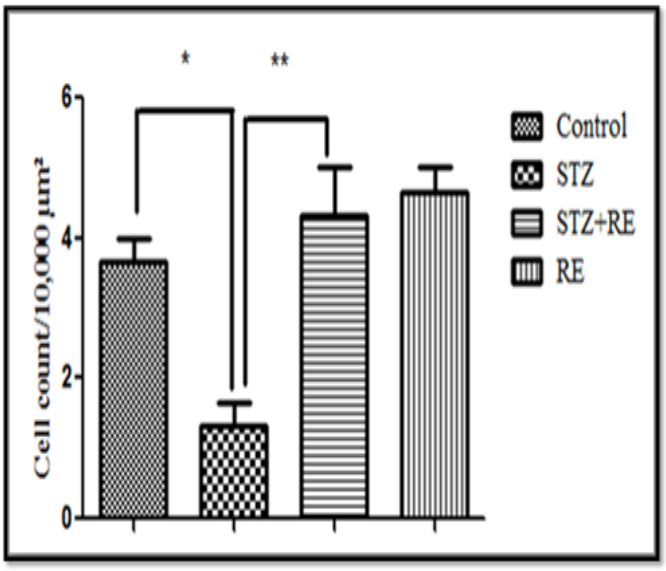

Figure 4.21 NeuN immunoreactive neuronal count in dentate gyrus of diabetic mice is significantly less than control mice *p<0.05. Diabetic mice receiving RE treatment show significantly higher NeuN immunoreactive neuronal count as compared to diabetic mice **p<0.01. Control mice receiving RE treatment does not show any significant difference in NeuN immunoreactive neuronal count as compared to controls.

CHAPTER 5

DISCUSSION

In the present study, it is demonstrated that diabetes negatively affects the hippocampal neurogenesis in concert with cognitive deficits in mice receiving HFD and STZ. However *R. officinalis* has positive consequences on neurogenesis, learning and memory as well as the cognitive function.

Hippocampal neurogenesis believed to have a quantitative relationship with learning and memory (Roosalijazi et al., 2015). Decline of learning and memory was observed in mouse model for T2DM along with a decrease in neurogenesis process in dentate gyrus of hippocampus. In another investigation similar to our data, Morris water maze test results show decrease of spatial memory along with a hippocampal neurogenesis decline in diabetic mice (Stranahan et al., 2008). Another similar study conducted on mice receiving HFD also shows a decline of spatial memory and learning in radial arm maze test and this pattern was followed by decreased hippocampal cell proliferation (Boitard et al., 2012). The current study shows significant improvement in learning and memory after administration of *R. officinalis* extract treatment (50mg/kg p/o for 21 days) in diabetic mice. However no significant difference in learning and memory was observed in control mice receiving *R. officinalis* treatment. Results of current studies for control animals are similar to research carried out by Roosolijazi et al. Their research does not reveal any significant difference in spatial memory and learning at 50mg/kg *R. officinalis* extract treatment. Results of the mentioned studies revealed that *R. officinalis* extract (40% carnosic acid) may show improvement in memory score and oxidative stress activity in middle aged rats in a dose dependent manner, especially at 100mg/kg *R. officinalis* extract treatment.

In order to study hippocampal neurogenesis in dentate gyrus of hippocampus in diabetic mice and effect of *R. officinalis* treatment on neurogenesis in diabetic mice, histochemical studies and immunohistochemistry techniques were applied. Decreased neuronal density in dentate gyrus of hippocampus was observed in diabetic mice upon H & E staining. Diabetic mice receiving *R.*

officinalis treatment showed improved neuronal density as compared to diabetic mice upon H & E staining. Control mice receiving RE treatment did not show any significant difference in neuronal count in dentate gyrus as compared to control mice. A research conducted by Roosalijazi reflected that *R. officinalis* had no significant effect on the density of neurons in the CA1 region of the hippocampus in normal healthy rats. Thereby, it can be concluded here that *R. officinalis* treatment improves the neuronal density or improves the neurogenesis that is impaired due to hyperglycemia.

To investigate the plaque formation, congo red staining was carried out. Plaque formation associated with neurodegeneration was observed in the dentate gyrus of diabetic mice as well as the diabetic mice receiving *R. officinalis* treatment reflecting no role of *R. officinalis* on plaque clearance at mentioned dose.

Immunohistochemical studies were brought about to observe the effects of diabetes and *R. officinalis* treatment in diabetes on neuronal density in neurogenesis stages. Neuronal proliferation (studied through the expression of Ki67), maturation (found through expression analysis of NeuN) was significantly reduced in diabetic brain indicating the extent to which hyperglycemia affects the normal functioning of our brain. These findings about NeuN are similar to research group (wang et al., 2009). Diabetic group receiving *R. officinalis* treatment showed improvement in neuronal density at proliferation stage as well as maturation stage of neurogenesis. In present study the results of immunohistochemistry reflect that *R. officinalis* treatment improves the neuronal count at proliferation and maturation stage of neurogenesis in dentate gyrus of T2DM mouse model. As it has been already reported that in diabetes reduced neurogenesis in dentate gyrus which can be responsible for impairment of memory and learning (Ho et al., 2013). It can thus be concluded here that *R. officinalis* improves memory and learning by improving hippocampal neurogenesis.

5.1 Conclusion:

Our findings suggest that T2DM causes memory impairment that can be attributed to its ability to negatively affect the hippocampal integrity and neurogenesis. Improvement in learning and memory were observed in diabetic mice after receiving *R. officinalis* extract treatment by up regulating hippocampal neurogenesis. In conclusion it can be suggested that *R. officinalis* can be a potential drug for improvement of learning and memory in diabetes, however further studies are also required to explore the active constituents of *R. officinalis* that will further strengthen the preliminary finding of the present study.

CHAPTER 6

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