Screening of natural molecules for their neuromodulatory potential through *in-vitro* and *in-vivo* models

Project report submitted in partial fulfillment of the requirement for the degree of Bachelor of Technology

In

BIOTECHNOLOGY

By

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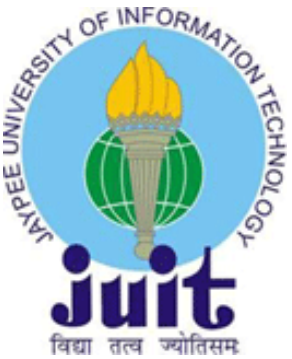
Under the supervision of

Dr. Udayabanu M.

To



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CERTIFICATE

This is to certify that the work entitled **"Screening of natural molecules for their neuromodulatory potential through *in-vitro* and *in-vivo* models"** pursued by Prerika Mathur (131562) in partial fulfillment for the award of degree of Bachelor of Technology in Biotechnology from Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other university or institute for the award of any degree or diploma.

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ACKNOWLEDGEMENT

It gives me immense pleasure to recollect the whole time and effort utilized in making this project a success. It was a great pleasure and privilege to work under the guidance of Dr. Udayabanu M., who inculcated in me the quality of dedication and value of hard work. His constant care, endless encouragement, positive criticism and experimental guidance have been the major driving force throughout the project.

I would like to pay most sincere thanks to Dr. R.S.Chauhan, Head of Department, Department of Biotechnology and Bioinformatics, JUIT, Waknaghat for providing me the opportunities and facilities to carry out the project.

I am also thankful and indebted to Mr. Vineet Mehta, Mr. Arun Parashar and Mr. Arun Sharma for giving me a chance to work and providing me encouragement along with incessant help throughout my work.

Also, I wish to express my gratitude towards Jaypee University of Information Technology (JUIT), Waknaghat for providing the excellent facilities for my work. It is a place where seekers of knowledge come and benefit from research facilities and excellent atmosphere.

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SUMMARY

Diabetes Mellitus, a chronic metabolic disorder is at present been extensively researched upon in order to find out if there is some link between the pathogenesis of Alzheimer's disease and type 2 Diabetes Mellitus. Alzheimer's disease, which is defined by impaired cognitive efficiency and loss of memory, is suspected to be type 3 diabetes. Type 2 Diabetes Mellitus is been assumed to cause neurodegeneration, which is a part of the pathogenesis of Alzheimer's disease. In the present study, natural molecules such as Berberine, Piperine, Quercetin and Rutin were screened via *in-vitro* and *in-vivo* studies to find out the most effective natural molecule that has the efficiency of restoring the memory and halting or preventing neurodegeneration. Among the above mentioned natural molecules, quercetin was found to be the most effective drug in restoring memory and enhancing cognitive efficiency.

LIST OF ABBREVIATIONS

AD: Alzheimer's Disease

DM: Diabetes Mellitus

IL: Interleukin

T2DM: Type 2 Diabetes Mellitus

APP: Amyloid Precursor Protein

NFT: Neurofibrillary Tangles

PHF: Paired Helical Filaments

FAD: Familial AD

LOAD: Late Onset AD

PS: Presenilin

LRP: Lipoprotein Receptor Related Protein

VaD: Vascular Dementia

RAGE: Receptor for Advanced Glycation End Products

MAP: Microtubule Associated Protein

IDE: Insulin Degrading Enzyme

NEP: Neprilysin

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Alzheimer's disease (AD) is now been considered to be the sixth leading cause of death in the US. AD is now been referred to as "Type III Diabetes" due to reported glucose metabolism dysfunction. A number of preclinical studies indicate the inverse relation between the level of insulin and the AD pathology. With an increase in the level of insulin, decrease in the pathogenesis of AD has been observed. Alzheimer's disease (AD) is a chronic neurodegenerative disorder of the central nervous system associated with progressive cognitive and memory loss. AD accounts for around 50-60% of dementia cases and is thus considered to be the most common cause of age-related. It is more common in women [Bassil et. al., 2012]. Apart from age, lifestyle and genetics also considered to be risk factors [National Institutes of Health, 2016]. Type 2 diabetes mellitus (T2D) is yet another factor that greatly enhances the risk of developing this disorder by 50-60% [Chami et. al., 2016; Mittal &Katare, 2016].

1.1 Characterization of Alzheimer's disease

The cognitive symptoms of Alzheimer's can be characterized by the following A's (Alzheimer's Foundation of America, 2016):

Amnesia: It is a condition in which there is loss of short term memory and after that long term memory with the disease progression. It is the major contributing factor to the development of the other three "A's."

Aphasia: It is defined as the inability to effectively communicate. Individuals suffering from AD find it difficult to choose the appropriate words for communication.

Apraxia: It is defined as the inability to carry out pre-programmed motor tasks, and in AD, is caused by memory loss of motor skills learned during development.

Agnosia: It is defined as the inability of the individual to properly interpret sensory signals.

The patient might no longer recognize a person's face or understand the meaning of the sensation of a full bladder. As a result of these symptoms the individual finds it difficult to complete day-to-day activities without considerable assistance.

1.2 Major Molecular Hallmarks of AD

It is characterized by molecular hallmarks such as deposition of the beta amyloid peptide, further leading to the formation of senile plaques, cholinergic deficit, neurofibrillary tangles that contain hyper phosphorylated tau protein, widespread neuronal loss and synaptic changes in the hippocampus and cerebral cortex, which are essential for cognitive and memory functions.

1.2.1 Senile Plaques

Neuritic plaques were first reported by Blocq and Merinesco in 1882 and neurofibrillary tangles were detected in year 1904 by Fragnito [Amtul et. al., 2002]. The production and deposition of the β-amyloid peptide (Aβ) is widely believed to drive the pathogenesis of Alzheimer's disease (AD). Amyloid plaques are formed in the spaces between the brain's nerve cells. They are mainly composed of insoluble toxic protein peptide deposits which are known as beta-amyloid protein. The ~4 kDa Aβ peptide derived from the larger amyloid precursor protein (APP) by sequential proteolytic cleavage was firstly isolated as the chief constituent of amyloid deposits in the brain and cerebro-vasculature of Alzheimer's disease and Down's syndrome patients.

1.2.2 Neurofibrillary Tangles (NFTs)

Neurofibrillary tangles are yet another major hallmark of AD. They are basically large collections of twisted protein threads that are found inside the nerve cells. Tau protein is the major component of these neurofibrillary tangles. A number of phosphate molecules are bound to tau protein, which further help in the stabilization of microtubules. But in AD, a great number of additional phosphate molecules bind to tau, leading to tau hyper phosphorylation, giving rise to tau disengagement from the microtubules. The so formed tau threads form structures which are known as paired helical filaments, which get tangled with one another, thus forming tangles within the cell. This leads to collapse of the neuron's internal transport network as the microtubules disintegrate in the process which disables the internal communication between the neurons.

1.2.3 Cholinergic Deficit

Another important hallmark of an AD brain is the presence of excessive acetylcholinesterase (AChE) which is commonly associated with the development of β-amyloid plaques and neurofibrillary tangles (NFT). Cholinergic neuronal systems play a major role in cognitive functions. In Alzheimer's disease brain, a significant deficiency of the neurotransmitter acetylcholine (ACh) and a decrease in activity of enzyme choline acetyltransferase (ChAT) has been detected [García-Ayllón et. al., 2016].

1.3 Epidemiology of Alzheimer's disease

Global prevalence of dementia, including Alzheimer's disease (Fig. 1), is around about 24.3 million people, with 4.6 million fresh cases occurring per year [Ferri et. al., 2005]. This number is expected to rise roughly by fourfold by the year 2050 unless suitable interventions are found.

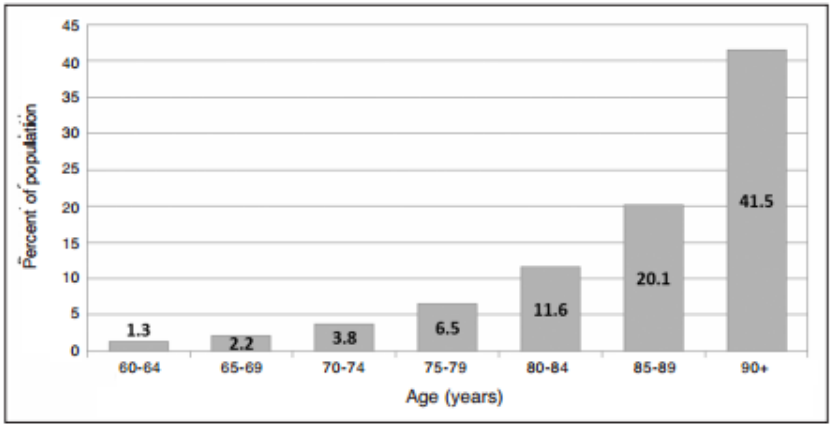


Fig. 1 Global epidemology of Alzheimer's disease [Ferri et al., 2005].

As per the prediction made by World Health Organization (WHO), by 2025, approximately 75% of the estimated 1.2 billion people who are aged 60 years and older will stay in developing countries [WHO, 2002]. According to WHO, dementia patients will double every 20 years to 42.3 million in 2020. Highest rate (around 336%) of growth will be observed in India, China, South Asia, and western Pacific regions. [WHO, 2004].

1.3.1 Prevalence of dementia in Different States of India

In India, different regions have different prevalence rates (Table 1) of dementia [Rajkumaret. al., 1997; Chandra et. al., 1998; Raina et. al., 2010; Vas et. al., 2001]. This may be due to adoption of different methodologies, defining criteria, screening instruments, multi-ethnicity, multicultural factors and different environmental factors. The occurrence of dementia among rural population present in South India when compared with in North India depicted a widely varying rate between 3.39 % and 0.84%, respectively [Chandra et. al., 1998]. There are very few urban studies from different regions of India showing quite similar varying rates: between 2.44 % and 4.1 % in Western India [Vas et. al., 2001; Saldanhaet. al., 2010], 0.9 % - 1.83 % in Northern India [Raina et. al., 2010], 0.8 % and 1.28% in Eastern India [Banerjee et. al., 2014] and 3.6 % in

Region	Study	Age/gender	Number of subjects	Prevalence rates (%)		Instruments used	Remarks
				All dementia	AD		
South India	Shaji *et al.*, 1996[6]	≥60	2067	3.39	1.31	Screening: MMSE and CAMDEX Confirmation: Clinical and DSM IV	Rural South Indian population in Kerala
		Male	965	2.8	0.73		
		Female	1102	3.54	1.81		
	Rajkumar *et al.*, 1997[7]	≥60	750	3.5		Geriatric Mental State Examination	Rural South Indian population in Madras
	Shaji *et al.*, 2004[8]	>65		3.36			Urban South Indian population
North India	Chandra *et al.*, 1998[9]	≥55	5126	0.84		Screening: Hindi MMSE Confirmation: CDR and DSM IV	Rural North Indian population
		≥65		1.36			
		Male		1.8	0.77		
		Female		1.25	0.46		
	Raina *et al.*, 2010[10]	>60	1856	1.83		MMSE and EASI	Migrated population in Jammu region of J and K
West India	Vas *et al.*, 2001[11]	>40	24,488	0.43	0.25	Sandoz clinical assessment geriatric scale and MMSE for screening. CDR and DSM IV for diagnosis	Urban western Indian population in Mumbai
		≥65		2.44	1.5		
		Male	11,875		0.2		
		Female	12,613		0.3		
	Saldanha *et al.*, 2010[12]	>65	2145	4.1		Community screening instrument	Urban population in Pune
East India	Das *et al.*, 2008[13]	60	5430	0.8	0.38 VaD: 0.33	BMSE and KCB-Kolkata cognitive battery	Urban Kolkata
	Banerjee *et al.*, 2008[14]	≥50	6129	0.62		Screening questionnaire for cognitive dysfunction, KCB-Kolkata cognitive battery	Urban Kolkata
		≥60	2720	0.1.28	0.34		

Table 1: Epidemiology of dementia in different regions of India

Southern India [Shajiet. al., 2005]. The differences could be true on considering the multiethnic, multicultural, and environmental differences.

1.4 Early and Late Onset of Alzheimer's disease

AD can be classified as either early onset AD, which is even known as familial AD (FAD) or late onset AD (LOAD). FAD is the one which is genetically based, while the late onset AD (LOAD) is the sporadic form of AD and probably involves polygenetic factors and environmental factors. FAD leads to an early onset of AD with approximately around 45 years of age. Gene concerned in FAD codes for amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2) [Van et. al., 2010]. Mutations in these genes give rise to around 50% of all FAD cases. Mutations in the above mentioned genes have been shown to amplify the production and aggregation of Aβ. Majority of the AD patients (~95%) suffer from LOAD and the disease generally develops after 65 years of age. Apolipoprotein E allele ε4 (ApoEε4) expression is one of the risk factors which has been identified for LOAD.

1.5 Aβ Generation, and clearance

Amyloid precursor protein (APP), the starting point for amyloid plaques, is a cell membrane associated protein and acts as a barrier that encloses the cell. This protein is snipped or cleaved by specific enzymes into discrete fragments. These enzymes are known as alpha- secretase, beta-secretase, and gamma-secretase. APP processing can undergo any one of two pathways which have very different consequences for the cell. This clearly depends on the enzyme which is involved and its site of cleaving [Iwata et. al., 2001].

1.5.1 Different Pathways of APP Processing

In the benign pathway, toxic amyloid beta is not formed. Amyloid Precursor Protein molecule is cleaved by alpha secretase such that the portion which is quite potent of becoming beta-amyloid gets cleaved. Thus, this clearly removes the chances of beta-amyloid peptide generation and plaque buildup. As a result of this proteolytic cleavage by alpha-secretase, sAPPα, a neuron fragment is released, which is essential for the neuron. It facilitates neuronal survival and growth. Remaining APP fragment is then snipped off by gamma-secretase enzyme.. Among the resulting fragments, the smaller one is released outside the neuron, while the larger one stays within the neuron and keeps interacting with the factors present inside the nucleus.

In case of the harmful pathway, beta-secretase enzyme first cleaves off the APP molecule at one specific end of the beta-amyloid peptide, thus releasing sAPPβ fragment from the cell. Another enzyme, gamma-secretase then cuts the resultant APP fragment, which is still there in the neuron's membrane. Beta- amyloid peptide on being cleaved on both sides gets out into the space outside the neuron and sticks to other beta-amyloid peptides. Aggregates of proteins are formed. These small and soluble beta-amyloid peptides aggregates are called oligomers. These oligomers can be of varying sizes and it can be that certain specific sizes of theirs might be responsible for interacting with receptors present on neighboring cells and synapses, thus affecting their ability to function.

1.5.2 Beta Amyloid Degradation and Clearance from Brain

Beta amyloid degradation and clearance is of utmost importance to the neuron. Two chief enzymes which facilitate this process are Neprilysin (NEP) and insulin degrading enzyme [Iwata et. al., 2000; Iwata et. al., 2001; Marr et. al., 2003]. They both are metalloproteases but Neprilysin is plasma membrane bound and only does the extracellular degradation of toxic peptides. Whereas,, IDE is active both intra- and extracellularly. It has roughly a much greater affinity for insulin as compared to Aβ, but it hydrolyzes insulin at a much slower pace. Thus, insulin is known to be a better inhibitor of the IDE-dependent cleavage of Amyloid beta. Both the enzymes decrease in normal aging and in disease-affected regions in case of AD [Maruyama et. al., 2005].

Even after extensive catabolism in the brain, a major sum of Aβ still remains undegraded. Two mechanisms are involved in the movement of soluble amyloid beta across the Blood Brain Barrier. They are the low-density lipoprotein receptor-related protein (LRP) present on the abdominal (brain) side and the receptor for advanced glycation end products (RAGE) on the luminal (blood) side. The degree of cerebral amyloid burden is predicted by the net efflux of Aβ across the BBB [De Mattos et. al., 2002]. Disruptions of these mechanisms may contribute quite significantly to the effective development of amyloid pathology [Zlokovic et. al., 2005].

1.6 Tau pathology

Microtubule-associated protein (MAP) tau, MAP1 (A/B) and MAP2 are the chief microtubule-associated proteins of a normal mature neuron. These three MAPs perform quite similar functions, i.e., maintaining the stability of microtubules. In Alzheimer disease (AD) and related disorders called tauopathies, tau is generally abnormally hyper-phosphorylated and is accumulated as intraneuronal tangles of paired helical filaments (PHF) [Mandelkowet. al., 2007]. Tau is abnormally hyper-phosphorylated in an AD brain and is the chief protein subunit of PHF/SF. PHF form neurofibrillary tangles, a hallmark lesion of this disease. The abnormal hyper-phosphorylation of tau provides it resistance to proteolysis by the calcium activated protease and most likely it is due to this that levels of tau are several-fold increased in AD [Rosenberg et al., 2008].

1.7 Neuromodulatory Effect of Natural Molecules

A number of natural molecules have been found to have neuromodulatory properties. Rutin is a major flavonoid and is mainly synthesized through the phenylpropanoid metabolic pathway in plants. It has a number of pharmacological properties such as antioxidant properties [Sharma et. al., 2010], which have been quite extensively exploited in human medicine and nutrition. Other flavonoids such as quercetin and Berberine also have antioxidant properties. Quercetin and Berberine are prominent flavonoids having antioxidant [Zhang et. al., 2011], anti-inflammatory and anti-cancer properties [Joshi et. al., 2011]. Piperine, an alkaloid, found in the roots of *Piper nigrun* and *Piper longum* species of Piperaceae family is also extensively used for research and medicinal purposes due to its antioxidant and anti-inflammatory properties [Xiu et. al., 2012].

1.8 Diabetes Mellitus

Type 2 diabetes mellitus is a condition that is characterized by elevated blood glucose level resulting from greater hepatic glucose production, impaired insulin production by pancreatic β-cells, impaired insulin release in response to insulin resistance. Thirst, polyuria, weight loss and blurring of vision are some of the characteristic symptoms of Diabetes mellitus. It is a common metabolic disorder and is associated with cognitive decline, anatomical measures of brain aging

and with an elevated risk of stroke and vascular dementia (VaD) [Kharroubi and Darwish, 2015]. Diabetes has also been found to be associated with important vascular disorders, such as retinopathy, cardiovascular disease, neuropathy, nephropathy, and cognitive impairment [Umegaki, 2012]. Insulin dependent diabetes (diabetes type 1, DT1), which accounts for 5-10 % of total diabetic patients, and non-insulin-dependent diabetes (diabetes type 2, DT2), which accounts for the majority of the remaining diabetic patients are the two major clinical forms of Diabetes Mellitus. In addition to that there are various other types of diabetic conditions which prevail, such as gestational, genetic syndrome, monogenic diabetes, cases associated with disease of the exocrine pancreas, and cases induced by hormones or other drugs [Brands et. al., 2005; Kharroubi and Darwish, 2015].

1.8.1 Epidemiology of Diabetes Mellitus

T2DM has become an observably global public health problem. As per the recent statistical data T2DM has shown an increase in developed nations, such as United States and Japan. As per the predictions, it will keep increasing in the next twenty years with greater than 70 % of the patients appearing in developing nations [Darwish et. al., 2015]. Most of the countries with the highest number of diabetes patients are lower middle-income countries, such as India, Brazil, Russia, China, Indonesia, Pakistan, and Bangladesh [Darwish et. al., 2015]. The prevalence rates among them are 12.1% and 9.7 % in India and China, respectively [Brands et al., 2005]. Even though age poses as a risk factor for T2DM but increasing rates of obesity in children has given rise to T2DM getting common in children, teenagers and adolescents [Whitmer et. al., 2005].

1.9 Correlation between Type 2 Diabetes Mellitus and Alzheimer's disease

In mid-1980s, it was found that the hippocampus and some specific parts of the cerebral cortex, contained elevated densities of IR β [Gammeltoft et. al. 1985; Baskin et. al. 1987]. This marked the beginning of correlating insulin receptor β-subunit and AD. Insulin has remarkable effects on

human cognition [Strachan et. al., 1997], and the actions of insulin and insulin-sensitizing peroxisome proliferator-activated receptor- γ (PPAR γ) agonists have been explored in AD patients [Watson and Craft 2003]. The risk of AD and memory dysfunction is elevated by hyperinsulinemia and resistance to insulin, characteristics of T2DM [de la Monte 2009; Carlsson et. al., 2010], which is linked to an amplified risk of AD [Sims et. al., 2010]. These observations and gave rise to the hypothesis that the cognitive deficits observed in AD may arise, in part, from insulin insensitivity in the brain.

A number of different mechanisms have been observed and reported which link the two diseases together. Oxidative stress, formation of advanced glycation end products (AGES) and impairments in CNS insulin signaling [Krasuski et. al., 2014] are all linked to diabetes and AD. Also, deregulation of cellular processes like glucose metabolism, apolipoprotein processing, cholesterol metabolism, mitochondrial activity, calcium homeostasis, and second messenger signaling factors are also suspected for contributing to both the diseases [Matsuzaki et. al., 2010].

1.10 Need for alternative therapeutic strategy

Drugs which are currently used in the clinical settings for the management of neurological complications, especially dementia, provide only symptomatic relief from the ailment. These drugs are neither capable of reversing neurological complications nor they halt their progression. Further, a number of severe side effects are associated with these drugs which limit their use on full potential. Need of the day is to develop some alternative and safer therapeutic strategies which can not only halt the disease progression but also reverse the damage. For this small molecules of natural molecules may be the potential solution as they are comparatively safer than synthetic molecules and many of them are known possess neuromodulatory potential.

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Pathology of Alzheimer's disease

In Alzheimer's disease, a delayed immune response and an elevated level of apoptosis [Mandrekar & Landreth, 2010] is observed in the brain. Also, Aβ peptides and PHF-tau protein aggregates that form Aβ plaques and NFTs precede the cognitive and behavioral symptoms of AD [Wang et. al., 2015]. Although protein aggregation is observed in a number of parts of the brain, but the majority of severe pathology is found in the hippocampus, the region in the brain predominantly responsible for learning and memory. Certain specific sub-regions of the hippocampus are also linked with elevated AD pathology [Willette et. al., 2015]. Plaques were first observed to form inside the subiculum and then extend to the remaining portions of the brain. As the disease progresses, pathology spreads firstly to areas in the limbic system, such as the hippocampus and amygdala, which contributes to memory loss, disorientation, and behavioral changes [George et. al., 2014].

2.1.1 Amyloid Beta Plaques

As per the amyloid cascade hypothesis, the primary cause of AD is the dysregulation between the generation and removal of both internal and external Aβ plaques [Zou et. al., 2015]. This hypothesis suggests that the formation of Aβ plaques is the primary event in AD pathogenesis and is caused by the aggregation of the peptides, specifically peptides Aβ40 and Aβ42, caused by improper cleavage of natural cellular proteins. The aggregation of these peptides leads to the initiation of a cascade of negative effects which contribute to AD [Tong et. al., 2015; Hardy & Selkoe, 2002]. These amyloid beta peptides are the result of modified APP proteolysis. APP is an integral membrane protein which is accumulated around the synapses of neurons that, when functioning properly, serves as an important modulator of synapse formation, neural plasticity, and iron export [Turner et. al., 2003]. APP is broken down by enzymes α- secretases and γ-secretases to generate peptide fragment sAPPα. It has both neurotrophic and neuroprotective effects [Zhang et. al., 2012]. But in case of AD, an altered pathway is followed, in which amyloid precursor protein is cut by β-secretase and gives rise to Aβ protein formation. These Aβ plaques do not just cause toxicity to the neurons, but also impose indirect effects on AD

pathology. These effects include triggering cascades of neuroinflammation, oxidative stress, tau hyperphosphorylation, and tangle formation, ultimately resulting in major NCD [Collins-Praino et. al., 2014].

2.1.2 Neurofibrillary Tangles

Apart from the improperly cleaved proteins that contribute to Aβ plaques, another effective contributor to AD pathology is the hyperphosphorylation of the structural protein tau. Tau is a soluble protein which is found in neurons of the CNS. It binds to and stabilizes the microtubules, aids the transport of nutrients, neurotransmitters, and cellular materials down the axon of a cell. Tau also acts as a protein scaffold and participates in different signal transduction cascades [Peric & Annaert, 2015]. Tau is usually phosphorylated in order to fine-tune its function, but in case of AD, it is hyperphosphorylated. Hyperphosphorylated tau form Paired Helical Filaments (PHF) and lead to formation of insoluble proteins. Insoluble tau self-aggregates and yields NFTs, which compromises tau's cytosolic functions, dysregulates intracellular homeostatic mechanisms and leading to neuronal death [Mudher & Lovestone, 2002]. Also cognitive decline associated with Aβ aggregation occurs only when there are elevated levels of PHF-tau, which clearly suggests that Aβ is an upstream modulator of tau hyperphosphorylation [Peric & Annaert, 2015].

2.1.3 Inflammation

Inflammation is an immune response in which affected tissue responds to harmful stimuli. Inflammatory mediators help in increasing the blood flow and in accumulating defense cells in the affected areas [Institute for Quality and Efficiency in Health Care, 2015]. Microglia are the brain immune cells that act quite similar to macrophages in recognizing Aβ plaques as a signal of brain tissue damage [Mandrekar & Landreth, 2010]. On activation, microglia generates chemokines which signal for greater microglia activity for responding to the toxic Aβ plaque signal [Yoon & Kim, 2015]. Although microglia facilitates the maintenance of homeostasis under normal physiological conditions, long-term microglial activation may have some

detrimental effects on neurons. In the presence of a manageable amount of Aβ plaques, microglia is able to successfully clear those plaques via phagocytosis and without generating a large inflammatory response [Mandrekar & Landreth, 2010]. However, once plaque development reaches severe pathological levels, the phagocytic capabilities of microglia diminish. Although the Aβ plaques can still be internalized but the digestion of these dense plaques becomes difficult within the phagocytic microglia [Yoon & Kim, 2015; Mandrekar & Landreth, 2010]. Thus, not all phagocytic activity completes Aβ plaque digestion, which can lead to a cascade of microglial aggregates surrounding tissue regions with high plaque content. This leads to the initiation of a positive inflammatory feedback loop as the microglia recruited to digest the amyloid beta plaques fail and start sending signals of distress, which attract even more microglia. Microglia generates a pro-inflammatory environment in the brain by generating chemokines, thus diminishing its phagocytic capabilities and leading to Aβ aggregation [Mandrekar & Landreth, 2010].

2.1.4 Apoptosis

Brain atrophy is yet another major hallmark of AD. Aβ plaques and NFTs give rise to prominent levels of apoptosis [Xu et. al., 2012]. Although it is necessary for removing damaged cells or unnecessary cells but its elevated levels deplete the healthy brain cells that are important for proper functioning of the brain. Two operating pathways of apoptosis are present and both of them are quite relevant to AD. The first one gets activated by the cell-surface death receptors and is known as the extrinsic pathway. The second one is the intrinsic pathway, which requires signals from mitochondria [Xu et. al., 2012].

In the extrinsic pathway, Fas ligand binds to the Fas death receptor present on the surface of the cell, thus triggering the development of death-inducing signaling complex (DISC) in response to external apoptotic signals, such as Aβ plaques. Effector caspases are able to induce apoptosis via a caspase cascade which is triggered by initiator caspases within the DISC. Caspase 8, an initiator caspase activates the effector caspases which then follow a cascade in order to activate caspases 3, 6, and 7. Executioner caspases such as these then activate the target proteins which break down the cell from within [Andersen et. al., 2005]. The intrinsic apoptosis pathway is

mediated by mitochondria and initiated by the presence of cytoplasmic cytochrome-C, which is suggestive of mitochondrial dysfunction and oxidative stress. Mitochondria are needed for the regulation of the cell death pathways, fulfilling the high-energy requirements of the brain and in stimulating apoptosis as and when required. Thus, mitochondria are considered to be quite appropriate indicators of neuron survival. Oxidative stress also gives rise to mitochondria becoming larger and more disorganized, especially with age [Moreira et. al., 2012].

2.1.5 Oxidative Stress

Oxidative stress also has a fundamental role in AD pathogenesis leading to neuronal degradation and cell death. The level of oxidative markers has been found to be directly related to the degree of cognitive impairment. Oxidative stress increases in the AD brain due to the DNA and protein oxidation, greater lipid peroxidation, diminished level of cytochrome c oxidase and enhanced glycosylation end products [Fleming et. al., 2012]. Due to brain's high metabolic rate, it is much more susceptible to the damage caused by reactive oxygen species. Protein nitration acts as a biological marker for protein oxidation and it is seen to be elevated in the hippocampus and neocortex of individual with AD [Chen et. al., 2014].

AD has mostly been categorized as a multifaceted disorder, thus implicating a number of aberrant signaling cascades to be involved in its pathogenesis. One such factor is insulin resistance which is known to affect a number of different cascades of known importance to AD [Craft et. al., 2013; Zhu et. al., 2005]. De la Monte et al. was the first one to report cerebral insulin and Insulin-like growth factor (IGF) production. They further observed that even in AD there were impairments in energy metabolism and glucose utilization mechanisms [Zhu X et. al., 2005]. Insulin receptors (IRs), insulin, and IGF deficiency in AD brain further implicated insulin resistance in AD neuropathology [Straten et. al., 2006]. Braak et. al. have demonstrated and reported an inversely proportional relationship of AD pathology and insulin expression. AD patients have shown an 80 % decline in insulin receptors [Andersen et. al., 2011]. Insulin's ability to attach to its receptors was found to diminish. Moreover, amyloid beta pathology, cholinergic system dysfunction, tau hyperphosphorylation, pro-apoptotic and pro-inflammatory events have all also been reported to be due to impaired insulin signaling [Becker et. al., 2012].

2.2 Possible Biological Mechanisms Underpinning the Association between Diabetes Mellitus and Alzheimer's disease

2.2.1 Glucose Toxicity

Hyperglycemia has the potential to lead to decrements in working memory and attention [Sommerfield et. al., 2004]. Chronic hyperglycemia has been found to be potent enough of causing cognitive impairment and irregularities in synaptic plasticity [Biesselset. al., 1996]. Glucose toxicity is mediated and regulated by certain factors, such as an increase in flux of glucose via the polyol and hexosamine pathway, an accumulation of advanced glycation end-products and an increase in production of oxidative stress [Brownlee et. al., 2001]. These processes have the potential of causing vascular damage and affecting the onset of neurodegenerative disorders in brain. As per a study, firm glycemic control majorly reduces the pace of brain atrophy as compared with the standard glucose treatment, but no clear difference was found in the cognitive function between the treatment groups. [Launer et. al., 2011].

2.2.2 Hypoglycemia

A number of studies reported that severe hypoglycemia could be a risk factor for cognitive impairments in type 2 diabetes patients. Patients with major hypoglycemia have an enhanced risk of cognitive impairment [Whitmeret. al., 2009; Lin et. al., 2013; deGalan et. al., 2009]. Severe hypoglycemia can induce irreversible neural damage including neuronal cell death [Fanelli et. al., 2004] and cause an amplification in platelet aggregation and fibrinogen formation [Wright et. al., 2008], thus accelerating cognitive dysfunction. Older patients are suspected to have lesser brain plasticity as compared with younger patients. Thus hypoglycemia might lead to neurologic changes which cause an older patient much more susceptible to dementia [Artola et. al., 2002].

2.2.3 Modifications in Insulin Sensitivity

Insulin resistance and hyperinsulinemia are used for characterizing type 2 diabetes mellitus' early stages. On the other hand, a number of evidences have suggested that insulin and insulin receptors do play major roles in the regulation of cognitive performance by modification of the actions of both excitatory and inhibitory postsynaptic receptors and by activating specific signaling pathways [Zhao et. al., 2001; Zemva et. al., 2011; Salkovic-Petrisic et. al., 2009]. Insulin receptors are significantly expressed in many brain regions such as the cortex and the hippocampus [Zhao et. al., 2001]. As per a study, prolonged hyperinsulinemia has the potential of inducing an impaired response to insulin via an alleviated expression of insulin receptors at the blood brain barrier and brain, and thus inhibits the insulin transport into cerebrospinal fluid and brain tissues [Neumann et. al, 2008]. Such changes have the potential of causing deficits in learning as well as memory formation. This might be due to an energy crisis of neuroglial cells [Zhao et. al., 2001; Craft et. al., 1998]. Also, patients with Alzheimer's disease have less or disrupted brain insulin sensitivity. This is evident by decreased insulin levels in cerebrospinal fluid, elevated plasma insulin levels, and drastically diminished densities of insulin receptor in the brain as compared to the healthy adults [Craft et. al., 1998]. Due to elevated levels of plasma, amyloid accumulation takes place due to limited degradation of amyloid beta protein which is due to the direct competition for the insulin degrading enzyme. It has the potential of degrading both insulin and amyloid beta protein [Neumann et. al., 2008]. Also, insulin and insulin-like growth factor-1 initiate and facilitate the transportation of amyloid beta carrier proteins such as albumin and transthyretin into cerebrospinal fluid and the elimination of amyloid beta protein from the brain. However, lesser insulin levels in cerebrospinal fluid and the impaired response to insulin and insulin-like growth factor-1 inhibit the transportation of these carrier proteins and reduce the clearance and removal of amyloid beta protein [Craft et. al., 2004].

2.2.4 Inflammation

Chronic inflammation has been reported to be a part of the initiation of insulin resistance and in the pathogenesis of diabetes mellitus [Mandrekar & Landreth, 2010]. Type 2 diabetes mellitus patients have been reported to have elevated levels of circulating inflammatory markers

[Akiyama et. al., 2000]. The associations between the inflammatory markers and impaired cognitive skills and decline among community-dwelling elderly have been extensively studied [Dhawan et. al., 2012; Lue et. al., 2001]. In an AD brain, inflammatory response of microglial cells has been observed to be activated [Querfurth & LaFerla, 2010]. Elevated levels of interleukin-1, tumor necrosis factor-α, interleukin-6, C-reactive protein, granulocyte macrophage colony-stimulating factor and eotaxin have been reported in brain tissue from patients with Alzheimer's disease [Freeman et. al., 2002]. A cross-sectional study has shown that prominent circulating levels of inflammatory markers were linked to poor cognitive efficiency in people with diabetes mellitus. These findings suggest that chronic inflammation may possibly play a role in increasing cognitive impairment, either through a direct effect on the brain, or by influencing the development of vascular disease [Takeda et. al., 2010].

2.2.5 Diabetes Mellitus and Tau Pathology

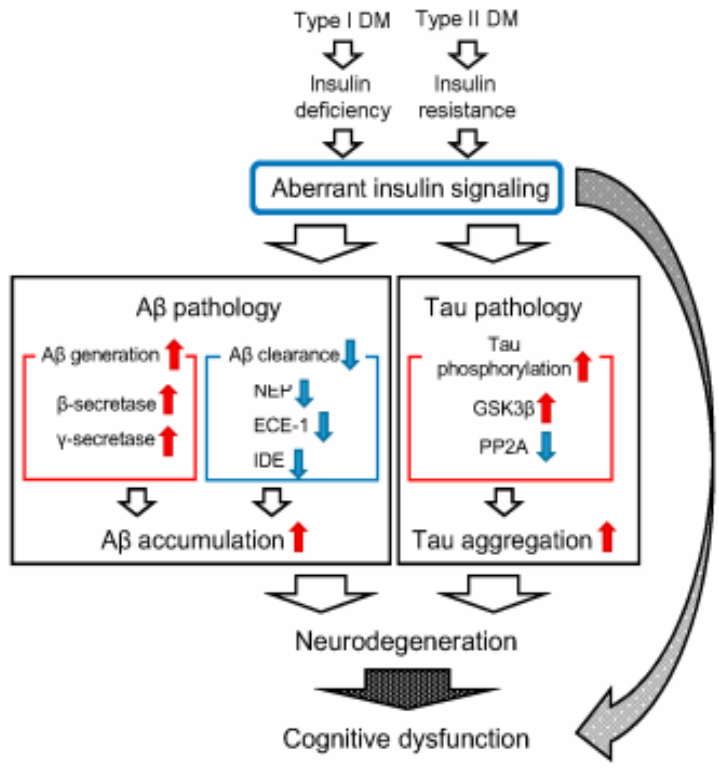


Fig.2

The relationship between Type 2 DM, tau pathology and dementia in AD patients has been well depicted in fig.2. Insulin and insulin-like growth factors bind to insulin receptor and are involved in intracellular signaling pathways. This leads to the auto phosphorylation and activation of insulin receptor. Insulin receptor tyrosine kinases phosphorylate the IR substrate molecules which results in the activation of phosphoinositide-3 kinase (PI3K)/Akt signaling. The activation of this pathway leads to the phosphorylation of Ser9 of GSK3β and inhibition of its kinase activity.

Thus, insulin resistance has the potential of inducing abnormal activation of GSK3β. A number of studies have demonstrated the activity of GSK3β is much improved in both type I and type II DM models, further leading to the buildup of hyper phosphorylated tau [Chami et. al., 2016]. The activity of protein phosphatases is also necessary and is required for mediating tau phosphorylation level. In an AD brain, decreased activity of protein phosphatases, such as protein phosphatase 2A (PP2A) was found [Matsuzaki et. al., 2008].

2.3 Quercetin

Quercetin is a prominent bioflavonoid that has antioxidant [Zhang et. al., 2011], anti-inflammatory and anti-cancer properties [Joshi et. al., 2011]. Frequently it occurs as glycosides (sugar derivatives). Quercetin is termed the aglycone or sugarless form of rutin and is a key molecule in fighting several chronic neurodegenerative diseases. A number of *in-vivo* and *in-vitro* studies are evident enough and are indicative of the neuroprotective properties of quercetin. For an instance, quercetin on being orally administered (50 mg/kg body weight) to male Wistar rats (0.12–0.14 kg) was found to greatly reduce the elevated oxidative stress in the hippocampus and striatum of rats which were exposed to forced swimming [Ishisaka et. al., 2011]. In a study conducted by Sabogal-Guaqueta et. al., triple transgenic mice were taken and 25 mg/kg of quercetin was intraperitonially injected into them and that too in every 48 hours. This was done for 3 months. A significant cell density loss was observed in the subiculum of those mice. After 3 months an increase in the cell density was observed in the subiculum. Also, apart from enhancing cell density, quercetin effectively reduced the amount of beta amyloid deposition and NFTs in the hippocampus and amygdala of those mice. Astrocyte activation and microglial

activation which are considered to be hallmarks of AD were decreased in quercetin treated 3xTg-AD mice [Sabogal-Guaquetaet. al., 2015]. Quercetin also provided neuroprotection against the neuronal injury induced by cadmium in frontal cortex of Sprague-Dawley rats [Unsal et. al., 2013]. The neurodegeneration caused by aluminium has been well documented. ROS are generated by aluminum, which leads to impaired cellular antioxidant defense and releases cytochrome c from mitochondria into the cytosol. This leads apoptosis. As per a study, 10mg/kg body weight/day quercetin was administered to male albino rats (Wistar strain). Quercetin did not only decrease ROS production but also increased mitochondrial superoxide dismutase activity. In addition to that, it even prevented aluminum induced translocation of cytochrome-c, upregulated Bcl-2, downregulated Bax, p53, activated caspase 3 and reduced DNA fragmentation. Quercetin effectively obstructs neurodegenerative changes rats [Sharma et. al., 2016]. Quercetin has also been found to protect against the neurodegeneration induced by metals such as lead, tungsten and methyl mercury. It was also found to be neuroprotective in intracerebral hemorrhage models of rats [Lucio et. al., 2016]. Quercetin also enhances memory, enhances cognitive efficiency and prevents anxiogenic-like behavior, which is induced by STZ-diabetes. In also prevents the decrease in the NTPDase and increases the adenosine deaminase (ADA) activities in SN from cerebral cortex of STZ-diabetes. Quercetin, by specifically activating macroautophagy and proteasomal degradation pathways, proved able to prevent beta amyloid aggregation and paralysis in *Caenorhabditis elegans* [Charlotte et. al., 2014].

Quercetin (40 mg/kg) reduced scattered senile plaques, alleviated amyloid beta-induced mitochondrial dysfunction, and improved cognitive impairment in APPswe/PS1dE9 transgenic mice. It shows therapeutic promise in an AD transgenic mouse model, and it may have potential as an AD therapeutic agent [Dong-Mei et. al., 2014].The appearance of 3-O-β-glucuronide (Q3GA) has been observed in rat plasma after administering quercetin via oral route. Q3GA could thus be partly held responsible for elevating the antioxidant activity obtained through the intake dietary quercetin-rich foods [Moon et. al., 2001]. Thus, quercetin metabolites also contribute towards the antioxidant defense in blood circulation. Q3G has also been reported to be the major active component in plasma and inside the tissue after the orally administering quercetin or Q3G [Yang et. al., 2016].

It has been shown to strongly inhibit Aβ fibril formation, and protect HT22 murine neuroblastoma cells from Aβ oxidative attack [Kim et. al., 2005; Bastianetto et. al., 2002]. As per a study, Hsp68 was induced in C6 rat glioma cells by increased oxidative stress. Quercetin treatment has also proved to improve the viability of P19 cells against oxidative injury caused by hydrogen peroxide. Quercetin attenuated the generation of reactive oxygen species, prevented hydrogen peroxide-induced nuclear condensation, increased the caspase 3/7 activity and elevated the poly(APD-ribose) polymerase expression [Jembrek et. al., 2012]. It has been reported via *in vitro* studies carried out in neuronal cell lines and in primary neurons that quercetin, at low micromolar concentrations, combats cellular toxicity which is induced by various oxidants (e.g., hydrogen peroxide, linoleic acid hydroperoxide) and other neurotoxic molecules which are believed to act by inducing oxidative stress (e.g., 6-hydroxydopamine and N-methyl-4phenyl-1,2,3,6-tetrahydropyridinium) [Mercer et. al., 2005]. Multiple glucuronidated, sulfated and methylated quercetin metabolites have demonstrated neuroprotective actions in vitro [Shirai et. al., 2006; Yeh et. al., 2011].

CHAPTER 3

AIM AND OBJECTIVE

3. AIM AND OBJECTIVE

In the present project we aimed to screen small molecules of natural origin for their potential to alleviate neurological complications (dementia) which is commonly observed to be associated with and Alzheimer's disease and Diabetes Mellitus. For this we used *in-vitro* and *in-vivo* experimentation approach and fabricated our project into following broad objectives.

1. To screen small molecules of natural origin for their ability to modulate cholinergic signaling (Acetylcholinesterase and Butylcholinesterase inhibition) through *in-vitro* screening.
2. To inducing Alzheimer's disorder in male Wistar rats through stereotaxic intrahippocampal injection of amyloid beta (1-42).
3. To evaluate effect of Quercetin treatment of amyloid induced memory dysfunction.
4. To induce diabetes in Swiss albino mice and evaluate the effect of quercetin on associated memory dysfunction.
5. To understand association between memory impairment and neuronal morphology during diabetic state and to evaluate the effect of Quercetin in this scenario.

CHAPTER 4

MATERIAL AND METHODS

4. MATERIALS AND METHOD

4.1 MATERIALS

Apart from very common laboratory reagents, materials, which include chemicals, reagents and drugs, used in the current study are listed in a table below in Table 2.

Table 2: List of drugs, chemicals and natural compounds

S. No.	Name of Chemical/Item	Brand
1	DTNB	Sigma Aldrich
2	BUTYRYLCHOLINESTERASE	Sigma Aldrich
3	ACETYLCHOLINESTERASE	Sigma Aldrich
4	DMSO(dimethyl sulfoxide)	Loba Chem
5	QUERCETIN, RUTIN, ROSIGLITAZONE, BERBERINE, PIPERINE	Sigma Aldrich
6	BRADFORD REAGENT	HiMedia
7	BETA AMYLOID	Sigma Aldrich
8	KETAMINE	Medindia
9	XYLAZENE	Medindia
10	EVANS BLUE	Sigma
11	GOLGI COX SOLUTION	Sigma
12	ANTIBODIES	Santa Cruz Biotech

Apart from very common laboratory instruments, apparatus used in the current study are listed in a table below in Table 3.

TABLE 3: List of apparatus

S. No.	APPARATUS	Brand
1	SDS-PAGE assembly unit	Medox
2	WESTERN BLOT assembly unit	Bio-Rad
3	SPECTROPHOTOMETRE	Systronics

4	STEREOTAXIC	Kopf
5	HAMILTON SYRINGE	Sigma
6	PHOTOACTOMETRE	Medox

4.2 METHODS

4.2.1 *In-vitro* Acetylcholinesterase and Butyrylcholinesterase Inhibition Assays

Principle

Acetylcholinesterase and butyrylcholinesterase enzymes effeciently catalyses the hydrolysis of acetylthiocholine–sulphur analog of the natural substrate of these enzymes. After hydrolysis the substrate analog gets reduced to acetate and thiocholine. In the presence of highly reactive dithiobisnitro- benzoate (DTNB) ion, thiocholine generates a yellow color, which is quite visible and is observed via spectrophotometer at 512 nm wavelength.

Experimental Protocol

A master mix solution comprising of DTNB ((5,5-dithio-bis-(2-nitrobenzoic acid), phosphate buffer saline (PBS; pH 7.4) and either acetylcholinesterase (AChE) or butyrylcholinesterase (BChE) enzyme at 12.5 mU was prepared for different reaction volumes in the concentrations given in Table.4:-

CONCENTRATION	PBS BUFFER (µl)	DTNB (µl)	BChE (µl)
1000	375	450	75
500	450	450	75
250	487.5	450	75
125	506.25	450	75
62.5	515.625	450	75

Table 4: Recipie for *in-vitro* butylcholinesterase and acetylcholinesterase assay

For every reaction an equal volume of the master mix was pipetted into 5 wells of the 96 flat bottom well plate.The control taken was DMSO and it was added to the first well that was containing the master mix. Quercetin, Rutin, Berberine and Piperine were respectively added to rest of the wells. Absorbance was taken at 512nm wavelength. After that Butyrlythiocholine iodide was pipetted to all the five wells and absorbance was then recorded four times with a time interval of 60 seconds. Same protocol was followed for Acetylcholinesterase assay.

4.2.2 WESTERN BLOT (GAPDH expression in Cortex)

Protein Estimation Using Bradford Assay

Principle

Bradford assay is based on the principle of an absorbance shift of the Coomassie Brilliant Blue dye G-250. Once the protein binds to the dye, it stabilizes the Coomassie dye. Greater is the amount of the complex which is present in solution, greater will be the absorbance reading and thus greater will be the estimated protein concentration. The cationic (unbound) form of the dye has an absorption spectrum maximum at 465 nm. The anionic form of the dye has absorption spectrum maxima at 595 nm. The increase of absorbance at 595 nm is proportional to the amount of bound dye, and thus to the amount (concentration) of protein present in the sample.

Experimental Protocol

1.5 ml eppendorf was taken for the sample collection. The eppendorf was labeled for the collection of supernatant. The sample aliquots were taken from -80. They were thawed and centrifuged for 30 mins at 160 rpm. Aliquots were put back in the ice bucket. The supernatant was then taken from it without resuspending it. 1200µl Bradford reagent and 397 µl water were then added in the eppendorf. Protein sample was then added to the eppendorf. Mixing was done appropriately in order to dissolve all the contents properly. 300µl from the mixture was taken and pipetted in duplicates in the 96 well plate. Absorbance was taken at 595nm. On the basis of absorbance, the concentration taken was 30 µg, as is given in Table 5.

SAMPLE	AB1	AB2	AVG	SLOPE	AMT=S/AVG	CONC=30/AMT
AD2	0.11	0.153	0.1315	0.0338	3.89053	10.14
ADQ2	0.125	0.132	0.1285	0.0338	3.80178	10.45
ADR1	0.114	0.0982	0.1061	0.0338	3.13905	13.59
AZ1	0.131	0.134	0.1325	0.0338	3.92012	10.03
AZQ2	0.141	0.157	0.149	0.0338	4.40828	8.62
AZR4	0.13	0.163	0.1465	0.0338	4.33432	8.81
CD6	0.275	0.249	0.262	0.0338	7.75148	4.39
CDQ2	0.258	0.279	0.2685	0.0338	7.94379	4.27
CDR1	0.217	0.259	0.238	0.0338	7.04142	4.91
CZ1	0.281	0.241	0.261	0.0338	7.72189	4.41
CZQ6	0.318	0.349	0.3335	0.0338	9.86686	3.35
CZR6	0.342	0.398	0.37	0.0338	10.9467	2.99

Table 5: Protein concentrations quantified in different samples using Bradford assay

4.2.3 SDS PAGE WESTERN BLOT

Principle

Sodium Dodecyl Sulphate (SDS) is an anionic detergent that binds uniformly to the protein molecule and denatures it. It imparts a uniform negative charge to the protein molecule. Since all the protein molecules carry the same charge, they are thus separated on the basis of their respective molecular weights. Denatured samples are loaded into the wells present in stacking gel (pH: 6.8) which is present above the resolving gel (pH: 8.8) and are separated by the application of a suitable voltage. The stacking gel has a larger pore size as compared to the resolving gel. This gives rise to a sieving effect which results in a solely weight-dependent separation of proteins as smaller proteins migrate faster than larger molecules through the gel.

The importance of various components of SDS-PAGE is as follows:

Sodium Dodecyl Sulphate: SDS is an anionic detergent that denatures proteins and provides them uniform negative charge.

Acrylamide: Polymerization forming a meshwork.

Tetra ethylene diamine (TEMED): Catalyses the acrylamide to form free radicals & stabilizes the free radicals and improves polymerization.

Ammonium Per-Sulfate: Accelerates the TEMED

Experimental Procedure

Aliquots of protein samples were prepared by adding 6µl bromophenol blue dye to the amount of protein (given in table 4) in 1.5ml eppendorf. Resolving and stacking gel (10%) were prepared as per the Table 5 given below:

	RESOLVING GEL	STACKING GEL
WATER	4ml	3.4ml
TRIS-base	2.5ml(1.5M)	630µl (1M)
30% ACRYLAMIDE	3.3ml	830µl
10% SDS	100µl	50µl
10% APS	100µl	50µl
TEMED	6µl	5µl

Table 6: Recipe for the preparation of 10 % resolving gel and 4 % stacking gel for SDS-PAGE

Assembly was checked for leakage. Resolving gel was then poured followed by n-butanol (to reduce bubbles and avoid evaporation from surface). Gel formation was allowed without disturbing and was washed 3-4 times with autoclaved water. The level of separating gel was marked. Then 5ml stacking gel was added after removing the n-butanol. The comb was then inserted and was kept undisturbed until gel formation. The comb region was marked and gently

removed after the gel formation. Running buffer was then added to the assembly. The sample and ladder (GAPDH) were then loaded in the wells so formed. Gel was run at 100V till the dye got drained out of the gel.

4.2.4 Sample Preparation

Sample was mixed with 6 µl of loading dye/buffer. It was then heated at 95 degree centigrade in boiling water for 5 mins to denature proteins and then centrifuged for 10 sec at 8000 rpm. 30 µg samples were then loaded into the wells in the gel. Gel was allowed to run at 100V and 50A current until the loading dye almost reached the end of the gel.

4.2.5 TRANS BLOT

Principle

Once the proteins have been separated via SDS PAGE, then they are transferred to a polyvinylidene fluoride (PVDF)/nitrocellulose membrane in the presence of an electrical field. The gel is sandwiched between the layers of membrane and filter pads and a suitable voltage is provided, which enables the transfer of proteins from the gel to the membrane. Once the proteins are bound to the membrane, they are detected with the help primary and secondary antibodies.

Experimental Procedure

Two filter pads, each of 4.5 cm by 6.5 cm were taken and kept soaked in transfer buffer for about half an hour. Gel was removed and the extra gel was cut. The gel was immersed in transfer buffer and placed on an equal sized nitrocellulose membrane on the top of it. Transblot transfer apparatus was wiped with 70 % ethanol/isopropanol. Transfer buffer was then poured in it. Firstly, a filter pad was placed and small amount of transfer buffer was added onto it. An equal size whatmann paper was placed onto it prior to placing it in transfer buffer. The gel was placed on it and adjusted according to the size of the pad. Two soaked whatmann filter paper were placed on it and a little more buffer was poured onto it. The lid of the apparatus was then closed. The current was set and gel was allowed to run for 1 hr. After 1 hr the lid was lifted to see transfer of spots/bands to membrane. The membrane was soaked in 3% BSA in PBS and rocked it for 2 hrs at room temperature or at 4 ºC overnight. After incubation, primary antibody (goat polyclonal IgG) (1:2500) was added to it and kept at 4ºC overnight. Then it was kept on the

rocker for 1 hour at room temperature. After that the primary antibody was removed and membrane was washed 3 times with PBS and 2 times with PBST (0.1 % tween 20), 5 time each. Secondary antibody (donkey anti-goat HRP) (1:5000) was then added to the membrane and kept on the rocker for 2 hours at room temperature. After 2 hours, the secondary antibody was removed and washes were given (5 times PBS and 3 times with PBST) of 5 min each. To visualize the bands, DAB (6.8 mg/10 ml PBS), H_2O_2 (10 µl) and $CoCl_2$ (100 µl) were added to the membrane. Bands were visualized, membrane was then washed with PBS, bands were scanned and optical density was determined by image J software.

4.3 Animal Model for Alzheimer's disease (Amyloid Beta Induced Alzheimer's disease)

Standardization of Stereotaxic Surgery

Stereotactic surgery is a surgical procedure wherein a three-dimensional coordinate system is used for locating targets inside the body and performing on them some actions such as ablation, biopsy, stimulation, injection, lesion removal, implantation, radiosurgery (SRS), etc.

Protocol for Standardization of Stereotaxic Surgery

Wistar rats were anesthetized by giving intraperitoneal injection of 90 mg ketamine and 5mg xylazene. Depilatory cream was applied on the head to remove hair. Betadine/PBS was applied onto the head. Ear bars were inserted and the animal was made to fix. Following an excision, the skin above the skull was opened up. AP (-4.8mm), ML (±3.5mm) AND DV (3 mm) coordinates were marked. Drilling was done using needle. The required distance was marked on the Hamilton syringe. 5 µL Evans blue was injected in 5-10 mins duration and the syringe was kept in that place for 10 mins and then removed slowly. Dental cement was then applied and the animal was allowed to stand for 30 mins. The animal was then sacrificed and the brain was isolated. Brain of the animal was cut into different section to visualize the injection sight and spreading of dye to confirm whether the dye have been injected into the hippocampus or not.

4.4 Morris Water Maze (Learning and Memory evaluation)

Animals were grouped into 4 groups as follows: Group 1: Control (no surgery was done), Group 2: Sham operative (ICH surgery and saline injected), Group 3: Aβ control (ICH surgery + Aβ injected) and Group 4: (ICH surgery + Aβ injected + Quercetin treatment). Stereotaxic surgery was performed and animals were allowed to recover for 14 days. After which learning and memory was evaluated via Morris Water Maize test. Briefly, apparatus consisted of a circular pool filled with water. Pool was divided into 4 hypothetical quadrants, in one of the randomly selected quadrant a escape platform was provided such that it remained invisible to the animals. Learning was given for 4 days, with 4 learning sessions each day during which ability of animal to find hidden platform was recorded. Cut off time was set to 60 sec. On day 5, memory index was evaluated by removing platform. Rats were then sacrificed and their hippocampus was isolated for further study of protein expression analysis.

4.4 Streptozotocin induced diabetic model in Swiss albino mice

In order to evaluate the effect of Quercetin treatment in memory dysfunction associated with type II diabetes mellitus, we induced type II diabetes in Swiss albino mice by injecting them with 50 mg/kg Streptozotocin (STZ) intraperitoneally in ice cold citrate buffer (pH 4.5). Diabetes was confirmed by taking blood glucose from tail vein using Accu check Glucometer. Animals were divided into 6 groups: I: Control (received vehicle), II: Received 15 mg/kg Quercetin, III: Received 5mg/kg Rosiglitazone, IV: Received STZ + Vehicle, V: Received STZ + Quercetin and VI: Received STZ + Rosiglitazone. Treatments were provided for 2 months after which memory dysfunction was evaluated using Passive Avoidance Step Down task.

4.5 Passive Avoidance Step Through Latency (Memory evaluation)

Passive Avoidance Step Through apparatus consisted of a light and dark chamber. Light chamber is brightly illuminated with 100 W bulb and is connected to dark chamber with small opening through which animal can freely move to dark chamber. Dark chamber has grid floor which can deliver foot shock to the animals. On day 1 of the test, learning is given. During this animal was placed inside light chamber and connecting door was opened. Time taken by animals to enter dark chamber was recorded. As soon as the animal enter dark chamber, door was closed and a foot shock was given to the animal. After 24 h, ability of the animal to remember foot shock was

noted as a measure of memory index in term of time taken to enter dark chamber. Experiment was repeated and time taken to enter dark room was recorded. However, this time no shock was given to the animals. Results were recorded as inflexion ratio calculated as (time taken to step through on day 1 – time taken on day 2)/ time taken on day 1.

4.6 Protocol of Golgi Staining

In order to evaluate the effect of diabetes and Quercetin treatment on neuronal morphology, we performed Golgi-Cox staining. Animals were sacrificed by cervical dislocation. The brain was washed with chilled water followed by ice cold Golgi Cox solution. Blocks of the brain were prepared such that hippocampus is almost visible. Those blocks were then placed in freshly prepared Golgi-Cox solution in dark for 24 hrs at 37ºC. Sections of 200 μm thickness were then cut. They were then rinsed twice with distilled water for 5 mins and then dehydrated in 50% ethanol for 5 mins. The blocks were then kept in ammonium solution (3:1; ammonia: water) for 5-10mins. Then they were rinsed twice with water for 5 mins each. After that they were kept in 5% sodium thiosulphate for 10mins in dark and then rinsed twice with distilled water for 2mins. They were later dehydrated twice (5-10mins each) in 70%, 80%, 95% ethyl alcohol and 99% 1-butanol, cleared in toulene and fixed in DPX on gelatinised slides and visualized under 400X and 1000X magnification of light microscope.

CHAPTER 5

RESULTS AND DISCUSSIONS

5 RESULTS AND DISCUSSION

5.1 *in-vitro* Butylcholinesterase (BChE) and Acetylcholinesterase (AChE) inhibition assay

In-vitro inhibition of Butylcholinesterase (BChE) and Acetylcholinesterase (AChE) enzyme activity was performed. These enzymes play an important role in the cholinergic signaling as they are responsible for the degradation of Acetylcholine (Ach). ACh is a neurotransmitter which is essential for the activation of cholinergic signaling. Cholinergic signaling is crucial for memory formation and retention, besides playing important role in neuronal survival. During dementia, cholinergic signaling is downregulated, and therefore, drugs which are capable of inhibiting these enzymes, such as donepezil, are used clinically to control dementia. Here we screened natural molecules for their ability to inhibit these enzymes through in-vitro assay. Results are depicted in the Figure 3 below. Natural molecules, especially Quercetin, efficiently inhibited these enzymes and IC50 value of the BChE inhibition was found to be in the order Quercetin < Berberine < Piperine < Rutin and IC50 value of the AChE inhibition was found to be in the order Piperine < Berberine < Rutin < Quercetin. These resukts suggest that natural molecules can efficiently inhibit the activity of these molecules and therefore can be crucial I upregulating cholinergic signaling during dementia.

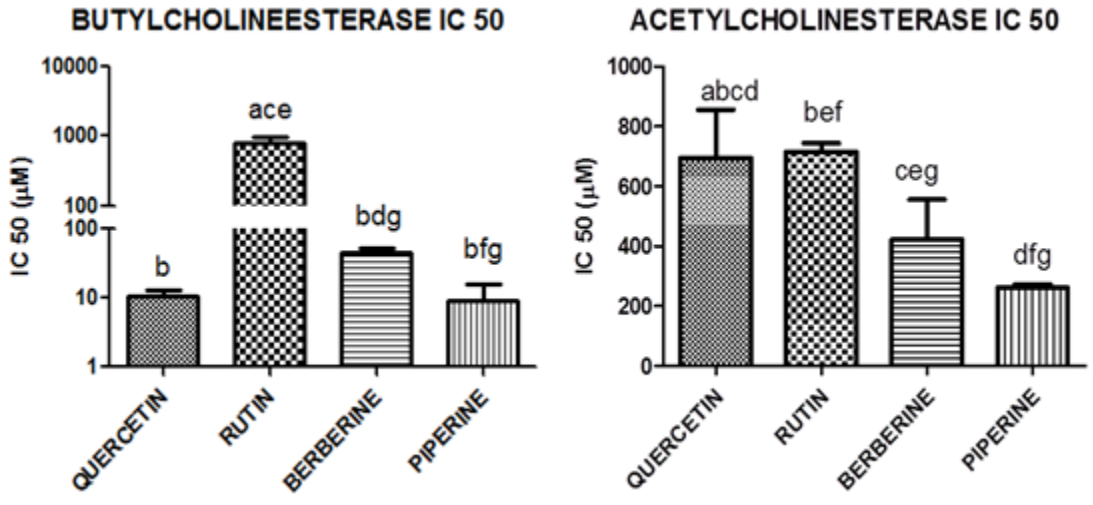


Fig. 3: In-vitro inhibition of BChE and AChE activity in terms of IC50 values

The bars in Fig.3 having same alphabetical denomination are non-significantly different from each other and bars having different alphabetical denomination are significantly different. These bars represent the IC50 values of the different drugs used i.e. of Quercetin, Rutin, Berberine and Piperine.

5.2 Protein quantification

Amount of protein in different samples was evaluated by Bradford assay using standard curve of Bovine serum albumin. Final concentration of the proteins in the samples was adjusted to 3 µg/µl. To run every blot 30 µg or 10 µl sample was loaded. Table demonstrated the protein concentration in different samples tested.

Table 7: Protein quantification using Bradford assay

SAMPLE	Abs 1	Abs 2	Avg.	Slope (S)	AMT = S/Avg.	Conc. = 30/AMT
CTRL-1	0.11	0.153	0.1315	0.0338	3.89053	10.14
CTRL+Q-1	0.125	0.132	0.1285	0.0338	3.80178	10.45
CTRL+ROSI-1	0.114	0.0982	0.1061	0.0338	3.13905	13.59
STZ-1	0.131	0.134	0.1325	0.0338	3.92012	10.03
STZ+Q-1	0.141	0.157	0.149	0.0338	4.40828	8.62
STZ+ROSI-1	0.13	0.163	0.1465	0.0338	4.33432	8.81
CTRL-2	0.275	0.249	0.262	0.0338	7.75148	4.39
CTRL+Q-2	0.258	0.279	0.2685	0.0338	7.94379	4.27
CTRL+ROSI-2	0.217	0.259	0.238	0.0338	7.04142	4.91
STZ-2	0.281	0.241	0.261	0.0338	7.72189	4.41
STZ+Q-2	0.318	0.349	0.3335	0.0338	9.86686	3.35
STZ+ROSI-2	0.342	0.398	0.37	0.0338	10.9467	2.99

5.3 <u>Western Blot (Standardization using protein samples from the cortex region of the brain)</u>

Western blot is a common technique used to evaluate expression levels of different proteins and can be used to evaluate expression of different proteins in the brain samples of diabetic animals and effect of drug treatment on it. However, before proceeding the entire protocol needs to be standardized. Herein we standardized western blot using samples from the cortex region of the brain and determined expression of housekeeping protein GAPDH. Results are demonstrated in the Figure 4 below. Image above represent the visualized bands on nitrocellulose membrane and graph represent the expression levels in term of optical density calculated form Image J software. These results suggest that the entire procedure is successfully standardized, besides suggesting that there is no significant difference present in the expression of housekeeping protein between groups.

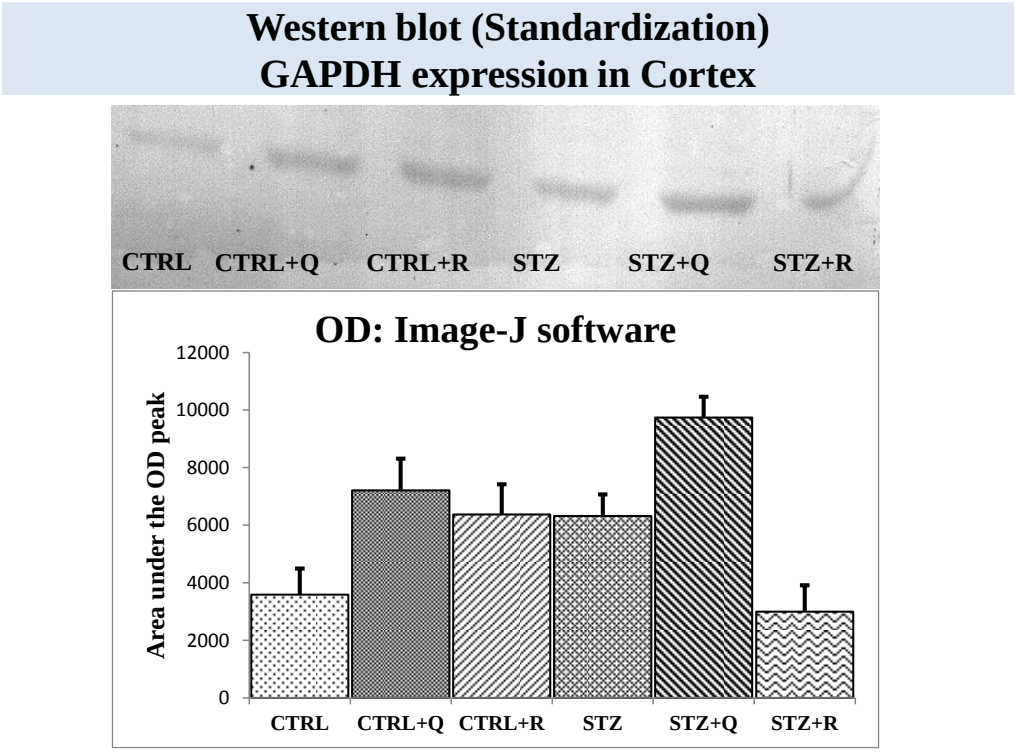
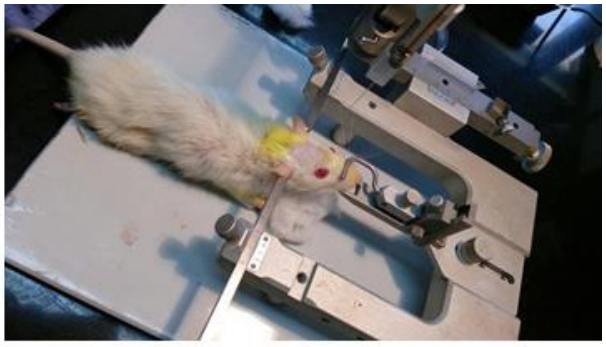


Fig. 4: Western blot expression of GAPDH in the cortex of STZ model animals

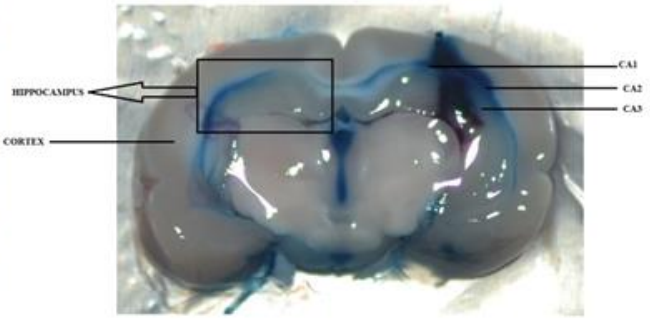
5.4 Standardization of stereotaxic surgery and ICH injection

We have successfully standardized the stereotaxic surgical procedure in our lab and located the injection site of ICH injection such that injected dye/drug spread uniformly throughout the hippocampus region of the brain. Results are depicted in the Figure 5 below. Figure 5a and 5b depicts the stereotaxic apparatus and animal mounted on it along with injection using Haemilton syringe. Figure 5c depicts the injection site and uniform spreading of the Evan's blue dye in the hippocampus using section of brain.

Stereotaxic apparatus and surgery



Stereotaxic apparatus and surgery



ICH injection and spreading of Evan's blue

Fig.5: Stereotaxic surgery, ICH injection and visualization of injection site in hippocampus using Evan's blue dye

5.5 Morris Water Maze (MWM)

Results of the MWM are depicted in Figure 6. As indicated by the given figure, control, and sham operated demonstrated normal learning ability as there was no significant difference between these animals to find the hidden platform across the trials. These results suggests that surgery is not having any significant impairing effect on the learning in animals. Further, amyloid injection to the hippocampus resulted in significantly impaired learning ability in animals and they struggled to find the hidden platform. Treating animals with quercetin efficiently improved learning and significant ($p < 0.001$) improvement was observed. These results suggest that Quercetin has beneficial effect in improving amyloid induced learning and memory dysfunction and therefore can prove to be a beneficial molecules in the management of dementia.

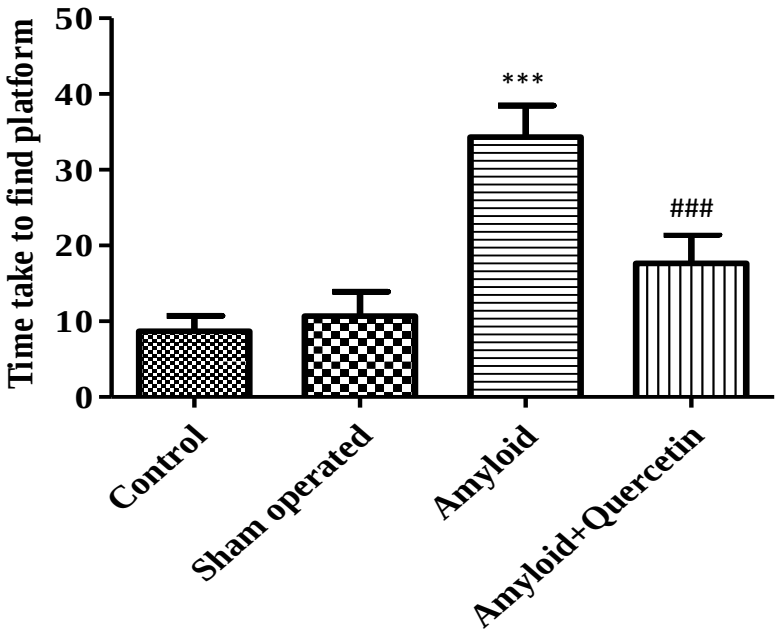


Fig. 6: Time taken by animals to find the hidden platform (sec)

5.6 Passive Avoidance Step Through test

Effect of type II diabetes and Quercetin treatment on memory was evaluated using Passive Avoidance Step Through test and results are depicted in Figure 7 in terms of inflexion ratio. These results suggest that diabetes induced significant ($p < 0.001$) impairment in the memory of animals. Treating animals with quercetin resulted in a significant ($p < 0.001$) improvement in memory performance, suggesting that quercetin is capable of alleviating diabetes associated memory dysfunction. Rosiglitazone (taken as standard drug) also showed significant ($p < 0.01$) improvement in the memory, however results were not as pronounced as those observed in Quercetin treated animals. These results suggest that Quercetin is capable of modulating memory dysfunction in diabetic animals.

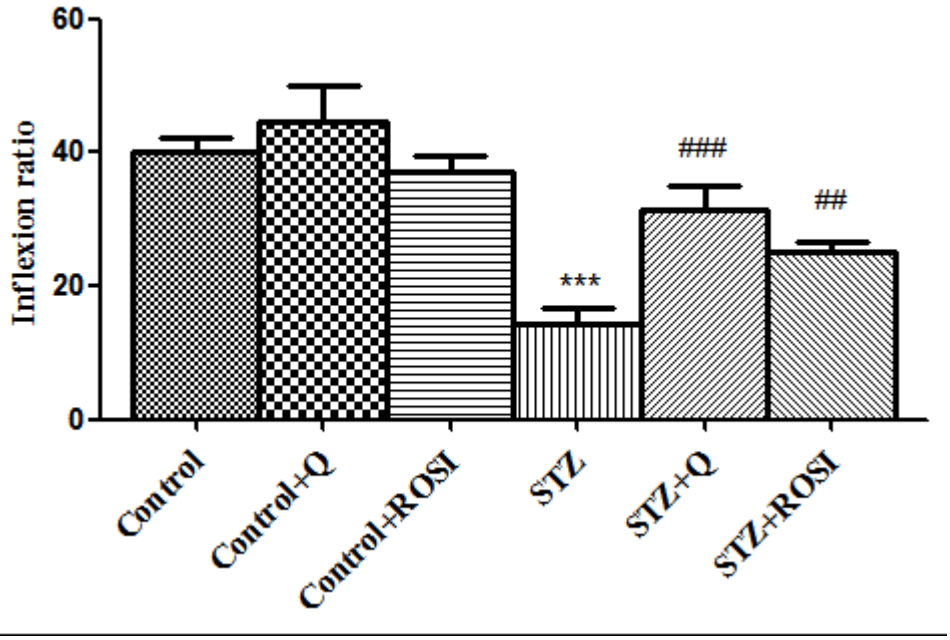
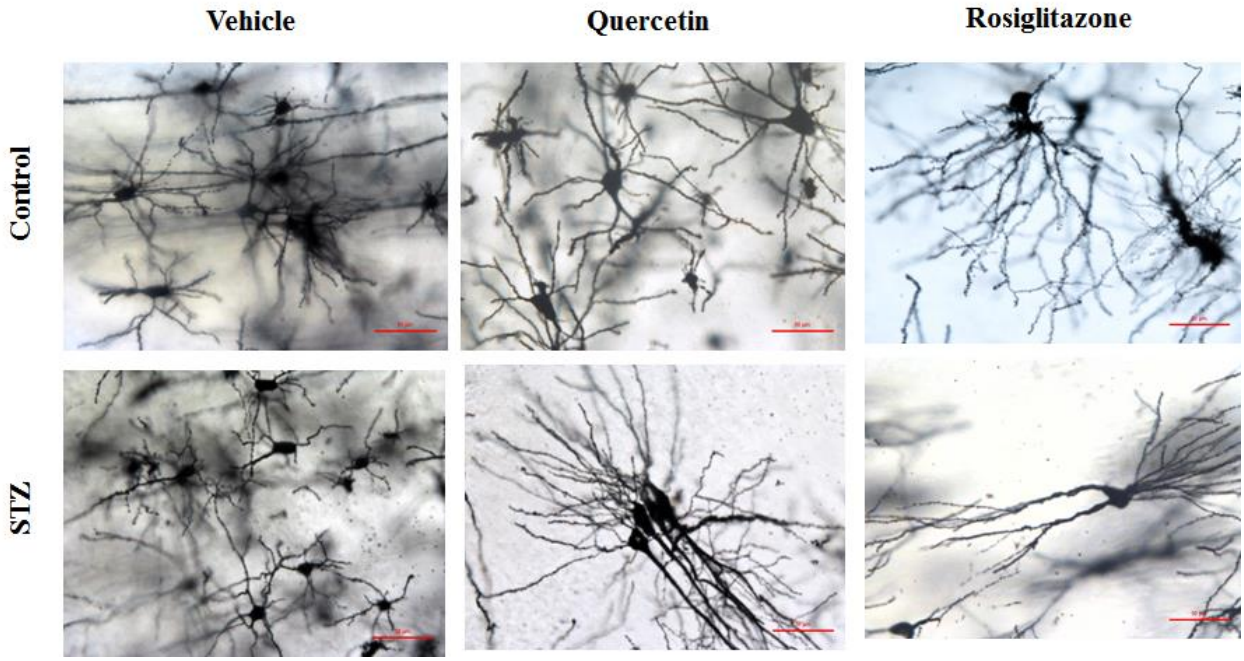


Fig. 7: Effect of diabetes and Quercetin treatment on memory performance of Swiss albino mice

5.7 GOLGI COX (Neuronal morphology; 400 X magnification)

We evaluated the effect of diabetes and quercetin treatment on neuronal morphology using Golgi-Cox staining procedure. Results are depicted in Fig. 8 below. It was observed that diabetes induces significant degeneration to the neurons as neurons in STZ+ vehicles appeared to be damaged with very few dendritic branching. Treating animals with Quercetin and Rosiglitazone improved neuronal morphology, neurons appeared to be healthy with numerous dendritic branching. These results suggests that impaired memory performance may be because of the neurodegeneration observed in diabetic animals and memory improvement in quercetin treated animals may be because it have prevented neurodegeneration and maintained healthy state of neurons.



5.8 GOLGI COX (Spine density; 1000 X magnification)

Numbers of spines in the dendrites are directly related to the number of new networks/connections it can establish with the other neurons, i.e. more the spike density more connection the neuron will form with other neurons. It was observed that number of dendritic spikes was significantly lower in diabetic animals which may explain the lower branching

observed in these neurons. Treatment with quercetin and Rosiglitazone improved neuronal morphology and significantly higher number of spines were observed. These neurons showed significantly greater branching and interneuronal connections, which may be explained by the higher spike density, observed in these animals.

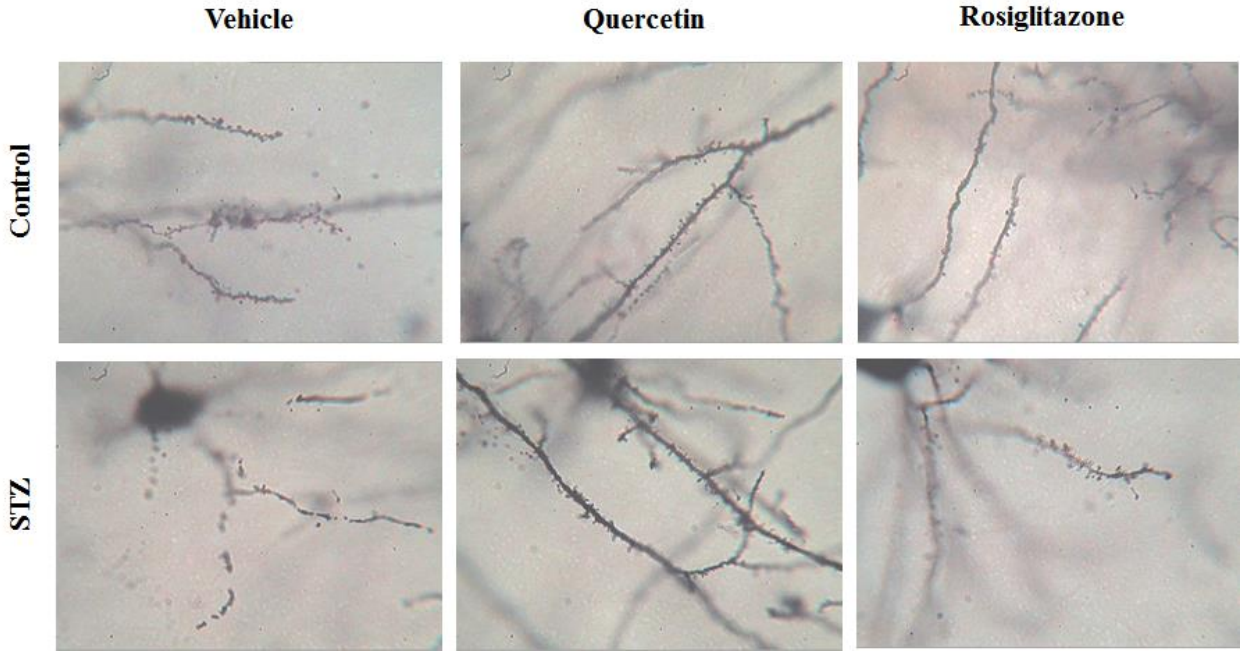


Fig. 8: Dendritic spikes visualized at 1000 X magnification (Golgi-Cox staining)

CHAPTER 6

CONCLUSION

6. CONCLUSION

In this study we demonstrated that natural molecules, especially quercetin, have good potential to inhibit the activity of Acetylcholinesterase and Butylcholinesterase enzyme activity and therefore may be beneficial in managing dementia by upregulating cholinergic signaling. Therefore, we further evaluated whether or not Quercetin is able to alleviate memory dysfunction through two animal models, i.e. ICH amyloid beta induced Alzheimer's model and STZ induced diabetic model. WE concluded that Quercetin is having good potential to improve memory performance in both these models and therefore may find an application in the management of learning and memory dysfunction associated with Alzheimer's or Diabetes. Further, we wished to understand why memory dysfunction is being observed in animals and what probably Quercetin might have done to improve it. Therefore, we performed Golgi-Cox staining to observe neuronal morphology, neurodegeneration and inter neuronal connections. We concluded that memory dysfunction is associated with the neurodegeneration, low spike density and low inter-neuronal connections. Quercetin treatment improved memory as a result of improved neuronal morphology, connection and survival. Therefore, it can be concluded that beneficial effects of Quercetin are partly related to its potential to improve neuronal survival. Quercetin may find a clinical application in the management of neurological complications, however, prior to that a rigorous screening in this respect need to be done further.

CHAPTER 7

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REFERENCES

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