Comparative Effects of Methylphenidate and Rosmarinic acid on $AlCl_3$ Mouse Model of Alzheimer's Disease

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MS Healthcare Biotechnology 2019

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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Healthcare Biotechnology

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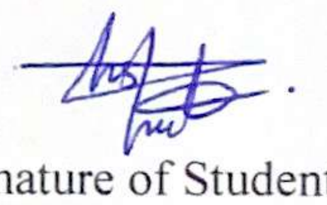

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Dedicated to

Almighty Allah

&

My Beloved Parents

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All praise to Almighty Allah, the Creator of opportunities and Authorities of knowledge, who gave me the strength and perseverance to accomplish this work. All regard to Holy Prophet Hazrat Muhammad (SAW) the greatest educator, the everlasting source of guidance and knowledge of humanity.

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Nishat Malik

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ABBREVIATIONS

AD	Alzheimer's disease
Aβ	Amyloid beta plaques
NFTs	Neurofibrillary tangles
APP	Amyloid Precursor Protein
ROS	Reactive Oxygen Species
SOD	Superoxide dismutase
RNS	Reactive Nitrogen Species
PD	Parkinson disease
HD	Huntington disease
MPH	Methylphenidate
ADHD	Attention Deficit Hyperactivity Disorder
CNS	Central Nervous System
RA	Rosmarinic Acid
UA	Ursolic Acid
CA	Carnosic Acid
AChE	Acetylcholine Esterase
PRDX6	Peroxiredoxin-6
BACE1	Beta Site Amyloid Precursor Protein Cleaving Enzyme 1
PSEN	Presenilin
TNF-α	Tumor Necrosis Factor Alpha
IL-6	Interleukin 6
NF-κB	Nuclear Factor Kappa B
SPs	Senile Plaques
FAD	Familial AD
SAD	Sporadic AD
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
NO	Nitric Oxide
GPx	Glutathione Peroxidase

HNE	4-hydroxynonenal
AGEs	Advanced Glycation End Products
RAGE	Receptor for Advanced Glycation End Products
H2O2	Hydrogen Peroxide
NMDAR	N-Methyl-D-aspartate receptors
STEP	Striatal-Enriched Protein Tyrosine Phosphatase
MnSOD	Manganese Superoxide Dismutase
PTMs	Post Translational Modifications
PFC	Prefrontal Cortex
BDNF	Brain Derived Neurotrophic Factor
NGF	Nerve Growth Factor
MWM	Morris Water Maze
FST	Forced Swim Test
EPM	Elevated Plus Maze
OFT	Open Field Test
PFA	Paraformaldehyde
DTT	Dithiothreitol
PMSF	Phenyl Methyl Sulfonyl Fluoride

ABSTRACT

Alzheimer's disease (AD) is an irreparable progressive neurodegenerative disorder which develop gradually and leads to cognitive decline affecting memory, thinking and behavior. It is a multifactorial disease involving amyloidogenesis, neuroinflammation and oxidative stress as the predominant pathogenic events. There is currently no treatment to cure or halt the progression of neurodegenerative diseases. Donepezil is one of the FDA approved drugs for symptomatic treatment however accompanied with various side effects. The current study investigated the interaction potential of the Rosmarinic acid (RA), a bioactive compound of *Rosmarinus officinalis*, and Methylphenidate (MPH) with different target proteins crucially implicated in AD pathogenesis, in comparison to the standard drug, Donepezil. Docking results revealed that Donepezil has the highest and RA has a comparable binding affinity with the enzymes acetylcholinesterase (AChE), presenilin-1 (PSEN-1) and beta-secretase 1 (BACE1). RA has exhibited the strongest binding with pro inflammatory cytokines (IL6, TNF-α, NF-κB, p50) followed by MPH and then Donepezil. Donepezil and RA has the highest and almost similar binding affinity with the antioxidant enzymes (SOD1, SOD2, Prdx6). Furthermore, drug likeness analysis indicated that RA showed desirable drug like criteria. The overall results have shown that RA potentially target multiple pathways involved in AD pathogenesis and can be considered as a potential drug candidate to combat AD associated consequences and apathy. Moreover, *in-vivo* studies carried out on mouse models for AD to understand the potential outcomes of Donepezil, *R. officinalis* and MPH on memory, anxiety and depression through behavioral analysis. The animals were separated into eight groups (each with n=8). 2-Dimensional Gel Electrophoresis was carried out to assess the differential protein expression in $AlCl_3$ +

Donepezil, $AlCl_3$ + *R. officinalis* and $AlCl_3$ + MPH-treated groups compared to control and $AlCl_3$-treated groups. Additionally, Western Blot was performed to compare protein acetylation and Congo red staining was performed for histological assessment. Donepezil, MPH and *R. officinalis* significantly improved memory in $AlCl_3$ induced mice model. All the tested compounds showed comparable effects on cognition. *R. officinalis* significantly reduced depression in $AlCl_3$-treated mice as compared to other treatment groups. Donepezil displayed the strongest anxiolytic like behavior followed by *R. officinalis* while MPH did not show significant anxiolytic effects in AD model. Furthermore, a marked difference in protein expression and protein acetylation was observed between control and diseased groups which was modified by drugs treatment. The results showed that Donepezil, MPH and *R. officinalis* have a significant role in modifying protein expression and protein acetylation in $AlCl_3$ treated mice. Histopathological assessment showed that AlCl3 exposure led to the formation of amyloid beta plaques in hippocampus but none of the tested drugs caused significant reduction in amyloid burden at the selected doses. In conclusion, *R. officinalis* is best in treating all aspects of AD as it is a natural compound which would have minimal side effects. Additionally, these findings provide a preliminary data set of the targeted proteins crucial for AD therapeutics and can be helpful to uncover the complex molecular mechanisms encompassing AD pathology.

Chapter 1

INTRODUCTION

Alzheimer's disease (AD) is an irreparable and gradually developing neurodegenerative disorder which leads to cognitive decline affecting memory, thinking as well as behavior (Ossenkoppele et al., 2015; Förstl & Kurz, 1999; Lu et al., 2018). It is the most prevalent form of dementia that is an extensive term defining deterioration in cognition apart from the general expected consequence of biological aging and that affects a person's ability to do everyday life activities ("2021 Alzheimer's disease facts and figures," 2021). AD is accompanied with the loss of memory, changes in the personality and mood, aphasia, infection in early dementia, performance disorders and eating problems. These symptoms affect the AD patients to the extent that they result in reducing the life quality and increasing the cost of patient's care that would be a major public health problem (Cao et al., 2018; Chaney et al., 2019; Heneka et al., 2015; Kinney et al., 2018; Lin et al., 2018; Newcombe et al., 2018; Zhu et al., 2018).

Alois Alzheimer was the first to report AD in 1907 (Hoyer, 1986) and this devastating disorder is now considered by World Health Organization as the major cause of dementia, adding to almost 60 to 70% of dementia cases ("2021 Alzheimer's disease facts and figures," 2021). According to the recent calculations, approximately 6.2 million Americans of age 65 or older are having Alzheimer's dementia in 202 (Rajan et al., 2021) and this figure is anticipated to raise to 13.8 million by 2060 ("2021 Alzheimer's disease facts and figures," 2021; Rajan et al., 2021). It is estimated that more than one in nine people of the age 65 and older are living with Alzheimer's dementia in the total American population

(Rajan et al., 2021). AD is officially recorded as the 6th major cause of mortality in the United States of America as well as is the 5th major cause of mortality for the older population of age 65 and more than 65 (Ahmad et al., 2021).

The greatest risk factors for developing AD are age, genetics and family history. Age is the greatest of all these factors. The percentage of people having AD increases as the age increases. Alzheimer's Association 2021 AD facts and figures stated that 5.3 percent of people aged 65-74, 13.8 percent of people aged 75-84 and 34.6 percent of people aged 85 and older are living with AD ("2021 Alzheimer's disease facts and figures," 2021; Rajan et al., 2021). Young people can also be affected by AD, but it is very rare with uncertain prevalence. AD is a gradually progressive neurodegenerative illness that start many years before the symptoms appear. Early symptoms are usually short-term memory loss such as facing hurdle in remembering names, recent events or conversations. It can worsen to disorientation, communication problems, behavioral changes that eventually lead to difficulty in speaking, walking and swallowing ("2021 Alzheimer's disease facts and figures," 2021).

AD is a multiplex disease. Amyloid beta (Aβ) plaques, neurofibrillary tangles (NFTs), neuronal loss as well as synaptic loss especially in cortex and hippocampus is the main neuropathologic features of AD. Aβ form extracellular plaques while hyperphosphorylated tau protein is responsible for intracellular NFTs (Lin et al., 2018). Aβ is derived from amyloid precursor protein (APP) which is basically a transmembrane protein and undergo cleavage by the action of α, β and γ secretase proteases. Successive cleavage of AAP by β-secretase and γ-secretase enzymes generate insoluble and neurotoxic Aβ that aggregates and forms plaques in brain tissues (Heneka et al., 2015). Hyperphosphorylation of tau

protein give rise to NFTs (Kinney et al., 2018; Al Mamun et al., 2020; Newcombe et al., 2018; Zhu et al., 2018).

Along with above mentioned pathologic characters, high neuroinflammation and oxidative damage are also seen in AD. These pathological events work together leading towards continuous neuronal destruction and cognitive decline. Primarily, a vicious circle grows amid Aβ, NFTs, inflammation as well as oxidative stress. Microglia are activated by Aβ plaques and NFTs which leads to the production of reactive oxygen species (ROS) and inflammatory cytokines. ROS and inflammatory cytokines on the other hand, operate directly on neurons increasing the synthesis of Aβ and NFTs (Broussard et al., 2012; Glass et al., 2010; Luque-Contreras et al., 2014). As a result, finding effective treatments that target several targets is extremely desirable (Frautschy & Cole, 2010).

Some isoforms of the Aβ peptide are formed by APP cleavage and the cleavage of APP occurs by the action of β- and γ-secretase enzymes. These isoforms can clump together to form senile plaques (O'brien & Wong, 2011). Therefore, targeting Aβ amyloidogenesis is a promising approach not only to slow but to stop further neurodegeneration (Zhao et al., 2008; Hardy & Selkoe, 2002). Beta secretase-1 which is called as beta site amyloid precursor protein cleaving enzyme 1 (BACE1) is considered as a primary target for drug development because Aβ production can be blocked by inhibiting the enzyme BACE1. Along with BACE1, inhibition of γ-secretase is also a clear approach to inhibit the production of Aβ. Despite being a protein complex, the development of secretase inhibitors capable of crossing the blood-brain barrier has been quite clear (Laras et al., 2005; Tomita, 2009; Zhao et al., 2008). Presenilin (PSEN) is the catalytic subunit of γ-secretase (Ahn et al., 2010; Steiner et al., 1999). Two isoforms of the PSEN, PSEN-1 and PSEN-2 are

implicated in the cleavage of APP but PSEN-1 has been shown to produce Aβ42 (relative to Aβ40) which is more aggregation prone as compared to PSEN-2 (Lee et al., 2011; De Strooper et al., 1998).

Cholinergic deficit is considered as a constant and preliminary finding in AD. Acetylcholinesterase (AchE) is regarded as the most feasible target for symptomatic treatment. Different inhibitors of acetylcholinesterase including donepezil, galantamine and tacrine etc have been developed for improving symptoms in AD (Mehta et al., 2012; Orhan et al., 2009).

Neuroinflammation is another major event in AD where elevated levels of pro-inflammatory cytokines are found (Morales et al., 2014). Increased levels of proinflammatory cytokines including tumor necrosis factor alpha (TNF-α) ae well as interleukin 6 (IL-6) were found in brain tissues as well as in serum of AD patients as compared to controls (Fillit et al., 1991; Strauss et al., 1992). In addition to being neurotoxic, Aβ aggregation can be aided by activated microglia and astrocytes. Many studies conducting in mouse models have exhibited that inflammatory conditions caused increase in Aβ deposition (Guo et al., 2002). TNF-α-activated nuclear factor kappa B (NF-κB) signal also appear to enhance the transcription of BACE1 leading to enhanced Aβ generation (Chen et al., 2012). TNF-α and IL6 are the proinflammatory cytokines that promote inflammatory responses.

AD pathogenesis has also been observed to be closely linked with oxidative stress induced toxicity (Uddin et al., 2020a; Uddin et al., 2020b; Uddin et al., 2020c; Zhang et al., 2004). Several enzymes including superoxide dismutase (SOD), peroxiredoxin-6 and small molecules are the parts of the antioxidant defensive mechanism. In human AD

investigations, amounts of these antioxidant enzymes are observed to be lowered in the brain of patients (Mota et al., 2015; Ramsey et al., 2007). AD brains are vulnerable to ROS assaults due to decreased levels of the essential antioxidant enzymes. ROS causes irreparable and gradual brain damage in such circumstances therefore key therapeutic strategy can delay or inhibit the oxidative stress via enhancing the activity of endogenous antioxidants (Feng & Wang, 2012), like antioxidant enzymes including superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2) and peroxiredoxin-6 (PRDX6) or halting the generation of ROS.

Methylphenidate (MPH) is a psychostimulant that has been approved by the FDA for the treatment of Attention Deficit Hyperactivity Disorder (ADHD) (Cheng et al., 2014; Thorpy et al., 2014; Trenque et al., 2014). It is a reuptake blocker of dopamine and norepinephrine and increases the concentration of these neurotransmitters in synaptic cleft by inhibiting reuptake transporters in the presynaptic neurons (Capp et al., 2005). In general, it creates a standard stimulant effect in the central nervous system (CNS) particularly within the prefrontal cortex. It is a derivative of phenethylamine and benzylpiperazine (Viggiano et al., 2004).

Rosemary is the common name for *Rosmarinus officinalis (R. officinalis)*, a member of Family Lamiaceae. It has been an important constituent of culinary and orthodox medicine since centuries, for a variety of purposes. European union has approved *R. officinalis* as a natural anti-oxidant for food preservation. In addition, *R. officinalis* serves as an anti-bacterial, reduces inflammation and holds anti-fungal properties. Aromatherapy also makes use of this multi-purpose aromatic herb. It reinstates proliferation ability of adult hippocampus in stress conditions (Rasoolijazi et al., 2015) and promotes hippocampal

neurogenesis mainly within the dentate gyrus region. *R. officinalis* has been reported to mitigate as well as improve the cognitive decline (Pengelly et al., 2012). Pharmacological effects produced by *R. officinalis* have been linked to its active compound rosmarinic acid (RA), ursolic acid (UA) and carnosic acid (CA) that have been manifested in in-vivo as well as in-vitro models. Presently the mild to moderate AD is treated by the available symptomatic therapeutics that improve cognitive performance. Such therapeutics are basically acetylcholine esterase (AChE) inhibitors and Donepezil is one of these (Eskandary et al., 2018; Kimura et al., 2005; Birks & Harvey, 2018). Donepezil is often utilized as positive control in research focusing on various elements of Alzheimer's disease and other neurological disorders to compare the efficacy of different drugs and natural chemicals (Adlimoghaddam et al., 2018).

Neurodegenerative diseases are becoming a global threat with the passage of time. There is presently no therapy available that can cure or halt the progression of neurodegenerative diseases. A combination treatment will be more effective to obtain the best therapeutic results by targeting multiple mechanism at a time. A latest study exhibited the comparative outcomes of MPH and *R. officinalis* extract on $AlCl_3$ induced AD mice model. It was found that *R. officinalis* extract acts as an anti-inflammatory and promising neurogenic and neuroprotective by regulating synaptic gene expression while MPH caused increase in inflammatory markers in healthy mice but reduced the expression of inflammatory markers in $AlCl_3$ induced mice (Khalid et al., 2020). This study was designed to find out the molecular targets of MPH and RA in multiple pathways of brain involved in AD by taking Donepezil as a standard drug, as well as to elucidate the comparative effects of Donepezil,

MPH and *R. officinalis* on hippocampal differential protein expression and differential acetylation in $AlCl_3$ induced mouse model of Alzheimer's disease.

1.1 RESEARCH OBJECTIVES:

This study intended to explore various aspects of AD pathology and effects of Donepezil, MPH and RA. The specific objectives were:

- Elucidation of the interaction patterns of Donepezil, MPH and RA with BACE1, PSEN-1, AChE, inflammatory markers (IL6, TNF-α and NF-κB p-50) and oxidative stress markers (PRDX6, SOD1, SOD2) through an *Insilico* approach.
- Investigation of the comparative outcomes of Donepezil, MPH and *R. officinalis* on anxiety, depression and memory of $AlCl_3$-induced AD mouse model through behavior tests.
- Elucidation of the comparative effects of Donepezil, MPH and *R. officinalis* on hippocampal protein expression in $AlCl_3$ -induced AD mouse model.
- Elucidation of the comparative outcomes of Donepezil, MPH and *R. officinalis* on hippocampal post-translational modifications in $AlCl_3$-induced AD mouse model.
- Histopathological assessment of hippocampus treated with Donepezil, MPH and *R. officinalis.*

Chapter 2

LITERATURE REVIEW

2.1 Alzheimer's Disease

Alois Alzheimer, a well-known neurologist exhibited the neuropathological features of the disease on 3rd November 1906, at the 37th meeting of the society of Southwest German Psychiatrists in Tübingen, Germany (Alzheimer, 1911). He described his observations about a female patient whose age was 51 years. The patient was having severe progressive cognitive deficits accompanied with brain atrophy, senile plaques (SPs) as well as neurofibrillary tangles (NFTs). The disease was named after him later by Emil Kraepelin (Kraepelin, 1913).

SPs are formed extracellularly by the abnormal aggregation of amyloid β (Aβ) protein while the NFTs are intracellular abnormal aggregates of hyperphosphorylated microtubule associated tau protein (Perrin et al., 2009; Al Mamun et al., 2020; Braak & Braak, 1991). The most affected areas in brains of AD include cortex, olfactory bulb, and hippocampus. SPs and NFTs are spatiotemporally disconnected: SPs appear in the cerebral neocortex around ten years before NFTs which are more prevalent in the entorhinal cortex. Aβ plaques and NFTs are pathological requirements of AD (Nelson et al., 2012). The correlation between them is still not understood but changes in the amyloid metabolism are considered to be involved in the disease processing (Hardy & Selkoe, 2002). AD is classified as familial (FAD) and sporadic (SAD). Familial is caused by genetic mutations in the genes of presenilin 1, presenilin 2 and the amyloid precursor protein (APP) (Cacace et al., 2016; Pimenova et al., 2018), whereas sporadic is caused by environmental as well

as genetic factors and is accountable for more than 95% of all AD cases (Karch et al., 2014; Kim et al., 2014).

2.1.1 Amyloid Cascade Hypothesis

Different cells of the body along with neuronal and glial cells have amyloid precursor protein that is a normal transmembrane protein. Successive cleavage of amyloid precursor protein (APP) leads to the generation of peptides of Aβ (Santana et al., 2015; Francis et al., 2005). Cleavage is done by secretase enzymes **(**α-, β-, γ-) which are actually protein complexes comprising presenilin and nicastrin (Haass et al., 2012). Normally, α-secretase cleaves APP and give rise to soluble sAPPα chunk and C83 chunk (83 amino acid chunk at carboxy terminal). sAPPα continue to live outside the cell while C83 is implanted in the cell membrane (Castello & Soriano, 2013; Haass et al., 2012)**.** In AD pathology, APP breakdown by β-secretase-1 (BACE-1) first generate sAPPβ and C99. Then C99 breakdown by γ-secretase give rise to peptide fragments of Aβ (1-40) or Aβ (1-42) which are considered to be accountable for plaque development (Haass et al., 2012). sAPPα is neuroprotective which has beneficial role in learning, memory, regulating action potential, enhancing synaptic plasticity and protecting against oxidative stress while fragments of Aβ can reduce synaptic plasticity, enhance oxidative stress, disrupt energy metabolism and calcium homeostasis (Haass et al., 2012; Mucke & Selkoe, 2012)**.** According to amyloid hypothesis, development, accumulation and precipitation of Aβ particularly the Aβ (1-42) is basically responsible for the diseases processing which then leads to toxicity and neurodegeneration (Hardy & Selkoe, 2002; Allan Butterfield, 2002; Haass et al., 2012). Enhanced Aβ (1-42) accumulation outside the cell triggers hyperphosphorylation of Tau

protein forming NFTs and ultimately brain inflammation (Castello & Soriano, 2014; Nalivaeva et al., 2008; Nussbaum et al., 2012)**.** So Aβ aggregation is induced by any of these three factors which are increased Aβ formation and increased formation of aggregation susceptible entities (regarding FAD) or decreased clearance of Aβ in AD brain (regarding SAD) (Selkoe & Hardy, 2016).

2.1.2 Cholinergic deficits:

Cholinergic hypothesis of AD describes that the loss of cholinergic neurons within the basal forebrain causing cholinergic transmission deficit. Such deficit adds in AD symptoms including both cognitive as well as non-cognitive (Bartus et al., 1982; Giacobini, 2003; Cummings & Back, 1998). The AD patients have revealed the reduced activities of both choline acetyltransferase as well as AChE in brain tissues for the first time in 1976 and 1977 (Bowen et al., 1976; Davies & Maloney, 1976; Perry et al., 1977). Synthesis as well as the degradation of acetylcholine is contributed by both these enzymes. Decreased levels of these enzymes in AD indicated that cholinergic neurons were being selectively destroyed (Knopman et al., 2001). Post mortem investigation of brains of the patients having early dementia mainly supported the acetylcholine deficit hypothesis (Perry, 1986; Gil-Bea et al., 2005; Bartus et al., 1982; Whitehouse et al., 1986).

2.2 Inflammation and AD:

Along with Aβ and NFTs, inflammation is another fundamental mechanism in the pathophysiology of AD. Though the link between Alzheimer's disease and neuroinflammation has been known for more than 30 years but there is yet an ambiguity

whether it is a cause or an outcome of AD (Chaney et al., 2019; McGeer et al., 2016). Inflammation is necessary for healing process and it usually resolve on its own. However, if inflammation lasts for a long time, it turns into chronic inflammation that can have deleterious effects on brain functioning because of the release of cytotoxic substances in an excessive or persistent manner. Many neurodegenerative diseases including AD have been seen to involve the chronic over activation of pro-inflammatory reactions (Chaney et al., 2019). A lot of evidence has shown a key role of neuroinflammation in the etiology of AD (Cao et al., 2018). Microglia as well as the astrocytes are primarily responsible for the production of cytokines in AD. Cytokines have a key part in the progression of neuroinflammation (Heneka et al., 2015). Microglia are the CNS's resident immune cells. They are crucial for maintaining brain homeostasis and surveillance (Chaney et al., 2019). Microglia may play both useful and harmful roles and have negative consequences (Heneka et al., 2015). Microglia are neuroprotective in physiological conditions, playing a crucial function in the release of phagocytosis and neurotrophin. They have a key role of maintaining healthy brain environment. On the other hand, when there is a disease, injury or inflammation, these microglia become activated and produce a proinflammatory response by inducing the synthesis and release of inflammatory cytokines such as interleukin-1α (IL-1α), interleukin-1β (IL-1β), tumor necrosis factor-α(TNF-α), as well as reactive oxygen and nitrogen species. Microglia is activated by proinflammatory mediators including interleukin-1β, interleukin-6 as well as TNF-α during pre-symptomatic stage of AD, resulting in synaptic dysfunction as well as death of neurons (Cao et al., 2018; Perry et al., 2010). Various studies have found the enhanced microglial activation as well as cytokine expression in AD (Kinney et al., 2018; Zhu et al., 2018). Aβ has been shown to

activate the complement system along with microglia, resulting in releasing anaphylatoxins as well as proinflammatory cytokines, further increasing inflammation. APP production is stimulated by cytokines, and as the APP amount increases, the amount of Aβ produced increases too (Blasko et al., 2000; Hu et al., 1998; Zhu et al., 2018). Activated microglia have been observed to induce the formation of free radicals, nitric oxide, as well as neurotoxic chemicals that damage neurons, in cell culture studies (Cao et al., 2018; Lin et al., 2018).

T cells in the brain parenchyma become activated, releasing inflammatory cytokines including IL-1, γ –interferon, IL-6 as well as TNF-α in AD (McGeer et al., 2016). The existence of activated microglia around amyloid plaques, as well as elevated amount of proinflammatory cytokines within the peripheral as well as central nervous systems (CNS), aid the inflammation's major part in Alzheimer's disease (Chaney et al., 2019). Neuroinflammation is normally not the cause of the disease like other hereditary causes and risk factors of AD. It is often a result of risk factors, or the consequence of AD pathologies linked with Alzheimer's disease. It worsens the disease condition through worsening the pathologies of both Aβ as well as tau (Kinney et al., 2018). Today, neuroinflammation is a hot topic in brain science. Inhibiting neuroinflammation could be a promising therapy option for a variety of chronic neurological illnesses, including AD.

2.3 Significance of oxidative stress in AD

Oxidative stress generally defined as a condition where oxidative system overpower the antioxidant defense system as show in figure 2.1, in case of Alzheimer's disease. Oxygen as well as nitrogen based molecule having unpaired electrons generate free radicals that is

reactive oxygen species (ROS) eventuating in oxidative stress (Chauhan & Chauhan, 2006).

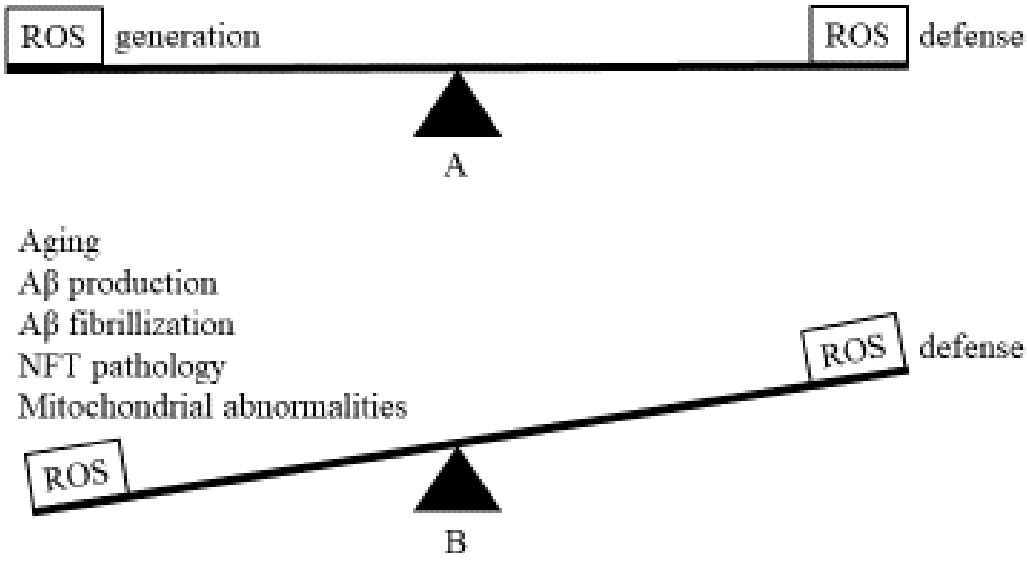

Figure 2. 1: Suggested mechanism of oxidative stress in AD. ROS are neutralized through ROS defense mechanism under normal circumstances (A). When ROS generation in AD overpowers its defense system due to age, high production and fibrillization of A, NFT Pathology as well as mitochondrial abnormalities, an imbalance occurs, resulting in oxidative stress (B) (adapted from (Chauhan & Chauhan, 2006).

Superoxide dismutase (SOD), glutathione peroxidase (GPx), as well as catalase are the three prime enzymes of the antioxidant defense system. These antioxidant enzymes are implicated in cellular defense opposed to damage caused by oxygen derived free radicals (Chauhan & Chauhan, 2006). Superoxide dismutase (SOD) has two types (Corvo et al., 2015). Superoxide dismutase 1 (SOD1) is found in the cytoplasm of eukaryotic cells and it contain active site for copper and zinc therefore also called as copper-zinc SOD (Okado-

Matsumoto & Fridovich, 2001). Superoxide dismutase 2 (SOD2) is specific to mitochondrial matrix where it removes $O2^{\bullet -}$ generated in the matrix of the mitochondria. SOD2 has manganese (Mn) active site instead of copper and zinc therefore also known as MnSOD (Fridovich, 1995).

Oxidative stress has been shown to have a significant part in Alzheimer's disease development (Chen et al., 2012). Oxidative stress is a prominent element in the pathogenesis of Alzheimer's disease that has been identified and debated for over two decades, in several review articles showing the crucial part of ROS in the pathology of Alzheimer's disease (Cassidy et al., 2020). There is generally considerable oxidative damage to neuronal tissue in the atrophied brains of people with AD (Gella et al., 2009; Valko et al., 2007). Progression of Alzheimer's disease is also suggested to result from oxidative stress mainly by three processes that include macromolecule peroxidation, redox potential of Aβ metal ion, as well as dysfunction of mitochondria. All of these factors affect Aβ accumulation and p-tau accumulation cell homeostasis, ROS production, and accumulation of Aβ and p-tau accumulation (Chen et al., 2012; Gella et al., 2009; Hawking, 2016).

Both Aβ production as well as tau phosphorylation have been demonstrated to be upregulated by oxidative stress. Proteolytic function of BACE1 as well as the expression of BACE1's mRNA increases that correlates with increased biomarkers of oxidative stress for example HNE (4-hydroxynonenal) in hypoxia (Guglielmotto et al., 2009). BACE1 is upregulated when AGEs (advanced glycation end products) bind to RAGE (receptor for advanced glycation end products) and NF-κB signaling pathway activates. This leads to transcription and production of BACE1 that eventually leads to Aβ formation (Kuhla et al.,

2015; Tamagno et al., 2012). Elevated synthesis along with aggregation of Aβ and p-tau into oligomers because of oxidative stress and generation of ROS result in destructive gain of function through species movement towards dendritic spines. These events disturb the functions of the receptors that locate postsynaptic membrane ultimately activating proteins that are responsible for the collapse of dendritic spine as well as synapse loss (Nisbet et al., 2015). Aβ oligomers have the ability to effect N-methyl-D-aspartate receptors (NMDAR) that causes inflow of Ca2+ into neuronal cells leading to increased kinases activation. Activation of these kinases is responsible for the phosphorylation of tau protein. (Parsons & Raymond, 2014). High phosphorylation of tau leads to binding of p-tau with Fyn kinase which causes p-tau to move towards dendritic spines of neurons (Nisbet et al., 2015). Moreover, Aβ along with p-tau that is bound to Fyn, affect synergistically leading to reduced surface NMDAR which ultimately causes dendritic spine's death as well as synapse loss. As a result of this, hippocampal long term potentiation inhibits which hence affects memory consolidation (Musardo & Marcello, 2017). Eventually, Aβ stimulates the enzyme striatal-enriched protein tyrosine phosphatase (STEP) which is involved in the inactivation of Fyn. Inactivation of Fyn forms a cascade of following events that result in dendritic spine collapse Mairet-Coello et al., 2013). Positive feedback loop is triggered by oxidative stress which leads to enhance ROS formation, Aβ production, as well as phosphorylation of tau protein, resulting in even more oxidative stress. In the end, this pathological neurodegeneration cascade causes significant neuronal damage (Gella et al., 2009). Hence, the interactions among oxidative stress and the hall mark proteins of AD including Aβ as well as p-tau are critical for clinical symptoms seen in AD patients (Cassidy et al., 2020).

2.3.1 Antioxidant enzymes in AD:

High amount of oxidative stress and dramatically increased Aβ accumulation as a result of impaired antioxidant defense system have been seen in transgenic mice overexpressing mutant APP (Li et al., 2004; Zhao & Zhao, 2013; Nishida et al., 2006), whereas burden of Aβ plaques as well as the oxidized protein levels in the brain are found to be reduced by dietary antioxidants such as curcumin (Lim et al., 2001). Furthermore, antioxidant supplementation may alleviate the elevated Aβ deposition as well as the accompanying earlier onset and extreme cognitive impairment caused by a deficiency in the antioxidant defense system (Nishida et al., 2006). In Tg19959 transgenic mice overexpressing mutant APP, upregulation of manganese superoxide dismutase (MnSOD) lowered the oxidation of protein along with boosting the antioxidant defense potential within the brains, meanwhile lowering Aβ burden and repairing cognitive loss (Dumont et al., 2009). Additionally, deletion of copper/zinc superoxide dismutase (SOD1) has resulted in enhanced Aβ aggregation and accelerated decline in spatial memory in Tg2576 AD mice model as compared to control AD mice. This indicated that oxidative damage contributes potentially in Aβ aggregation. These findings imply the potential part of oxidative stress in the beginning and progression of AD by increasing Aβ production as well as Aβ oligomerization (Murakami et al., 2011).

2.4 Differential Proteome in AD:

Proteomics has proven to be a remarkable tool for the study of neurodegenerative diseases. The only approach currently available for isolating and separating the hundreds of individual proteins that make up a tissue proteome is 2-D gel electrophoresis. Because of

the ability to map, screen, quantitate, and identify many proteins readily and reliably, expression proteomics has allowed researchers to compare and differentiate varied situations. Such concepts have been used to detect particular variations in the protein expressions of distinct areas of the AD brain when analyzed in comparison to the control brain. This can aid in the understanding of the disease's causes as well as the effect of the testing therapeutics. A significant amount of this research has been published. Scientists have discovered a number of protein expression alterations in brains of people with AD, which may assist in explaining disease pathophysiology. Aside from the disease's well-known pathophysiological markers, AD is linked to loss of synapse, impaired glucose metabolism, oxidative stress, deficits of mitochondria, excessive protein misfolding, as well as reduced protein clearance. Changes in various proteins expression that are implicated in various pathways aid in the development of testable theories of neurodegenerative mechanisms in the AD brain (Butterfield et al., 2003).

Oxidative damage to proteins and DNA, as well as lipids was proposed as a key initial step in the etiology of Alzheimer's disease. Protein carbonyl derivatives are formed as a result of ROS-mediated interactions with proteins and presented as a marker for protein damage occurred due to ROS. The link of oxidatively damaged protein to ageing as well as Alzheimer’s disease was discovered using this marker (Stadtman & Berlett, 1997). Oxidative stress induced oxidation of proteins as well as peroxidation of lipids has been proved in APP/presenilin 1-transgenic mice (Abdul et al., 2004) When placed into their specific biochemical pathways, Aβ 1-42-induced oxidative stress in rat brain replicates few of the proteins oxidized in Alzheimer’s disease brain, providing understanding about brain

damage which could cause neurodegeneration in Alzheimer's disease (Boyd-Kimball et al., 2005).

2.5 Post Translational Modifications:

The complex and diverse functions of the biological system are largely dependent upon the regulated functions of the proteins. After translation, proteins must undergo various stages of chemical modifications which are called as post translational modifications (PTMs). These PTMs can modify structures via changing physicochemical characteristics of primary sequences of proteins as well as adjust protein folding through modifying charge of the proteins (Marsh & Forman-Kay, 2010). Furthermore, PTMs can affect the transition of a protein's state (Bah & Forman-Kay, 2016). Methylation, acetylation, glycosylation, ubiquitination, and phosphorylation are the most common PTMs (Adaniya et al., 2019). Posttranslational modifications determine the protein functionality and have a vital contribution not only in the normal nervous system development (Cole & Hart, 2001) but also in brain injury as well as in neurodegeneration including AD (Castegna et al., 2002; Charlwood et al., 2001; Espinosa et al., 2003; Liu et al., 2002; van Rensburg et al., 2000).

2.5.1 Protein Acetylation and AD

Protein acetylation is considered as one of the most advanced subjects in PTM research. The primary mechanism of protein acetylation involves the transfer of acetyl groups to protein via acetyltransferase catalysis (Drazic et al., 2016). Acetylation of lysine is the most common type of acetylation. Histone acetylation and non-histone protein acetylation are the two types of acetylation (Verdin & Ott, 2015).

Protein acetylation has been studied for more than 50 years. Vincent Allfrey discovered and postulated lysine acetylation in histones in 1964 (Allfrey et al., 1964), and reported its role in the regulation of gene transcription (Verdone et al., 2005). Positive charge of histone is attributed to the basic groups like lysine and arginine. When lysine residues are acetylated, it will no longer have positive charge. As a result, DNA binding with histone is loosen allowing for easier gene transcription (Allfrey et al., 1964).

In 2009, researchers found over 1,000 different forms of acetylated non-histone proteins by investigating various species metabolic pathways (Choudhary et al., 2009). Gene transcription is regulated by histone acetylation and deacetylation by altering transcriptional factor assembly and chromatin structure. Main protein element of chromatin and eukaryotic cell nuclei are Alkaline histones. In general, gene transcriptional activation is linked with histone acetylation, whereas histone deacetylation is linked to gene transcriptional repression.

Histone acetylation dysregulation is crucial for various signaling pathways, like cell death and differentiation, vascular remodeling, inflammation and immunological responses, neural plasticity, as well as metabolic reprogramming (Barnes, 2009; Gräff et al., 2012; Keenen & de la Serna, 2009; Mihaylova & Shaw, 2013; Pons et al., 2009; Powell et al., 1999; Vickers, 2017). Reversible acetylation of specific, conserved lysine(s) in the protein MnSOD enzymatic activity is also found to be regulated by the. These findings imply that mitochondria have bidirectional posttranslational signaling networks, like those found in the cytoplasm and nucleus.Altered lysine acetylation effects MnSOD enzymatic activity. Alteration in the acetylation of nuclear as well as cytoplasmic proteins like tau (Irwin et al., 2012; Mills et al., 2011), alpha tubulin (Perez et al., 2009), NF-κB (Chen et al., 2001),

and p53 (Barral et al., 2000) has also been found to be linked with AD, giving another degree of regulation for molecular pathways involved in AD.

During the last ten years, the identification of the link between impaired histone acetylation homeostasis and memory loss has resulted in a significant advance in our understanding of cognitive decline in neurodegenerative illnesses. According to certain research, Aβ deposits caused hyperacetylation in neuroblastoma cells (Gu et al., 2013; Guo et al., 2011; Lu et al., 2014). Certain genes involved in APP metabolism are influenced by Histone acetylation. In Swedish mutant APP transfected with N2a cells, it was discovered that there is hyperglycation of H3 in PSEN1 and BACE1 promoters. (Lu et al., 2014). In triple transgenic mice APP/PS1/tau, BACE1 promoters has been observed to have same H3 hyperacetylated promoter (Marques et al., 2012). These studies indicated the significance of aberrant histone acetylation in AD pathophysiology. A superior information on these issues will probably prompt the advancement of fruitful AD medicines.

2.6 Psychostimulants

Psychostimulants belongs to the wide varieties of drug which are used as the performance enhancing agents attributed to their vigor inducing properties (Spencer et al., 2015). These drugs are majorly known for their potential as a substance of abuse, associated with chronic use. They have been used casually to enhance performance and induce wakefulness, for centuries. Students use these drugs to enhance focus on academic tasks and improve overall academic performance.

Stimulants initiate the sympathetic nervous system, a part of autonomous nervous system, which identifies the body in fight or flight situation, cause dilation of eye pupil and raise

heartbeat and blood pressure. They are classified as direct or indirect sympathomimetic and non-sympathomimetic. It includes drugs of abuse, cocaine and methamphetamine, and therapeutic drugs like amphetamine, methylphenidate etc. However, their therapeutic/abuse potential is completely dose dependent.

Their low doses are reported to improve prefrontal cortex (PFC) associated behavioral task in healthy and diseased individuals. There is sufficient evidence, which suggest that PFC id the prime site for cognition enhancing activity of psychostimulants (Casey et al., 2007). Among primates, rats have a heterogeneous PFC associated with cognitive activity and incentive-based behaviors. However, it works in coordination with other regions of brain e.g. striatum, to perform higher cognitive tasks.

There is a little knowledge available regarding use of stimulants for treatment of depression. They are reported to have a transient effect on treatment of depressive signs and symptoms. Further research is required to identify their long term benefits and mechanism of action in depression (Malhi et al., 2016).

2.6.1 Methylphenidate

MPH, FDA approved drug, is available in the market by brand name ‘Ritalin’ with variable durations of action. It is a drug of choice for the treatment of ADHD and narcolepsy in children (Wood et al., 2014). Its prescription has increased many folds since the acceptance of ADHD diagnose in 1960. ADHD is characterized by high levels of inability to focus, impulsiveness and aggression (Scheffler et al., 2009) with an underlying mechanism of neurotransmitter reuptake (dopamine) at post-synaptic terminals. The drug serves as an antagonist to this mechanism and blocks the dopamine transporter to maintain a sufficient

extracellular concentration of dopamine, which enhances motor function and aids to regain focus. There is a little knowledge about its mechanism of action in the developing brain but it has been reported to increase the concentration of norepinephrine in juvenile rodent's hippocampus. Similarly, it raises dopamine levels in various brain region besides hippocampus where it has a major regulatory role in neurogenesis during brain development. In that phase, formation of synaptic concentrations which are reliant on trophic factors like nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) is essential to neural cell survival. Chronic administration of MPH has reported to alter the synaptic connections by reducing BDNF and NGF expression. Moreover, it has been reported that the long-term exposure to MPH results in the behavioral impairments and the loss of astrocytes and neurons (Schmitz et al., 2017).

2.7 Medicinal Plants

Medicinal plants are widely used throughout the world to cure or control progress of a number of diseases. Effective utilization of therapeutic phytochemicals from Kingdom Plantae can lead to development of novel drugs. Several plants and their extract from the tradition medicine have anti-inflammatory (Otimenyin, 2018), antibacterial, antifungal, anti-diabetic and antioxidant potential (Andrade et al., 2018). They are used as immuno-stimulants (Van Hai, 2015) and for female healthcare in many Southeast Asian rural areas (De Boer & Cotingting, 2014). Many plant species are under consideration for their potential anticancer attributes (Greenwell & Rahman, 2015). Number of studies have reported active ingredients from plant sources to exhibit anticonvulsant activity (Rabiei, 2017). Vast amount of plant derived pharmaceutical products (De Boer & Cotingting,

2014) are the area of interest for researchers because of their increasingly proven significant therapeutic potential.

Therapeutic potential of varieties of medicinal plants have been studied with reference to memory and learning. Findings have reported use of *Boswellia spp, Ficus carica, Silybum marianum, Cannabis sativa, Nigella sativa, Glycine max, Origanum vulgare L.,* and *Melissa officinalis.* Their effectiveness is attributed to their antioxidant potential and phytochemical compounds (Nikfarjam et al., 2016). High production of phenolic and flavonoids within medicinal plants play a role in preventing oxidative stress linked age-related disorders (Azwanida, 2015). A memory enhancing effect was observed in chronically stressed mice when treated with extract containing *Anchusa italic* (Al-Snafi, 2015). Al-Snafi has also reported prophylactic action of neurological ailments like AD, Parkinson's disease, depression, anxiety and cognitive impairment using *Antirrhinum majus*. Likewise, research has suggested use of *Caesalpinia crista* extract to be beneficial for cognitive recovery (Al-Snafi, 2015).

With reference to AD, six Chinese medicinal plants namely *Coptis Chinensis Franch, Ganoderma, Rhizoma Curcumae Longae, Panax Ginseng Herba Epimedii, Green tea* are suggested as promising agents in development of potential AChE1s to treat AD (Jiang et al., 2017). Aqueous extract of *Caesalpinia crista* has also delivered promising results in inhibition of Aβ-42 aggregates and fibrils, a major pathology in AD (Al-Snafi, 2015).

2.7.1 *Rosmarinus officinalis L.*

Rosmarinus officinalis L. (Rosemary) (figure 2.2), a perennial plant, is a commonly used ancient aromatic herb. It belongs to Lamiaceae family and has applications in traditional

medicine and culinary since centuries (Ribeiro-Santos et al., 2015). Like other plants, many benefits are traditionally associated with Rosemary as well. Studies have reported its activity as a CNS stimulant (Filiptsova et al., 2017). *R. officinalis* essential oil spray has also been found to yield statistically significant results in the improvement of short-term memory (Filiptsova et al., 2018). According to a study in AD patients, 28-day aromatherapy with rosemary essential oil improved cognitive activity task speed. (Filiptsova et al., 2017; Jimbo et al., 2009). However, it has no significant effect on the mood (Moss & Oliver, 2012). It also ameliorates the memory in dementia instances (Duke & therapies, 2007). Main components of *R. officinalis* demonstrate potent neuroprotective effect against neurodegeneration diseases i.e. Parkinson's disease and AD, through their antioxidant activity. These includes carnosic acid (CA) and rosmarinic acid (RA) (Park et al., 2010). Furthermore, it is reported that the plant extracts augmented the release of neurotransmitter in the brain (Sasaki et al., 2013) which prompts depression reversal and mood alterations (Machado et al., 2012).

Figure 2.2: *Rosmarinus Officinalis* (Rosemary) used as a source for plant material in this research work.

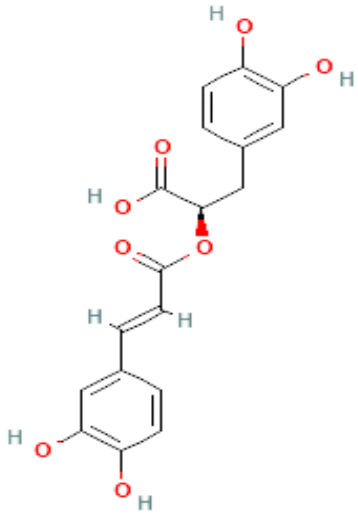

Figure 2.3: 2D structure of Rosmarinic acid

2.7.1.1 Rosmarinic acid

Rosmarinic acid (RA) (figure 2.3) is a major active constituent of *Rosmarinus officinalis L.*, rosemary. (Fazel Nabavi et al., 2015; Gonçalves et al., 2019; Ribeiro-Santos et al., 2015). It constitutes of caffeic acid ester and 3,4-dihydroxyphenyl-lactic acid and have reported powerful antioxidant activity amid the simple phenolic compounds (Petersen & Simmonds, 2003). RA has been shown to reduce oxidative damage, neuronal death in vitro and have neuroprotective properties (Choi et al., 2002; Lee et al., 2008; Qiao et al., 2005), in addition to anti-inflammatory effects in ischemic stroke experimental models (Bigford & Del Rossi, 2014; Luan et al., 2013). RA also demonstrated protective role against oxidative stress-inducing agents in SHSY5Y human neuroblastoma cells (Fallarini et al., 2009).

Chapter 3

MATERIALS AND METHODS

3.1 In-silico Analysis

The structure of the ligands and protein were retrieved from the PubChem and PDB databases, respectively, for the study. Ligand and proteins were prepared by Open Babel and UCSF Chimera respectively. Best binding sites of the proteins based on the best drug store were selected from DoGSiteScorer. Docking was performed by Autodock4 and the docked complexes were visualised using PyMol and Discovery Studio.

3.1.1 Ligand Retrieval:

The 3D structures of Donepezil (CID: 3152), RA (CID: 5281792) and MPH (CID: 4158) were obtained in SDF format from the PubChem compound database (https://pubchem.ncbi.nlm.nih.gov/) and converted to MOL2 format using Open Babel.

3.1.2 Retrieval of protein structures:

Protein Data Bank was used to retrieve and download protein structures (https://www.rcsb.org/). Targeted proteins with their PDB code are mentioned as follow.

AChE: 4BDT

PSEN-1: 5A63

BACE1: 6EJ3

IL-6: 1ALU

TNF-α: 6OP0

NF-κB-p50: 1SVC

SOD1: 4B3E

SOD2: 1N0J

Prdx6: 5B6M

The criteria for structure selection is based on retrieving the FASTA sequence form UNIPROT (https://www.uniprot.org/) followed by PDB advance BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) . Structure showing maximum score and query coverage in the BLAST was selected for each protein. Proteins were cleaned by removing duplicated structures and nonstandard residues using USCF Chimera.

3.1.3 Best binding site selection:

The best binding sites of each protein for a drug were selected, before performing docking analysis, from a server the DoGSiteScorerhttps://proteins.plus/. The pocket was selected on the basis of best drug score and the amino acids residues were noted.

3.1.4 Docking analysis:

. The docking analysis of Donepezil, RA and MPH to the indicated selected proteins was performed using Autodock4. Ligand-protein binding energies were found by setting the grid box based on residues of the best drug score. 20 runs were generated for each ligand and protein interaction. A complex of all the 20 possible ligand protein poses was made by running Cygwin commands. The best pose based on the lowest binding energy was selected

and exported from the complex using PyMol. Finally, the bonds and interactions between a ligand and a protein were identified and visualised by BIOVIA Discovery Studio.

3.1.5 Drug Likeness Analysis:

Drug likeness analysis of Donepezil, RA and MPH was performed using Lipinski filter (http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp). The Lipinski filter predicts drug likeness of a molecule on the basis of five rules. Orally active drugs should follow a minimum of four out of five rules. The criteria includes hydrogen bond acceptor, hydrogen bond donor, molecular mass, cLogP, as well as molar refractive index (Lipinski, 2004). Standard range of these criteria are mentioned in Table 4.7. PDB files of the molecules were submitted for Lipinski filter.

Furthermore, analysis of the adsorption, distribution, metabolism, excretion and toxicity (ADMET) properties of ligands was performed by admetSAR1 (http://lmmd.ecust.edu.cn/admetsar1/predict/?smiles=&action=A)which is an important tool for estimating pharmacokinetics properties of the molecules in order to predict their drug likeness. SDF files of the molecules were downloaded from PubChem and converted into smile format using Online Smiles Translator (https://cactus.nci.nih.gov/translate/). Smile files were then submitted on admetSAR1 to calculate the ADMET properties using default parameters.

3.2 *In-Vivo* Analysis

3.2.1 Chemical and Reagents:

Aluminum Chloride hexahydrate ($AlCl_3.6H_2O$) was bought from Scharlau (Product catalogue no. AL0770). Donepezil Hydrochloride (Donecept) and Methylphenidate (Ritalin) was purchased from local pharmacy of Islamabad, Pakistan. Pierce silver stain kit and Antibodies were brought from Thermo Fisher Scientific. Chemicals used for electrophoresis experiment were obtained from Sigma-Aldrich (USA). IGP strips and all the chemicals used for first dimension were obtained from BIO-RAD. Commonly available dried leaves of *Rosmarinus officinalis* were collected during Fall 2020 from vendors in local markets of Islamabad, Pakistan. Verification of plant was conducted by an experienced botanist prior to initiation of the procedure.

3.2.2 Animals:

Male Balb/c mice (6-8 weeks old) were chosen for the study. Animals were bred and resided in the Laboratory Animal House of Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Science and Technology (NUST). Mice were housed in standard metal cages under standard conditions of constant temperature (25±2 ºC) and a regular light-dark cycle (12-12 hours). Animals of same groups were accommodated in the same cages. Throughout whole experimental period animals were provided with distilled water *ad libitum* and standard diet comprising of (%): crude fiber 4, crude fat 9, crude protein 30 and moisture 10.

3.2.3 Ethical Statement:

The mice were kept in the Laboratory Animal House of Atta-ur-Rehman School of Applied Biosciences (ASAB), National University of Science and Technology (NUST), under a controlled environment. All experiments were carried out in accordance 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 Animal: Eighth Edition, 2011). Internal Review Board (IRB) of Atta-ur-Rahman School of Applied Biosciences, NUST approved the study protocol.

3.2.4 *Rosmarinus officinalis* Extract Preparation:

R. officinalis (500g) leaves were ground to fine powder form and were allowed to pass through 80 mesh sieve. It was followed by taking 10g of fine powder in thimble (thick filter paper) and loading thimble into Soxhlet extractor with a distillation flask containing 100% ethanol as extraction solvent. The process was run for 24 hours before filtrate was collected and concentrated using rotary evaporator (R200 rotavapour, Buchii) with pressure reduced to 68°C to attain a crude extract. Any remaining solvent was removed by incubation at 37°C. The extract was stored at 4°C until further use (Mirza et al., 2021; Rahbardar et al., 2017).

3.2.5 Diphenyl-1-Picrylhydrazy (DPPH):

The ability of *R. officinalis* to scavenge the reactive oxygen species is identified through DPPH assay. Powdered methanolic extract of the plant was diluted in varying concentrations of 20 to 100µg. Same concentration of the Ascorbic acid was prepared as it

was taken as a control. DPPH solution was diluted with methanol and the final concentration was made 2:50. The final solution was placed in dark and on ice. 1ml of both solutions (Extract and ascorbic acid) were taken in Eppendorf and 0.5ml of DPPH was added and mixed in both solutions. Solution was incubated for one hour in the dark at room temperature. The Optical density (OD) of extract was measured at 517 nm. The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\% \text{ age inhibition} = 100 * (Vt / Vc - 1)$$

Where, Vt = absorbance of test sample,

Vc = absorbance of control.

3.2.6 Development of AD Mouse Model:

To induce AD in the mice, the protocol described by (Khalid et al., 2020) was followed. Animals were divided into eight groups, 8 mice each. The groups for study are listed in Table 3.1. Aluminum chloride ($AlCl_3$) (300mg/kg) (Amber et al., 2018) was given in drinking water for 15 days to Group 2, 4, 6 and 8 (n=32). The animals of the control groups were given distilled water.

Table 3. 1 Group-wise division of animals for study

No.	Groups	Treatment	
		15 days	5 days (I.P. injections)
1	Control	Distilled H_2O	Normal saline
2	$AlCl_3$	300mg/kg $AlCl_3$	Normal saline
3	Donepezil	Distilled H_2O	Donepezil 2mg/kg
4	$AlCl_3$ + Donepezil	300mg/kg $AlCl_3$	Donepezil 2mg/kg
5	MPH	Distilled H_2O	MPH 10mg/kg
6	$AlCl_3$ + MPH	300mg/kg $AlCl_3$	MPH 10mg/kg
7	*R. officinalis*	Distilled H_2O	Rosemary extract
8	$AlCl_3$ + *R. officinalis*	300mg/kg $AlCl_3$	Rosemary extract

3.2.7 Study Design:

A 15 days long plan was designed to generate AD mouse model (n=32) by oral administration of $AlCl_3$ (300mg/kg). This was followed by 05 days of intraperitoneal (I.P.) injections of Donepezil-2mg/kg (Fitzgerald et al., 2020; Neishaboori et al., 2021), Ritalin-10mg/kg (Khalid et al., 2020) and *R. officinalis*-100mg/kg (Khalid et al., 2020). Behavior tests for anxiety, depression and spatial memory and learning were carried out next 10 days followed by histological analysis. Protein expressions and post translational modifications analysis were then performed through 2-Dimensional Gel Electrophoresis and Western Blot respectively (Figure 3.1).

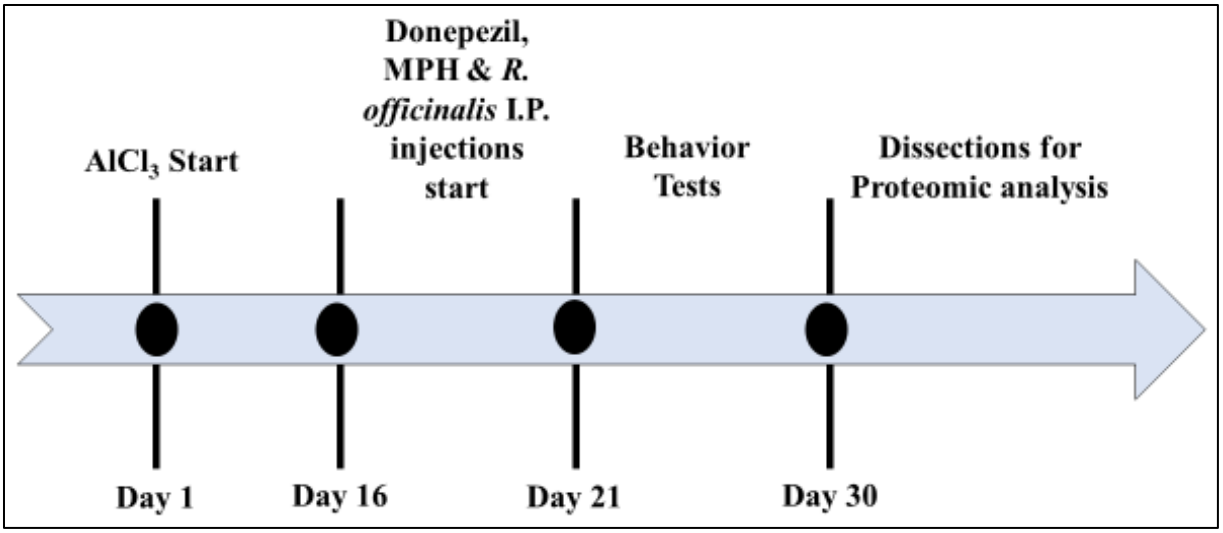

Figure 3. 1: Study Design. Timeline depicting a 15days period for the induction of AD, treated with Donepezil, MPH and *R. officinalis*, behavior analysis and decapitation of animals for proteomic studies.

3.2.8 Behavior Studies:

3.2.8.1 Elevated Plus Maze Test:

The Elevated Plus Maze (EPM) is one of the extensively used test to analyze anxiety in rodents. The goal of this model is to investigate rodents' aversion to open spaces. Thigmotaxis behavior was identified as a result of aversion, in which rodents constrain their movement to closed ends and avoid open spaces. Procedure described by (Arendash et al., 2004) with minimal modifications was used to carry out test. The apparatus comprised of four arms (50 × 10 cm each), two open and two closed, crossed perpendicularly to each other and a center area (10cm × 10cm). The apparatus is made up of opaque iron alloy and is elevated 75.5cm from the ground. It was smaller than that used for rats and had an immovable base. Each mouse was provided free access to all arms after releasing it on the center area with its head facing to one of the closed arms. Each mouse

underwent one 5-minute trial, which was recorded on camera. The videos were later analyzed to calculate the following parameters.

- Number of entries into the open arm
- The time spent in open arm

If two of the animal's paws and more than half of its body were in the respective arm, it was regarded into an arm. To minimize skewed results due to olfactory cues, the apparatus was cleaned with 70% ethanol after each trial.

3.2.8.2 Open Field Test:

This test determines the locomotor and exploratory activity as well as anxiety levels in mice. A square shaped arena (40 x 40 x 40) was used which was divided into center and periphery by drawing a boundary. Animal was placed in the center of a wooden box and was allowed to explore the box for 30 minutes. Their anxiety was monitored using a camera. The following parameters were assessed.

1. Time spent in center
2. Time spent in periphery

3.2.8.3 Forced Swim Test:

Forced swim test is used to analyze depressive behavior in rodents. The animals are subjected to stress by placing them in water container, making their escape impossible. This situation leads to the development of tendency of depression (Yankelevitch-Yahav et al., 2015). The protocol of the test was followed as described earlier (Fitzgerald et al., 2020) with a few modifications. Transparent container with diameter of 20cm and a height of 30

cm was filled with water 25±2 ºC. The depth of the water was adjusted according to the size of the mice, to prevent their hind limbs and tail from touching the bottom of the container. Mice were placed into the water and their activity was recorded by camera for 6 minutes. The water in the container was discarded and refilled for each mouse prior to testing. Following parameters were recorded.

- Latency to immobile
- Time spent immobile
- Number of immobile episodes

3.2.8.4 Morris Water Maze Test

The procedure described by (Bromley-Brits et al., 2011) was used with slight modification to conduct Morris Water Maze (MWM) Test. The test is used to establish animal's spatial learning and memory. A circular pool (120 cm × 60 cm) was filled with water (21 °C ±2 ºC). The pool was divided equally into four quadrants (north, south, east and west). A transparent platform (13 cm × 32 cm) was placed at the North-West quadrant. Five acquisition trial were carried out five times a day for five consecutive days with a minimum of 10min inter-trial interval for each mouse. The mice were released in water in each trial everyday according to the arrangement given in Table 3.2 with their heads facing the tank. The cut off time was identified as 90s and those who failed to find the platform in that time were manually placed on the platform for 20s. Those who located the platform before 90s were allowed to sit there for 5s. The average of acquisition trial and escape latency was calculated. One day 06, platform was removed and probe test was performed. In this test,

the mice were allowed to swim in the pool for 90s and the video was recorded. The animal's reference memory was monitored by calculating the following parameters.

- Time spent in target quadrant
- Number of crossings over the removed platform position

Table 3. 2 Starting positions for MWM test training and spatial probe trial test

No. of Days	**Direction of Release**				
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
01	West	South	North	East	South
02	North	West	East	West	South
03	North	East	West	South	North
04	East	South	West	East	North
05	West	South	North	East	South
06	Single trial without platform, direction of release: West				

3.2.8.5 Statistical Analysis:

Data was analyzed using GraphPad Prism 10 by applying one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test.

3.2.9 Brain Dissection:

Mice were put under anesthesia and euthanized through cervical dislocation. The head was stretched forward gently and a cut was made posterior to the ears, using surgical scissors. A small incision was made, starting from caudal point followed by cutting firmly through the anterior part of the skull. The parietal bone of both sides was tilted and broken off using narrow curved pattern forceps. The curved narrow pattern forceps were then slid under the

anterior part of the brain which was gently taken out of the skull. The removed brain was immediately transferred to a petri dish contained pre-chilled Phosphate Buffer Saline (PBS) such that 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 were then held in a closed position between the cerebral halves. The forceps were 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°, were directed counterclockwise and clockwise to separate the left and right hippocampus from the cortex respectively. The cortex and the dissected hippocampus were then transferred separately in a pre-chilled Eppendorf and stored at -80 °C until further downstream processing.

3.2.10 Histological Examination:

3.2.10.1 Tissue Perfusion for Histological Analysis:

Heart perfusion was performed to excise whole brain (Gage et al., 2012). The whole brain was washed with Phosphate Buffer Saline (PBS) and transferred to 4% Paraformaldehyde (PFA) solution immediately for 24hrs before further processing. After 24 h in 4 % PFA, brain tissue was dehydrated with 70%, 95% and 100 % isopropanol for 1hr each before paraffin permeation. The tissue was then subjected to xylene incubation for 4 hrs. Afterwards, the tissue was put in molten paraffin and paraffin embedding was performed for 4hrs at 60°C. The temperature in this duration was maintained at 60°C. It was left to solidify in the mold at 4°C, preliminary to cutting.

3.2.10.2 Congo Red Staining:

The Congo red stain (working solution: 49.5 mL Congo Red (Stock) and 0.5 mL 1% NaOH) was poured on the de-paraffinized brain sections and retained for 20 minutes. Double distilled water (ddH_2O) and alkaline alcohol was used to wash sections for 2 minutes. The sections were then counterstained by haemotoxylin for 30 seconds and further washed with 70% isopropanol for 6 min and then with dd H_2O. After air-drying (1 hr) the slides were mounted by cover slips and later visualized by Optika vision lite2.1 at 40 X, 10X and 4X resolution. The images were captured by Optika Vision Lite2.1 image analysis software.

3.2.11 Protein Expression Studies:

3.2.11.1 Protein Extraction:

The entire tissue lysates were prepared by suspension (50mg tissue) in 100 µl of ice-cold lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 10 mM Phenyl methyl sulfonyl fluoride (PMSF), 1% Dithiothreitol (DTT)), superseded by sonication utilizing an UP400S Ultrasonic Processor (Hielscher Ultrasound Technology). To increase dissolubility, the homogenates were placed at room temperature for 1 hour and then centrifuged at 14000 rpm at 4 ºC for 10 min. After centrifugation, the supernatant was collected and stored at -20 ºC. To maximize the yield, 50 µl lysis buffer was added to the pellet and the treatment was recapitulated. The two suspensions were then pooled and centrifuged at 14000 rpm for 90 minutes. The last supernatant was stored at – 80 ºC until further use.

3.2.11.2 Protein Quantification:

Bradford's Assay was employed to measure the concentration of proteins extracted from the cortex and hippocampus tissues. Bovine Serum Albumin (BSA) (1mg/1ml) serial dilutions were prepared with ddH2O. The samples were diluted with ddH2O (1:20) in duplicate. The total volume of every standard/example was 20 µl and 1 ml of Bradford's reagent was included, followed by a spin in the vortex. The samples were incubated at room temperature for 10 min. Absorbance of every sample was measured at 595 nm reagent blank using OPTIMA 300 spectrophotometer. A standard curve was inferred by plotting standard absorbance against its concentration. This curve was used to quantify the protein concentration against the observed absorbance.

3.2.11.3 Two-Dimensional Gel Electrophoresis (2-DE):

3.2.11.3.1 Isoelectric focusing:

Proteins were separated on the basis of their isoelectric point by isoelectric focusing in the first dimension. Protein sample (200 µg) was taken and the total volume of the sample solution was made 125 µl with rehydration buffer (7M Urea, 2MThiourea, 4% CHAPS, 0.002% Bromophenol Blue, 50mM Dithiothreitol (DTT), 0.2% (w/v) Bio-Lyte®3/10Ampholyte). Sample solution was pipetted carefully along the center of the channel in the 7cm i12 rehydration tray without introducing air bubble. IPG Strips (7cm/PH 3-10; BIO-RAD Ready Strip™) were used and gently placed onto the sample solution in the channel preventing air bubble. The "+" and pH 3-10 end positioned at the left side of the tray. Mineral oil (1 ml) was overlaid the strip after one hr of rehydration. Tray was covered with the lid and left on the bench overnight to rehydrate the sample. On the next

day strip was removed using forceps and was carefully hold vertically for almost 10 seconds so mineral oil could drain off. Then rehydrated IPG strip with the gel side up was transferred to a channel in the 7 cm i12 Protean IEF focusing tray positioning positive "+" end of the strip on the positive side of the focusing tray. Two electrode wicks per IPG strip were wet with nanopure water and placed at each end of the strip followed by overlaying mineral oil (1 ml). Positive and negative electrodes were assembled in the focusing tray and the tray with the rehydrated strip was placed in the Peltier platform of the BIO-RAD PROTEAN® i12™ IEF Cell followed by connecting electrodes with the instrument. Cover was closed and protocol was run by selecting lanes and assigning procedures to lanes. PROTEAN IEF Cell was programmed using three step protocol as shown in Table 3.3 Default cell temperature of 20ºC with maximum current of 50 µA per strip and Yes Rehydration conditions were used and finally start button was pressed to start the electrophoresis run. After the completion of the electrophoresis run the IPG strip was removed from the focusing tray and the excess oil is drained by holding vertically using forceps. Strip was then transferred to a cleaned 7 cm rehydration tray. The tray was covered with the lid, wrapped in plastic and stored at - 80.

Table 3. 3 Running protocol for IEF

7cm IPG Strip	**Voltage**	**Time**	**Volt-Hour**	**Ramp**
Step 1	250	20min	…..	Linear
Step 2	4,000	2hr	…..	Linear
Step 3	4,000	…..	10,000 V-hr	Rapid
Total		5hr	14,000 V-hr	

3.2.11.3.2 Equilibration:

On the next day, Equilibration buffers were prepared 15 minutes before use by adding DTT and Iodoacetamide to the equilibration base buffer ((6M Urea, 2% SDS, 30% Glycerin, 50mM Tris). IPG strip was removed from - 80 were left 10- 15 minutes to thaw. Then strip was transferred to falcon tube (15ml) containing 5ml of equilibration buffer I (6M Urea, 2% SDS, 30% Glycerin, 50mM Tris, 1% DTT, Trace of Bromophenol blue) and placed on the shaker to equilibrate for 30 minutes. After first completion of first equilibration the equilibration buffer I was discarded by carefully from the tube. The equilibration buffer II (6M Urea, 2% SDS, 30 % Glycerin, 50 mM Tris, 4 % Iodoacetamide, Trace of Bromophenol blue) was poured in the tube containing strip and the tube was again placed on the shaker to complete second equilibration for 30 minutes. Equilibration buffer II was discarded after the completion of second equilibration.

3.2.11.3.3 Second Dimension-SDS PAGE:

After isoelectric focusing, proteins were further separated on the basis of molecular weight using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) in the second dimension. Before starting equilibration step, SDS-gel was prepared. Resolving gel 12.5% (distilled water; monomer solution; 1.5M Tris-HCl pH 8.8; 10% SDS; 10% ammonium per sulphate (APS); TEMED) was prepared and poured quickly between the glass plates. Isopropanol was added to the top and the gel was left to polymerize for 45 minutes. This was followed by the preparation of 5% stacking gel (distilled water; monomer solution; 1.5 M Tris-HCl pH 6.8; 10%SDS; 10%APS; TEMED). A thin layer of Stacking gel was poured on top of the polymerized resolving gel. The gel was left for

another 45 minutes to allow polymerization. Meanwhile the equilibration was started and after equilibration, the IPG strip was removed from the falcon tube and dipped briefly into 100 ml graduated cylinder containing 1X electrode tank buffer. The strip was adjusted on the top of the stacking gel with the plastic side of the gel onto the back plate. Agarose gel 0.5% (0.5% agarose in 1X electrode tank buffer) was immediately overlaid on the strip into the glass plates and was allowed to polymerize for 5mins. Then the glass plates were moved to the electrophoresis tank, which was loaded with 1X electrode tank buffer. Electrophoretic separation procedure was carried out at 100 volts for 90mins.

3.2.11.3.4 Silver Staining:

After complete running of the gel, the gel was washed with distilled water for two times (5 minutes each). 30% ethanol: 10% acetic acid solution (6:3:1 water: ethanol: acetic acid) was used to fix gel and it was left in fixing solution overnight. On the next day gel was washed with 10% ethanol two times (5mins each). It was again washed with distilled water for two times, 5 minutes each. Gel was incubated in sensitizer working solution (50 µl sensitizer in 25 ml water) for one minute followed by two washings with distilled water for one minute each. Then the gel was incubated in stain working solution (0.5 ml enhancer in 25 ml stain) for 30mins. Gel was washed with two changes of distilled water for 20 s each. Developer working solution (0.5 ml enhancer in 25 ml developer) was immediately added onto the gel and incubated until protein band appear (2-3 min). Stop solution (5% acetic acid) was immediately added when the desired intensity of the protein bands appeared. Gel was washed briefly and replaced with stop solution. ChemiDoc MP (BioRad, USA) was

used to detect the protein spots. Images were acquired and gel analyzed using Delta 2D image analysis software (Decodon, Germany).

3.2.11.3.5 Imaging Analysis

The 2DE gel images were analyzed with Decodon Delta 2D software for the quantification of protein spots. The relative quantity of each spot was assessed to calculate differential expression of protein.

3.2.11.4 Analysis of Post Translational Modifications:

3.2.11.4.1 Western Blotting:

SDS-PAGE gels were prepared according to the protocol described previously in section 3.2.11.3.3 with 50ug protein sample. Stacking gel was poured on top of the polymerized resolving gel followed by immediate insertion of the comb into the gel. The gel was left for another 45 minutes to allow polymerization. After polymerization, the combs were removed and the glass plates were moved to the electrophoresis tank, which was loaded with 1X electrode tank buffer. Samples (50 µg protein) were prepared by the addition of sample diluting buffer (0.125M Tris-HCl pH 6.8; 20% Glycerol; 10% 2- Mercaptoethanol) in 1:1 ratio. The samples were boiled at 100ºC for 3 min and given a short spin at 14000 rpm for 1 min. The samples were then loaded into the wells and the electrophoretic separation procedure was carried out at 100 volts for 90 mins. After complete running of the gel, it was soaked in transfer buffer (0.25 M Trizma base, 1.92 M lycine, 20% Methanol) for 5 min. Filter paper and Nitrocellulose membrane were cut according to the size of gel and also soaked in transfer buffer after which the sandwich was assembled and

placed in the Trans –Blot SD Semi-Dry Transfer Cell (Bio-Rad, USA). The transfer blot was allowed to run at 30mA for 90 minutes. The blot was incubated in 15 ml of Ponceau S. stain to check the quality of transferred proteins and later washed for 3 times (10 minutes each) in TBS-T buffer (50Mm Tris. 0.5M NaCl, 0.1 % Tween 20, PH 7.4) to destain. It was then placed in blocking buffer (5 % BSA, 0.5 % Tween and 1^{x} TBS) for 1 hour at room temperature and then washed with TBS-T twice (five min each). Anti-Acetylated Lysine Antibody (1:1000 dilution) was diluted in 1% BSA TBST-T and the blot was incubated in this solution for overnight at 4 ºC. The blot was washed for 5 times (15 min each) with TBS-T and then incubated for 1 hr at room temperature in HRP conjugated anti-rabbit IgG (1:15000 dilution). It was then subjected to washing (4X) in TBS-T (15 min each) and a final washing with TBS (5 minutes).

3.2.11.4.2 Imaging and Detection:

The blot was incubated in 1:1 Amershan ECL detection reagent (GE Healthcare, UK) for 3 min. The excess reagent was drained and the blot was placed in a plastic sheet. ChemiDoc MP (BioRad, USA) was employed to detect bands after 30s exposure.

3.2.11.4.3 Image Analysis:

Image Lab software was employed to select and analyze individual bands. Density was measured in terms grey value (maximum and minimum) and results were evaluated. The results were tabulated as expression graphs and analyzed using One-way ANOVA. The expression graphs were generated using GraphPad Prism 10.

Chapter 4

RESULTS

4.1 *IN-SILICO* ANALYSIS

4.1.1 Molecular Docking

Docking analysis was performed to explore the interaction potential of Donepezil, MPH and RA with the proteins crucially involved in the pathophysiology of AD. All the three ligands were able to interact with the selected proteins. The interaction between ligands and proteins is exhibited by the binding energy (BE), inhibition constant (KI) as well as Hydrogen bonds. The lower the binding energy (BE) the higher the binding affinity. The inhibition constant (KI) is an indicator of how potent the drug is. The lower the KI, the higher the binding affinity and the smaller amount of drug is required to produce the desired effect (Sung et al., 2003). Binding parameters of Donepezil, RA and MPH with AChE, PSEN-1 and BACE1 are listed in Table 4.1. Binding energies of these ligands with AChE are -13.08, -12.66 and -9.47 kcal/mol, respectively. Their estimated KI values are 258.97 pM, 527.49 pM and 113.88 nM, respectively. Being an acetylcholine esterase inhibitor Donepezil showed highest binding affinity with AChE. RA has slightly lower than Donepezil but suggesting a comparable binding of RA with the enzyme AChE. Interacting residues and hydrogen bonds along with Hydrogen bond lengths are mentioned in Table 4.2. In the selected highest drug score binding site, Donepezil has shown to make three hydrogen bonds while RA was able to make seven hydrogen bonds. Interacting residues in the Table 4.2 shows that ASP74, TRP86 and ASN87 are the common residues of AChE interacting with Donepezil and RA. MPH has lowest binding energy interacting through

hydrophobic interactions with AChE without making any hydrogen bond. Visual representation of AChE interaction with the ligands is depicted in figure 4.1.

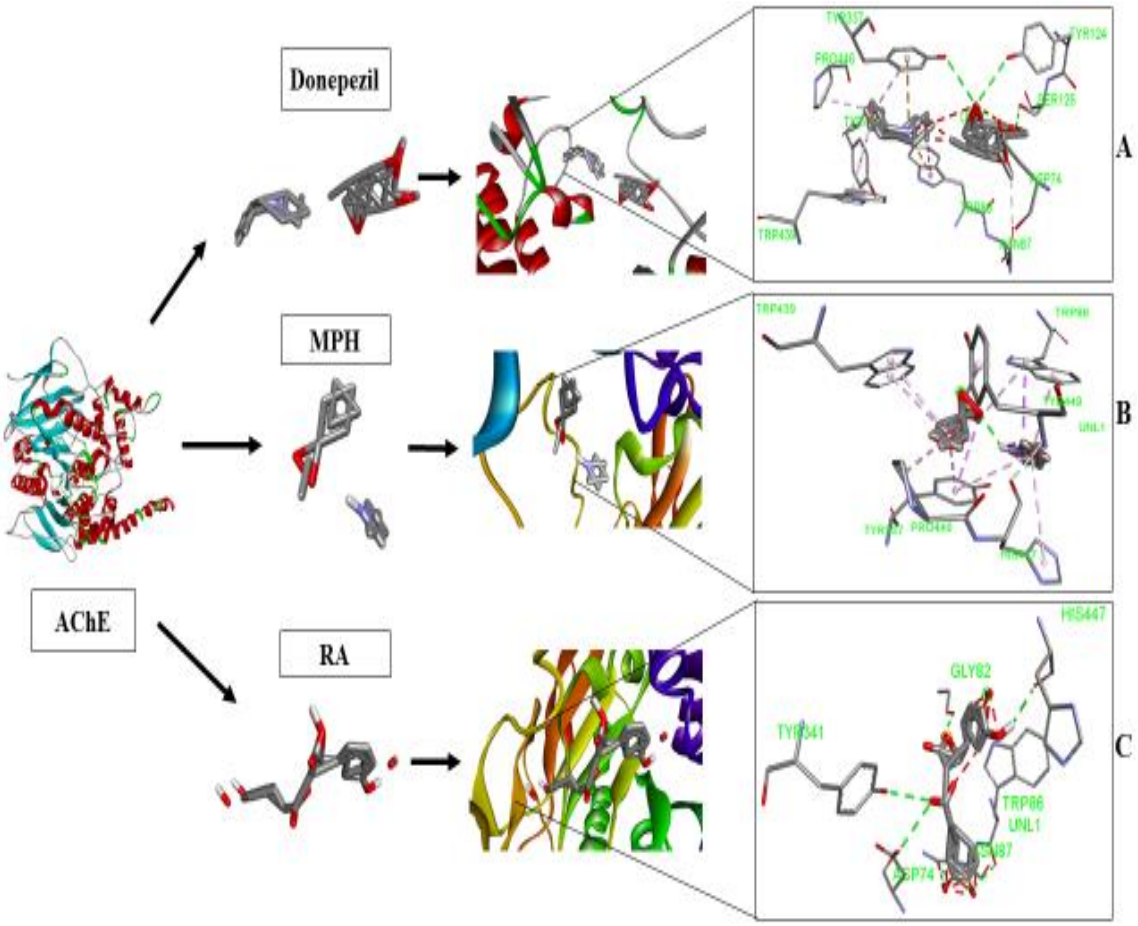

Figure 4. 1: Molecular docking of AChE with Donepezil (A), MPH (B) and RA (C) visualized by BIOVIA Discovery Studio. AChE: Acetylcholinesterase. MPH: Methylphenidate. RA: Rosmarinic Acid. Structure of AChE downloaded from PDB and viewed in BIOVIA Discovery Studio. Structures of Donepezil, MPH and RA downloaded by PubChem and viewed in BIOVIA Discovery Studio. A: Best docking pose between AChE and Donepezil generated by Autodock4 and interacting residues of AChE with Donepezil shown in 3D model. B: Best docking pose between AChE and MPH generated by Autodock4 and interacting residues of AChE with MPH shown in 3D model. C: Best docking pose between AChE and RA generated by Autodock4 and interacting residues of AChE with RA shown in 3D model.

Estimates of binding energies of Donepezil, RA and MPH with BACE1 are -10.84, -9.97 and -9.07 kcal/mol respectively, and the KI values are 11.40 nM, 49.52nM and 223.68 nM respectively (Table 4.1). Donepezil has lowest binding energy indicating the strongest binding affinity among all followed by RA and then MPH. Interacting residues of BACE1 with all the ligands showed that, Donepezil was able to make one hydrogen bond with BACE1 while RA interacted through 6 Hydrogen bonds. GLY11 was the common amino acid among Donepezil and RA making Hydrogen bond. Though RA and MPH showed close binding energy values but the interactions are different in the selected pocket (Table 4.2). MPH was able to make only hydrophobic interactions and no hydrogen bond with BACE1. BACE1 interaction with the ligands is depicted in figure 4.2.

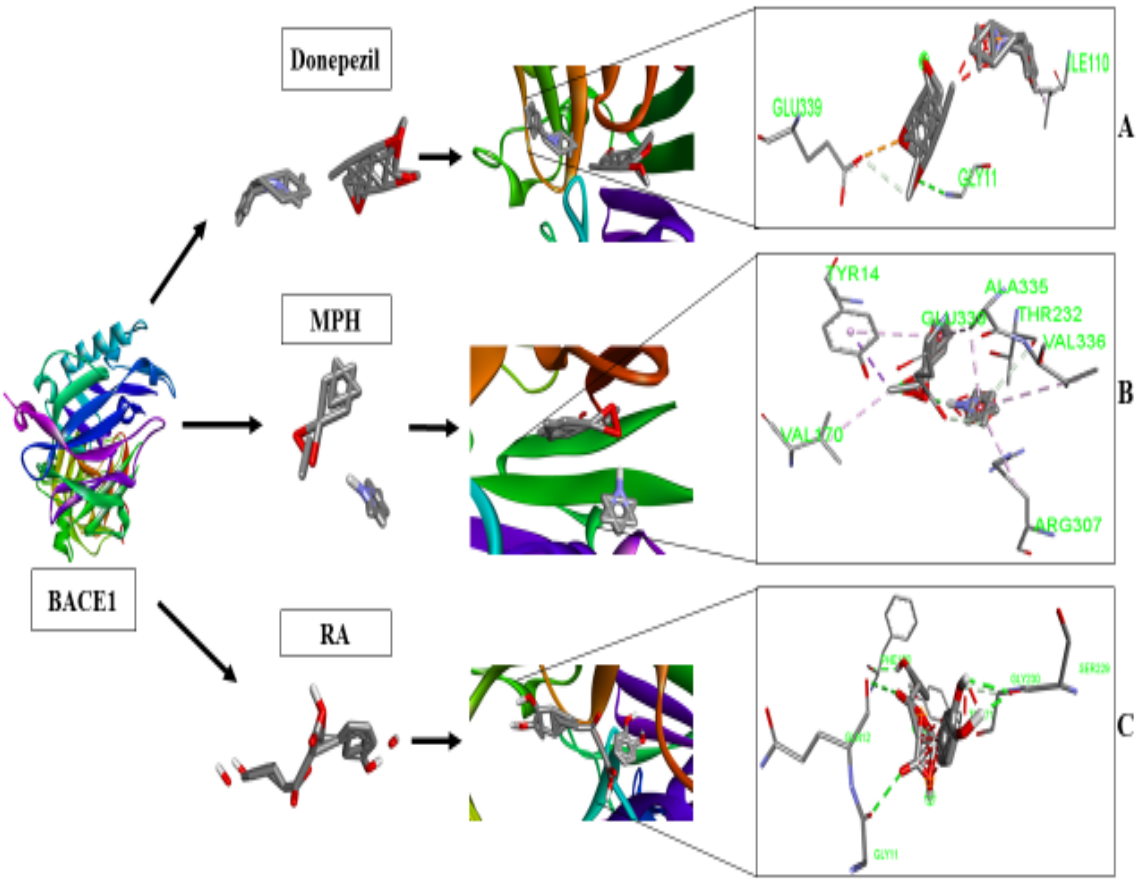

Figure 4. 2: Molecular docking of BACE1 with Donepezil (A), MPH (B) and RA (C) visualized by BIOVIA Discovery Studio. BACE1: Beta secretase-1. MPH: Methylphenidate. RA: Rosmarinic Acid. Structure of BACE1 downloaded from PDB and viewed in BIOVIA Discovery Studio. Structures of Donepezil, MPH and RA downloaded by PubChem and viewed in BIOVIA Discovery Studio. A: Best docking pose between BACE1 and Donepezil generated by Autodock4 and interacting residues of BACE1 with Donepezil shown in 3D model. B: Best docking pose between BACE1 and MPH generated by Autodock4 and interacting residues of BACE1 with MPH shown in 3D model. C: Best docking pose between BACE1 and RA generated by Autodock4 and interacting residues of BACE1 with RA shown in 3D model.

Binding energies of Donepezil, RA and MPH with PSEN-1 are -10.18, -10.78 and -7.73 kcal/mol respectively, and their KI values are 34.29 nM, 12.55 nM and 2.15 uM

respectively (Table 4.1). Donepezil and RA showed good and almost same binding with a very slight difference in binding energies while MPH has lowest among three indicating the lowest binding affinity of MPH towards PSEN1. Interacting amino acids of PSEN1 with the ligands are listed in Table 4.2. RA interacted with PSEN-1 through 5 Hydrogen bonds while Donepezil did not make hydrogen bond and formed hydrophobic interactions with HIS81 and LEU85. MPH was able to make one hydrogen bond with LEU381 which is a common amino acid making hydrogen bond with RA (Table 4.2). Interactions of PSEN-1 with the ligands are shown in figure 4.3.

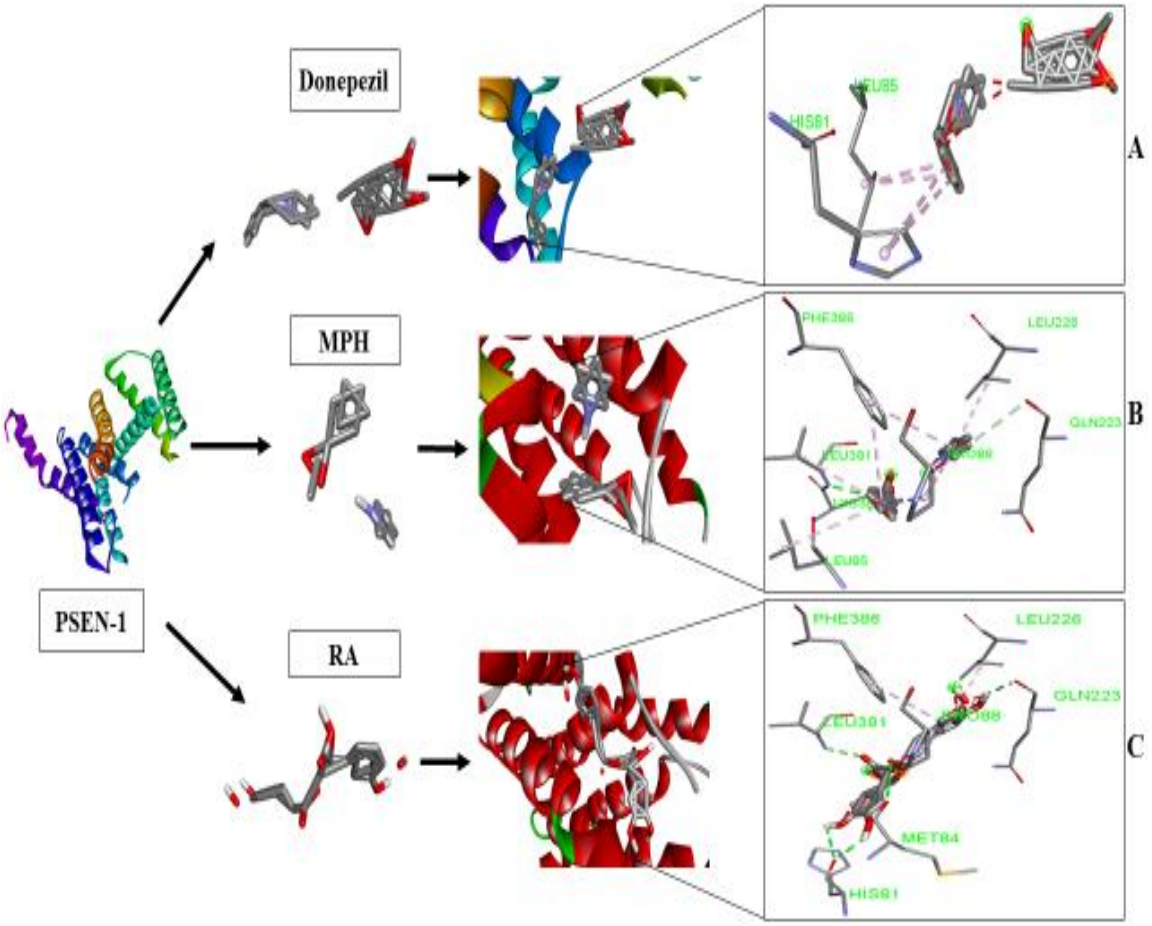

Figure 4. 3: Molecular docking of PSEN-1 with Donepezil (A), MPH (B) and RA (C) visualized by BIOVIA Discovery Studio. PSEN-1: Presenilin-1. MPH: Methylphenidate. RA: Rosmarinic Acid. Structure of PSEN-1 downloaded from PDB and viewed in BIOVIA Discovery Studio. Structures of Donepezil, MPH and RA downloaded by PubChem and viewed in BIOVIA Discovery Studio. A: Best docking pose between PSEN-1 and Donepezil generated by Autodock4 and interacting residues of PSEN-1 with Donepezil shown in 3D model. B: Best docking pose between PSEN-1 and MPH generated by Autodock4 and interacting residues of PSEN-1 with MPH shown in 3D model. C: Best docking pose between PSEN-1 and RA generated by Autodock4 and interacting residues of PSEN-1 with RA shown in 3D model.

Table 4. 1 Binding energies (BE) and Inhibition constants (KI) for the binding of ligands with AChE, PSEN-1 and BACE1 enzymes

Protein	**Ligand**	**Binding Energy (BE) (Kcal/mol)**	**Inhibition Constant (KI)**
AChE	Donepezil	-13.08	258.97 pM
	RA	-12.66	527.49 pM
	MPH	-9.47	113.88 nM
PSEN-1	Donepezil	-10.18	34.29 nM
	RA	-10.78	12.55 nM
	MPH	-7.73	2.15 uM
BACE1	Donepezil	-10.84	11.40 nM
	RA	-9.97	49.52 nM
	MPH	-9.07	223.68 nM

Table 4. 2 Interacting residues & Hydrogen bonds between ligands & protein (AChE, PSEN-1, BACE1 enzymes)

Protein	**Ligand**	**Interacting residues**	**H-bonds**	**Bond length**
AChE	Donepezil	ASN87, ASP74, SER125, TRP86, TYR337, TRP439, TYR449, PRO446, TYR124	TYR 337 TYR 124 SER 125	3.23 A 3.25 2.85
	RA	ASP74, TYR341, GLY82, HIS447, TRP86, ASN87,	ASP74 TYR341 GLY82 HIS447 TRP86 ASN87 ASN87	3.03 2.91 2.42 2.19 2.53 2.96 1.81
	MPH	TYR337, TRP86, TYR449, TRP439, HIS447, PRO446		
BACE1	Donepezil	GLY11, GLU339, ILE110	GLY11	2.92
	RA	TYR71, PHE108,	TYR71 PHE108	2.45 2.07

		GLY11,	GLY11	2.73
		GLN12,	GLN12	3.32
		SER229,	SER229	2.33
		GLY230	SER229	1.84
	MPH	VAL170,		
		TYR14,		
		ALA335,		
		GLU339,		
		THR232,		
		VAL336,		
		ARG307		
PSEN1	Donepezil	HIS81, LEU85		
	RA	GLN223,	GLN223	2.24
		MET84,	MET84	3.25
		LEU381, HIS81,	LEU381	2.70
		LEU226,	HIS81	2.56
		PHE386,	HIS81	2.16
		PRO88, LEU85		
	MPH	LEU381,	LEU381	3.06
		LYS380,		
		LEU85, PRO88,		
		PHE386,		
		GLN223,		
		LEU226		

Binding parameters of pro-inflammatory cytokines IL6, TNF-α and the transcription factor NF-κB, p50 with the ligands are listed in Table 4.3. Binding energies of Donepezil, RA and MPH with IL6 are -7.25, -8.30 and -8.32 kcal/mol respectively and KI values are 4.86 uM, 821.08 nM and 801.61 nM respectively. Values of binding energies showed that MPH

and RA has good and almost similar binding with IL6 while Donepezil has the weakest binding. Interacting residues between all ligands and IL6 are shown in Table 4.4. Only RA was able to make Hydrogen bonds with IL6. RA made two hydrogen bonds with LYS171 and HIS164 while interacting with IL6. (Table 4.4, Figure 4.4).

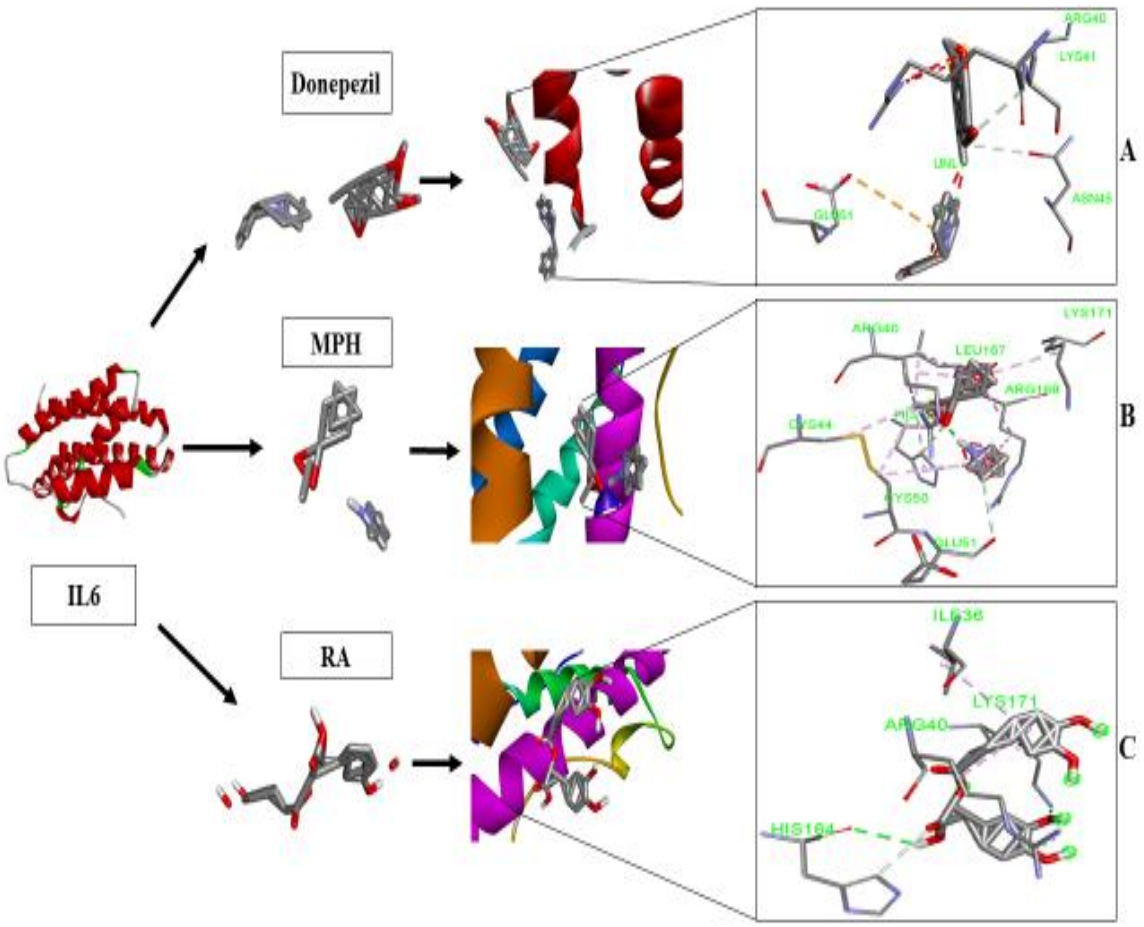

Figure 4. 4: Molecular docking of IL6 with Donepezil (A), MPH (B) and RA (C) visualized by BIOVIA Discovery Studio. IL6: Iterleukin-6. MPH: Methylphenidate. RA: Rosmarinic Acid. Structure of IL6 downloaded from PDB and viewed in BIOVIA Discovery Studio. Structures of Donepezil, MPH and RA downloaded by PubChem and viewed in BIOVIA Discovery Studio. A: Best docking pose between IL6 and Donepezil generated by Autodock4 and interacting residues of IL6 with Donepezil shown in 3D model. B: Best docking pose between IL6 and MPH generated by Autodock4 and interacting residues of IL6 with MPH shown in 3D model. C: Best docking pose between IL6 and RA generated by Autodock4 and interacting residues of IL6 with RA shown in 3D model.

Binding energies of Donepezil, RA and MPH with TNF-α are -8.44, -9.32 and -7.31 kcal/mol respectively, and Ki values are 655.08nM, 148.33 nM and 4.38 uM respectively

(Table 4.3). RA showed the strongest binding affinity with TNF-α followed by Donepezil and then MPH. All the ligands were able to interact with TNF-α except Donepezil (Table 4.4). RA and MPH interacted with the selected pocket of TNF-α by making six and two Hydrogen bonds respectively. Common residues of TNF-α making Hydrogen bonds with RA is 134ALA (Table 4.4). Interactions of TNF- α with RA and MPH are shown in figure 4.5.

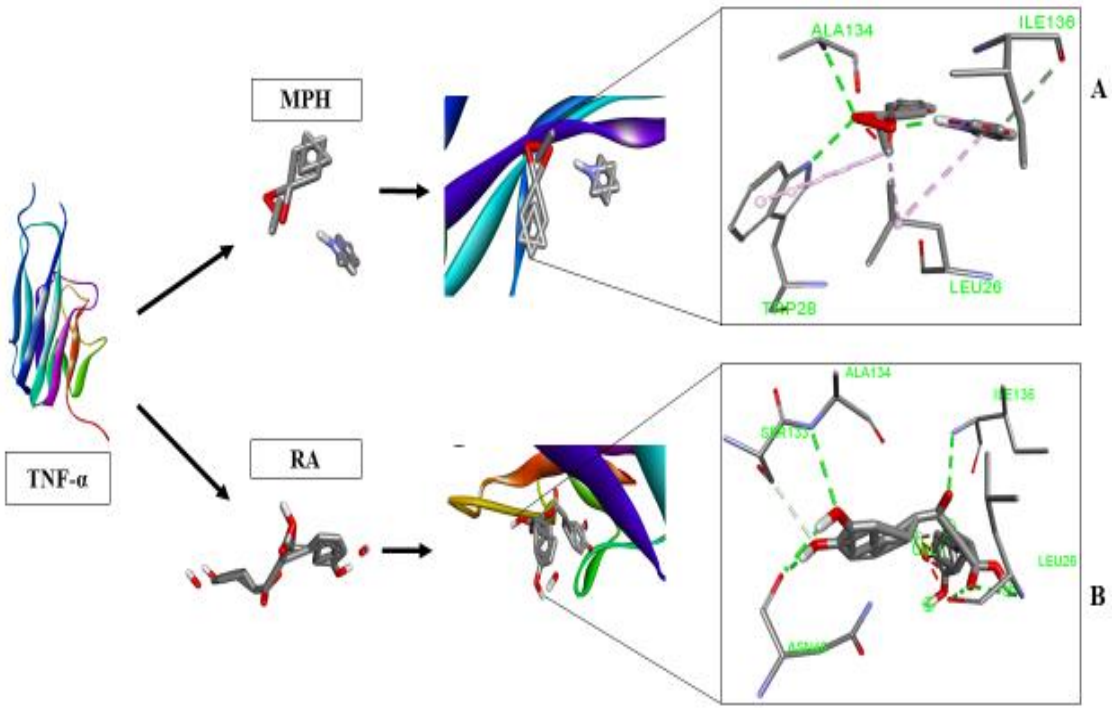

Figure 4. 5: Molecular docking of TNF-α with MPH (A) and RA (B) visualized by BIOVIA Discovery Studio. TNF-α: Tumor necrosis factor-alpha. MPH: Methylphenidate. RA: Rosmarinic Acid. Structure of TNF-α downloaded from PDB and viewed in BIOVIA Discovery Studio. Structures of MPH and RA downloaded by PubChem and viewed in BIOVIA Discovery Studio. A: Best docking pose between TNF-α and MPH generated by Autodock4 and interacting residues of TNF-α with MPH shown in 3D model. B: Best docking pose between TNF-α and RA generated by Autodock4 and interacting residues of TNF-α with RA shown in 3D model.

Values of binding energies for the interaction of Donepezil, RA and MPH with NF-κB, p50 are -8.95, -9.23 and -9.05 kcal/mol respectively, and the Ki values are 275.64 nM, 171.92 nM and 231.13 nM respectively (Table 4.3). RA was able to make strongest binding with NF-κB, p50 along with IL6 and TNF-α. MPH has also shown a very close binding energy value to RA showing its similar potential to interact with NF-κB, p50. Interacting residues of all the ligands are shown in table 4.4. RA and MPH was able to bind through

hydrogen bonds while Donepezil interacted without making hydrogen bond. RA and MPH has made five and one Hydrogen bond with NF-κB, p50, respectively where 138ALA was the common residue making Hydrogen bond (Table 4.4). Docked complexes of NF-κB, p50 with the ligands are visualised in 4.6.

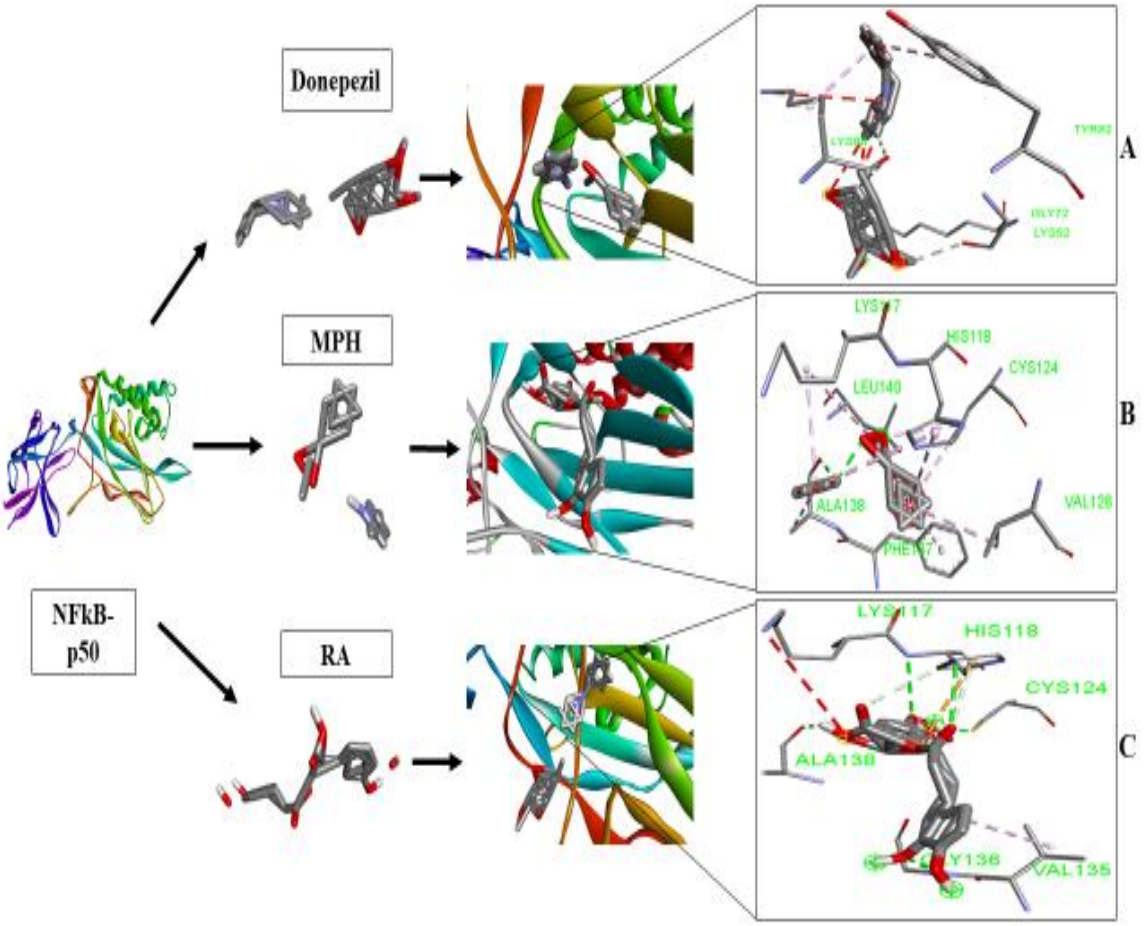

Figure 4. 6: Molecular docking of NF-κB-p50 with Donepezil (A), MPH (B) and RA (C) visualized by BIOVIA Discovery Studio. NF-κB-p50: Nuclear factor kappa B-p50. MPH: Methylphenidate. RA: Rosmarinic Acid. Structure of NF-κB-p50 downloaded from PDB and viewed in BIOVIA Discovery Studio. Structures of Donepezil, MPH and RA downloaded by PubChem and viewed in BIOVIA Discovery Studio. A: Best docking pose between NF-κB-p50 and Donepezil generated by Autodock4 and interacting residues of NF-κB-p50 with Donepezil shown in 3D model. B: Best docking pose between NF-κB-p50 and MPH generated by Autodock4 and interacting residues of NF-κB-p50 with MPH shown in 3D model. C: Best docking pose between NF-κB-p50 and RA generated by Autodock4 and interacting residues of NF-κB-p50 with RA shown in 3D model.

Table 4. 3 Binding energies (BE) and Inhibition constants (KI)for binding of ligands with pro inflammatory cytokines

Protein	**Ligand**	**Binding Energy (BE) (Kcal/mol)**	**Inhibition Constant (Ki)**
IL6	Donepezil	-7.25	4.86 uM
	RA	-8.30	821.08 nM
	MPH	-8.32	801.61 nM
TNF-α	Donepezil	-8.44	655.08 nM
	RA	-9.32	148.33 nM
	MPH	-7.31	4.38 uM
NF-κB,	Donepezil	-8.95	275.64 nM
p50	RA	-9.23	171.92 nM
	MPH	-9.05	231.13 nM

Table 4. 4 Interacting residues & Hydrogen bonds between ligand & proinflammatory cytokines

Protein	Ligand	Interacting residues	H-bonds	Bond length
IL6	Donepezil	GLU51, ASN45, LYS41, ARG40		
	RA	LYS171, HIS164, ILE36, ARG40, HIS164	LYS171 HIS164	2.93 2.86
	MPH	LYS171, ARG40, LEU167, ARG168, CYS44, CYS50, HIS164, GLU51		
TNF-α	Donepezil			
	RA	ALA134, ASN46, ILE136, LEU26, SER133	ALA134 ASN46 ASN46 ILE136 LEU26 LEU26	3.10 2.11 1.92 3.00 3.21 2.68
	MPH	ALA134, TRP28, LEU26, ILE136	ALA134 TRP28	2.88 2.79
NFkB-50	Donepezil	LYS80, TRP82, LYS52, GLY72		
	RA	ALA138, HIS118, CYS124,	ALA138 HIS118 HIS118	2.29 3.22 3.06

	GLY136, LYS117, VAL135	CYS124	2.64
		GLY136	2.66
MPH	ALA138, LYS117, HIS118, LEU140, CYS124, VAL126, PHE137	ALA138	2.27

Interactions of all the three ligands with the antioxidant enzymes (SOD1, SOD2, Prdx6) are shown by binding parameters in table 4.5. Binding energies of Donepezil, RA and MPH with SOD1 are -8.88, -8.57 and -6.70 Kcal/mol respectively, and their inhibition constants are 310.55 nM, 525.77 nM and 12.27 µM respectively. Values of binding energies show that Donepezil and RA has almost equal binding affinity with SOD1 while MPH has shown comparatively weaker binding affinity. Interacting residues of SOD1 with all the ligands are listed in table 4.6. Six hydrogen bonds were shown to form between RA and the selected pocket of the SOD1 enzyme while two hydrogen bonds formed by MPH with the enzyme (Table 4.6). Though Donepezil has shown the lowest binding energy while interacting with SOD1 but the interactions are not strong enough to make any hydrogen bond table (4.6). Interactions are depicted in figure 4.7.

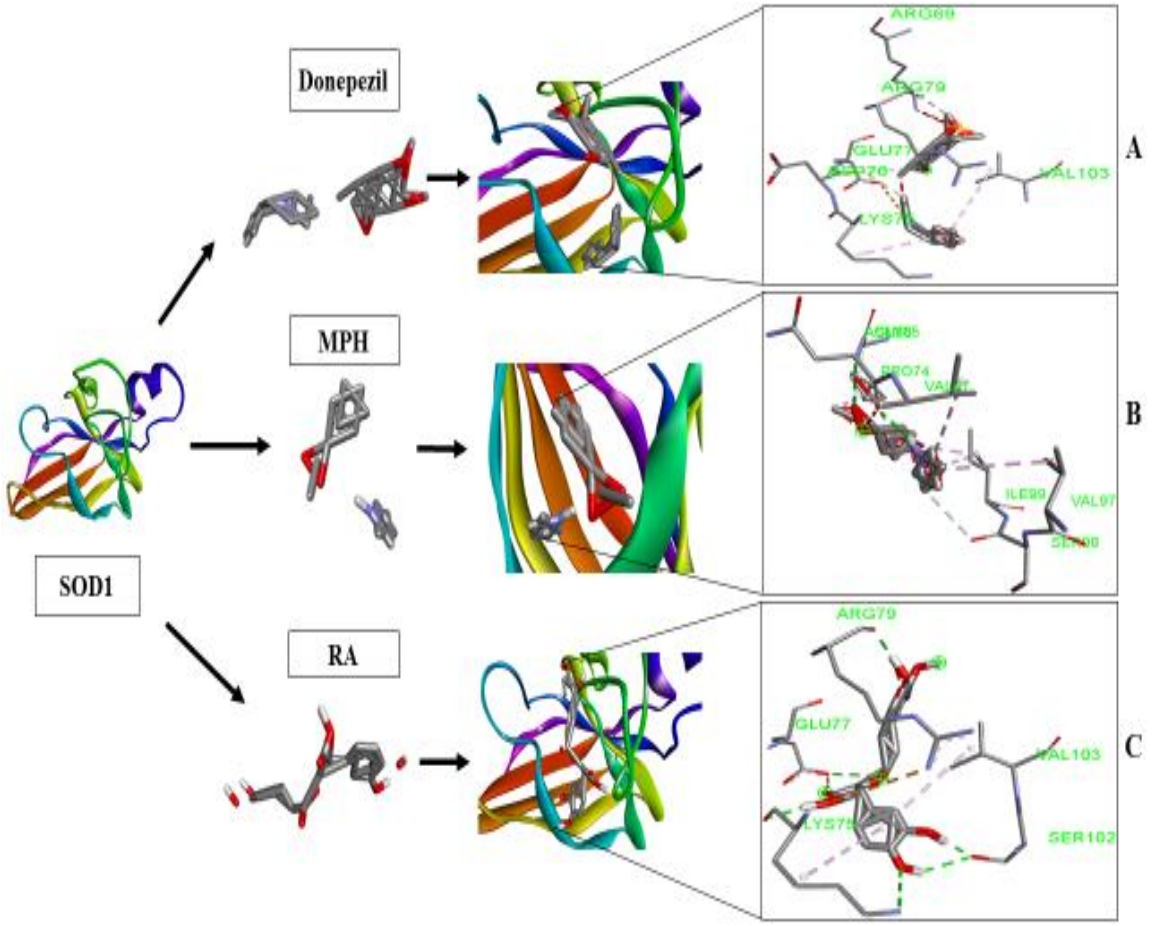

Figure 4. 7: Molecular docking of SOD1 with Donepezil (A), MPH (B) and RA (C) visualized by BIOVIA Discovery Studio. SOD1: Superoxide dismutase 1. MPH: Methylphenidate. RA: Rosmarinic Acid. Structure of SOD1 downloaded from PDB and viewed in BIOVIA Discovery Studio. Structures of Donepezil, MPH and RA downloaded by PubChem and viewed in BIOVIA Discovery Studio. A: Best docking pose between SOD1 and Donepezil generated by Autodock4 and interacting residues of SOD1 with Donepezil shown in 3D model. B: Best docking pose between SOD1 and MPH generated by Autodock4 and interacting residues of SOD1 with MPH shown in 3D model. C: Best docking pose between SOD1 and RA generated by Autodock4 and interacting residues of SOD1 with RA shown in 3D model.

Energies of binding for the interaction of Donepezil, RA and MPH with SOD2 are found to be -8.76, -9.28 and -7.60 Kcal/mol respectively along with their inhibition constant

378.46 nM, 158.11 nM and 2.67 µM respectively (Table 4.5). RA has the strongest binding followed by Donepezil and then MPH. All the ligands were able to interact with SOD1 (Table 4.6). One hydrogen bond was found to be present in 'Donepezil-SOD2 interaction' and six hydrogen bonds in 'RA-SOD2 interaction'. There was no hydrogen bond in 'MPH-SOD2 interaction' (Table 4.6, Figure 4.8).

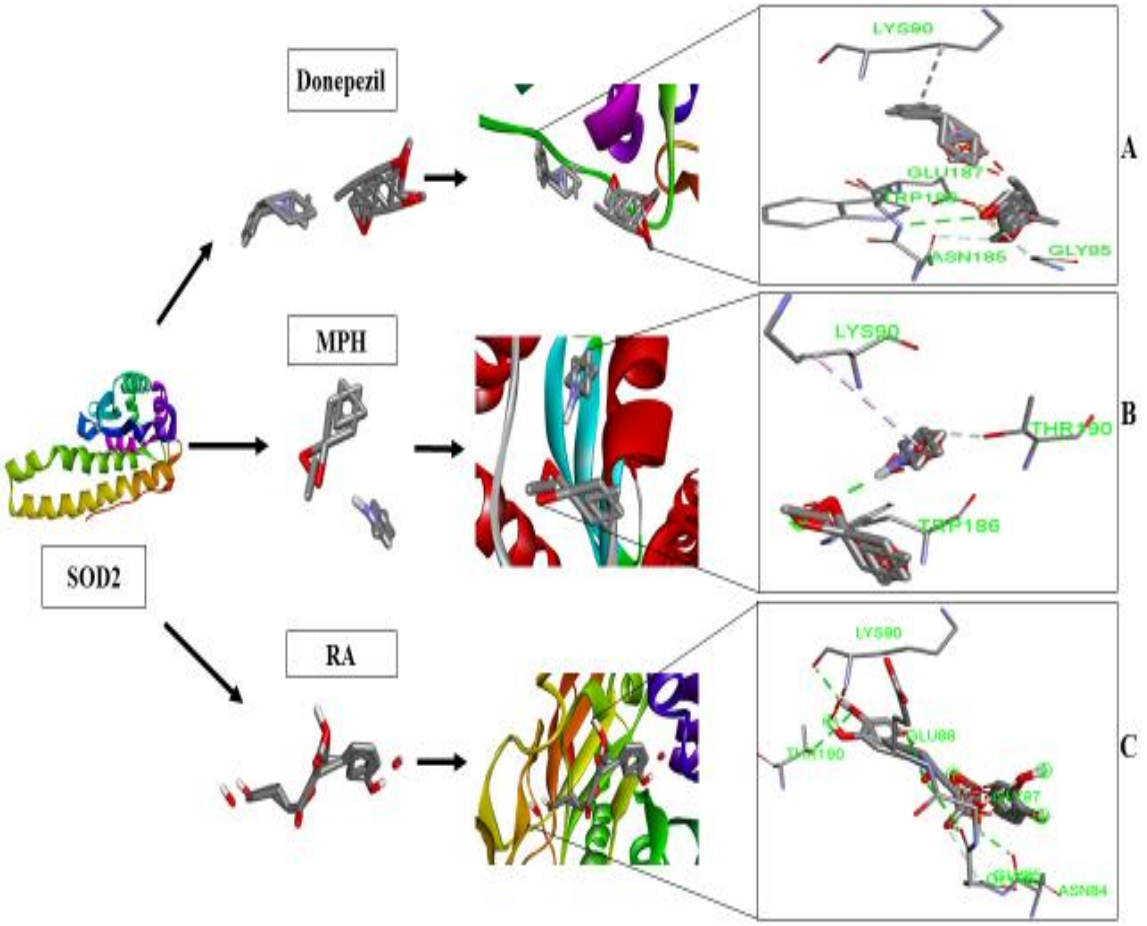

Figure 4. 8: Molecular docking of SOD2 with Donepezil (A), MPH (B) and RA (C) visualized by BIOVIA Discovery Studio. SOD2: Superoxide dismutase 2. MPH: Methylphenidate. RA: Rosmarinic Acid. Structure of SOD2 downloaded from PDB and viewed in BIOVIA Discovery Studio. Structures of Donepezil, MPH and RA downloaded by PubChem and viewed in BIOVIA Discovery Studio. A: Best docking pose between SOD2 and Donepezil generated by Autodock4 and interacting residues of SOD2 with Donepezil shown in 3D model. B: Best docking pose between SOD2 and MPH generated by Autodock4 and interacting residues of SOD2 with MPH shown in 3D model. C: Best docking pose between SOD2 and RA generated by Autodock4 and interacting residues of SOD2 with RA shown in 3D model.

Binding energies for the complex of Donepezil, RA and MPH with Prdx6 are -8.06, -8.02 and -7.24 respectively Kcal/mol and their inhibition constants are 1.23 µM, 1.33 µM and

4.97 µM, respectively (Table 4.5). Donepezil and RA has stronger and almost equal binding affinity with Prdx6 while MPH has comparatively weaker but good binding. All the ligands were found to interact with amino acids of the selected pocket of Prdx6 (Table 4.6). RA was able to form six hydrogen bonds while Donepezil formed only one hydrogen bond. Binding energy of MPH is slightly higher than the rest of the ligands but MPH was able to bind strongly by making three hydrogen bonds. 47CYS is the common residue of Prdx6 interacting through H-bond with RA and MPH (Table 4.6, Figure 4.9).

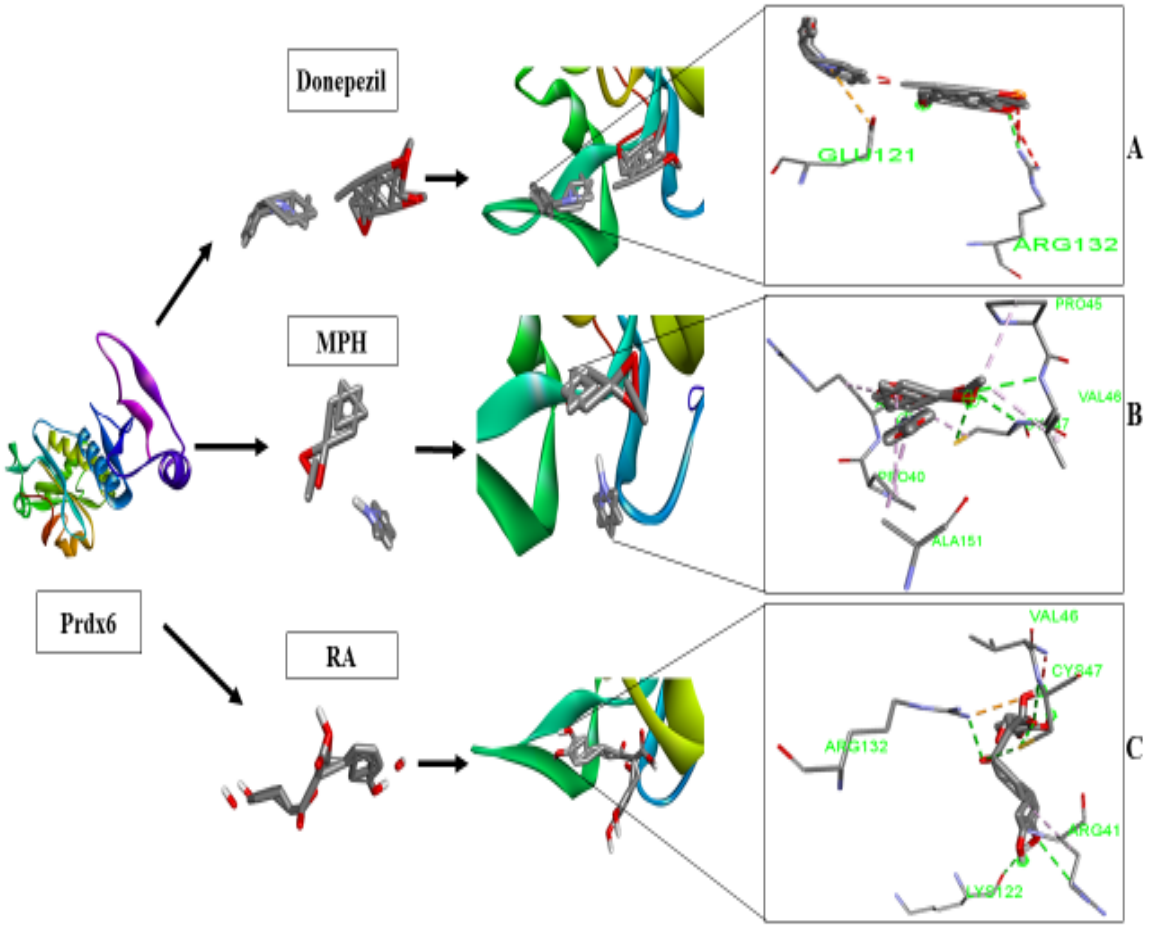

Figure 4. 9: Molecular docking of Prdx6 with Donepezil (A), MPH (B) and RA (C) visualized by BIOVIA Discovery Studio. Prdx6: Peroxiredoxin-6. MPH: Methylphenidate. RA: Rosmarinic Acid. Structure of Prdx6 downloaded from PDB and viewed in BIOVIA Discovery Studio. Structures of Donepezil, MPH and RA downloaded by PubChem and viewed in BIOVIA Discovery Studio. A: Best docking pose between Prdx6 and Donepezil generated by Autodock4 and interacting residues of Prdx6 with Donepezil shown in 3D model. B: Best docking pose between Prdx6 and MPH generated by Autodock4 and interacting residues of Prdx6 with MPH shown in 3D model. C: Best docking pose between Prdx6 and RA generated by Autodock4 and interacting residues of Prdx6 with RA shown in 3D model.

Table 4. 5 Binding energies (BE) and Inhibition constants (KI) for the binding of ligands with antioxidant enzymes (SOD1, SOD2, Prdx6)

Protein	**Ligand**	**Binding Energy (BE) (Kcal/mol)**	**Inhibition Constant (Ki)**
SOD1	Donepezil	-8.88	310.55 nM
	RA	-8.57	525.77 nM
	MPH	-6.70	12.27 uM
SOD2	Donepezil	-8.76	378.46 nM
	RA	-9.28	158.11 nM
	MPH	-7.60	2.67 uM
Prdx6	Donepezil	-8.06	1.23 uM
	RA	-8.02	1.33 uM
	MPH	-7.24	4.97 uM

Table 4. 6 Interacting residues & Hydrogen bonds between ligands & antioxidant enzymes (SOD1, SOD2 and Prdx6)

Protein	Ligand	Interacting residues	H-bonds	Bond length
SOD1	Donepezil	ARG79, ARG69, ASP76, GLU77, VAL103, LYS75		
	RA	ARG79, GLU77, LYS75, SER102, VAL103	ARG79 GLU77 LYS75 LYS75 SER102 SER102	1.82 3.27 2.05 3.04 2.64 1.99
	MPH	ASN86, GLY85, PRO74, ILE99, VAL87, VAL97, SER98	ASN86 ASN86	3.01 2.56
SOD2	Donepezil	TRP186, ASN185, GLY85, GLU187, LYS90	TRP186	3.34
	RA	LYS90, THR190, GLU88, GLY86, ASN84, LYS90, GLY87, GLY85	LYS90 THR190 GLU88 GLY86 GLY86 ASN84	2.18 3.10 2.96 3.22 3.24 2.27

	MPH	LYS90, THR190, TRP186		
Prdx6	Donepezil	ARG132, GLU121	ARG132	2.62
	RA	ARG41,	ARG41	3.12
		LYS122,	LYS122	2.63
		CYS47,	CYS47	3.58
		ARG132,	CYS47	2.85
		VAL46	CYS47	3.22
			ARG132	2.66
	MPH	VAL46, CYS47,	VAL46	2.95
		PRO45,	CYS47	3.29
		ALA151, PRO40, ARG41	CYS47	3.40

4.1.2. Drug Likeness Analysis

Drug likeness of all three ligands was investigated using Lipinski filter and by predicting pharmacokinetics properties (ADMET) of the ligands using admetSAR1. Lipinski filter and admetSAR1 tools are very helpful in predicting drug likeness as well as drug designing (F. Cheng et al., 2012). Results of Lipinski filter and ademtSAR1 (Table 4.7) shows that all the tested compounds on all the mentioned parameters exhibited values within the standard range representing their potential to be used as drugs for application in biological systems. Scores of the three drugs were comparable on all the parameters of Lipinski filter as well as ademtSar1 (Table 4a and 4b). Donepezil and MPH are already established drugs approved by the FDA and values of all the parameters suggested that RA qualified to be used as a drug.

Table 4. 7 Drug likeness of Donepezil, RA & MPH

a- Lipinski filter analysis

Lipinski filter	**Standard range**	**Donepezil**	**RA**	**MPH**
Molecular mass	<500 daltons	379	360	312
cLogP	<5	4.265309	1.355830	-0.053101
Hydrogen bond donors	<5	0	5	5
Hydrogen bond acceptors	<10	3	8	6
Molar refractivity	40-130	109.857483	81.292488	77.145782

b- ADMET analysis

Parameters	Donepezil	RA	MPH
Blood-Brain Barrier	BBB+	BBB+	BBB+
Human Intestinal Absorption	HIA+	HIA+	HIA+
Caco-2 Permeability	Caco2+	Caco2-	Caco2+
AMES Toxicity	Non AMES toxic	Non AMES toxic	Non AMES toxic
Carcinogens	Non-carcinogens	Non-carcinogens	Non-carcinogens
Rat Acute Toxicity	3.0123	2.3234	2.7718
Acute oral toxicity	III	III	II

4.2. IN-VITRO ANALYSIS

4.2.1 Anti-oxidant activity of *R. officinalis*

DPPH assay was carried out to examine the free radical scavenging potential of *R. officinalis* extract. *R. officinalis* extract showed a dose dependently high potential to hamper free radicals of the DPPH. Anti-oxidant activity of both control (ascorbic acid) and methanolic extract of *R. officinalis* were observed by their free radical scavenging activity. The anti-oxidant potential of *R. officinalis* was found to be lower than that of ascorbic acid

but the radical scavenging potential increased in a dose dependent manner as shown in figure 4.10.

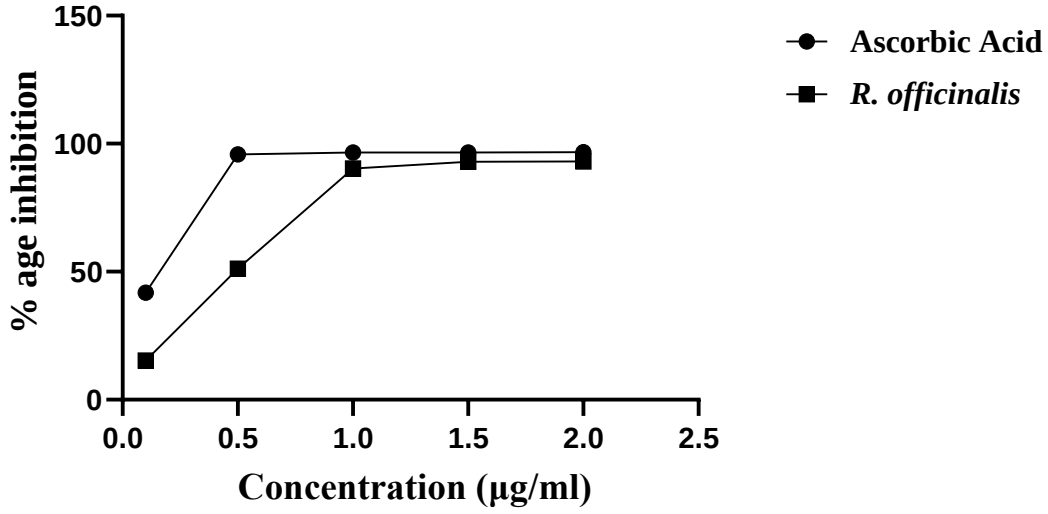

Figure 4. 10: Graphical representation of anti-oxidant potential of *R. officinalis* and ascorbic acid through DPPH assay.

4.2.2 Behavioural Analysis

4.2.2.1 Comparative Effect of Donepezil, MPH and *R.officinalis* on Cognition

MWM test was employed to assess the outcomes of MPH and *R. officinalis* on spatial learning and memory in comparison to Donepezil. Average escape latency to find out the platform directly indicates the outcome of the drugs on spatial memory. All groups showed significant improvements in memory following drugs administration in comparison to the $AlCl_3$ induced Alzheimer's disease model. $AlCl_3$-treated group (64.08 ± 5.54) showed significantly poor memory retention by demonstrating an escape latency of 64sec than the control group (8.06 ± 0.62, $p< 0.0001$) with an average escape latency of 8sec at 5^{th} day.

AlCl3 + MPH-treated group (4.96 ± 0.51) showed significant improvement ($p< 0.0001$) in spatial memory with an escape latency of almost 5sec than $AlCl_3$-treated group at day 5. AlCl3 + Donepezil-treated (8.97 ± 2.84) and AlCl3 + *R. officinalis*-treated (11.43 ± 1.34) groups also showed significant ($p< 0.0001$) restoration of memory by displaying an escape latency of almost 9sec and 11sec, respectively compared to AD model displaying an escape latency of 64sec at day 05. Better restoration of the spatial memory as compared to other drug treated groups was seen in AlCl3 + MPH-treated mice. Escape latency for five days is represented graphically in figure 4.11.

Probe trial was carried out for the assessment of reference memory. Exploration time taken by mice for the previously placed invisible platform was determined by measuring "time spent in the target quadrant". $AlCl_3$-treated mice spent the lowest time in the target quadrant as well as crossed even lesser over previously placed platform position, reflecting a significant decrease in reference memory. $AlCl_3$-treated mice (1.25 ± 0.16) significantly ($p< 0.0001$) made less number of crossings over the platform position compared to control group (3.37 ± 0.18). All $AlCl_3$ induced drug treated groups that is AlCl3 + Donepezil (2.75 ± 0.25), AlCl3 + MPH (3.25 ± 0.25) and $AlCl_3$ + *R. officinalis* (2.42 ± 0.20) showed significant improvement ($p\leq 0.001$), ($p< 0.0001$), ($p< 0.01$) respectively in reference memory with increased number of platform crossings than the $AlCl_3$-treated mice. Similarly, $AlCl_3$-treated (2.00 ± 0.50) group also showed significantly ($p< 0.0001$) less number of entries in the target quadrant than control (9.12 ± 0.50). Drug treatment significantly increased the number of entries in target quadrant in $AlCl_3$ + Donepezil (7.25 ± 0.45) ($p< 0.0001$), AlCl3 + MPH (7.62 ± 0.37) ($p< 0.0001$) and $AlCl_3$ + *R. officinalis* (4.25 ± 0.45) ($p< 0.05$) than $AlCl_3$-treated mice (figure 4.12). However, AlCl3 + MPH-

treated and AlCl3 + Donepezil-treated groups made almost same number of entries in the target quadrant (figure. 4.13). $AlCl_3$ + *R. officinalis*-treated group showed significant increase in number of entries than $AlCl_3$-treated group but significantly less ($p< 0.001$), ($p< 0.01$) entries than $AlCl_3$ + MPH-treated and $AlCl_3$ + Donepezil-treated groups respectively. Similarly, $AlCl_3$-treated group (27.50 ± 2.18) displayed significant decrease ($p< 0.001$) in time spent in the target quadrant than control group (43.00 ± 1.71) while drug treatment improved the memory with increase in time spent in target quadrant (figure 4.14). $AlCl_3$ + Donepezil-treated (41.29 ± 2.36), ($p< 0.01$) and AlCl3 + *R.* o*fficinalis*-treated mice (42.50 ± 2.63), ($p< 0.001$) showed significant increase in time spent in the target quadrant than $AlCl_3$-treated group while $AlCl_3$ + MPH (37.13 ± 2.41) did not show a significant increase in time spent. Overall, all the compounds were able to improve memory significantly in AD model but Donepezil and MPH showed more significant results compared to *R. officinalis*.

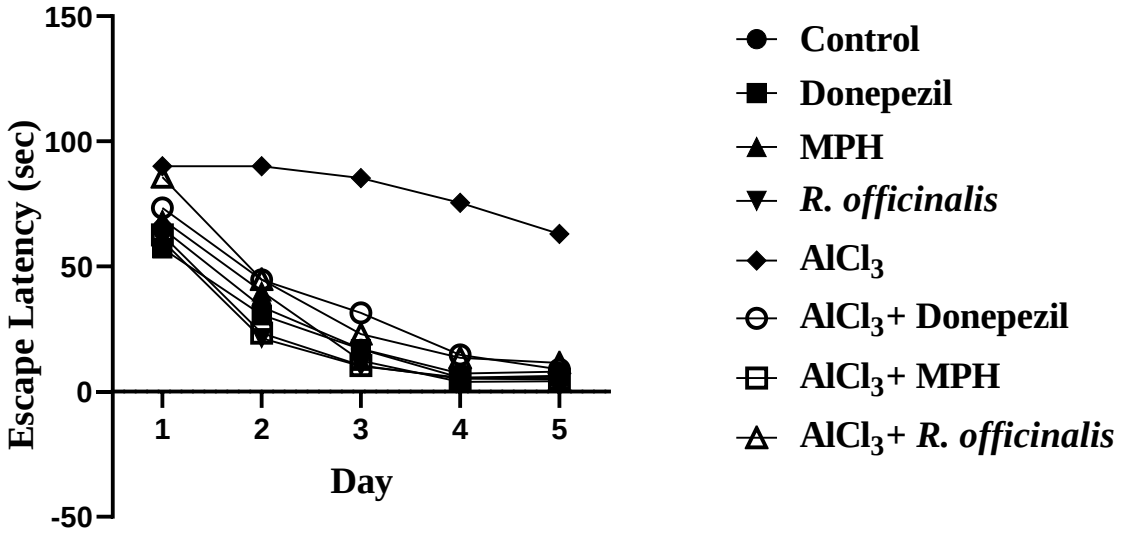

Figure 4. 11: Effect of Donepezil, MPH and *R. officinalis* on spatial learning and memory using Morris Water Maze Test.

Graph demonstrates escape latency (sec) to analyze spatial memory development between Control, Donepezil, MPH, *R. officinalis*, $AlCl_3$, $AlCl_3$ + *R. officinalis*, $AlCl_3$ + MPH and AlCl3 + Donepezil -treated groups. AlCl3-treated mice displayed decreased retention of spatial memory than Control group. The data indicated that $AlCl_3$ + MPH, AlCl3 + Donepezil and $AlCl_3$ + *R. officinalis*-treated groups found the platform much faster than $AlCl_3$-treated group after drug treatment. One-way ANOVA followed by Bonferroni comparison test (mean ± SEM) was applied to analyze the data using Graphpad Prism. n=8, $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$.

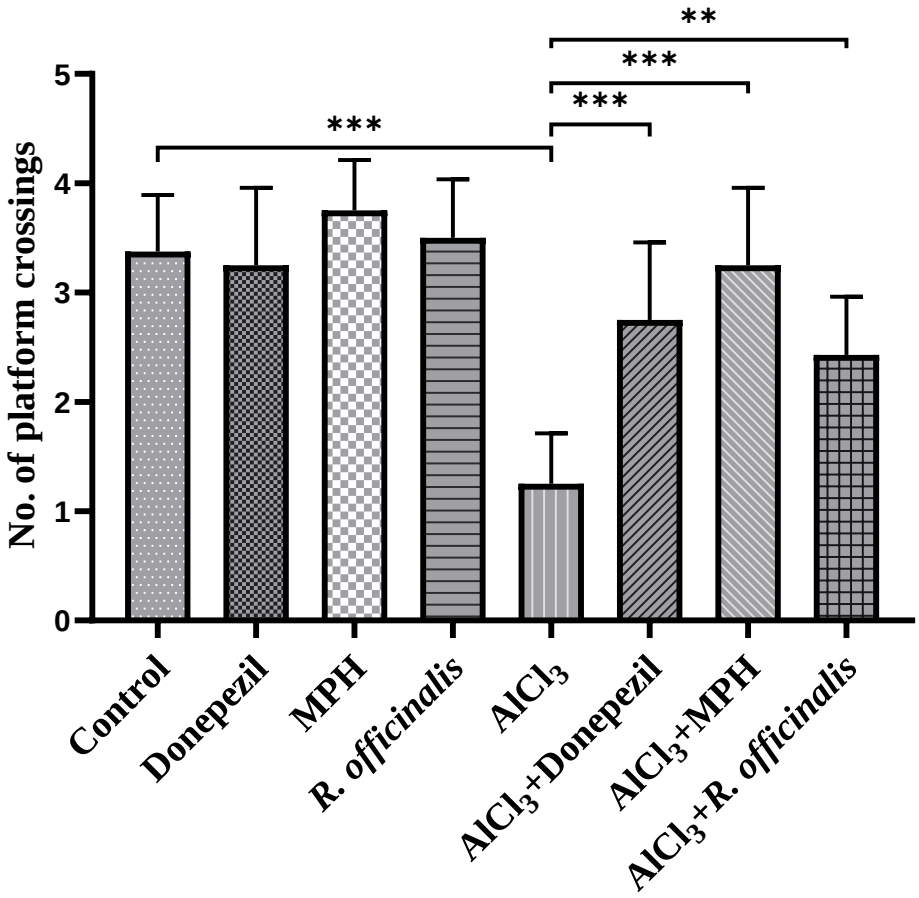

Figure 4. 12: Bar graph representing the effect of Donepezil, MPH and *R. officinalis* on number of platform crossings over the platform position by subjects in Morris Water Maze Test. $AlCl_3$-treated mice showed significantly decreased number of crossings compared to control group over the previously placed platform position. $AlCl_3$ + Donepezil, $AlCl_3$ + MPH, $AlCl_3$ + *R. officinalis* groups improved reference memory indicated by significant increase in number of platform crossings compared to $AlCl_3$-treated group after drug treatment. One-way ANOVA followed by Bonferroni comparison test (mean ± SEM) was applied to analyze the data using Graphpad Prism. n=8. $^{***}p<0.001$, $^{**}p<0.01$

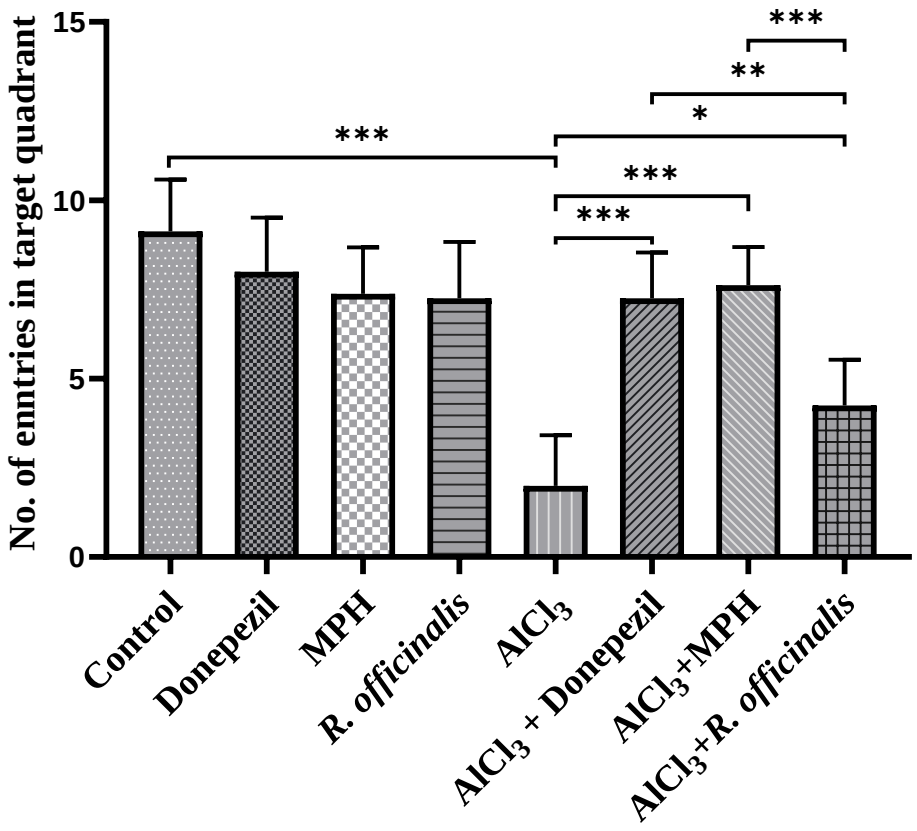

Figure 4. 13: Bar graph depicting the effect of Donepezil, MPH and *R. officinalis* on number of entries made by the subjects in target quadrant in Morris Water Maze Test. $AlCl_3$-treated group made significantly lesser entries in the target quadrant compared to control group. $AlCl_3$ + Donepezil, $AlCl_3$ + MPH, $AlCl_3$ + *R. officinalis* groups made significantly more entries compared to $AlCl_3$-treated group after drug treatment. $AlCl_3$ + Donepezil-treated and $AlCl_3$ + MPH-treated mice made almost same and significantly more entries than $AlCl_3$ + *R. officinalis*-treated mice. One-way ANOVA followed by Bonferroni comparison test (mean ± SEM) was applied to analyze the data using Graphpad Prism. n=8. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$.

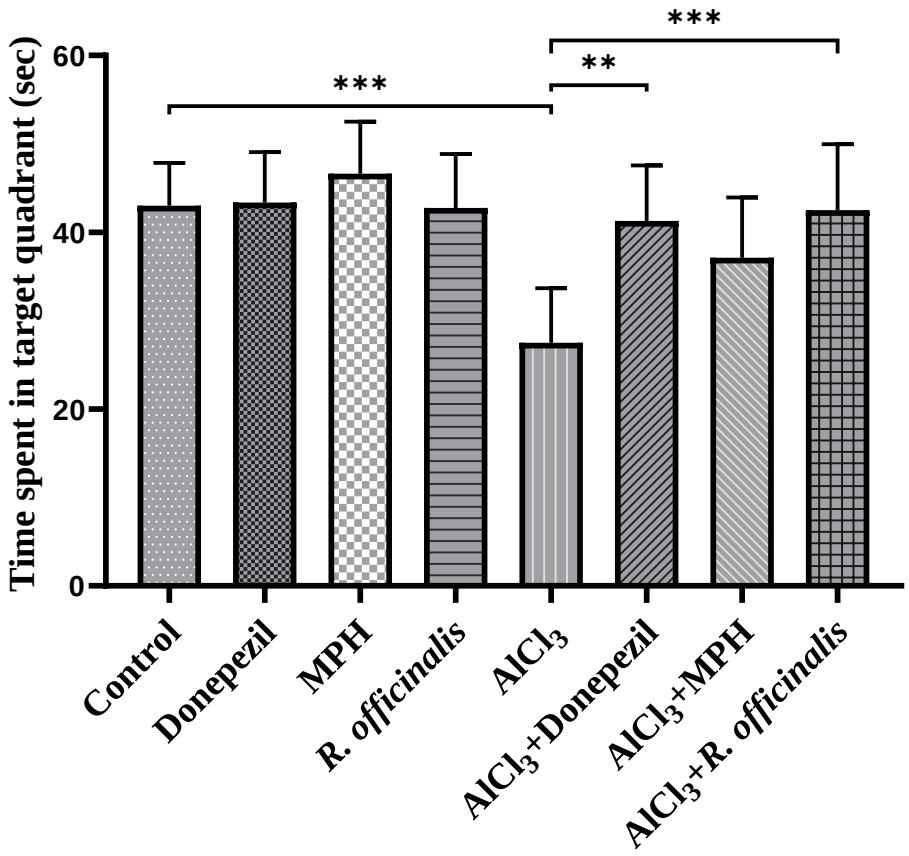

Figure 4. 14: Bar graph representing the effect of Donepezil, MPH and *R. officinalis* on time (sec) spent in target quadrant by subjects in Morris Water Maze Test. $AlCl_3$-treated mice spent less time in the target quadrant than control group. $AlCl_3$ + Donepezil and $AlCl_3$ + *R. officinalis* groups showed significant increase while $AlCl_3$ + MPH group spent insignificantly more time in the target quadrant after drugs treatment. One-way ANOVA followed by Bonferroni comparison test (mean ± SEM) was applied to analyze the data using Graphpad Prism. n=8. $^{***}p<0.001$, $^{**}p<0.01$.

4.2.2.2 Comparative Effect of Donepezil, MPH and *R. officinalis* on Depression

The forced swim test was performed to assess the antidepressant effect of Donepezil, MPH and *R. officinalis* on AD model having impaired cognitive functions. $AlCl_3$-treated group (2.50 ± 0.37) showed depressive behavior by showing significantly less ($p< 0.0001$) latency

to immobility than control group (19.13±0.29). All drug treated groups spent significantly more time in struggling and hence delayed latency to immobility except $AlCl_3$-treated group which displayed relatively more depression by taking early but prolonged immobility and high number of immobile episodes. All treated groups $AlCl_3$ + Donepezil (19.00 ± 0.78), $AlCl_3$ + MPH (10.06 ± 1.38) and $AlCl_3$ + *R. officinalis* (18.50 ± 1.21) significantly increased ($p< 0.0001$) latency to immobility than $AlCl_3$-treated group (2.50 ± 0.37) (figure 4.15). $AlCl_3$ + Donepezil and $AlCl_3$ + R. *officinalis* significantly ($p< 0.0001$) delayed more latency to immobility than $AlCl_3$ + MPH. Similarly, $AlCl_3$-treated (23.17 ± 1.49) group displayed significantly more ($p< 0.0001$) number of immobile episodes than control group (12.29 ± 0.56). After treatment, the $AlCl_3$ + Donepezil (12.86 ± 1.22), $AlCl_3$ + MPH (9.28 ± 0.64) and $AlCl_3$ + *R. officinalis* (12.83 ± 1.13) groups significantly decreased ($p< 0.0001$) the number of immobile episodes as compared to $AlCl_3$-treated group. But there was no significant difference in reducing number of immobile episodes in between all drug treated groups (figure 4.16). $AlCl_3$- treated (96.13 ± 3.85) group spent insignificantly more time being immobile than control (81.00 ± 4.81). Time spent immobile was significantly decreased by drugs treatment in treated groups $AlCl_3$ + Donepezil (74.38 ± 5.26) ($p< 0.05$), $AlCl_3$ + MPH (61.63 ± 4.66) ($p< 0.0001$) and $AlCl_3$ + *R. officinalis* (38.25 ± 0.82) ($p< 0.0001$) (figure 4.17). $AlCl_3$ + *R. officinalis*-treated group showed more significant decrease in time spent being immobile compared to $AlCl_3$ + Donepezil-treated ($p< 0.0001$) and $AlCl_3$ + MPH-treated ($p< 0.05$) groups.

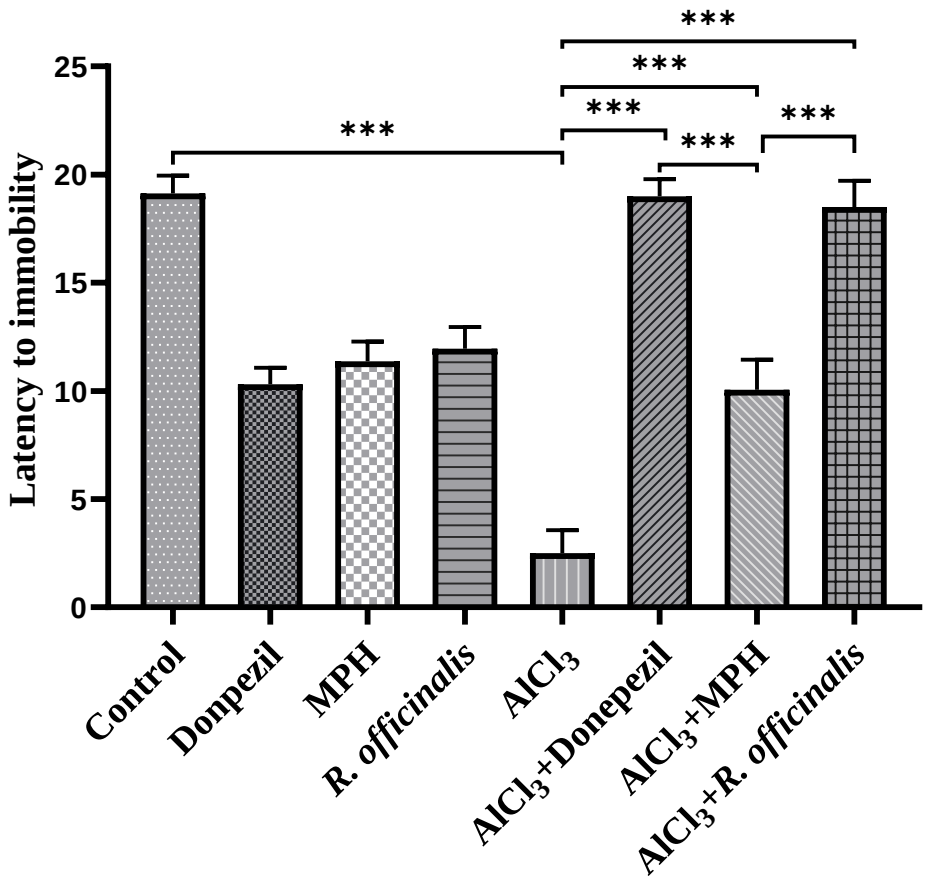

Figure 4. 15: Bar graph depicting the effect of Donepezil, MPH and *R. Officinalis* on Latency to Immobility in Forced Swim Test. $AlCl_3$-treated mice showed depressive behavior by displaying significantly less latency to immobility compared to control group. $AlCl_3$ + Donepezil, $AlCl_3$ + MPH and $AlCl_3$ + *R. officinalis* groups showed significant delay in latency to immobility after drug treatment. $AlCl_3$ + Donepezil-treated and $AlCl_3$ + *R. officinalis*-treated showed more significant increase in latency to immobility than $AlCl_3$ + MPH-treated group. One-way ANOVA followed by Bonferroni comparison test (mean ± SEM) was applied to analyze the data using Graphpad Prism. n=8. $^{***}p<0.001$.

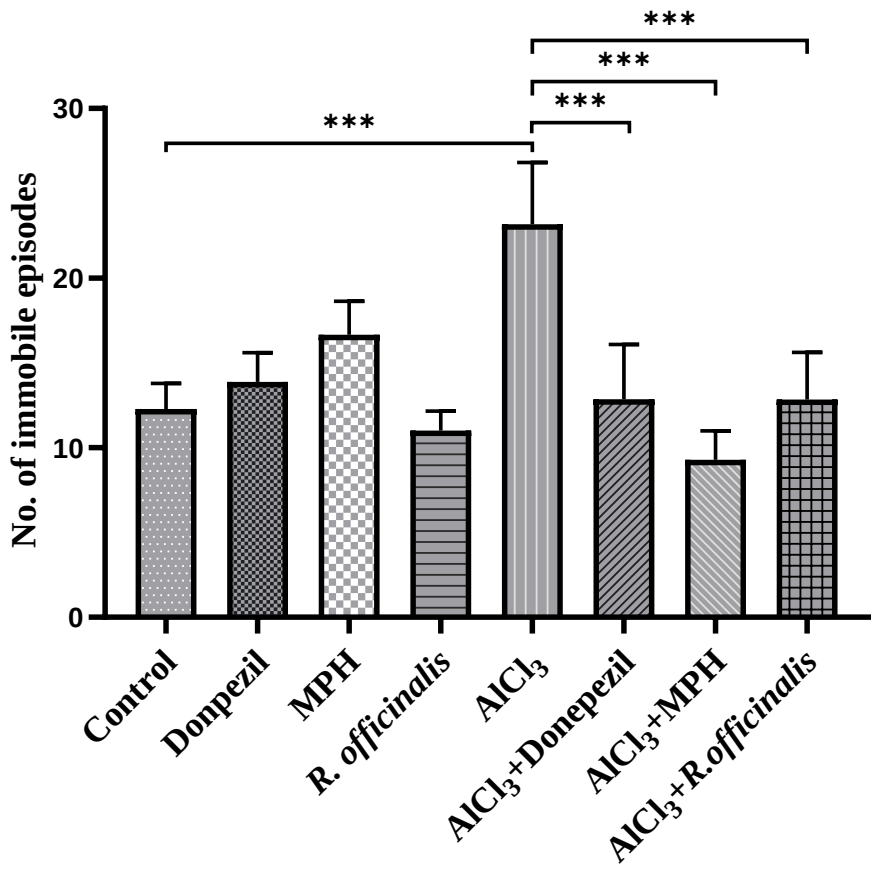

Figure 4. 16: Bar graph representing the effect of Donepezil, MPH and *R. Officinalis* on number of immobile episodes in Forced Swim Test. $AlCl_3$-treated mice showed significant increase in number of immobile episodes than control group. $AlCl_3$ + Donepezil, $AlCl_3$ + MPH and $AlCl_3$ + *R. officinalis* groups showed significant decrease in number of immobile episodes than $AlCl_3$-treated group after drug treatment. One-way ANOVA followed by Bonferroni comparison test (mean ± SEM) was applied to analyze the data using Graphpad Prism. n=8. $^{***}p<0.001$.

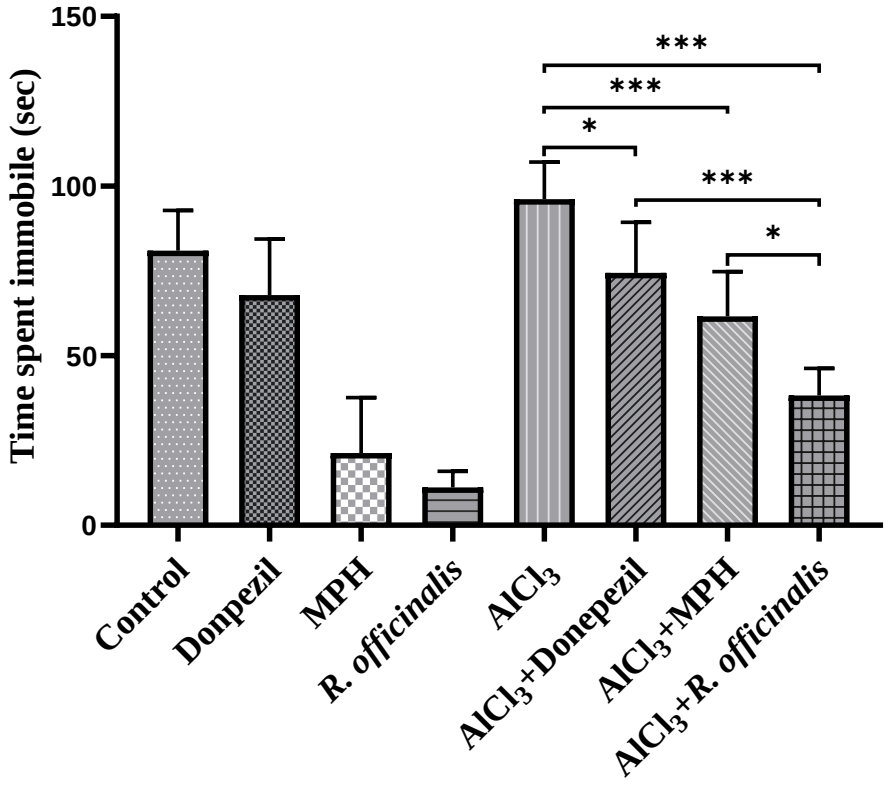

Figure 4. 17: Bar graph representing the effect of Donepezil, MPH and *R. Officinalis* on time spent immobile in Forced Swim Test. $AlCl_3$-treated mice spent insignificantly more time being immobile than control group. $AlCl_3$ + Donepezil, $AlCl_3$ + MPH and $AlCl_3$ + *R. officinalis* groups showed significant decrease in time spent immobile than $AlCl_3$-treated group after drug treatment. $AlCl_3$ + *R. officinalis*-treated mice showed more significant decrease in time spent immobile compared to $AlCl_3$ + Donepezil-treated and $AlCl_3$ + MPH-treated mice One-way ANOVA followed by Bonferroni comparison test (mean ± SEM) was applied to analyze the data using Graphpad Prism. n=8. $^{***}p<0.001$, $^{*}p<0.05$.

4.2.2.3 Comparative Effect of Donepezil, MPH and *R. officinalis* on Anxiety (Elevated Plus Maze Test)

Elevated Plus Maze is a behavioral test for analyzing anxiety and depression like behavior in animal models. Animal being less anxious tend to make more entries and spend more

time in open arms. $AlCl_3$-induced AD mice model (1.50 ± 0.28) made insignificantly less entries in the open arms compared to control group (4.25 ± 0.47). Donepezil-treated (14.25 ± 1.03) and $AlCl_3$ + Donepezil-treated group (9.75 ± 1.31) displayed a strong ($p< 0.0001$) anti-anxiety potential by making more entries in the open arms compared to control and $AlCl_3$-treated mice, respectively (figure 4.18, figure 4.19). AlC_3 + *R. officinalis* (8.25 ± 1.10) also showed significant ($p< 0.0001$) increase in number of entries in open arms compared to $AlCl_3$-treated group while AlC_3 + MPH (4.25 ± 0.75) showed insignificant increase in number of entries in open arms than $AlCl_3$-treated. In between the groups, $AlCl_3$ + Donepezil- treated mice significantly more entries in open arms compared to AlC_3 + *R. officinalis*-treated mice.

Same trend is observed in time spent in open arm. $AlCl_3$-treated group (3.33 ± 0.76) spent insignificantly less time in open arms compared to control group (5.50 ± 0.76). Donepezil-treated group (24.60 ± 1.93) and $AlCl_3$ + Donepezil-treated group (18.92 ± 1.92) again spent significantly ($p< 0.0001$) more time in open arms compared to control and $AlCl_3$-treated groups, respectively. AlC_3 + *R. officinalis* (11.33 ± 1.82) group showed significant increase ($p< 0.05$) while AlC_3 + MPH showed insignificant increase in time spent in open arms than $AlCl_3$-treated group. Comparison of the results revealed that $AlCl_3$ + Donepezil-treated group made comparatively more significant entries ($p< 0.01$) and spent more significant ($p< 0.05$) time in open arms than AlC_3 + *R. officinalis*-treated group while MPH did not show a significant increase in any parameter. Donepezil overall showed most significant anxiolytic potential.

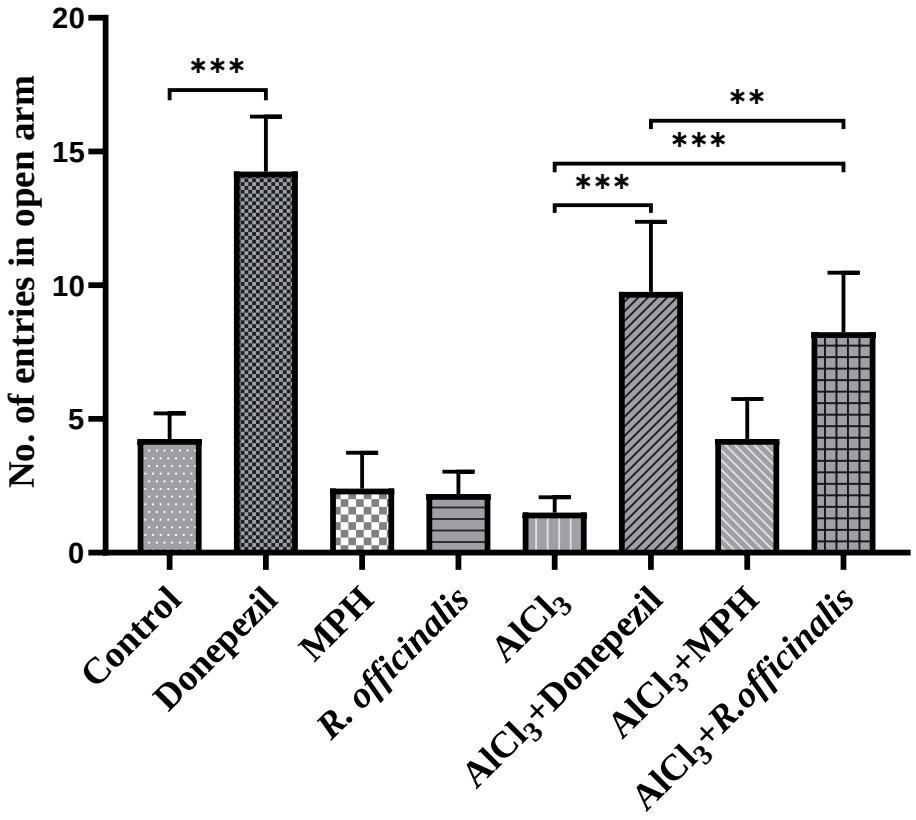

Figure 4. 18: Bar graph depicting the effect of Donepezil, MPH and *R. Officinalis* on number of entries in open arm in Elevated Plus Maze Test. $AlCl_3$-treated mice made insignificantly lesser entries in open arm than control group. Donepezil-treated group showed significant increase in number of entries in open arm compared to control. $AlCl_3$ + Donepezil and $AlCl_3$ + *R. officinalis* groups showed significant increase in number of entries in open arm than $AlCl_3$-treated group after drug treatment. $AlCl_3$ + Donepezil-treated group made significantly more entries than $AlCl_3$ + *R. officinalis*-treated group. One-way ANOVA followed by Bonferroni comparison test (mean ± SEM) was applied to analyze the data using Graphpad Prism. n=8. $^{***}p<0.001$, $^{**}p<0.01$.

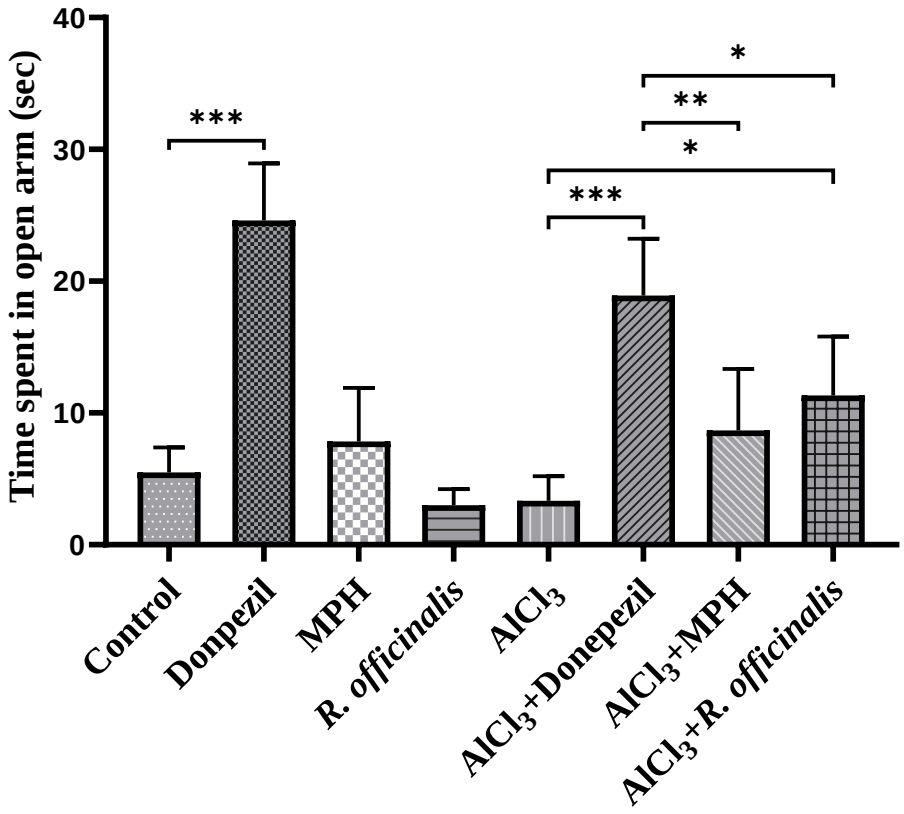

Figure 4. 19: Bar graph representing the effect of Donepezil, MPH and *R. Officinalis* on time spent in open arm in Elevated Plus Maze Test. $AlCl_3$-treated mice spent insignificantly lesser amount of time in open arms than control group. Donepezil-treated group showed significant increase in time spent in open arm compared to control. $AlCl_3$ + Donepezil and $AlCl_3$ + *R. officinalis* groups showed significant increase in time spent in open arms than $AlCl_3$-treated group after drug treatment. $AlCl_3$ + Donepezil-treated group spent significantly more time in open arms than $AlCl_3$ + *R. officinalis*-treated group. One-way ANOVA followed by Bonferroni comparison test (mean ± SEM) was applied to analyze the data using Graphpad Prism. n=8. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$.

4.2.2.4 Comparative Effect of Donepezil, MPH and *R. officinalis* on Anxiety and Exploratory behavior (Open Field Test)

The test was conducted to measure the anxiety as well as exploratory behavior of mice. Animal tend to spend more time in center is considered less anxious. $AlCl_3$-treated group showed more anxiety and decrease in exploratory behavior by spending less time in center and more time in periphery compared to control group. $AlCl_3$-treated group (155.5 ± 18.50) showed significant decrease ($p< 0.0001$) in time spent in center of the box compared to control group (741.0 ± 70.63). $AlCl_3$ + Donepezil-treated (491.8 ± 57.94) and $AlCl_3$ + *R. officinalis*-treated (704.5 ± 41.82) groups significantly ($p< 0.0001$) improved the performance by spending more time in center compared to $AlCl_3$-treated group which shows their comparable anxiolytic potential while $AlCl_3$ + MPH-treated group (86.75 ± 14.27) spent the least amount of time in the center of the box. Similarly, $AlCl_3$- treated group (1645 ± 18.76) showed significant increase ($p< 0.001$) in the amount of time spent in periphery of the box compared to control (1209 ± 147.8). Treatment with Donepezil and *R. officinalis* significantly ($p< 0.05$), ($p< 0.0001$) improved the exploratory behavior and reduced anxiety which is reflected by decrease in time spent in periphery in $AlCl_3$ + Donepezil (1308 ± 57.94) and $AlCl_3$ + *R. officinalis* groups (1096 ± 41.82) respectively compared to $AlCl_3$ treated group. $AlCl_3$ + MPH (1713 ± 14.27) showed the most anxious and least exploratory behavior by spending greatest amount of time in the peripheral region.

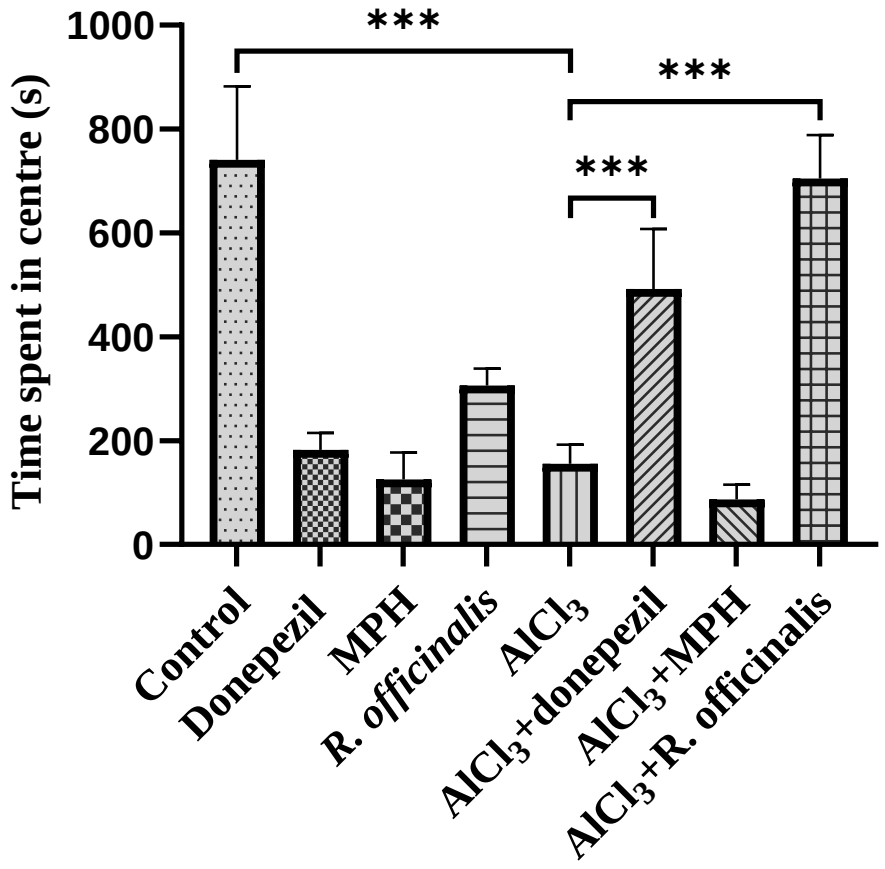

Figure 4. 20: Bar graph representing the effect of Donepezil, MPH and *R. Officinalis* on time spent in center in Open Field Test. $AlCl_3$-treated mice spent significantly lesser amount of time in center than control group. $AlCl_3$ + Donepezil and $AlCl_3$ + *R. officinalis* groups showed significant increase in time spent in center than $AlCl_3$-treated group after drug treatment. One-way ANOVA followed by Bonferroni comparison test (mean ± SEM) was applied to analyze the data using Graphpad Prism. n=8. $^{***}p<0.001$.

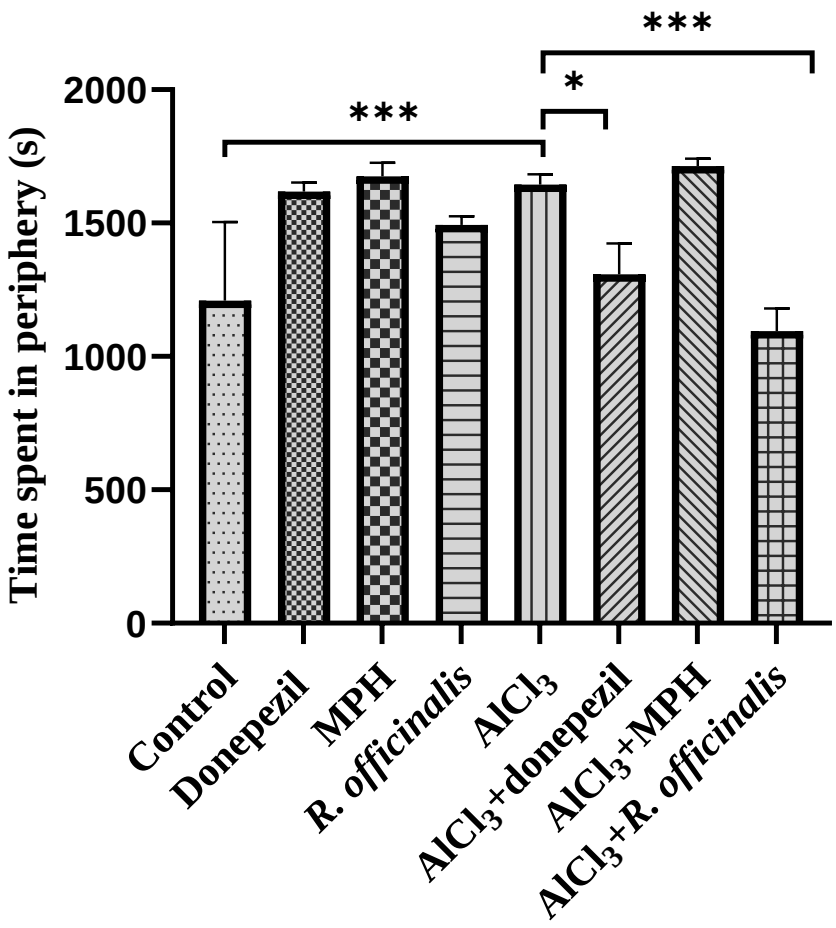

Figure 4. 21 Bar Graph depicting the effect of Donepezil, MPH and *R. Officinalis* on time spent in periphery in Open Field Test. $AlCl_3$-treated mice spent significantly more time in periphery than control group. $AlCl_3$ + Donepezil and $AlCl_3$ + *R. officinalis* groups showed significant decrease in time spent in periphery than $AlCl_3$-treated group after drug treatment. One-way ANOVA followed by Bonferroni comparison test (mean ± SEM) was applied to analyze the data using Graphpad Prism. n=8. $^{***}p<0.001$, $^{*}p<0.05$.

4.2.3 Comparative Histological Assessment of effects of Donepezil, MPH and *R. officinalis*

Congo Red staining of hippocampus showed the presence of amyloid beta plaques in $AlCl_3$-treated groups compared to control. Almost similar number of plaques are seen in

almost all $AlCl_3$-induced drug treated groups and $AlCl_3$-treated group. None of the tested compound could show a significant decrease in amyloid beta plaques in $AlCl_3$ + Donepezil-treated, $AlCl_3$ + MPH-treated and $AlCl_3$ +*R. officinalis*-treated groups.

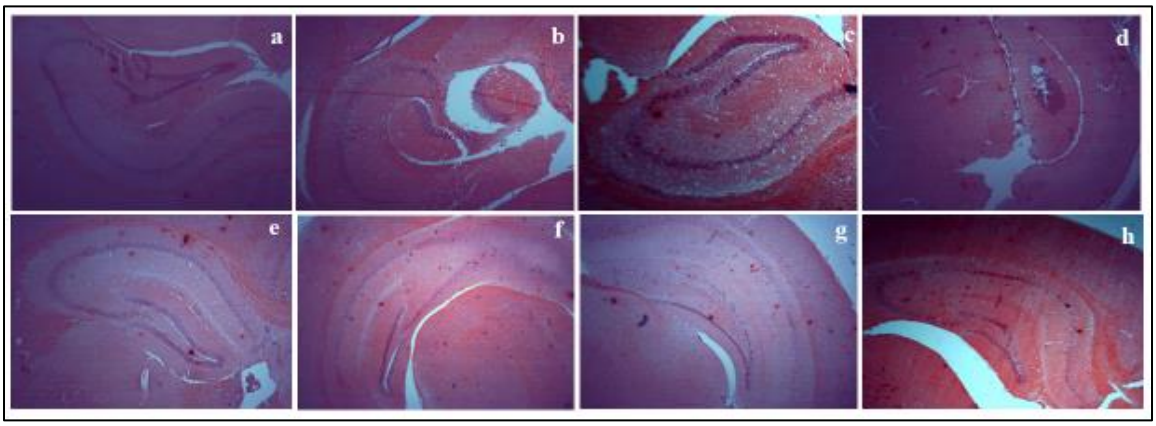

Figure 4. 22: Histological assessment hippocampal tissues sections stained with Congo Red (4X): a: control b: Donepezil-treated. c: MPH-treated. d: *R. officinalis*-treated. e: $AlCl_3$-treated. f: $AlCl_3$+Donepezil-treated. g: $AlCl_3$+MPH-treated. h: $AlCl_3$+*R. officinalis*-treated. magnification 4X.

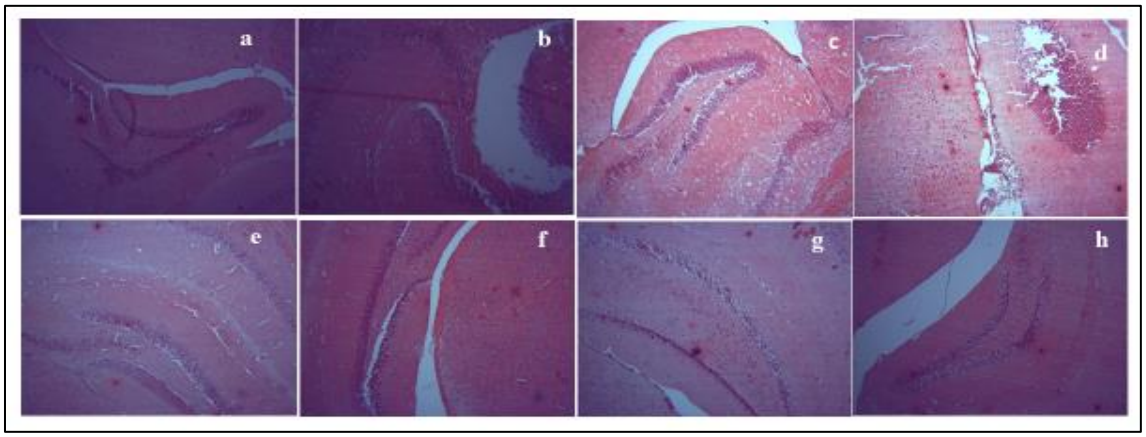

Figure 4. 23: Histological assessment hippocampal tissues sections stained with Congo Red (10X): a: control b: Donepezil-treated. c: MPH-treated. d: *R. officinalis*-treated. e: $AlCl_3$-treated. f: $AlCl_3$+Donepezil-treated. g: $AlCl_3$+MPH-treated. h: $AlCl_3$+*R. officinalis*-treated.

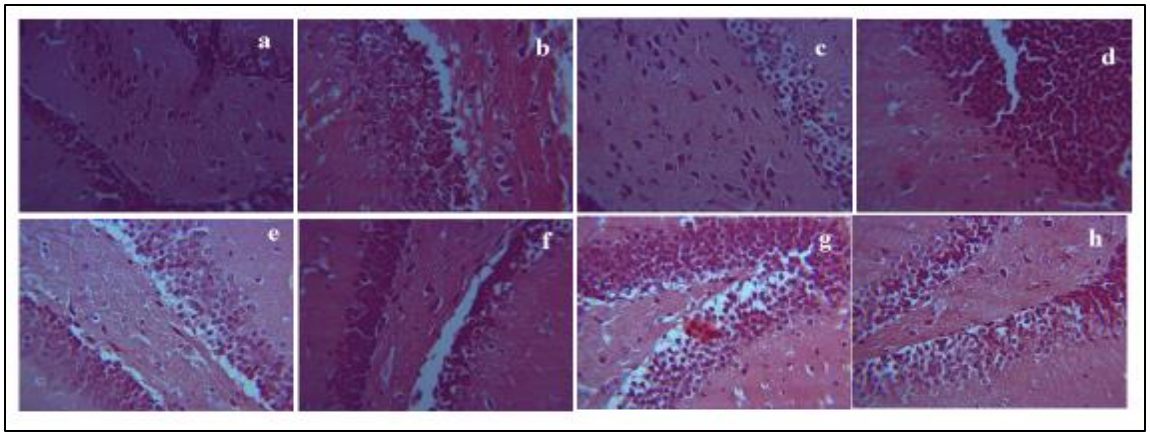

Figure 4. 24: Histological assessment hippocampal tissues sections stained with Congo Red (40X): a: control b: Donepezil-treated. c: MPH-treated. d: *R. officinalis*-treated. e: $AlCl_3$-treated. f: $AlCl_3$+Donepezil-treated. g: $AlCl_3$+MPH-treated. h: $AlCl_3$+*R. officinalis*-treated.

4.2.4 Protein Quantification

Protein concentration of each sample was measured by plotting the absorbance value of the colored reaction mixed product on the standard curve. The intensity of the color of the product is directly proportional to the protein content of the sample.

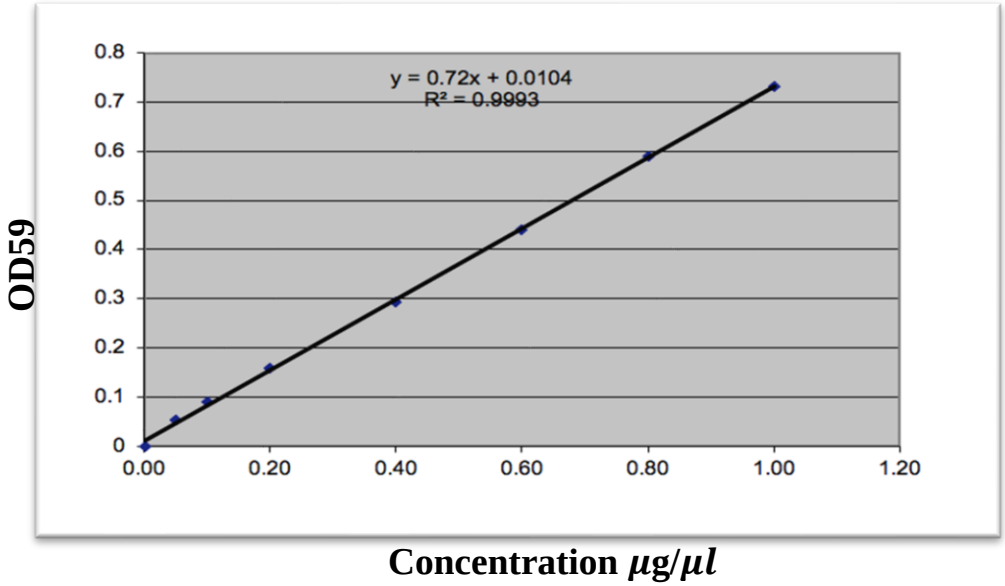

Figure 4. 25 Protein quantification curve through Bradford's Assay. Concentration was plotted on the x-axis (independent variable). Absorbance measured at 595 nm was plotted on the y-axis (dependent variable). This graph represents linear regression for the eight points. The obtained linear regression value was 0.9993 (R^2=0.9993).

4.2.5 Differential Hippocampal Proteome Profile

2-Dimensional Gel Electrophoresis is performed to identify differentially expressed proteins in the hippocampus of $AlCl_3$-induced AD model and drug treated groups ($AlCl_3$ + Donepezil, $AlCl_3$ + MPH and $AlCl_3$ + *R. officinalis*). A total of four spots were detected on 2D-GE showing differential expression of the proteins (figure 4.26). In the hippocampus of AD model, proteins are found significantly downregulated in spot 1 and 2 whereas significantly upregulated in spot 3 and 4 as compared to control. Donepezil and *R. officinalis* has shown to significantly upregulate the proteins in spot 1 but MPH failed to improve the expressions of altered proteins. Donepezil and MPH significantly enhance the expression level of the proteins in spot 2 compared to *R. officinalis* (figure 4.26). Donepezil showed comparatively much more significant effects than MPH. Proteins are found up-regulated in spot 3 and spot 4 in the hippocampus of $AlCl_3$-induced AD model than control group. Donepezil and *R. officinalis* are shown to significantly normalize the protein expression in spot 3 and spot 4 while MPH has down-regulated the protein expression compared to $AlCl_3$-treated but could not completely normalize the expression of proteins in both spots.

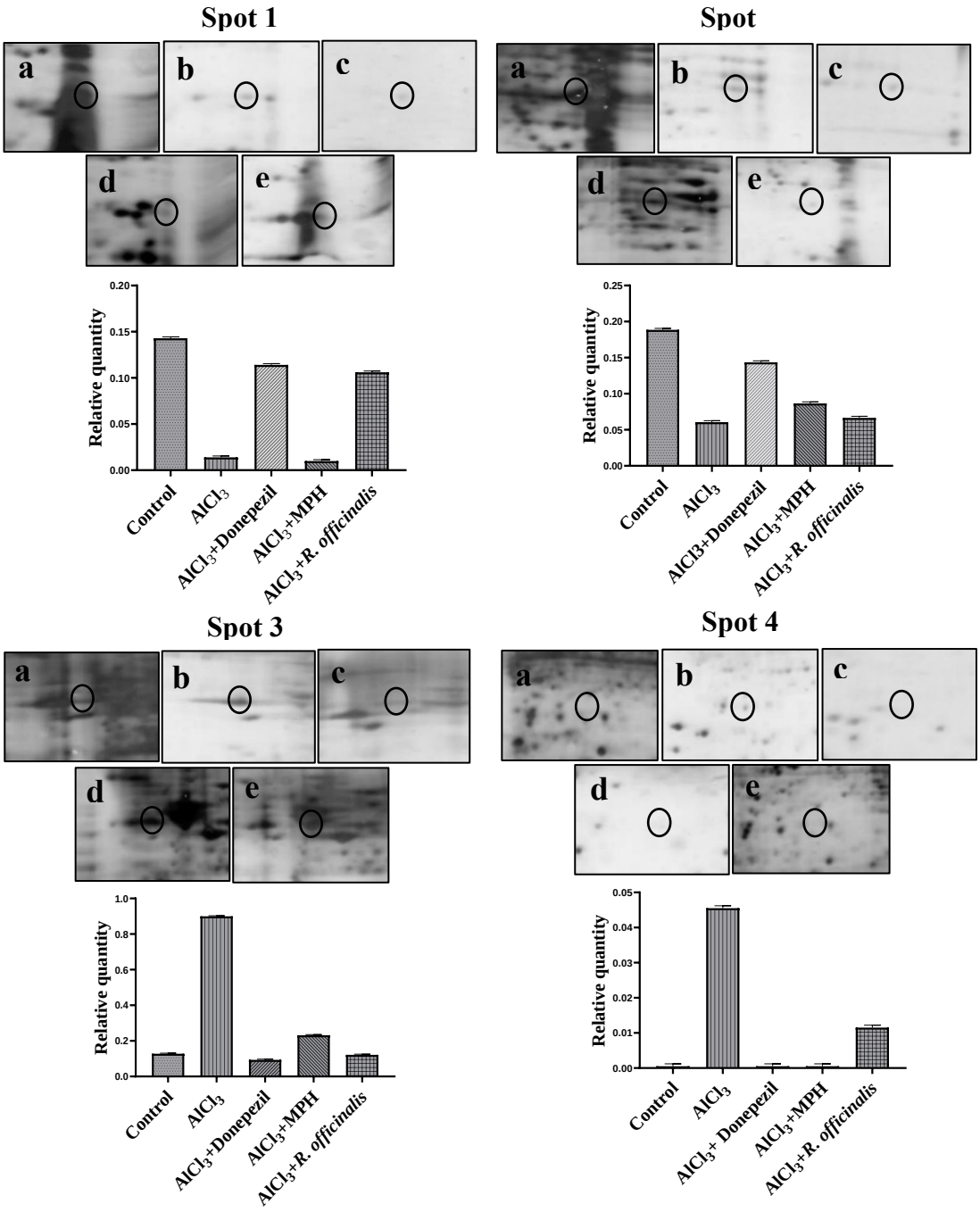

Figure 4. 26: 2-DE protein spots of differentially expressed hippocampal proteins in $AlCl_3$ -induced AD mouse model. (a) Control (b) $AlCl_3$ (c) $AlCl_3$ + Donepezil (d) $AlCl_3$ + MPH (e) $AlCl_3$ + *R. officinalis*-treated. Total protein extracted from hippocampus was separated from small 2-DE gel (7cm IPG strips, non-linear with pH 3-10) followed by silver stained. The spots were detected using Delta 2D image analysis software 4.0 (Decodon). Spots were selected based on normalized volume. One-way ANOVA was applied to analyze the data.

4.2.6 Differential Hippocampal Protein Acetylation

Western Blot analysis was performed to check the aberrant lysine-acetylation in the hippocampus of AD model. Four proteins are found to be differentially acetylated. There is decreased acetylation at two proteins of the molecular weight of 55 kDa and 23 kDa in $AlCl_3$-induced AD hippocampus. Acetylation level is found to be increased at 55 kDa and 23 kDa after treatment with Donepezil and MPH in AD + Donepezil and AD + MPH groups while *R. officinalis* did not show any improvement in AD + *R. officinalis.* Moreover, an increase in acetylation of the proteins in AD hippocampus is observed in two proteins having molecular weight of 47 kDa and 35 kDa. Drug treated groups (AD + Donepezil, AD + MPH and AD + *R. officinalis)* showed decrease in acetylation levels in the protein of 47 kDa molecular weight whereas only *R. officinalis* was able to normalize aberrant acetylation levels in the protein 35 kDa in AD + *R. officinalis* group.

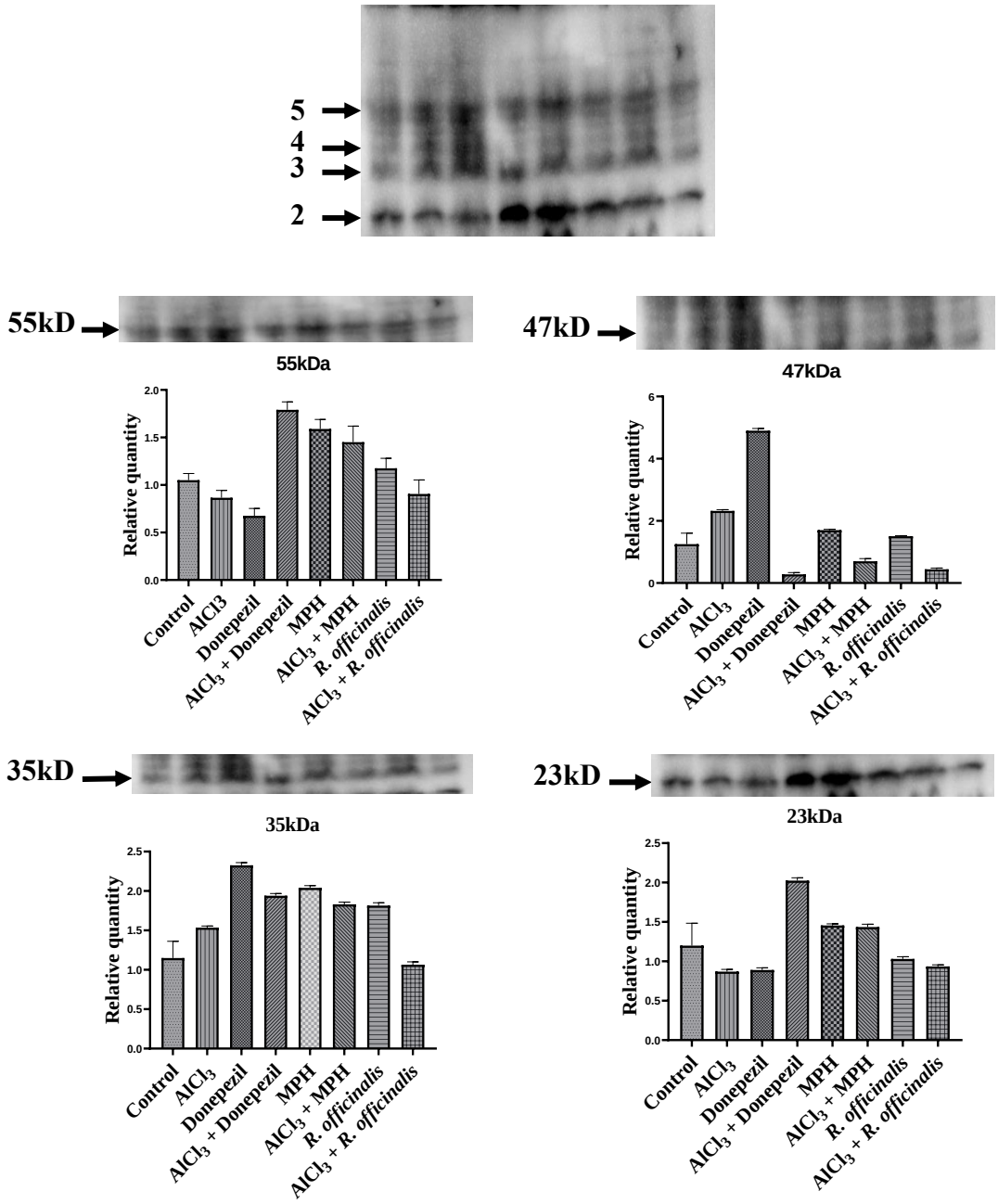

Figure 4. 27: Western Blot analysis for Lysine-Acetylation status of protein in hippocampus. The Gel analyzed from the image lab software shows five bands of 55KDa, 47KDa, 35KDa & 23KDa of differentially lysine-Acetylated protein. Differential protein acetylation was analyzed between Control, Donepezil, MPH, *R. officinalis*, $AlCl_3$, $AlCl_3$ + *R. officinalis*, $AlCl_3$ + MPH and AlCl3 + Donepezil -treated groups

Chapter 5

DISCUSSION

The current study sheds light on the potential interaction between Donepezil, RA and MPH to majority of the proteins involved in AD pathology. Rosmarinic acid has found to inhibit AChE to some extent (47.3%) at 1.0 mg/ml doses (Orhan et al., 2008). Molecular docking of rosmarinic acid with AChE has also shown the lowest binding energy in comparison to strong AChE inhibitors (Alam et al., 2018; Demirezer et al., 2015). MPH has been shown to have a major effect on decreasing activity of AChE enzyme in mice brain (Arunagiri & Balamurugan, 2016; Linhares et al., 2014; Tamilselvan et al., n.d.). We docked RA and MPH with AChE and compared with the standard acetylcholinesterase inhibitor, the Donepezil. Our results also showed the comparable binding energy of RA with Donepezil, and it is consistent with prior research. Though Binding energy of MPH with AChE is comparatively lower its value shows the potential to interfere with AChE suggesting a reason for its effects observed in cholinergic system.

Inhibition of BACE1 and PSEN1 is a clear therapeutic approach as they are directly involved in Aβ production. There is a growing interest in designing Donepezil analogues as dual inhibitors of both AChE as well as BACE1 by introducing amide linkers to the compounds that are able to make hydrogen bonds with BACE1 (Costanzo et al., 2016; Gabr & Abdel-Raziq, 2018a; Gabr & Abdel-Raziq, 2018b; Green et al., 2018). Comparison of the docking results showed that RA and MPH has comparatively lower but close binding energies to Donepezil. RA was able to make highest number of hydrogen bonds with BACE1 showing its potential to work as a dual inhibitor. RA also showed the lowest value

of binding energy and the highest number of hydrogen bonds while interacting with PSEN1.

Neuro-inflammation plays a major part in pathophysiology of AD. IL6 and TNF-α are clearly defined in initiation and regulation of inflammation, aggregation of Aβ plaques, neuronal loss and cognitive decline in AD (Chang and yee., 2017l Ringheim et al., 1995; Lai et al., 2017; Huell et al., 1995). Many in-vivo studies have suggested that inhibition of TNF-α and IL6 can stop or delay neural dysfunction in Alzhemier's disease (MacPherson et al., 2017; Current and O'Connor., 2003; Chu et al., 2018). RA has observed to reverse the memory impairment and neuro-inflammation along IL6 and TNF-α decreasing level in a dose dependent manner in lipopolysaccharide induced neuro-inflammation and memory impaired animal models (Hassanzadeh-Taheri et al., 2021; Thingore et al., 2021). TNF- α and IL6, both proinflammatory cytokines, have been shown to be reduced by RA. Molecular docking of RA has shown a great affinity towards TNF-α (Bhagat et al., 2020; Hegde & Samant, n.d.). Our results have also shown the higher affinity of RA towards TNF-α than the rest of the drugs. Furthermore, a comparatively higher and almost same binding affinity of RA and MPH towards IL6 is observed where RA interacted via hydrogen bonds while MPH through hydrophobic interactions.

Further essential factor in the pathogenesis of Alzheimer's disease is oxidative stress (Cassidy et al., 2020; X. Chen et al., 2012). Transgenic mice overexpressing mutated amyloid precursor protein (APP) were shown to enhance oxidative stress as well as accumulate significantly more Aβ as a direct consequence of an impaired antioxidant defense system (Nishida et al., 2006; Li et al., 2004). Antioxidant supplementation can reduce Aβ deposition as well as the accompanying earlier onset and extreme cognitive

impairment caused by a deficiency in the antioxidant defense system (Nishida et al., 2006). Up-regulation of SOD boosted antioxidant defense capability, lowered Aβ burden within the brains and repaired memory loss (Dumont et al., 2009). RA has observed to reduce the oxidative stress by enhancing SOD activity (Hassanzadeh-Taheri et al., 2021; Thingore et al., 2021). Protein-protein interaction study of RA has shown 64 potential targets including IL6, TNF-α, SOD1 and SOD2. Furthermore, molecular docking of the RA with SOD2 showed that RA can interact directly with the enzyme (Guan et al., 2021). The current study also showed the valuable binding energies for the interaction of RA with the antioxidant enzymes SOD1, SOD2 and Prdx6. Donepezil and RA has showed almost similar and higher affinities toward antioxidant enzymes than MPH.

In wet lab tests, the current research compared the effects of MPH and *R. officinalis* on various parameters including memory and learning, anxiety and depression, protein profiling as well as differential lysine acetylation of proteins in $AlCl_3$-induced AD mouse model.

In humans, acute administration of MPH leads to attention enhancement and improvement of the well-being while in rodents, this has a reinforcing impact over learning (Berke and Hyman., 2000). MPH as well as other psychostimulants are often used in therapy to enhance cognitive functions reduce impulsivity and induce wakefulness (Wood et al., 2014). MPH is generally used both on and off-label to improve long-term memory (Marshall et al., 2010; Rhodes et al., 2012The majority of the studies had already emphasized the significance of MPH in attention and working memory (Mehta et al., 2004; Eagle et al., 2007; Berridge et al., 2012) and few studies examined the effect of MPH on spatial memory (Kinney et al., 1979; Tian et al., 2009; Zeise et al., 2007). Our results have

showed the significant effect of MPH in improving spatial learning and memory which is consistent with previous research wherein MPH at such a dose of 10mg/kg improved significantly the memory and spatial learning (Carmack et al., 2014a; Schneider et al., 2015).

R. officinalis was previously compared with Donepezil (Mirza et al., 2021) and MPH (Khalid et al., 2020) separately where it showed *R. officinalis* to be more effective in cognitive enhancement than Donepezil and MPH in AD mice model (Mirza et a., 2021; Khalid et al., 2020. We compared the effects of MPH and *R. officinalis* with Donepezil and found comparable effects on cognitive enhancement with a slightly insignificant increase in spatial memory by MPH in Morris water maze test. *R. officinalis* showed more significant effects in reference memory especially in comparison to Donepezil in terms of time spent in target quadrant, so even though MPH has not shown a substantial increase in time being spent in target quadrant. Donepezil and MPH showed more significant effects in terms of number of platform crossings and number of entries in target quadrant.

Apathy, depression and anxiety are important neuropsychiatric symptom observed in AD (Jost & Grossberg, 1996; Marin, 1991; Siarkos et al., 2015). Therefore, effects of Donepezil, MPH and *R. officinalis* were assessed on anxiety and depression as well. MPH is suggested to decrease apathy in AD (Mintzer et al., 2021). *R. officinalis* as well as its bioactive compounds like rosmarinic acid have shown anti-anxiety and anti-depressant effects in several studies (Heinrich et al., 2006; Ito et al., 2008; Mirza et al., 2021).

Donepezil has reported to produce anti-depressant effects (Papp et al., 2016) in forced swim test in Swiss mice (Maurice et al., 2006) and C57BL/6J mice (Fitzgerald et al., 2020). Our results have also shown the antidepressant properties of Donepezil in BALB/c mice

treated with $AlCl_3$. Depression is among the most prevalent behavioral symptoms of AD (Siarkos et al., 2015) and Donepezil has been shown to enhance depression as well as anxiety-like symptoms in patients of AD (Gauthier et al., 2002). MPH has also shown to significantly improve depression symptoms in a study of 23 AD patients (Padala et al., 2010). Comparison of our forced swim results showed that all the tested compounds have significantly improved depression like symptoms in AD model but *R. officinalis* have shown significantly more anti-depressant potential compared to Donepezil and MPH by delaying more latency to immobility and spending more time being immobile. Our results suggest *R. officinalis*, a better option to treat psychological symptoms in AD with natural plant compound.

Anxiety has also been an important behavioral symptom in AD (Jost & Grossberg, 1996). Studies have reported that anxiety level varies from 25% to 75 % in AD patients (Harwood et al., 1998; Mendez et al., 1990; Sultzer et al., 1992; Tariot et al., 1995; Teri et al., 1992; Tractenberg et al., 2000). Though our results did not show significant anxiety level in AD model compared to control in elevated plus maze test, but it has shown the anxiety to some extent in AD model reflected when compared with the control in the open arms of the elevated plus maze, there were fewer entries and much less time spent. Furthermore, AD model has also spent less time in center of the open field box compared to control which reflects the anxious and decreased exploratory behavior of the animals.

Acetylcholinesterase inhibitors have varying effects on anxiety. AChE knockdown in the hippocampus resulted to enhance anxiety in elevated plus maze test (Mineur et al., 2013) but systematic administration of acetylcholinesterase inhibitors has also reported to show anxiolytic effects (Chen et al., 2011; Cutuli et al., 2008; Zarrindast et al., 2011). Donepezil

has found to reduce anxiety in AD patient with moderate to severe disease (Gauthier et al., 2002) as well as in animal models (Papp et al., 2016; Cutuli et al., 2008). Similarly, the effects of MPH on anxiety are still not clear. Anxiety has found not to be affected by MPH (Carmack et al., 2014b; Schneider et al., 2015) while some studies showed that MPH has either decreased (McFadyen-Leussis et al., 2004) or increased anxiety (Crawford et al., 2013; Ferreira et al., 2010). Our results showed that treatment with Donepezil and *R. officinalis* have significantly improved anxiety like behavior in $AlCl_3$-treated AD model by increasing the time spent in center arena of the open field box. The anxiolytic like effect of Donepezil and *R. officinalis* is also reflected in elevated plus maze test. Comparison of the results showed that Donepezil displayed the strongest anxiolytic potential. Though *R. officinalis* reflected comparatively less significant anxiolytic potential than Donepezil but its significant anxiolytic effects than $AlCl_3$-treated group suggest it a better option to treat neuropsychiatric symptoms.

MPH did not show significant anxiolytic effects in elevated plus maze and in the open field test, it exhibited increased anxiety-like behavior. Different effects of the MPH on anxiety can be attributed to the procedure applied. MPH has shown to produce opposite effects on anxiety depending upon the task applied. MPH treatment reflected decreased anxiety in novelty conditioned place preference test while displayed increase in anxiety in elevated plus maze test (Crawford et al., 2013). Producing consistent results on a single parameter in different test is not always universal. For example, exposure of the male chemical cues to sexually receptive females produced anxiolytic implications as measured by the elevated plus maze test, but an increased anxiety has been observed in light dark exploration test by the same cues (Behr et al., 2009). Similarly, phencyclidine induced anxiety like behavior

in rats when measured on elevated plus maze, yet phencyclidine reduced anxiety when assessed on light dark box test (Turgeon et al., 2011). Hence, procedural differences could be the possible reason for the varying results in different studies. On the other hand, it has been debated that these variations indicate the multidimensional and the complex nature of the emotional behaviors such as anxiety (Ramos, 2008). Thus, there is a possibility that the procedures applied to assess anxiety only measure a specific idiosyncratic domain of the tested emotion. Therefore, various experimental approaches would be needed to completely assess the effects of MPH on anxiety.

Additionally, MPH has shown to increase anxiety after sudden treatment break which has been linked to increased neural sensitization of anxiety-related regions of brainstem (Ferreira et al., 2010). So, withdrawal effects could be the possible reason for high anxiety in MPH treated group. Time can be another possible reason for a varying effect on anxiety in both tests. MPH did not significantly reduced anxiety compared to other groups in elevated plus maze test while in open field the rest of the tested compounds have again show anxiolytic effects but not MPH. So, time can be another possible reason for varying effects on anxiety in both tests and for producing high withdrawal effects in open field since open field test was conducted after elevated plus maze test.

Histopathological assessment revealed that all the tested compounds failed to reduce the amyloid beta plaques burden. A study showed that *R. officinalis* and Donepezil have reduced the amyloid beta plaques revealed by Congo red staining. Their tested doses were 300mg/kg, 400mg/kg for *R. officinalis* and 2.5mg/kg for Donepezil (Waly et al., 2019). Results of the present study indicate that our selected doses are not much effective to reduce the amyloid plaques. As the selected doses are comparatively lower and treatment duration

is shorter than the previous study and it could be the possible reason for not being effective in reducing amyloid burden.

At the molecular level a number of studies has identified alterations of the protein expressions in AD brain (Lubec et al., 1999). Our study has also identified some aberrantly expressed proteins which were also modified by drug treatment. But the exact mechanism and detailed study about the effects of the drugs on the specific proteins is needed. Similarly, alteration in the acetylation of nuclear as well as cytoplasmic proteins has reported to be associated with AD (Barral et al., 2000; Chen et al., 2001; Irwin et al., 2012; Min et al., 2010; Perez et al., 2009). We have also identified aberrant acetylation of the proteins in AD which was modified by our tested drugs. Donepezil and *R. officinalis* has positive effect in normalizing the acetylation.

CONCLUSIONS

When the as a whole docking results were compared, it was found that RA was better as compared to Donepezil and MPH on average in interacting with all the docked proteins suggesting that RA can be a new therapeutic option for AD treatment. RA, like other two drugs, has been predicted to meet drug likeness criteria; consequently, it's indeed explorative in terms of the both in-vivo and in-vitro tests actions on AD model to evaluate as well as optimise its treatment potential. As AChE inhibitors are used for symptomatic treatment of AD therefore inhibiting AChE along with the inhibition of amyloidogenesis and inflammation would open a way for AD treatment by targeting multiple pathways at a time. It could improve symptoms along with slowing down the progression of the disease. Memory loss along with neuropsychiatric symptoms in Alzheimer's disease lead to the requirement of the drug which can enhance memory and reduce behavioral symptoms. Donepezil, MPH and *R. officinalis* have comparable effects in enhancing memory. *R. officinalis* showed more anti-depressant like behavior than Donepezil and MPH. Donepezil displayed the strongest anxiolytic potential followed by *R. officinalis* while MPH did not show significant anxiolytic effects. Furthermore, all the tested compounds were able to modify the protein expression and protein acetylation. As donepezil is failed to stop the disease progression therefore *R. officinalis* which is natural compound would be more suitable to treat cognitive decline as well as psychological symptoms with minimal side effects. On average, *R. officinalis* was best in all the tested parameters so we suggest that it can be more suitable for AD treatment which have the potential to effect at the molecular level as well as to improve symptoms. Further study is needed to elucidate the effects of *R.*

officinalis and MPH at the proteomic level to specify the protein targets and explore their mechanism of action.

Chapter 6

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Appendix

APPENDIX

Nishat Malik, Saadia Zahid. Protein Targets and Cellular Mechanisms Defining Novel Therapeutic Approaches for Alzheimer's Disease. *Hippocampus (Submitted & Under Review).*

Abstract

Alzheimer's disease (AD) is one of the highly prevalent neurodegenerative disease worldwide. Although amyloid β and Tau protein has major contribution to the pathological hallmarks, the plaques and neurofibrillary tangles (NFTs) but altered expression of several physiologically important proteins, also contributes towards neurodegeneration and other AD associated consequences. In this review, we focused on the role of proteomics as a novel therapeutic approach aiming at differentially identified proteins along with established pathological markers Aβ and Tau protein, apolipoprotein, ubiquitin, amphiphysin-1 related proteins and complement activated proteins. The significance of adult hippocampal neurogenesis and stem cell therapy as a potential therapeutic regime for AD is also discussed.

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By

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MS Healthcare Biotechnology 2019

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