PHARMACOLOGICAL EVALUATION OF SELECTED PHYTOCONSTITUENTS IN COMBINATION WITH PROBIOTICS FOR ALZHEIMER'S DISEASE USING ANIMAL MODEL: A MECHANISTIC STUDY

Thesis Submitted for the Award of the Degree of

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By

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DECLARATION

I, hereby declared that the presented work in the thesis entitled **"Pharmacological evaluation of selected phytoconstituents in combination with probiotics for Alzheimer's disease using animal model: A mechanistic study"** in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision of Dr. Navneet Khurana, working as Professor, in the Department of Pharmacology, School of Pharmaceutical Sciences of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled **"Pharmacological evaluation of selected phytoconstituents in combination with probiotics for Alzheimer's disease using animal model: A mechanistic study"** submitted in fulfillment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the Department of Pharmacology, School of Pharmaceutical Sciences, is a research work carried out by Abhinav Anand, 41700258, is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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Errors or inadequacies are all mine

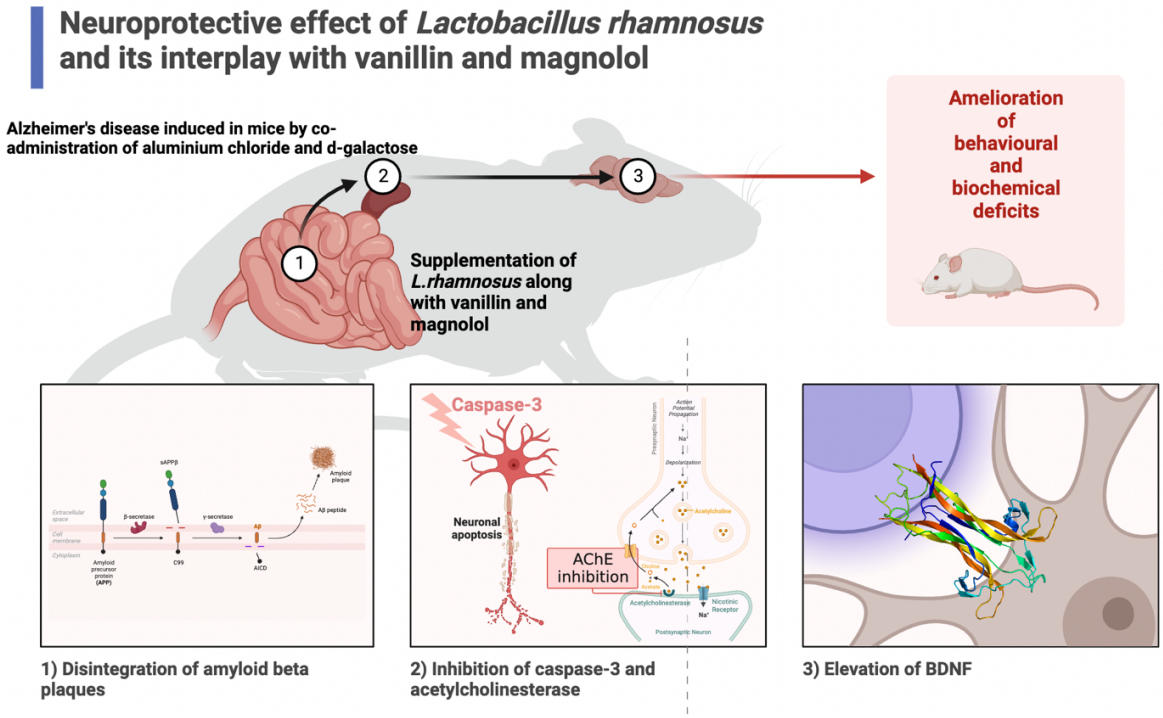
Abhinav Anand

ABSTRACT

Alzheimer's disease (AD) is a sinister neurodegenerative disorder that exacts a devastating toll on affected individuals and their families. It is characterized by the gradual and rather permanent loss of cognition, primarily attributed to the apoptotic death of neurons. The complexity of AD arises from a multitude of factors, including the enhanced expression of enzymes such as acetylcholinesterase (AChE), beta-secretase, and caspase-3, coupled with the accretion of neurotoxic amyloid plaques in the brain. The etiology of AD is multifaceted, but one of its central drivers is oxidative stress, which fuels both its initiation and relentless progression. At present, therapeutic approaches for AD are largely palliative, offering limited relief from symptoms and doing little to address the underlying pathological processes. Notably, AChE inhibitors like donepezil and rivastigmine have shown some promise in alleviating cognitive decline, albeit temporarily. However, there is a dire and pressing need of developing pharmacotherapeutic interventions that can truly modify the course of the disease, providing not only efficacy but also safety and cost-effectiveness in their application. This study focuses on two intriguing candidates for such intervention – vanillin and magnolol. Vanillin, a naturally occurring phytoconstituent, has been widely utilized in the food, beverage, and cosmetics industries as a flavouring agent. Remarkably, it has been safely ingested by humans for years. Previous *in vitro* studies have hinted at its potential as a neuroprotective agent. One such study reported that vanillin could reduce amyloid beta aggregation, a hallmark of AD. In conjunction with vanillin, another natural compound, magnolol, has been used. Magnolol, like vanillin, exhibits innate antioxidant properties. In earlier studies, magnolol has demonstrated its potential in mitigating pathological features resembling AD in a transgenic *C. elegans* model. This compound has shown promise in reducing Aβ accretion and enhancing the endocytosis and breakdown of Aβ in microglial cells. Additionally, it was also reported to

reduce amyloid beta induced cell death *in vitro* (PC12 cell lines). These findings suggest that magnolol and vanillin, individually and when coupled together, could contribute to a comprehensive anti-AD strategy. However, the study involves the novelty of introducing the Gut-Brain Axis (GBA) into the equation. GBA is a bidirectional communication system linking the gut microbiota with the central nervous system. In this intricate network, probiotics have emerged as influential players with demonstrated antioxidant and anti-inflammatory activities. Enter *Lactobacillus rhamnosus*, a probiotic with a well-established track record in maintaining gut health and, increasingly, in influencing brain function via the GBA. This comprehensive study employs a multifaceted approach to explore the individual and combined effects of vanillin, magnolol, and *L. rhamnosus* in the context of AD. A Swiss Albino Mouse model of AD is created by co-administering aluminum chloride and d-galactose, mimicking key aspects of the disease's pathological processes. The choice of these natural phenolic compounds, vanillin and magnolol, is grounded in their intrinsic antioxidant properties, offering potential neuroprotection against oxidative stress, a known driver of AD. The study's evaluations encompass a spectrum of parameters, providing a holistic assessment of the interventions' impact. Behavioural parameters, including transfer latency, escape latency, and novel object recognition, are measured to gauge cognitive function. Biochemical parameters, specifically antioxidant parameters and AChE inhibitory activity, offer insights into the compounds' effects at the molecular level. Molecular markers related to AD pathology, such as beta-secretase, caspase-3, $A\beta_{1-42}$, and brain-derived neurotrophic factor (BDNF), are analysed via enzyme-linked immunosorbent assay (ELISA). The results of this extensive investigation reveal an intriguing synergy between the interventions. Notably, the probiotic *L. rhamnosus* appears to enhance the anti-AD activity of both vanillin and magnolol. This enhancement can be attributed, in part, to the convergence of their common effects, particularly their antioxidant

properties. The diminution of oxidative stress is a pivotal factor in ameliorating AD pathology, and the combined approach seems to harness this effect effectively. It is important to underscore that the addition of a probiotic like *L. rhamnosus* to the regimen introduces a novel dimension to the treatment strategy. Probiotics have a rich history of therapeutic application for a variety of ailments, primarily gastrointestinal in nature. However, this study expands the growing body of research exploring the pharmacological effects of probiotics beyond the gut. The GBA is a fascinating area of investigation, and the findings here indicate that probiotics may potentially offer a promising avenue for modulating brain health and potentially influencing the trajectory of neurodegenerative diseases like AD. In conclusion, AD remains a formidable challenge in the area of neurodegenerative disorders. The current arsenal of treatments is insufficient to halt its relentless progression. This study introduces a novel approach by combining the natural compounds vanillin and magnolol with the probiotic *L. rhamnosus*. The results suggest that this combination exhibits enhanced anti-AD effects, likely through their collective antioxidant properties. The study also underscores the potential of probiotics to impact brain health via the GBA, opening up exciting possibilities for future research. While further investigation is warranted, these findings offer a glimmer of hope in the quest of developing efficacious and safe disease-modifying interventions for AD.



Key words: Alzheimer's disease, *Lactobacillus rhamnosus*, magnolol, neurodegeneration, vanillin

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ABBREVIATION	FULL FORM
Aβ	Amyloid beta
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
APP	Amyloid Precursor Protein
BACE	Beta secretase
BBB	Blood Brain Barrier
BDNF	Brain Derived Neurotrophic Factor
CAT	Catalase activity
DC	Disease control
DT	Donepezil treated
ELISA	Enzyme Linked Immunosorbent Assay
EL	Escape Latency
GBA	Gut-Brain Axis
GSH	Reduced glutathione
HO-1	Heme Oxygenase-1
i.p.	Intraperitoneal
MWM	Morris Water Maze
NFTs	Neurofibrillary tangles
NMDA	N-Methyl d-aspartate
NOX-4	NADPH Oxidase 4
Nrf2	Nuclear factor erythroid2-related factor 2
NOR	Novel Object Recognition
p.o.	Per oral
ROS	Reactive Oxygen Species
SCFA	Short Chain Fatty Acids
SEM	Standard Error of Mean
TBARS	Thiobarbituric Acid Reactive Substances
TNF-α	Tumour Necrosis Factor
TL	Transfer Latency
TS	Transverse Section
UPS	Ubiquitin Proteasome System
VC	Vehicle control

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CHAPTER-1

INTRODUCTION

1. Introduction

AD is the commonest and most widespread type of dementia. AD is a slowly progressing neurodegenerative condition marked by Aβ neuritic plaques and tau NFTs (Anand et al., 2018; Breijyeh and Karaman, 2020). According to reports, there were 43.8 million dementia sufferers worldwide in 2016, which shows an increase of 117% from the 1990 statistic of 20.3 million. It has been estimated that 152 million people will be suffering from AD and other dementias by the year 2050 (Li et al., 2022). The initial warning signs of AD include cognitive impairment and memory deficits. AD currently has no known cure, but there are treatments that can help with the symptoms (Kumar et al., 2019). The current pharmacotherapeutic approaches comprise of acetylcholinesterase inhibitor drug donepezil and NMDA glutamate antagonist drug rivastigmine (Anand et al., 2017a; Anand et al., 2017b). A major factor that accounts for initiation and development of AD is oxidative stress due to its neurodegenerative capability(Betteridge, 2000; Mori et al., 2013). Another lesser explored pathological pathway, pertaining to development of AD, which has gained research focus recently is via the GBA (Morais et al., 2021; Westfall et al., 2017). It refers to the bidirectional communication that exists between the enteric and central nervous systems (Carabotti et al., 2015). Gut dysbiosis is believed to lead to neuroinflammation which further augments the progression of AD (Anand et al., 2023b).

In the current research, the individual and combined effects of *Lactobacillus rhamnosus* (a probiotic), vanillin and magnolol (natural phytoconstituents) have been evaluated in a mouse based model of AD involving the co-administration of aluminium chloride and d-galacotse. Aluminum readily penetrates the brain, affecting slow axonal transports, inducing inflammation, structural abnormalities, and synaptic anomalies that eventually result in neurodegeneration (Chiroma et al., 2018; S. E. et al., 2019). AGEs i.e. Advanced Glycation

End Products are readily produced by the reactions between d-galactose, which is a reducing sugar, and free amines. D-galactose, when given over an extended length of time, results in alterations that mimic the natural process of senescence in animals, including genetic changes, oxidative stress, immune system impairment, and cognitive loss. Moreover, it results in mitochondrial dysfunction and raises acetylcholinesterase levels in the brain (Chiroma et al., 2018; Li et al., 2019; Shwe et al., 2018). In mice, it has been shown that administering D-galactose and aluminium chloride together for 90 days leads to the development of AD, which is marked by an increase in beta secretase, Aβ aggregation, and caspase-3 with a decrease in BDNF (Gao et al., 2015; Haider et al., 2020; Xiao et al., 2011; Xing et al., 2018).

Vanillin, derived from vanilla pods, is a phenolic compound known for its antioxidant activities attributed to its chemical composition. It has been widely utilized in various food, beverage, and cosmetic products for its delightful fragrance and taste. (Anand et al., 2019). Vanillin, in previously published reports, has been documented to possess an ameliorative impact in scopolamine induced dementia like cognitive deficits in mice (Anand et al., 2022) and in a mouse model of AD (Anand et al., 2023a).

Magnolol is a type of polyphenol, specifically a neolignan, which is sourced from the bark extract of *Magnolia officinalis*. These trees are predominantly found in Eastern and South-Eastern regions of Asia (Poivre and Duez, 2017). According to an *in vitro* study conducted on PC12 cell lines, it was found that magnolol exhibited a reduction in cell death induced by Aβ. (Hoi et al., 2010). Additionally, magnolol was found to mitigate pathological features resembling AD in a transgenic model based on *C. elegans*. Furthermore, it was observed to decrease the accumulation of Aβ and enhance the endocytosis and breakdown of Aβ in microglial cells. (Xie et al., 2020).

The probiotic used in the present study is *L.rhamnosus*. Due to its capacity for surviving and proliferating at stomach acid pH, in medium containing bile, and adhering to enterocytes. It has the capacity to release soluble compounds that have a positive impact on the intestinal environment. It supports the survival of intestinal crypts, reduce apoptosis in intestinal epithelial cells, and uphold the integrity of the cytoskeleton, in addition to a biofilm that can provides mechanical protection to the mucosa (Segers and Lebeer, 2014). *L.rhamnosus* has been demonstrated to have an antioxidant property (Seth et al., 2008). A study documented that *L. rhamnosus* (10^9 CFU/mouse/day) by gavage showed decrease in depression and anxiety with effects observed in hippocampal and cortical regions of brain (Cheng et al., 2019). This strengthens the rationale to use the probiotic for CNS disorders.

The assessment parameters were considered on the basis of endpoints reported in previously published literature. TL in Elevated Plus Maze paradigm (Itoh et al., 1990), EL in MWM paradigm (Bromley-Brits et al., 2011; Vorhees and Williams, 2006), and NOR (Antunes and Biala, 2012; Lueptow, 2017) behavioural tests were employed. To evaluate the antioxidant potential of various treatments, TBARS, GSH, CAT assays were performed (Anand et al., 2022; Habibyar et al., 2016). Furthermore, to understand the effect of the mentioned treatments on various pathological pathways of AD, their inhibitory activity was assessed on enzymes-acetylcholinesterase (AChE), beta secretase and caspase-3. The in vivo capability of various test substances to reduce the aggregation of $A\beta_{1-42}$ and elevate BDNF were also assessed. Histopathological analysis was employed to ascertain the neuroprotective effects of the chosen interventions.

Using natural phytoconstituents like vanillin and magnolol, alongside probiotics such as *Lactobacillus rhamnosus*, presents a promising approach to treat AD, potentially offering a novel therapeutic avenue for this neurodegenerative condition.

CHAPTER-2

REVIEW OF LITERARTURE

2. REVIEW OF LITERATURE

2.1 Dementia and its types

Dementia can be better understood as a collection of symptoms that have a significant impact on memory, cognition, and social abilities, rather than being considered a distinct disease. These disruptive effects on daily life stem from observable physical changes in the brain. Dementia can manifest in various forms, depending on the underlying pathological condition and the specific symptoms, as detailed in Table 2.1 (Alzheimer's Association, 2017a). Dementia conditions exhibit a gradual progression, often commencing with initial difficulties in short-term memory that eventually culminate in complete memory loss. While the manifestations of dementia can vary, it typically involves dysfunction in at least two of the following fundamental cognitive functions:

- Recollection or retention of learned things
- Linguistic expression and interpersonal interaction
- Capacity for staying attentive
- Capacity for logical thinking and evaluation
- Perception of visual stimuli (Alzheimer's Association, 2017b)

Apart from dementia, cognitive impairment may be manifested without any functional impairment. This syndrome is known as mild cognitive impairment (Petersen et al., 1999). Studies with community samples have revealed that its progression to dementia is about 12-15% annually, at an average (Tuokko and Frerichs, 2000; (Anand et al., 2017a; Anand et al., 2017b).

Table 2.1: Various types of dementia

Type of dementia	Underlying pathophysiology	Symptoms	Remarks	Reference
Alzheimer's disease	Deposition of amyloid beta plaques and Tau neurofibrillary tangles in brain	Begins with short term memory loss which progresses to disorientation, delirium, difficulty in speaking, swallowing, walking and other daily activities. Eventually, all social graces are lost	Most common form of dementia. It is responsible for about 60-80% of dementia cases	(Alzheimer's Association, 2017c)
Vascular dementia	Inadequate blood supply to brain tissue, usually following stroke	Impaired judgement, difficulty on decision making, organizing, planning contrary to memory loss	Less common in incidence. It is responsible for about 10% cases of dementia	(Alzheimer's Association, 2017d)
Dementia with Lewy bodies	Abnormal aggregation of alpha-synuclein in cortex	Memory loss, more prone to initial symptoms like sleep disturbances, well-structured visual	It is responsible for 10-25% of dementia cases	(Alzheimer's Association, 2017e)

		hallucinations, improper gait and slowness		
Mixed dementia	Abnormalities linked with more than one other types of dementia – commonly AD, dementia with Lewy bodies and Vascular dementia	Complications associated with more than one of the other types of dementia	The incidences are more common than previously thought	(Alzheimer's Association, 2017f)
Parkinson's disease dementia	Deposition of alpha-synuclein in substantia nigra pars compacta leading to neurodegeneration	Changes in memory, concentration, judgement, hallucinations, muffled speech, depression, irritability, anxiety, sleep disorders	About 50-80% of sufferers of Parkinson's disease develop this kind of dementia	(Alzheimer's Association, 2017g)
Frontotemporal dementia	No common distinguishing microscopic abnormality in all cases	Changes in personality, behaviour, language function	Sufferers survive for lesser years than those with AD	(Alzheimer's Association, 2017h)

Creutzfeldt-Jakob disease	Misfolding of prion protein throughout the brain causing malfunction	Impairment in memory, behavioural changes, causes mad cow disease in cattle	Rapidly fatal, occurs in about one in a million people worldwide, annually	(Alzheimer's Association, 2017i)
Normal pressure hydrocephalus	Accumulation of fluid in brain	Difficulty in walking, memory loss and inability to control micturition	Mainly affects people in a younger age i.e. 60-70 years than other dementias	(Alzheimer's Association, 2017j)
Huntington's disease	Single defective gene on fourth chromosome	Significant decline in reasoning and thinking, abnormal involuntary actions, mood swings, depression, irritability	Generally seen in very young age i.e. 30-50 years. May be seen at 2 years of age or even at 80 years of age	(Alzheimer's Association, 2017k)
Wernicke-Korsakoff Syndrome	Chronic severe deficiency of thiamine	Remarkable memory loss. Thinking, judgement and other	Rare in occurrence	(Alzheimer's Association, 2017l)

	social skills are generally normal

2.2 AD

2.2.1 Epidemiology

A German neuropathologist, Alois Alzheimer, initially documented AD in 1906. By the start of the 21st century, it was established as the commonest type of dementia among elderly population. In 2023, it has been estimated that nearly 55 million people globally are living with dementia, and the number of cases will be rising by approximately 10 million annually. (World Health Organization, 2023)**.** AD is a neurodegenerative condition typically manifesting in the later stages of adulthood. It is characterized by a gradual and rather permanent deterioration in memory and multiple other cognitive functions. In AD, there is a breakdown of neurons and a decline in neural connections within the cortical areas of the brain, leading to a significant decline in brain tissue mass (Perl, 2010). AD consistently advances and typically leads to fatality within a span of 5 to 10 years from its initial onset (Dwyer et al., 2009). Death typically occurs as a result of complications arising from the long-term illness. AD ranks among the top five leading reasons for mortality within the United States population. (Centers for Disease Control and Prevention, 2017). AD is primarily an ailment that affects the elderly, but there are rare instances of it occurring in people in their 40s and 50s. Clinical studies show that approximately 200,000 individuals under the age of 65 have AD, whereas about 5 million individuals aged 65 and older are afflicted by it. Speculations suggest that a new case of AD is projected to emerge roughly every 33 seconds by the year 2050. (Alzheimer's Association, 2014).

2.2.2 Etiology

The precise reasons contributing to the emergence of AD remain unclear, but several factors are recognized as significant contributors to the development of the condition. These factors encompass anomalies in the modification of tau protein mediated by phosphorylation, fluctuations in metabolism of calcium, increased turnover of ROS, neuro-inflammation, irregularities in energy metabolism, and the unwanted formation and aggregation of Aβ proteins, which are all thought to play crucial roles in the advancement of AD (Butterfield et al., 2002; Habibyar et al., 2016; Hardy and Selkoe, 2002).

2.2.3 Pathophysiology

AD is distinguished by the existence of two distinctive neuropathological features: extracellular Aβ plaques and intracellular Tau NFTs. The relatively less prominent familial form of AD which may present itself in early years of one's life is the consequence of a mutation in any of three genes namely APP, Presenilin 1 (PS-1), or Presenilin 2 (PS-2). The sporadic variant appears generally after 60-65 years of age and is responsible for a large majority of the cases. Most likely, it occurs from an association of both kinds of factors, the genetic and the environmental. The only definite risk factors for sporadic AD are the existence of the E4 allele of apolipoprotein E and senescence. Plaques in AD are primarily composed of the neurotoxic peptide called amyloid (Aβ). This peptide is produced through a series of enzymatic cleavages of a large precursor protein, the APP. These cleavages are carried out by two enzymes: β-secretase, commonly referred to as BACE1, and γ-secretase, which involves four proteins, including presenilin. It is noteworthy that Aβ doesn't form if APP first undergoes cleavage by α-secretase, instead of β-secretase. NFTs, on the other hand, predominantly consist of the protein tau, categorized as a microtubule-associated protein (MAP). In normal cellular function, tau plays a role in binding to microtubules, facilitating the neuronal transport system.

However, in the progression of AD, tau disengages from microtubules and forms aggregations known as tangles. This aggregation disrupts the transport process and leads to the disassembly of microtubules. The modification of tau by phosphorylation also plays a crucial role in this process. (Allen and Dawbarn, 2011).

Although AD is considered to be linked to several underlying pathophysiological mechanisms, a major part of pathogenesis has been attributed to oxidative stress. Oxidative stress also initiates and enhances the neurotoxicity due to Aβ oligomers and Tau tangles, both of which are characteristic neuropathological hallmarks for AD (Huang et al., 2016).

Ubiquitin is an oligopeptide which occurs in a highly conserved form in all eukaryotes. It is present in conjugation with the proteins that are to be targeted to the proteasome (Ciechanover and Schwartz, 1994). In two sequential steps, the Ubiquitin Proteasome System (UPS) clears the proteins i.e. a tagging reaction and the breakdown of the tagged proteins by the proteasome system (Oddo, 2008). UPS which is generally important for repair, degradation and turnover of proteins, is compromised in AD (Riederer et al., 2011). An elevation in the oxidative stress is a major reason for the declining function of UPS (Ross et al., 2015). This impairment in the UPS contributes to the accretion of Aβ aggregates and Tau tangles in the brain. A more recent hypothesis regarding mitochondrial dysfunction in the development of AD suggests that impaired brain mitochondria function contributes to amyloid accumulation, reactivation of the cell cycle, and the phosphorylation of tau proteins. It has been attributed to oxidative stress and UPS impairment. Evidence exists suggesting involvement of the neurochemical systems related to cholinergic and glutamatergic functions in the etiology of AD. ACh is a vital neurotransmitter that plays a key role in cognitive processes and the acquisition of knowledge. In individuals with AD, there is a reduction in both the quantity and effectiveness of ACh in the brain. This deficit and additional presynaptic cholinergic

restrictions, like deterioration of cholinergic neuronal network and reduced AChE activity, validate the cholinergic hypothesis of AD. Another neurochemical hypothesis for development of AD is the NMDA related glutamate-based hypothesis. Glutamate is a stimulatory neurotransmitter which acts on NMDA receptors, that are pivotal in learning and memory. However, in some circumstances, over stimulation of NMDA receptors by glutamate causes neuronal damage due to excitotoxicity (Francis, 2005).

2.2.4 Diagnostic methods

The diagnostic methods for AD involve questioning the patient and his/her family and/or friends. Radiodiagnostic techniques, including Computed Tomography (CT), Magnetic Resonance Imaging (MRI), or Positron Emission Tomography (PET) may be carried out as per the requirement, intensity of clinical presentation, and the gravity of the situation. Concrete confirmation of AD can be ascertained only posthumously, by correlating clinical data to an analysis of brain in an autopsy (NIH National Institute on Aging, 2017a).

2.2.5 Stages of disease progression and associated symptoms

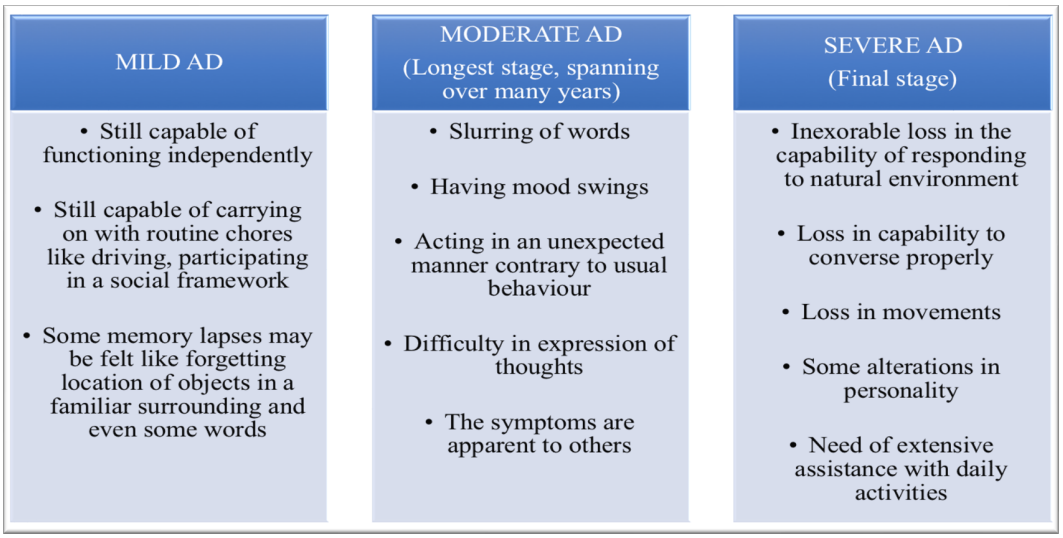


Fig. 2.1: Stages of AD progression and associated symptoms

The symptoms of AD get worse with time, although the pace at which the disease progresses is variable. Alterations in the brain related to AD commence years before any related clinical manifestations emerge. During this timeframe, which extends over several years, it is referred to as the preclinical stage of AD. The advancement of AD unfolds in three stages as given in Figure 2.1 (Alzheimer's Association, 2017m).

2.3 Animal models for testing potential Alzheimer's drug candidates preclinically

Researchers globally have utilized various animal models to assess drug candidates' effectiveness against AD. Commonly employed conventional AD animal models are outlined in Table 2.2.

Table 2.2: Commonly employed conventional AD animal models

Model	Animals used	Disease inducing agent(s)	Mode of disease induction	Reference
Aluminium chloride and d-galactose model	Mice	Aluminium chloride and d-galactose co-administration	Aluminium acts as a neurotoxin that enhances the APP expression whereas d-galactose replicates the normal aging phenomenon	(Xiao et al., 2011a)

Ibotenic acid model	Male rats	Ibotenic acid	Ibotenic acid acts as a potent activator of NMDA receptors, causing elevated glutamate levels, ultimately resulting in neuronal excitotoxicity	(Karthick et al., 2016)
β-amyloid model	Rats	Amyloid-β protein	Injecting neurotoxic Aβ peptide directly into the brain	(Nitta et al., 1994)
Senescence accelerated (SAMP8) model	Mice	NA	A mouse transgenic model with inherent accelerated aging, serving as a natural animal model	(Morley et al., 2012)

			for excessive production of APP and ROS	
Scopolamine induced dementia model	Rats/mice	Scopolamine	Scopolamine is both a neurotoxic substance and a cholinergic antagonist, leading to heightened oxidative stress and decreased levels of ACh and monoamines	(Habibyar et al., 2016; Haider et al., 2016)

2.3.1 Aluminium chloride and d-galactose induced AD in mice

The simultaneous administration of aluminium chloride and d-galactose has been profoundly used to mimic non-transgenic AD. Separately, these agents were known to mimic certain parameters of AD pathogenesis. D-galactose was known to induce subacute senescence (J. Gao et al., 2015) while Aluminium was known to be a neurotoxin (Kawahara and Kato-Negishi, 2011).

Both of these agents express the effect due to oxidative damage, mitochondrial dysfunction and elevation of AChE. After oral administration of Aluminium chloride, xanthine oxidase and glutathione peroxidase activities have been demonstrated to be increased and diminished, respectively, resulting in aggregation of intermediate neurotoxic products like H_2O_2 and OH^- radicals, which might be essential for Aluminium toxicity (Moumen et al., 2001). Following Aluminium chloride injection (intracerebroventricular), immune activity in phagocytic microglia and astrocytes, determined by estimation of glial fibrillary acidic protein and ED1, respectively, exhibited a greater inflammation in rat brain. Increase in inflammatory parameters and interactions with cholinergic neurons may contribute to the Aluminium caused cognitive and memory lapses (Platt et al., 2001). Aluminium ensues specific toxicity to cytoskeletal structures of brain neurons (Boni et al., 1976). Neurons express a typically greater sensitivity to Aluminium as compared to astrocytes. It has been demonstrated by the fact that neuronal-specific markers microtubule-associated protein type 2 (MAP2) and neurofilament light subunit (NF68KD) are restricted by lesser concentrations of Aluminium (IC50 180–630 mmol/L) than GFAP (IC50 700–1000 mmol/L) (Muller and Bruinink, 1994). Prolonged exposure to Aluminium decreases the base activity of guanylate cyclase and causes an impairment in the cGMP related processes *in vitro* and *in vivo*. Aluminum diminishes the levels of nitric oxide synthase and calmodulin in the cerebellum by 15% and 34%, respectively. Rats exposed to Aluminum also experience a 66% decrease in the baseline activity of soluble guanylate cyclase, along with a 50% reduction in the base cGMP levels in the extracellular space of cerebellum (Hermenegildo et al., 1999). Aluminium can bind to amyloid β-protein and form cross-links between them, which causes the proteins to aggregate into oligomers. These oligomers are toxic to neurons (Shunan et al., 2021). Exposure to aluminium leads to advancement of amyloidogenic pathway via activation of beta secretase and gamma secretase.

Also, it leads to inhibition of alpha secretase thereby retarding the non-amyloidogenic pathway (Skalny et al., 2021). Administration of aluminium chloride has also been reported to enhance caspas-3 mediated neuronal apoptosis in brains of rodents (Mesole et al., 2020). Aluminium chloride has also been linked to an increase in oxido-inflammatory burden while reducing the levels of BDNF (Abbas et al., 2022). D-galactose can replicate the effects of natural senescence in mice and Aluminium can stimulate the expression of APP in neurons, which may promote generation of Aβ. The individual effects of Aluminium and D-galactose were well established by several experiments. However, the combined effect was still not elicited. A modified protocol made the use of co-administration of Aluminium chloride orally and d-galactose subcutaneously for a period of 10 weeks. This method used for Kumming mice resulted in development of AD like lesions. Formation of structures similar to senile plaques and NFTs) was also reported. The behavioural and pathological changes lasted for a minimum of 6 weeks post withdrawal of Aluminium chloride and D-galactose administration (Xiao et al., 2011b). Another modified protocol made use of D-galactose (60 mg/kg day, i.p.) and Aluminium chloride (5 mg/kg day, p.o.) once daily for 90 days to develop AD like condition (L. Gao et al., 2015). The concurrent use of these substances is regarded as a simple and cost-effective method for creating an animal model (Wei et al., 2017).

The key mechanisms of aluminium chloride and d-galactose in development of AD have been illustrated in Figures 2.2 (Skalny et al., 2021) and 2.3 (Li et al., 2019), respectively:

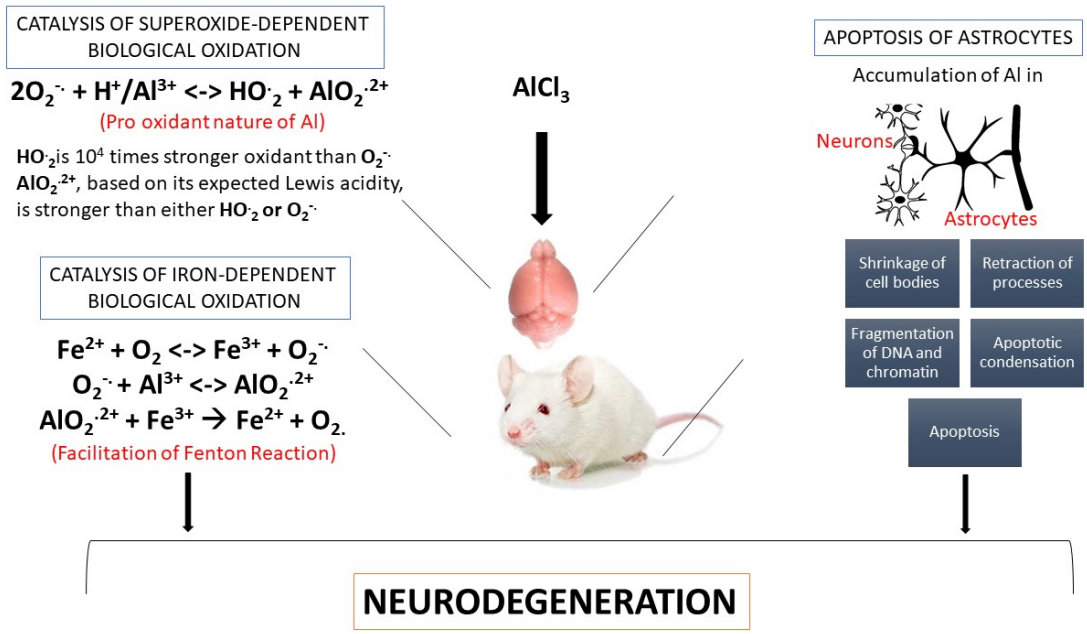


Fig. 2.2: Key mechanisms behind aluminium chloride induced neurodegeneration

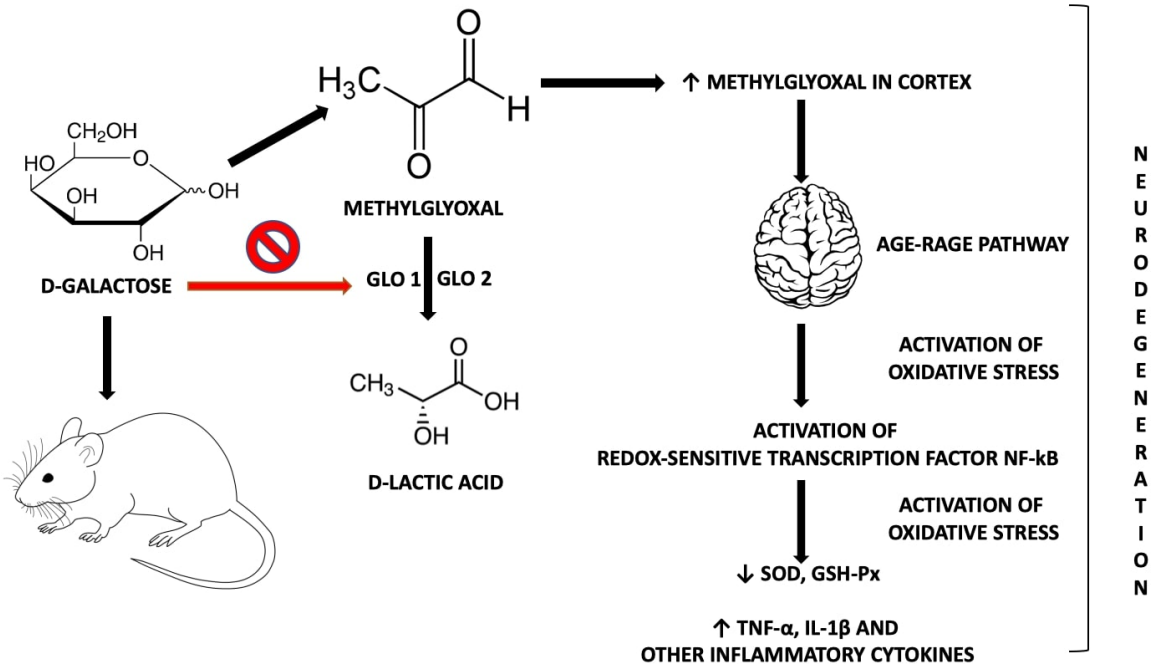


Fig. 2.3: Key mechanisms behind d-galactose induced neurodegeneration (GLO= Glyoxalase, AGE= Advanced Glycation Endproducts, RAGE= Receptor for Advanced

Glycation Endproducts, NF-kB= Nuclear Factor Kappa-B, SOD= Superoxide Dismutase, GSH-Px= Glutathione Peroxidase, TNF-a= Tumour Necrosis Factor alpha, IL= Interleukin)

A study reported an approximate decrease in viable neurons up to 71% in cornu ammonis 1, 78% in cornu ammonis 2 and 83% in cornu ammonis 3 regions of hippocampus following a chronic administration of aluminium chloride and d-galactose in rats for 10 weeks (Chiroma et al., 2018).

2.4 Pharmacotherapeutic approaches for AD

AD is a complex condition, and it is unlikely that any single drug or intervention can effectively cure it. Current pharmacological strategies aim to help individuals maintain cognitive functions, address behavioural symptoms, and slow down the disease's progression to delay the onset of symptoms. All the current therapies work by controlling the levels of specific brain neurotransmitters, primarily ACh and glutamate. These treatments can aid in preserving cognitive abilities, thinking processes, and communication skills, as well as partially alleviate certain behavioural problems. But these approaches do not treat the underlying cause of the disease. These treatments may not be universally effective for all AD patients and may provide varying degrees of benefit in terms of both effectiveness and duration. Several drugs are being marketed under the approval of the U.S. Food and Drug Administration (US-FDA) to provide symptomatic relief in AD (NIH National Institute on Aging, 2017b). The chief targets for pharmacological interventions in treatment of AD are given in Figure 2.4 (Kumar et al., 2016).

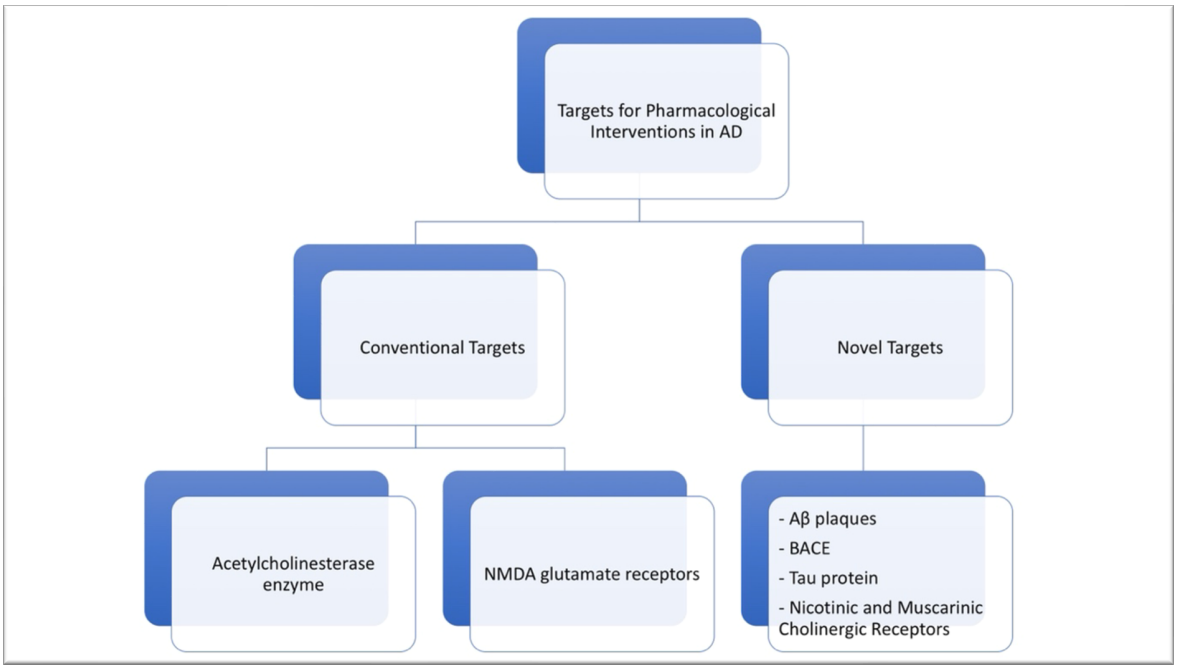


Fig. 2.4 The main targets for pharmacological interventions in AD

2.4.1 Conventional clinical management of AD

The current medications used in treatment of AD can be classified as:

a. AChE antagonists: Rivastigmine, Tacrine, Galantamine, Donepezil.

b. NMDA antagonist: Memantine

2.4.1.1 AChE antagonists: These medications exhibit a symptom improvement in AD that depends on the dosage, with different levels of cholinergic side effects throughout the body. Initial investigations in this drug category involved substances such as tacrine (tetrahydroaminoacridine), velnacrine and physostigmin. However, among these compounds, only tacrine progressed to extensive clinical trials and was subsequently introduced commercially in the United States and certain regions of Europe. Subsequently, newer drugs such as rivastigmine and donepezil were also introduced in the field (McGleenon et al., 1999).

In individuals with AD, there is an increase in the activity of AChE, which accelerates the breakdown of acetylcholine and results in reduced levels of ACh in the brain. This enzyme also plays a partial role in the formation of Aβ plaques and NFTs. ACh additionally acts as a facilitator by assisting in the aggregation of Aβ peptide fragments, forming complexes with the growing fibrils. These formed complexes are more harmful to cells than Aβ fibrils on their own (Alvarez et al., 1998, 1997; Singh et al., 2013).

These medications aim to raise the depleted ACh levels in the brains of individuals with mild to moderate AD by blocking the activity of the enzyme AChE, which is responsible for breaking down ACh, thus elevating its concentration. They are generally well-received by the majority of patients (Tabet, 2006).

2.4.1.2 NMDA antagonist: Glutamate is a primary neurotransmitter in the mammalian brain that participates in excitatory postsynaptic signalling via different types of glutamate receptors, including ionotropic and metabotropic ones. Glutamate-gated channels and a set of G-protein coupled receptors modulated by glutamate are responsible for releasing Ca^{2+} from internal reserves. These channels are named based on the compounds that activate them: amino 3-hydroxy 5-methyl 4-isoxazole-propionic acid (AMPA)-activated receptors, kainate-activated receptors, and NMDA receptors (Olivares et al., 2012).

NMDA receptors function as a coincidence detector in the regulation of synaptic plasticity. Only synapses that exhibit precise timing and spatial patterns of NMDA receptor activation undergo plastic changes, thanks to the influx of Ca^{2+} following the rapid unblocking of Mg^{2+}. This, in turn, plays a role in memory and learning processes. The voltage-dependent behavior of Mg^{2+} is so significant that, in pathological situations, it can exit the NMDA channel even with moderate depolarization, thereby impairing memory and learning (Koch et al., 2004).

Excessive activation of the glutamate system leads to a condition called excitotoxicity, causing harm to neurons by elevating the influx of calcium ions. Memantine serves as a non-competitive antagonist of NMDA receptors, countering the detrimental effects of elevated glutamate levels in the brain. Because of this attribute, it has established itself as a valuable therapeutic option for individuals with Alzheimer's disease. Memantine has been demonstrated to enhance cognitive functions and overall well-being in individuals with AD. It is currently available for the treatment of moderate to severe AD, authorized by the US FDA. (Reisberg et al., 2003). It is generally safe; however, there exists a possibility of experiencing mild and short-lived side effects. Due to its pharmacological characteristics, it has the potential to interact with specific other medications. Its mode of action can enhance the effects of levodopa, other dopamine agonists, and anticholinergics when taken simultaneously. Conversely, the effects of barbiturates and neuroleptic agents may be reduced. Concurrent use of memantine with other NMDA receptor antagonists with similar chemical properties (such as amantadine, budipine, ketamine, and dextromethorphan) may carry a risk of pharmacotoxic psychosis. Clinical trials have revealed that the most common symptoms reported were primarily dizziness, agitation, hallucinations, headaches, and fatigue. Less frequently reported symptoms included anxiety, vomiting, infections of urinary tract, and profuse perspiration. (Molinuevo et al., 2005).

2.4.2 Nutraceuticals for AD

Generally referred to as medical foods, these nutraceutical agents have found their use in improvement of conditions in patients having AD.

2.4.2.1 Tramiprosate: It is a small glycosaminoglycan mimicking product that can be taken orally. It gets bound to soluble Aβ and suppresses the aggregation of amyloid plaques and their subsequent deposition. Data of preclinical studies have proven that tramiprosate decreases the

levels of Aβ in brain and plasma, hinders fibril formation and poses a cytoprotective effect in the brain (Aisen et al., 2011; Wright, 2006). However, in 2007, a clinical trial using Tramiprosate for AD was halted in its phase III. An identical agent Homotaurine was later developed and released in the market by Neurochem Pharmaceuticals (now Bellus Health Inc.) as a neutraceutical (Malouf and Collins, 2009).

2.4.2.2 Phosphatisdyserine: Phosphatisdyserine, a lipid compound, is the chief component of neuronal cell membrane. It not only provides essential nourishment to the brain but also has the potential to enhance brain function, alleviate mental stress, and boost cognitive abilities, memory retention, and reaction speed. Hence, it is often referred to as a "brain nutrient." (Amaducci et al., 1986). FDA permits supplements containing phosphatisdyserine obtained from soy. Experts suggest that more research is required for phosphatisdyserine (Mattia et al., 1997).

2.4.2.3 Axona: It is dietary supplement which is use for treating AD and was studied in phase 2 clinical trials (Henderson et al., 2009). Caprylic acid is an active ingredient of Axona. It is a rich source of triglycerides and it can be also obtained from processed coconut oil. Caprylic acid undergoes hepatic metabolism to form β-hydroxybutyrate, a ketone body. These ketone bodies serve as substitute form of energy to brain cells. It finds its use in the form of a food supplement in AD (Thaipisuttikul and Galvin, 2012).

2.4.3 Plants of interest in treatment of AD

Over years, several plants and related products and/or extracts have been subjected to evaluation for anti-AD effects (Anand et al., 2017c).

- **Rosemary (*Rosmarinus officinalis* belonging to the Family Lamiaceae):** Abietane-type phenolic diterpenes are among the most prominent group of compounds obtained from

Rosemary. These diterpenes, having the basic skeleton of carnosic acid, are responsible for most of the antioxidant and many other pharmacological properties of the plant (Singhal et al., 2012). Recently, they have been reported to exert an inhibitory action on neuronal apoptosis because of various substances in controlled laboratory settings and within living organisms, thereby making it a promising candidate for neuroprotection in AD. Moreover, the involvement of rosemary diterpenes in influencing the formation, accumulation, and harmful effects of Aβ contributes to their extra benefit in addressing AD symptoms and associated issues (Habtemariam, 2016).

- **Magnolia bark (*Magnolia officinalis* belonging to the family Magnoliaceae):** In a study on neuronal apoptosis by Aβ in NGF-differentiated PC12 cell line, honokiol and magnolol (lignans), significantly controlled Aβ induced neuronal apoptotic cell death. The potential neuroprotective benefits may be achieved by lowering the production of ROS, reducing the elevation of intracellular calcium, and counteracting the activity of caspase-3 (Hoi et al., 2010).
- **Ashwagandha (*Withania somnifera* belonging to the family Solanaceae):** Active glycowithanolides of Ashwagnadha have an important antioxidant function, which is accomplished by potentiating the actions of certain enzymes (superoxide dismutase, catalase, and glutathione peroxidase). Ashwagandha is recognized as a nerve tonic with the ability to revitalize cells and enhance vitality. The evaluation of inhibitory effects on cholinesterase was performed employing a colorimetric method on the basis of Ellman's reaction and it was established that the *W. Somnifera* extract notably inhibited AChE in a dose dependent fashion (Kuboyama et al., 2005; Kumar et al., 2016; Zieneldien et al., 2022).
- **Guduchi (*Tinospora cordifolia* belonging to the family Menispermaceae):** It has been

demonstrated to have a nootropic effect on cognitive capabilities and learning in both normal and memory impaired animals. It improves the cognitive abilities by immune-stimulation and production of acetylcholine, thereby supplementing choline which in turn improves the cognitive function (Bairy et al., 2002).

- **Sting nettle (*Urtica dioica* belonging to the family Clusiaceae):** It has been in use over the years in the treatment of allergic conditions and associated symptoms, specifically hay fever. It comprises of compounds that help in reduction of inflammation. Also, it comprises of a fair quantity of boron that has been known to enhance the levels of estrogen. Estrogen, in turn, is advantageous in short-term memory. It has also been documented to elevate the mood in patients having AD (Singhal et al., 2012).
- **Garden angelica/ Wild celery (*Angelica archangelica* belonging to the family Umbeliferae):** It is a perennial herbaceous plant that has been in use since ages in the Traditional Chinese Medicine system for treating several cerebral disorders (Howes et al., 2003). An ethanolic extract obtained from the dehydrated roots of this plant, in a concentration dependent manner, was reported to be effective in the displacement of nicotine from the nicotine binding receptors (Perry et al., 1996). It was also reported to possess acetylcholinesterase inhibitory activity *in vitro* (Obulesu and Rao, 2011).
- **Sweet flag (*Acorus calamus* belonging to the family Araceae):** It is a perennial herb the rhizomes of which find place in the traditional Ayurvedic medicine system for treating memory loss. Ethanolic and hydroethanolic extracts of the rhizomes were reported to have sedative and neuroprotective effects, respectively, *in vivo* (Shukla et al., 2002; Vohora et al., 1990). It has also been reported to exert a valuable memory enhancing effect, along with an improvement in learning and behavioural adjustment. *A. Calamus* has also been shown to have an acetylcholinesterase inhibitory effect (Singhal et al., 2012).

- **Ginseng (*Panax ginseng* belonging to the family Araliaceae):** There have been reports suggesting a protective effect along with the beneficial trophic effects of ginseng in the cognition and memory in AD (Lee et al., 2008). Reports have indicated that ginseng may be clinically effective in preserving cognitive functions in individuals inflicted with AD. In a study, the effect of Korean Red Ginseng on cognition persisted for a two-year follow-up, suggesting feasible efficiency of long-term follow-up for AD (Heo et al., 2011).
- **Liquorice (*Glycyrrhiza glabra* belonging to the family Leguminoseae):** Glabridin, an isoflavonoid, obtained from the roots of Liquorice has been reported to possess a remarkable brain cholinesterase inhibitory activity, thereby making it viable to be employed in the management of AD (Cui et al., 2008). Another study suggested that the memory deficit alleviation effects of Liquorice may be because of its antioxidant and anti-inflammatory activities (Chakravarthi and Avadhani, 2013).
- **Guggulu (*Commiphora spp.* belonging to the family Burseraceae):** Gugulipid extracted from Guggul resin has been documented to have a remarkable preservative impact in the streptozotocin-induced dementia model. It has been suggested that the protective effect is due to its capability to reduce cholesterol along with significant antioxidant, and anti-acetylcholinesterase activity. Reduced levels of cholesterol in neurons hinder the amyloidogenic pathway thereby decreasing the formation of beta-amyloid complex. This effect may be attributed to the removal of Amyloid Precursor Protein from cholesterol and other membrane microdomains comprising of sphingolipids (Rao et al., 2012).
- **Turmeric (*Curcuma longa* belonging to the family Zingiberaceae):** Being lipophilic, Curcumin efficiently penetrates the BBB to get bound to the plaques. Reportedly, the binding of curcumin to Aβ leads to a blockade in the self-assembly of the plaques. Also, Curcumin has been documented to have a disintegrating influence on the Aβ plaques to

form fibrillar $A\beta_{40}$ (Mishra and Palanivelu, 2008). The advantageous effects of Curcumin in the improvement of conditions in the patients having AD have been attributed to blockade of Aβ aggregation, disintegration of amyloid beta plaques, antioxidant potential, metal chelation effect, reduced microglia formation and anti-inflammatory effect (Yao and Xue, 2014).

- **Firmoss/Toothed clubmoss (*Huperzia serrata* belonging to the family Huperziaceae):** Huperzine A, a sesquiterpene alkaloid, is a natural inhibitor of acetylcholinesterase. Therefore, it has high prospects to be employed in the symptomatic treatment of AD. However, the compound did not yield significant *anti* AD effect in phase II clinical trials (Rafii et al., 2011).
- **Sacred lotus (*Nelumbo nucifera* belonging to the family Nelumbonaceae):** The leaves, embryos, stamens, rhizomes of the plant have been evaluated for their comparative anti AD effects. Out of these, the embryonic extract was found out to have remarkable BACE inhibitory activity, Butyrylcholinesterase inhibitory activity and scavenging activity against peroxynitrite ions (Jung et al., 2015). The embryo of the plant also exhibited potent neuroprotective effect against excitotoxicity in neurons due to glutamate in the HT22 cell line (Kim et al., 2014).
- **Himatanthus (*Himatanthus lancifolius* belonging to the family Apocynaceae):** Uleine, an indole alkaloid extracted from Himatanthus has been reported to have a good acetylcholinesterase inhibitory activity on the basis of *in vitro* studies (Seidl et al., 2010), In further studies, uleine exhibited significant acetylcholinesterase and butyrylcholinesterase and BACE inhibitory activity. Also, it has been reported to exert a remarkable inhibitory effect on the self-aggregation of amyloid beta (Seidl et al., 2016).

- **Galangal (*Alpinia officinarum and Alpinia galangal* belonging to the family Zingiberaceae):** Galangin, a flavonoid obtained from Galangal has been documented to exhibit a good AChE antagonistic activity in an *in vitro* study. Nevertheless, it remains uncertain whether galangin directly binds to AChE or shares a binding site similar to its substrate (Guo et al., 2010).
- **Vanilla (*Vanillus planifolia* and *Vanillus tahitensis* belonging to the family Orchidaceae):** Vanillin has been documented to ensue a disintegratory effect on amyloid beta plaques *in vitro* (Song et al., 2016a). Additionally, vanillin has been reported to have antioxidant effects *in vivo* and neuroprotective effects *in vitro* (Dhanalakshmi et al., 2016, 2015a).

2.5 Gut Brain Axis and AD

GBA refers to the mutual interaction between the enteric and the central nervous systems (ENS and CNS), creating a link between the emotional and cognitive seats of the brain along with the peripheral intestinal functions (Anand et al., 2023b; Carabotti et al., 2015). Some reports have documented the role of microbiota in affecting anxiety and depression-like behaviours. Moreover, dysbiosis has also been linked to autism. The autistic patients express specific changes in the microbiota as per the severity of the disease (Foster and McVey Neufeld, 2013; Mayer et al., 2014; Naseribafrouei et al., 2014; Song et al., 2004). With the aim of exploring the role microbiota in modulation of GBA, various studies have been conducted. Studies on Germ Free (GF) animals have exhibited that bacterial colonization of the gut plays an essential role in the maturation and development of CNS and ENS, both (Barbara et al., 2005; Stilling et al., 2014). Some studies have also documented memory dysfunction in GF animals probably attributed to the changes in the expression of BDNF, one of the key factors involved in memory (Al-Qudah et al., 2014; Gareau et al., 2011). Changes in the composition of microbiota by

using probiotics has been reported to enhance the expression of BDNF and attenuation of senescence-related changes in the hippocampal region (Distrutti et al., 2014). A report documented that pre-incubation of HT-29 cells with *L. plantarum* strain 299v markedly decreased the percentage of caspase3-7^{+} cells. It suggests a caspase-3 and -7 inhibition by the probiotic strain which led to the decreased caspase mediated apoptotic cell death (Dykstra et al., 2011).

There has been convincing evidence that the interaction between the brain and the microbiota is mediated by the vagal innervation, that carries information to the CNS from the luminal environment. Moreover, behavioural and neurochemical impacts were not reported in vagotomized mice, thereby solidifying the notion that the vagus nerve comprises the chief constitutive modulatory pathway for communication between the brain and the microbiota (Bravo et al., 2011). A study also explored the association between the composition of microbiota in the faeces and quality of life by making the use of data obtained from more than 1,000 people. They conducted metagenomic analyses which showed that the potential of microbes to contribute to synthesis of some neuroactive metabolites may be correlated with mental well-being (Bray, 2019).

A linkage between gut dysbiosis and neurodegeneration is mainly rested upon the results obtained by certain pre-clinical evaluations. However, the clinical data are rather limited. The most accepted clinical proof of changes in the healthy microbial composition in a neurodegenerative condition arises from studies of patients inflicted with Parkinson's disease (Keshavarzian et al., 2015; Scheperjans et al., 2015). Some investigations have been carried out in populations of AD patients. It was observed that the microbial diversity was diminished in the faecal matter of AD patients contrasted to the sex and age-matched controls. It was reported that there was a decline in the numbers of *Actinobacteria* and *Firmicutes* and an

elevated level of *Bacteroidetes*. The relative abundance of bacterial genera was observed to have a correlation with the biomarkers of AD in the cerebrospinal fluid (Vogt et al., 2017). Another study evaluated the association of brain amyloidosis and selected bacterial taxa in patients having impaired cognition. Deposition of Aβ plaques in brain was found to be associated with low levels of anti-inflammatory taxon *Eubacterium rectale* and elevated levels of pro-inflammatory taxa *Shigella* and *Escherichia*, in stool samples. These changes were reported to have a correlation with the peripheral state of inflammation (Cattaneo et al., 2017). A more elaborate investigation in AD subjects showed more abundant presence of genus *Bilophila* in the phylum *Proteobacteria* and *Gemellaceae, Bacteroidaceae, Rikenellaceae*, and decreased presence at the familial level for *Ruminococcaceae, Turicibacteraceae, Peptostreptococcaceae, Clostridiaceae, Bifidobacteriaceae*, and *Mogibacteriaceae. Corresponding* correlations between relative abundance of these bacteria and cerebrospinal fluid biomarkers like $A\beta_{42}/A\beta_{40}$ and phosphorylated tau exhibited consistency in patterns, where genera identified as more prevalent in AD are associated with increased AD pathology (Janakiraman and Krishnamoorthy, 2018). The loss of balance between the production of Aβ and its clearance has been considered to be factor that leads to accumulation of Aβ and the consequent neuronal dysfunction. There is accumulating evidence that suggests the role of neuro-inflammation in the disrupted clearance of Aβ. It may even accentuate the pathological process of AD (Heneka et al., 2015; Kamer, 2010; Regen et al., 2017). This further opens up avenues for research on the possible causal relation between amyloidosis and the gut microbiota-related inflammation.

Recently, a growing body of evidence has suggested that gut dysbiosis triggers a systemic immune response which results in an increased inflammatory response in AD brain Click or tap here to enter text.(Chen et al., 2022; Montacute et al., 2017; Varesi et al., 2022).

Toll-like receptors (TLRs) are a part of the first line of defence that participates in the process of recognition of molecules widely shared by activated immune system and pathogens. TLRs play a role in upkeep of homeostasis, commensal colonization, and the integrity of the intestinal barrier. Aβ acts as a ligand for TLRs in addition to several gut bacteria and their metabolic waste products which may trigger the inflammatory process in the brain and the gut that leads to development of AD among other neurodegenerative disorders (Gómez-Llorente et al., 2010; Rakoff-Nahoum et al., 2004).

2.5.1 Role of probiotics in AD

2.5.1.1 The anti-inflammatory role of probiotics

Consumption of probiotics has been documented to improve the senescence associated modifications of immunological features in AD brain. It has been reported that probiotic interventions can alleviate the immunological reactions by regulating production of cytokines, bettering the function and distribution of macrophages, natural killer cells, T cells, and granulocytes. This leads to enhancement of the immune responses at both the systemic and mucosal levels. Given the immunomodulatory action of probiotics, it has been estimated that the probiotics may have an ameliorative effect in AD by regulating the inflammatory reactions triggered by deposition of Aβ and other risk factors, including inflammaging (Arunachalam et al., 2000; Chiang et al., 2000; Di Giosia et al., 2022; Gill et al., 2001; Sharma et al., 2014). There have been reports about probiotics directly mitigating neuronal inflammation characterized by reduced levels of pro-inflammatory cytokines in systemic circulation and microglia activation. In a d-galactose mouse model of accelerated senescence, inflammation was demonstrated to be diminished following consumption of probiotic *L. pentosus var. plantarum* C29. It was reported to have an inhibitory effect on activation of transcription factor NF-κB, M1 macrophages and the pro-inflammatory cytokine TNF-α (Jeong et al., 2015).

Another study documented the ameliorative effect of *B. breve* A1 on Aβ mediated toxicity that prevented cognitive dysfunction in a mouse based model of AD via its regulatory effect on the neuronal inflammation (Kobayashi et al., 2019). Another pathway concerning neuronal inflammation known as the 'kynurenine pathway' has gained attention of researchers recently. It is the pathway that is responsible for metabolism of 95% of the body's tryptophan. Its dysfunction has been documented to play a crucial role in the onset of neuronal inflammation. (Figure 2.5) (Arora et al., 2020; Savitz, 2020).

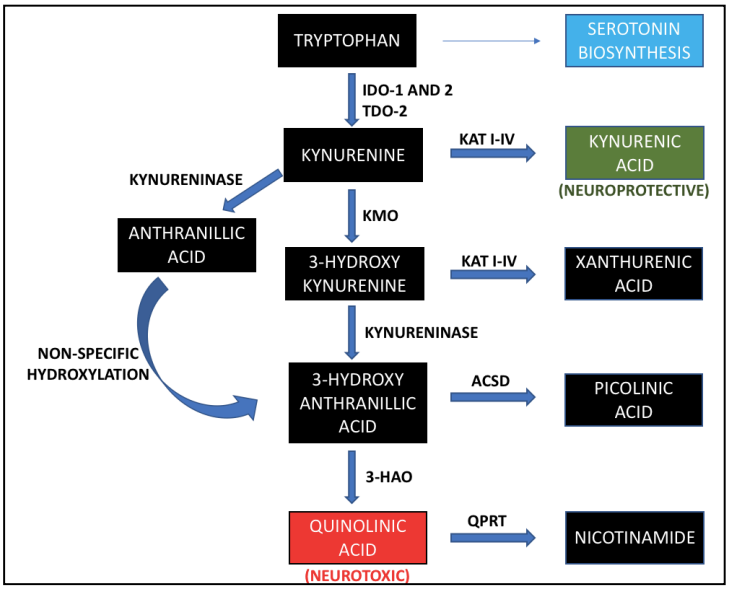


Fig. 2.5: Illustration of kynurenine pathway (IDO= Indoleamine 2,3- deoxygenase, TDO= Tryptophan deoxygenase, KAT= Kynurenine aminotransferase, ACSD= 2-amino-3-carboxymuconic-6-semialdehyde decarboxylase, HAO= Hydroxyanthranilate deoxygenase, QPRT= Quinolinate phosphoribosyltransferase)

The enzyme IDO-1 forms a subset of the enzymes responsible for the metabolism of tryptophan in the kynurenine pathway. It is stimulated by the pro-inflammatory cytokine IFN-γ. Also, the enzyme has been documented to have an essential role in co-localization with Aβ plaques (Desbonnet et al., 2008). Quinolinic acid has an agonistic activity on the NMDA receptors

which also inhibits the process of glutamate reuptake by the astrocytes thereby leading to excitotoxicity (Stone and Perkins, 1981). It has also been reported to cause neurotoxicity by several other mechanisms like disruption of BBB, generation of ROS and promoting phosphorylation of tau. Accumulation of quinolinic acid in the hippocampus has also been reported in AD brain (Guillemin, 2012). Administration of *L. johnsonii* has been reported to diminish the endogenous levels of IDO in germ-free mice along with a fall in levels of levels of kynurenine and an elevation in the serotonin levels, in serum (Valladares et al., 2013). Another study reported an elevation in plasma levels of the neuroprotective kynurenic acid accompanied by a reduction in pro-inflammatory cytokine release in rats treated with *B. infantis* (Desbonnet et al., 2008; Garcez et al., 2019).

2.5.1.2 The antioxidant role of probiotics

Various probiotic strains have been reported to have an antioxidant effect (Wang et al., 2017b) which can be helpful in alleviation of neurodegeneration in AD. Probiotics can act as chelating agents by capturing metal ions and thereby rendering them unavailable to catalyze the oxidation reactions. Several probiotic strains have been studied for their antioxidant property out of which *S. thermophilus* 821 showed the best chelation activity with Cu^{2+} and Fe^{2+} ions (Lin and Yen, 1999). Similar chelating effect was shown by *L. casei* KCTC 3260 in a separate study (Lee et al., 2005). Another study documented that the Fe^{2+} ion chelation effect of *L. helveticus* CD6 (Ahire et al., 2013). The mechanism has been ascribed to the inhibition of the enzyme catalysed phosphate ester displacement reactions by the ions of transition metals, thereby decomposing hydroperoxides to alkoxyl and peroxyl radicals. However, the exact mechanism is not well understood (Halliwell et al., 1995). Some studies have reported that the probiotic strains like *L. fermentum* E-3 and E-18, engineered *L. casei* BL23 are capable of

employing superoxide dismutase to resist the oxidative stress (Kullisaar et al., 2002; LeBlanc et al., 2011).

Probiotics can also have a stimulatory effect on body's own antioxidant enzyme mechanisms (Aluwong et al., 2013; Ejtahed et al., 2012; Wang et al., 2009, 2017a). Probiotics have also been known to produce ferulic acid by the group of enzymes known as ferulic acid esterases. It has been reported to drive the proliferation of neuronal stem cells accompanied by increasing the production of BDNF and nerve growth factor. *B. animalis*, *L. fermentum* NCIMB and *L. plantarum* NCIMB 8826 have been demonstrated to produce large quantities of the antioxidants (Lindsay, 1988; Meng et al., 2018; Nabavi et al., 2015; Westfall et al., 2017). In a transgenic mouse model of AD, treatment with ferulic acid has been reported to ameliorate neuronal inflammation accompanied by diminished levels of Aβ in cortex and hippocampus. The exact mechanism is not fully established but it has been believed that ferulic acid partially inserts itself between the Aβ in the presence of carboxyl and hydroxyl species which allow the formation of hydrogen bonds between these peptides (Ono et al., 2005; Sgarbossa et al., 2015). Ferulic acid has also been documented to modulate β-secretase (Mori et al., 2013). Another potential antioxidant mechanism of probiotics has been attributed to sirtuin-1 protein deacetylase which regulates various antioxidant genes to provide a neuroprotective effect (Bonfili et al., 2018).

2.5.1.3 Probiotics for enhanced cognition

Several studies have reported the improvement in cognition due to probiotics (Afzal et al., 2020; Deng et al., 2020; Kim et al., 2021; Marotta et al., 2019). It has been suggested by some investigations that there is a reduction in levels of potassium and sodium ions in the neurons due to formation of Aβ in the cerebellum and frontal cortex. At the cell periphery, it reduces the level of Na^+/K^+ ATPase transporters. These transporters are responsible for regulation of

the proton gradient across the cell membrane which in turn is responsible for the cell's capability to attain and propagate action potentials. The reduced levels of ATPases may have a contribution towards initiating AD pathogenesis. In an accelerated senescence mice model based on administration of d-galactose, usage of *L. plantarum* MTCC1325 was found to restore the ATPase level in hippocampus and the cerebral cortical region (Kairane et al., 2002; Mallikarjuna et al., 2016; Nimgampalle and Kuna, 2017).

Pre-clinical evidence indicates that products of microbial metabolism such as short chain fatty acids (SCFA) could be used as signalling molecules by gut microbiota to exert action on the CNS (Erny et al., 2015). Their interplay with the anti-inflammatory properties and the immune system makes them therapeutically interesting candidates for neurodegenerative disorders including but not limited to AD. Modifications in the diet and lifestyle are effective in producing chronic changes in the gut microbiota. Another modifiable parameter i.e., exercise is believed to promote the gut microbial diversity. Also, it enhances metabolic functions of the gut microbiota which have been proven advantageous in improvement of cognitive performance (Cerovic et al., 2019; Ticinesi et al., 2019). A preventive therapeutic approach involving dietary changes and increased physical exercise is likely to be the most promising practice for slowing the cognitive deterioration and ameliorating neuroendocrine, metabolic and vascular anomalies that often occur in precedence while remarkably contributing to cognitive decline.

2.6 Therapeutic interventions employed in the present study

2.6.1 Vanillin

In recent times, there has been a growing focus on the study of natural food components, particularly in relation to ingredients like flavourings and preservatives. Among the diverse range of naturally derived flavouring agents in use today, vanilla holds a significant position

in various industries. It has found widespread application in the production of cakes, soft drinks, ice creams, chocolates, alcoholic beverages, fragrances/perfumes, pharmaceuticals, and nutraceuticals (Ranadive, 1992). Natural vanilla comprises a combination of constituents extracted from the cured pods of various Vanilla species.: *Vanillus planifolia* and *Vanillus tahitensis* (Ramachandra Rao and Ravishankar, 2000). However, *V. planifolia* is highly prized primarily because of its pod quality and yield (Sinha et al., 2008).

Vanilla's flavor profile contains over 200 components, but only 26 of them are found in quantities exceeding 1 mg/kg. The predominant aroma and taste of vanilla extract are primarily attributed to the presence of vanillin, which occurs in a range of 1-2% w/w in cured vanilla pods (Gallage and Møller, 2015; Jadhav et al., 2009).

Moreover, recent *in vitro* research has shed light on another promising aspect of vanillin's potential therapeutic application in AD. This study has revealed that vanillin possesses the remarkable ability to disintegrate the Aβ plaque aggregates, which are hallmark pathological features of AD. By targeting and disrupting these aggregates, vanillin could help mitigate the progression of the disease, offering hope for improved outcomes in AD patients. Furthermore, the fact that vanillin can traverse the BBB is of paramount importance in the context of AD treatment. The BBB presents a formidable barrier that restricts the entry of many substances into the brain, making it challenging to deliver therapeutic agents directly to the site of action. Vanillin's capacity to cross this barrier underscores its potential as a viable candidate for AD therapy, as it can access the brain and exert its beneficial effects on neuronal health. In light of these compelling findings, the usage of vanillin in AD preclinical development appears to be a promising avenue. Its antioxidant properties, plaque disaggregation capabilities, and BBB-penetrating ability collectively support the rationale for further investigation and

development of vanillin-based treatments for Alzheimer's disease. (Dhanalakshmi et al., 2015b; Song et al., 2016b).

2.6.1.1 Pharmacokinetic parameters of vanillin

Pharmacokinetics of vanillin following p.o. administration have been established by a study involving rats. The parameters are listed in Table 2.3 (Beaudry et al., 2010).

Table 2.3: Pharmacokinetic parameters of vanillin (p.o.)

Parameter	Value
PEAK CONCENTRATION IN PLASMA	290.24 ng/mL
TIME TAKEN TO ACHIEVE PEAK CONCENTRATION IN PLASMA	4h
RELATIVE CLEARANCE	62.17 L/h/kg
PLASMA HALF-LIFE	10.3h
BIOAVAILABILITY	7.6%

2.6.1.2 Safety and toxicity profile of vanillin

U.S. FDA enlists vanillin as a 'generally recognized as safe' (GRAS) substance (U.S. FDA, 2016). However, the safety and toxicity profile of vanillin has also been established (Table 2.4).

Table 2.4: Safety and toxicity profile of vanillin

Parameter	Description
LD50 (Acute; Oral)	'1580 mg/kg' (Rat), '3925 mg/kg' (Mouse), '1400 mg/kg' (Guinea pig)

LD50 (Acute; Dermal)	5010 mg/kg (Rabbit)
Health hazards (Acute)*	Dangerous in cases of contact with eyes, of oral consumption, of inhalation. Slightly dangerous in case of dermal contact
Health hazards (Chronic)**	Slightly hazardous in case of dermal contact. No data available for carcinogenicity, mutagenicity, teratogenicity, developmental toxicity

*It may lead to irritation in eyes, mucous membrane, respiratory tract and skin. It may be absorbed dermally. Ingestion may lead to irritation in gastrointestinal tract and may also adversely influence the cardiovascular system, respiratory system, liver (causing jaundice), urinary system, nervous system (causing muscle weakness, somnolence, coma).

**Repeated or prolonged inhalation may ensue undesirable effects on the brain and blood (alterations in the count of white and red blood cells). Repeated or prolonged ingestion may adversely influence the urinary system, liver, heart, and metabolism (causing weight loss) (NLM Toxnet Toxicity Data Network, 2015).

2.6.2 Magnolol

Magnolol (2-(2-hydroxy-5-prop-2-enylphenyl)-4-prop-2-enylphenol) is a polyphenolic binaphthalene compound obtained from the root and stem bark of *Magnolia officinalis*, *Magnolia obovate*, *Magnolia grandiflora* (Family: Magnoliaceae)- traditional Chinese and Japanese herbal product ("Magnolol-PubChem," 2020; Ranaware et al., 2018). It has been employed in traditional medicine system for managing the anomalies of nervous system (Alexeev et al., 2012). Table 2.5 enlists various reported pharmacokinetic properties of magnolol (Zhang et al., 2019). Table 2.6 enlists the toxicological profile of magnolol.

Table 2.5: Pharmacokinetic characteristics of magnolol

Pharmacokinetic Process	Pharmacokinetic Parameter	Description	Experimental Model	Reference
ABSORPTION	Mean peak plasma concentration (after oral administration)	426.4±273.8ng/mL	Rat	(Sheng et al., 2014)
	Bioavailability (after oral administration)	17.5±9.7%	Rat	(Sheng et al., 2014)
	Absolute bioavailability	4%	Rat	(Lin et al., 2011)
DISTRIBUTION	Tissue distribution	Liver, lungs, kidneys, heart, brain	Rat	(Sheng et al., 2014)
METABOLISM	Major biotransformation pathway	Glucuronic acid conjugation via several isoenzymes UGT_{1A1}, UGT_{1A3}, UGT_{1A7}, UGT_{1A8}, UGT_{1A9}, UGT_{1A10}, and UGT_{2B7}	*In vitro* analysis in human liver	(Zhu et al., 2012)

	Accessory biotransformation pathway	Human intestinal microsomal enzymes $UGT1_{A10}$ and UGT_{2B7}	*In vitro* analysis in human intestine	UGT1A10 and UGT2B7
OTHERS	AUC_{0-t} (after oral administration as a solid dispersion with polyvinylpyrrolidone)	80.1%	Rabbit	(Lin et al., 2014)
	C_{max} (after oral administration as a solid dispersion with polyvinylpyrrolidone)	44.7%	Rabbit	(Lin et al., 2014)

Table 2.6: Toxicological profile of magnolol

Parameter	Description	Model Animal	Reference
LD_{50} (oral)	2,200 mg/kg	Mouse	(U.S. National Library of Medicine, 2020)

In an *in vitro* study involving PC12 cell lines magnolol was reported to reduce Aβ induced cell death (Hoi et al., 2010). Magnolol was also reported to attenuate an AD-like pathology in a transgenic *C. elegans* model. It was also reported to reduce Aβ deposition and enhance the phagocytosis and degradation of Aβ in microglia cells (Xie et al., 2020). Additionally, magnolol has been reported to have an oral LD_{50} value of 2,200 mg/kg (in mouse). Several *in silico* models have also predicted appreciable anti-AD effects of magnolol (Anand et al., 2021).

2.6.3 *Lactobacillus rhamnosus*

Many parts of the human body, most notably the female reproductive tract and the digestive system, including the oral cavity, are colonised by lactobacilli (Heeney et al., 2018). A "generally recognised as safe" (GRAS) designation from the U.S. Food and Drug Administration (USFDA) and a "qualified presumption of safety" (QPS) designation from the European Food Safety Authority (EFSA) make it relatively simple to utilise lactobacilli in the production of food. These qualities make Lactobacillus species the best possible probiotic candidates (Kechagia et al., 2013). Although *L. rhamnosus* GG is one of the strains that has been the subject of the most research, as in the present study, *L. acidophilus* is more frequently found in commercial products.

It has the capacity for surviving and proliferating at stomach acid pH, in medium containing bile, and adhering to enterocytes. In addition, *L. rhamnosus* can release soluble substances that benefit the gut by promoting intestinal crypt survival, reducing intestinal epithelial apoptosis, and maintaining cytoskeletal integrity in addition to a biofilm that can provides mechanical protection to the mucosa. Since then, it has been one of the probiotic strains that has undergone the most extensive research and is present in a range of commercially accessible probiotic products. Its advantageous properties have been thoroughly investigated in clinical trials and human interventions (Segers and Lebeer, 2014).

According to a clinical study, it showed significant antioxidant activity under conditions of high physical stress. The capacity of *L.rhamnosus* to boost antioxidant levels and counteract the effects of free radicals, especially the reactive oxygen species may be advantageous for athletes who are subject to oxidative stress (Wang et al., 2017c). A report documented that it produced the soluble proteins p40 and p75 that, through a MAPK-dependent mechanism, were able to reduce the breakdown of the epithelial barrier caused by H_2O_2 exposure (Seth et al., 2008).

CHAPTER-3

RESEARCH ENVISAGED AND RESEARCH METHODOLOGY

3. RESEARCH ENVISAGED AND RESEARCH METHODOLOGY

3.1 Rationale and scope of the study

As per the US Centers for Disease Control and Prevention (US-CDC), AD, a progressive neurological condition, is among the top 10 leading causes of mortality (US-CDC, 2022). It is the commonest form of dementia which is accompanied by a depletion in the levels of cortical and hippocampal acetylcholine, elevated expression of acetylcholinesterase, aggregation of Aβ plaques and tau NFTs along with neuroinflammation. In AD, what begins as short-term memory lapses evolves to rendering the patient functionally dependent and eventually leading to death. The onset and progression of AD are significantly influenced by oxidative stress due to its neurodegenerative capability (Anand et al., 2017b). The pharmacological interventions employed for the management of AD involve donepezil, galantamine, rivastigmine (acetylcholinesterase inhibitors) and memantine (NMDA glutamate antagonist), both of which provide nothing more than symptomatic relief (Anand et al., 2017a; Reisberg et al., 2003). Disease modifying and safer agents for treating and/or managing AD are ardently sought.

In the present research, AD was inflicted in mice by co-administering aluminium chloride and d-galactose. Aluminium permeates the brain easily and influences slow axonal transports, causes inflammation, and induces structural and synaptic abnormalities which ultimately lead to neurodegeneration (Chiroma et al., 2018; S. E. et al., 2019). D-galactose (a reducing sugar) readily undergoes reactions with free amines to yield Advanced Glycation End Products (AGEs). When administered over a prolonged period, D-galactose causes the alteration which resemble the natural senescence in animals including cognitive decline, oxidative stress, diminished immunological capability along with genetic changes. It also leads to mitochondrial dysfunction and an elevation in the level of brain acetylcholinesterase (Chiroma et al., 2018; Li et al., 2019; Shwe et al., 2018). Co-administration of aluminium

chloride and D-galactose for a period of 90 days has been reported to develop AD characterised by elevation of beta secretase, Aβ aggregation and caspase-3 while reducing BDNF in rodents (Gao et al., 2015; Haider et al., 2020; Xiao et al., 2011; Xing et al., 2018).

In the present research, the individual and combined effects of *Lactobacillus rhamnosus* (a probiotic), vanillin and magnolol (natural phytoconstituents) have been evaluated in aluminium chloride and D-galactose inflicted AD in mice.

Vanillin is a phenolic compound obtained from vanilla pods that has an antioxidant potential due to its chemical nature. It has been used for long in foods, beverages, cosmetics for its pleasant aroma and flavour (Anand et al., 2019). Vanillin has been reported to have an ameliorative effect in scopolamine induced cognitive impairment in mice (Anand et al., 2022). Also, there is *in vitro* evidence of vanillin causing the disintegration of Aβ (Song et al., 2016). In the present study, vanillin has been tested in aluminium chloride and D-galactose induced AD mouse model.

Magnolol is a polyphenol (neolignan) obtained from the extract of *Magnolia officinalis* bark. The trees are mainly found in Eastern and South-Eastern regions of Asia (Poivre and Duez, 2017). There are several traditional Chinese and Japanese herbal formulas which contain *Magnolia* like Banxia Houpo Tang and Saiboku-To (Iwasaki et al., 2000; Kuribara et al., 2000). *M. officinalis* has been used in Asian traditional medicine for treating anxiety, sleep disorders, nervousness, etc. It implies that magnolia bark extract holds the capability to elicit central effects. It has been listed in the Japanese Pharmacopoeia XIV (English Edition, 2001) and Pharmacopoeia of the People's Republic of China (English Edition, 2005) (Koetter et al., 2009). In an *in vitro* study involving PC12 cell lines magnolol was reported to reduce Aβ induced cell death (Hoi et al., 2010). Magnolol was also reported to attenuate an AD-like pathology in a transgenic *C. elegans* model. It was also reported to reduce Aβ deposition and

enhance the phagocytosis and degradation of Aβ in microglia cells (Xie et al., 2020). Additionally, magnolol has been reported to have an oral LD_{50} value of 2,200 mg/kg (in mouse). Several *in silico* models have also predicted appreciable anti-AD effects of magnolol (Anand et al., 2021).

The probiotic used in the present study is *L.rhamnosus*. Due to its capacity for surviving and proliferating at stomach acid pH, in medium containing bile, and adhering to enterocytes. Additionally, it can release soluble substances that benefit the gut by promoting intestinal crypt survival, reducing intestinal epithelial apoptosis, and maintaining cytoskeletal integrity in addition to a biofilm that can provides mechanical protection to the mucosa (Segers and Lebeer, 2014). *L.rhamnosus* has been documented to have antioxidant property (Seth et al., 2008). A study reported that *L. rhamnosus* (10^9 CFU/mouse/day) by gavage showed decrease in depression and anxiety with effects observed in hippocampus and prefrontal cortical region (Cheng et al., 2019). This strengthens the rationale to use the probiotic for CNS disorders.

As per the reported endpoints in the literature, TL in the Elevated Plus Maze paradigm (Itoh et al., 1990), EL in MWM paradigm (Bromley-Brits et al., 2011; Vorhees and Williams, 2006), and NOR (Antunes and Biala, 2012; Lueptow, 2017) behavioural tests were employed to assess the memory enhancing and disease ameliorating effects of vanillin. To evaluate the antioxidant potential of vanillin, thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), catalase activity (CAT) assays were performed (Anand et al., 2022; Habibyar et al., 2016). Furthermore, to understand the effect of vanillin on various pathological pathways of AD, its inhibitory activity was assessed on enzymes- acetylcholinesterase (AChE), beta secretase and caspase-3. The *in vivo* capability of vanillin to reduce the aggregation of $A\beta_{(1-42)}$ and elevate BDNF was also assessed.

Histopathological assessment of cortical and hippocampal regions was also done using eosin and haematoxylin staining.

Probiotics along with vanillin and magnolol can provide a multifactorial therapeutic approach in animal model of AD. Various pathways leading to development and progression of AD like GBA, amyloid pathway, caspase-3 pathway, oxidative stress pathway can be targeted with the combination which may be able to provide a significant benefit in therapeutic terms. The chosen therapeutic interventions have not been evaluated in the chosen animal model either alone or in combination which makes the proposed work novel.

3.2 Aim of the study

To evaluate the neuroprotective effects of vanillin, magnolol and *L.rhamnosus*, alone and in various combinations, in aluminium chloride and d-galactose induced Alzheimer's disease in mice.

3.3 Objectives

- To evaluate and compare the ameliorative effect of vanillin, magnolol and probiotics in AD using animal model
- To evaluate the combination effect of vanillin, magnolol and probiotics in AD using animal model

3.4 Plan of work

Plan of work has been illustrated in figure 3.1.

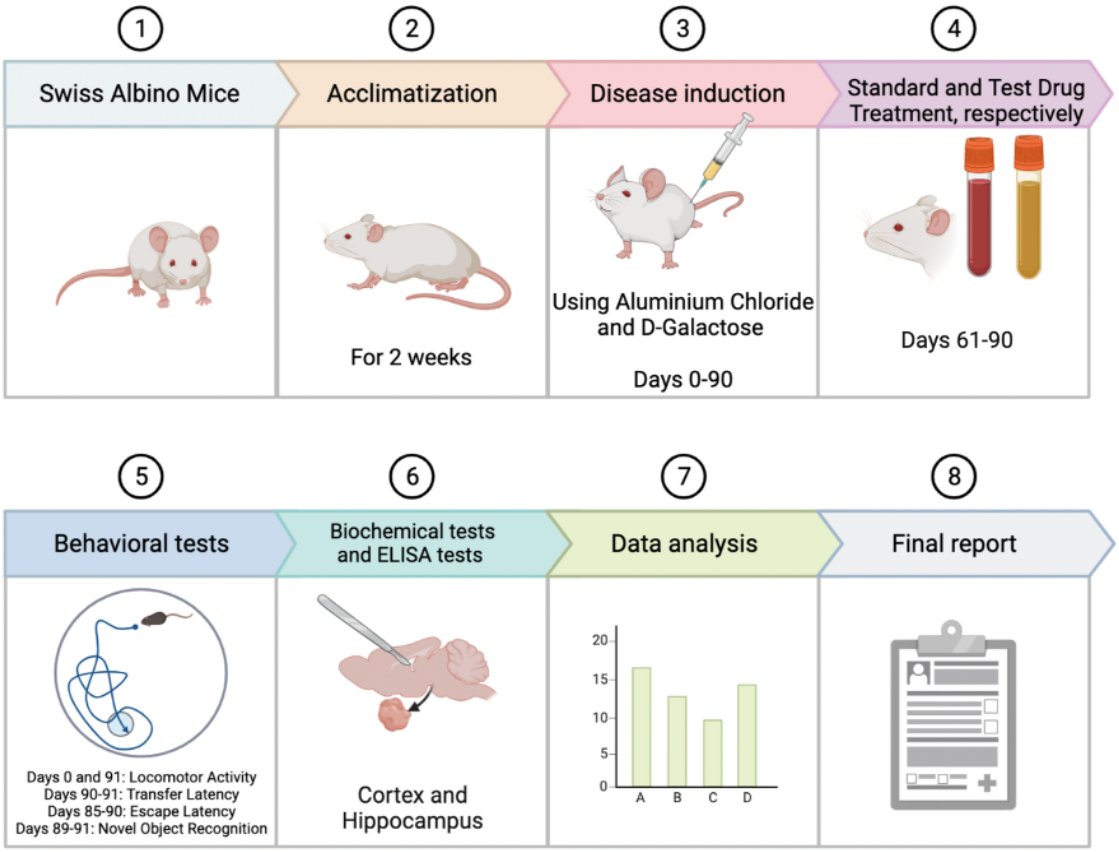


Fig 3.1: Plan of work

CHAPTER-4

EXPERIMENTAL WORK

4. EXPERIMENTAL WORK

4.1 Material

4.1.1 Equipment

The equipment used for the experimental work have been mentioned in Table 4.1

Table 4.1: Equipment used for experimental work

Equipment	Manufacturer
UV-Visible spectrophotometer	Shimadzu Corp., Japan
Centrifuge	Remi Instruments, India
Cooling centrifuge	Remi Instruments, India
Hot air oven	Navyug Udyog, India
Digital weighing balance	Contech Instruments Ltd., India
Deep freezer	Blue Star Ltd., India
Photoactometer	INCO Pvt. Ltd., India
Rotarod Apparatus	INCO Pvt. Ltd., India
Refrigerator	Kelvinator International, India
iMark^{TM} Microplate Reader	Bio-Rad

4.1.2 Chemicals and kits

The chemicals used for the experimental work have been listed in Table 4.2. The kits used for experimental work are listed in Table 4.3.

Table 4.2: Chemicals used for experimental work

Chemical	Manufacturer
(5,5-dithio-bis-(2-nitrobenzoic acid)	CDH Pvt. Ltd.
1,1,3,3 tetramethoxy propane	CDH Pvt. Ltd.
Acetic acid	LobaChemie Pvt. Ltd.
Acetylcholine hydrobromide	TCI Chemicals
Aluminium chloride	Molychem
Disodium hydrogen phosphate	LobaChemie Pvt. Ltd.
D-galactose	LobaChemie Pvt. Ltd.
Donepezil hydrochloride monohydrate	Sun Pharmaceutical Industries Ltd. (received *ex gratis*), Gurugram
Ethanol (absolute)	Chong Yu Hi-Tech Chemicals, China
Hydrochloric acid	Lobachemie Pvt. Ltd.
Hydrogen peroxide	LobaChemie Pvt. Ltd.
***Lactobacillus rhamnosus* GG**	Swiss Garnier Life Sciences (received *ex gratis*)
Magnolol	Xi'an Pincredit Bio-tech Co., Ltd., Shaanxi, (China)
n-butanol	CDH Pvt. Ltd.
Normal saline	Aculife Healthcare Pvt. Ltd.
Potassium dihydrogen orthophosphate	LobaChemie Pvt. Ltd.
Pyridine	CDH Pvt. Ltd.
Sodium Carboxy Methyl Cellulose	CDH Pvt. Ltd.
Sodium chloride	LobaChemie Pvt. Ltd.
Sodium dodecyl sulphate	CDH Pvt. Ltd.
Sodium hydroxide pellets	LobaChemie Pvt. Ltd.
Standard GSH reagent	CDH Pvt. Ltd.
Thiobarbituric acid	CDH Pvt. Ltd.
Titanium dioxide	LobaChemie Pvt. Ltd.
Tri-Sodium citrate (Dihydrate)	LobaChemie Pvt. Ltd.
Trichloroacetic acid	CDH Pvt. Ltd.
Vanillin	Sigma-Aldrich

Table 4.3: Kits used for experimental work

Kit	Manufacturer
Mouse Amyloid Beta (1-42) and Mouse Caspase-3 ELISA kits	Cusabio Biotech Co. Ltd. (Wuhan, China)
Mouse Beta Secretase and Mouse BDNF kits	Shanghai Korain Biotech Co. Ltd. (Shanghai, China)
Total Protein kit	Erba Mannheim

4.2 Methods

4.2.1 Animal study

NIPER i.e. The National Institute of Pharmaceutical Education and Research, SAS Nagar, Punjab (a breeding facility registered with Committee for the Control and Supervision on Experiments on Animals i.e. CCSEA) provided adult Swiss albino mice (either sex; 20-30 g each). The animals were divided into fifteen groups (n=6) as shown in table 4.4. To reduce stress during transport, the animals were driven in the institutional van. The animals were housed in polypropylene cages which were the right size to allow for comfortable, unrestricted movement and provided safety from harm. To make sure that they had enough food and, more importantly, water throughout the transit, food and water were provided in suitable containers and forms. They were housed in the CAHF i.e. Central Animal House Facility at Lovely Institute of Technology (Pharmacy), Lovely Professional University (Phagwara, Punjab) registered with the CCSEA. The animals were housed in conditions of ambient temperature and humidity with 12-hour cycles of light and darkness. *Ad libitum* availability of water and food was ensured. All procedures were carried out in accordance with CCSEA regulations. The IAEC i.e. Institutional Animal Ethics Committee of Lovely Professional University gave its approval to the animal usage procedures involved in the study vide protocol number LPU/IAEC/2020/75.

4.2.2 Experimental protocol

Table 4.4: Dosing regimen

Group	Treatment
Group 1: Vehicle control (VC)	Vehicle (10 ml/kg; orally, 5 ml/kg; intraperitoneally)
Group 2: Vanillin per se	Normal saline (5 ml/kg; intraperitoneally), Vanillin (40 mg/kg; orally)
Group 3: Magnolol per se	Normal saline (5 ml/kg; intraperitoneally), Magnolol (40 mg/kg; orally)
Group 4: Probiotic per se	Normal saline (5 ml/kg; intraperitoneally), *L. rhamnosus* (10^9 CFU; orally)
Group 5: Disease control (DC)	Aluminium chloride (5 mg/kg; orally), d-galactose (60 mg/kg; intraperitoneally)
Group 6: Standard treatment (DT)	Aluminium chloride (5 mg/kg; orally), d-galactose (60 mg/kg; intraperitoneally), Donepezil (5mg/kg, orally)
Group 7: Vanillin low dose treated group	Aluminium chloride (5 mg/kg; orally), d-galactose (60 mg/kg; intraperitoneally), Vanillin (20 mg/kg; orally)
Group 8: Vanillin high dose treated group	Aluminium chloride (5 mg/kg; orally), d-galactose (60 mg/kg; intraperitoneally), Vanillin (40 mg/kg; orally)
Group 9: Magnolol low dose treated group	Aluminium chloride (5 mg/kg; orally), d-galactose (60 mg/kg; intraperitoneally), Magnolol (20 mg/kg; orally)
Group 10: Magnolol high dose treated group	Aluminium chloride (5 mg/kg; orally), d-galactose (60 mg/kg; intraperitoneally), Magnolol (40 mg/kg; orally)
Group 11: Probiotic treated group	Aluminium chloride (5 mg/kg; orally), d-galactose (60 mg/kg; intraperitoneally), *L. rhamnosus* (10^9 CFU; orally)
Group 12: Vanillin low dose + Magnolol low dose treated group	Aluminium chloride (5 mg/kg; orally), d-galactose (60 mg/kg; intraperitoneally), Vanillin (20 mg/kg; orally) + Magnolol 20 mg/kg; orally)
Group 13: Vanillin low dose + Probiotic treated group	Aluminium chloride (5 mg/kg; orally), d-galactose (60 mg/kg; intraperitoneally), Vanillin (20mg/kg; orally) + *L. rhamnosus* (10^9 CFU; orally)
Group 14: Magnolol + Probiotic treated group	Aluminium chloride (5 mg/kg; orally), d-galactose (60 mg/kg; intraperitoneally), Magnolol (20 mg/kg; orally) + *L. rhamnosus* (10^9 CFU; orally)
Group 15: Vanillin low dose + Magnolol low dose + Probiotic treated group	Aluminium chloride (5 mg/kg; orally), d-galactose (60 mg/kg; intraperitoneally), Vanillin (20 mg/kg; orally) + Magnolol (20 mg/kg; orally) + *L. rhamnosus* (10^9 CFU; orally)

Aluminium chloride, vanillin, donepezil hydrochloride and *L.rhamnosus* were formulated with purified drinking water, respectively, and each was administered orally. D-galactose was formulated into a solution using sterile normal saline as a vehicle and it was administered intraperitoneally. Magnolol was suspended using 0.5% w/v sodium CMC in purified drinking water. The dose of vanillin was chosen as per previously published studies involving neurological disorders like amnesia (Anand et al., 2022) and epilepsy (Almostafa et al., 2024). The dose for magnolol was selected on the basis of previously published study involving neuropathology associated behavioural impairments (Xian et al., 2020). For the probiotic *L.rhamnosus*, the dose was selected on the basis of available literature (Han et al., 2021; Ma et al., 2021).

4.2.3 Evaluation

4.2.3.1 Behavioural evaluation

4.2.3.1.1 Locomotor activity

For evaluating the spontaneous locomotion of each test animal, an actophotometer (manufactured by Inco, India) was utilized. The apparatus comprised of a square-shaped activity chamber, which had a detachable opaque lid to facilitate easy placement and removal of the animals. The base of the activity chamber had twelve holes with photoelectric emitters and receivers installed in close proximity. The sensors were positioned in such a way that any obstruction in at least one of the light beams due to the movement of animal would get registered by the digital meter. Each mouse was placed individually in the activity chamber for 300 seconds. On the 0th and 91st days of treatment, respectively, the locomotor activity of the animals was recorded (Anand et al., 2022; Habibyar et al., 2016; Nazari et al., 2013). 0^{th} day refers to the day before the first day of aluminium chloride and d-galactose administration.

4.2.3.1.2 Transfer latency (TL)

The time that each animal took to traverse from one of the open arms of the elevated plus maze equipment to one of its enclosed arms (using all four paws) was noted as TL. The method, protocol, and endpoint were consistent with previous studies (Anand et al., 2022; Dhingra and Kumar, 2012; Parle et al., 2004). The equipment was made of two enclosed arms (16 cm × 5 cm × 15 cm) and corresponding open arms (16 cm × 5 cm) extending from a centrally located platform (5 cm × 5 cm), placed 25 cm above the ground. In the experiment, every animal was separately positioned at the boundary of an open arm, facing away from the centrally located platform, and TL was recorded on the 90th day of the protocol (acquisition). To assess the retention of memory the procedure was reiterated 24 hours after the acquisition trial. If an animal could not enter the enclosed arm within the 90-second cut-off time during the acquisition phase, it was gently assisted to reach the enclosed arm. After that, it was given 120 seconds to explore the maze before being returned to its home cage. A reduction in TL indicates an improvement in memory.

4.2.3.1.3 Novel Object Recognition (NOR)

The experiment utilizes the natural inclination of rodents for spending more time to explore unfamiliar objects when they are allowed to explore both a familiar and an unfamiliar (novel) object. It took place over three days: the habituation day, training day, and testing day. The methods, procedures, and endpoint were developed based on prior research (Antunes and Biala, 2012; Leger et al., 2013; Lueptow, 2017). The equipment consisted of a black non-porous plastic chamber measuring 40 cm x 40 cm x 40 cm which was divided into nine squares using non-toxic white paint. During the habituation test, each mouse was allowed to explore the activity area for 5 minutes without any objects present. During the training test, two similar unbreakable objects were placed diagonally, and a diffused light of 20 lux illuminated the

center of the chamber. Either of the objects was exchanged with a novel i.e. unfamiliar object during the retention test, which was different in shape and size but similarly complex. The duration during which each animal investigated the new and familiar objects was documented. The test was conducted for 5 minutes for each animal, provided the animal spent at least 20 seconds with each object. If the time spent with either object was less than 20 seconds, the test was continued until the exploration time for each object exceeded 20 seconds. Investigation time was calculated using the following formula:

$$(T_n\text{-}T_f/T_t)*100$$

;where T_n is the time spent in exploring the unfamiliar i.e. novel object, T_f is the time spent in exploring the familiar object, T_t is the total exploration time.

If the investigation time is less than 50%, it indicates that the animal favors the familiar object more. An investigation time equal to 50% means that the animal has an equal preference for both the familiar and novel objects. Finally, if the investigation time is greater than 50%, it signifies that the animal prefers the novel object more.

4.2.3.1.4 Escape Latency (EL)

The EL in the MWM was established as a method to measure the memory of test animals. It is defined as the time it takes for an animal to swim to the hidden platform in the water maze and climb onto it (Morris, 1981). The tendency for rodents to escape from water is generally not influenced by factors like physical movement or body mass, thereby making it apt for various screening models. The methods, protocol, and endpoint of the experiment were based on previous studies (Anand et al., 2022b; Bromley-Brits et al., 2011; Vorhees and Williams, 2014). The equipment used for the MWM comprised of a circular chamber or pool with a radius

of 75 cm and a depth of 50 cm. The inside of the pool was painted black using a non-toxic paint to create a contrast with the colour of the test animals. The pool was segmented into four sections using a thick thread, and spatial cues in the form of high contrast vivid images were placed both inside the pool (above the water level) and in the surrounding room. A fixed location in the pool housed a 10 cm x 10 cm platform, which could move vertically through a channel. The water temperature was maintained at a constant 22°C throughout the experiment.

The training trials for the MWM typically took place over a duration of five days. On the first day, the platform was placed above the water surface and a flag was placed over it to make it visible. The animals were introduced in the pool and were allowed to find the platform. If they were unable to ascend the platform within 60 seconds, they were gently guided to it. For the following four days, the platform was positioned beneath the surface of water and the flag was removed. The animals were placed in the pool from different quadrants, and the time each animal took to find the platform was recorded. On the sixth day, a probe trial was conducted during which the platform was taken out and the animals were placed in the pool. The amount of time that the animals spent in the quadrant where the platform was previously located was recorded. The results of the MWM can be used to evaluate the acquisition of spatial learning and memory of the animals. Animals that were able to reach the quadrant where the platform was located previously and spend more time in that quadrant were considered to have better spatial learning and memory.

4.2.3.2 Biochemical evaluation

At the conclusion of the protocol, all animals were humanely euthanized through decapitation following cervical dislocation. Post the removal of brains, the cortical and hippocampal regions were separated and placed in nearly frozen phosphate buffered saline (PBS) with a pH of 7.4 (10%w/v). The cortex and hippocampus were then homogenized in PBS and the resulting

homogenate was subjected to centifugation at 10,000g for 15 minutes at 4°C in a cooling centrifuge. The supernatant, the liquid portion after centrifugation, was collected and used for measuring TBARS levels (Ohkawa et al., 1979), GSH levels (Beutler et al., 1963), CAT (Aebi, 1984), and AChE (Ellman et al., 1961). The total protein content was determined using the Biuret kit method. All measurements were taken in triplicate.

4.2.3.2.1 Thiobarbituric Acid Reactive Substances (TBARS)

To measure the concentration of TBARS, a mixture of 0.2 mL of tissue homogenate supernatant, 200 µL of sodium dodecyl sulfate, 1500 µL of glacial acetic acid (pH 3.5), and 1500 µL of thiobarbituric acid was prepared. The total volume was then brought to 4000 µL by adding deionized water. The mixture was stored at 95°C for 1 hour. After incubation, the mixture was cooled and 5000 µL of a blend of n-butanol and pyridine (in a 15:1 v/v ratio) was added, followed by an additional 1 mL of deionized water. The mixture was centrifuged for 10 minutes at 4,000g and 4°C. The contents of the tubes developed a pink color, and the intensity of this color was recorded at 532 nm using a double beam UV spectrophotometer. A standard calibration curve was created using serial dilutions of 1,1,3,3-tetramethoxypropane. The concentration of TBARS was reported as nM/mg of protein.

4.2.3.2.2 Reduced glutathione (GSH)

To measure the concentration of glutathione (GSH), a mixture of 300 µL of supernatant from the tissue homogenate and 300 µL of 10% w/v ice-cold trichloroacetic acid was prepared. The mixture was then subjected to centrifugation at 1,000g for 10 minutes at 4°C using a cooling centrifuge. The supernatant was then poured into a new test tube and blended with 2000 µL of 0.3M disodium hydrogen phosphate and 500 µL of the supernatant. The test tube was filled with a freshly prepared 0.001M solution of [5,5-dithiobis(2-nitrobenzoic acid)] (DTNB) in 250

μL of sodium citrate (1% w/v).. The absorbance of the mixture was measured at 412 nm using a double beam UV spectrophotometer. A standard plot was created using serial dilutions of glutathione ranging from 10-100 μM. The concentration of GSH was reported as μM/mg of protein.

4.2.3.2.3 Catalase activity (CAT)

To assess catalase activity, a blend was created by combining 50 μL of tissue homogenate supernatant, 1950 μL of 50 mM phosphate buffer with a pH of 7, and 1000 μL of 30 mM hydrogen peroxide within a cuvette. The absorbance of the final solution was recorded at 240 nm using a double beam UV spectrophotometer at intervals of 15 seconds for a duration of 30 seconds. The following formula was utilized to calculate CAT:

$$\text{Catalase activity} = \delta \text{ O.D.} / \varepsilon \times V \times \text{protein content (in mg)}$$

;where δ O.D. = change in the absorbance per minute, ε = hydrogen peroxide's extinction coefficient (0.071 mM $^{cm-1}$), V = the volume of the sample in mL.

4.2.3.2.4 Activity of Acetylcholinesterase (AChE)

A colorimetric assay was used to assess the activity of the enzyme AChE. The test evaluates the rate at which thiocholine is produced from acetylcholine halide when AChE is present. Thiocholine reacts with dithiobisnitrobenzoate ions to form a yellow-colored product. The absorbance of the yellow product is recorded at 420 nm using a double beam UV spectrophotometer. The assay was performed by mixing 100 μL of tissue homogenate supernatant with a freshly prepared solution of DTNB (0.1 mg/mL) in pH 8 Sorenson's phosphate buffer. A separate test tube containing donepezil hydrochloride was used as a blank. The shift in absorbance per minute was measured at 420 nm at one-minute intervals over a

period of two minutes. The rate of thiocholine development is proportional to the activity of AChE. The following formula was used to calculate the AChE activity:

$$R = \delta \text{ O.D.} \times V / \varepsilon \times \text{mg of protein}$$

;where R = rate of enzymatic activity in 'n' mole of acetylcholine halide hydrolysed per minute per mg of protein, δ O.D. = change in absorbance per minute and ε = the extinction coefficient (13,600/M/cm).

4.2.3.2.5 Total protein

The total protein content of all samples was evaluated using the biuret method, according to the instructions provided in the package insert of the total protein estimation kit by Erba Mannheim. The total protein was calculated using the following formula:

Total protein (g/dL)= (Absorbance of test/Absorbance of standard) x Concentration of standard (g/dL).

4.2.3.3 ELISA

All assays were based on double enzyme sandwich method of assay.

4.2.3.3.1 Beta secretase

Beta secretase has become a coveted target for screening of potential anti-AD drugs. It is an enzyme which cleaves amyloid precursor protein and begins the production of Aβ (Vassar et al., 2009). Beta secretase inhibitory activity is highly desirable for any potential anti-AD drug to ensue a disease modifying effect.

All the reagents in the package were allowed to attain room temperature and the assay was performed at room temperature. Serial dilutions were made by reconstituting the standard (1600 pg/mL) with the supplied standard diluent. The plain standard diluent served as the zero standard (0 pg/mL). 40 µL sample was added to each well of the 96 well plate and then 10 µL of anti-beta secretase antibody was added to each sample well. The anti-beta secretase antibody was not added to the standard wells as the standard solution contained biotinylated antibody. Further, 50 µL of streptavidin-horseradish peroxidase was added to all wells except the blank control well. The plate was sealed and incubated at 37°C for 60 minutes. Then the plate was unsealed and rinsed 5 times with the supplied wash buffer (0.35 mL of wash buffer with a contact time of 30-60 seconds). 50 µL of substrate solutions A and B were poured into each well, sequentially. The plate was then sealed with a fresh supplied sealer and incubated in dark at 37°C for 10 minutes. 50 µL of stop solution was poured into each well turning the blue colour into yellow instantly. Optical density (OD) of each well was recorded immediately (within 10 minutes of adding the stop solution) at 450 nm using a microplate reader. Individual values for each well were calculated by using the standard plot (Bioassay Technology Laboratory, 2022a).

4.2.3.3.2 Caspase-3

Caspase-3 mediates apoptotic cell death which in turn does not only lead to neurodegeneration but also aids the progression of the underlying pathological mechanisms of AD. Several studies have reported synaptic increase in caspase-3 levels in AD patients. Inhibition of caspase-3 enzyme can be seen as a bright new avenue for research on anti-AD drug development (D'Amelio et al., 2010; Louneva et al., 2008; Rohn, 2010).

The contents of the kit were brought to room temperature before use. The vial containing the standard solution was centrifuged at 6,000-10,000 rpm for 30 seconds. The

given standard solution was reconstituted with 1 mL of sample diluent to prepare a stock solution of 20 ng/mL concentration. Serial dilutions were made by adding sample diluent to the stock solution in various proportions as per the procedure mentioned in the kit's literature. Sample diluent alone served as the zero standard (0 ng/mL). 100 µL of standard and sample was poured into the designated wells of the 96 well plate, respectively. The plate was sealed with the supplied adhesive sealing sheet and it was incubated at 37°C for 120 minutes. Later, the liquid was removed from all wells (without washing) and 100 µL of biotin antibody (1x) was introduced to each well. The plate was again sealed with a fresh adhesive sheet supplied in the package and it was allowed to incubate at 37°C for 1 hour. After the incubation was complete, each well was aspirated and washed thrice by filling each well with 200 µL of wash buffer and providing a contact time of 120 seconds. 100 µL of horseradish peroxidase-avidin (1x) was introduced to each well and the plate was sealed with a fresh adhesive sheet and it was allowed to incubate at 37°C for 1 hour. 90 µL of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was introduced to each well and the plate was lightly tapped to ensure complete mixing and removal of any air bubbles. It was incubated at 37°C for 15-30 minutes in dark. 50 µL of stop solution was poured into each well and the OD of each well was recorded within 5 minutes at 450 nm and 540 nm using a microplate reader. The OD at 540 nm were subtracted from those at 450 nm to correct for optical imperfections in the plate. Individual values for each well were calculated by using the standard plot (CUSABIO, 2022).

4.2.3.3.3 $A\beta_{1-42}$

Accumulation of $A\beta_{1-42}$ in brain leads to disruption of synaptic plasticity which further deteriorates memory function (Izzo et al., 2014; Sadigh-Eteghad et al., 2015). Aβ accumulation also leads to mitochondrial dysfunction which further leads to the evolution of

AD (Anand et al., 2017). The anti Aβ aggregatory activity is highly desirable for any potential anti-AD drug.

The contents of the kit were brought to room temperature before use. The vial containing the standard solution was centrifuged at 6,000-10,000 rpm for 30 seconds. The given standard solution was reconstituted with 1 mL of sample diluent to prepare a stock solution of 1000 pg/mL concentration. Serial dilutions were made by adding sample diluent to the stock solution in various proportions as per the procedure mentioned in the kit's literature. Sample diluent alone served as the zero standard (0 pg/mL). 100 µL of standard and sample was introduced to the designated wells of the 96 well plate, respectively. The plate was sealed with the supplied adhesive sealing sheet and it was allowed to incubate at 37^{o}C for 120 minutes. Later, the liquid was removed from all wells (without washing) and 100 µL of biotin antibody (1x) was introduced to each well. The plate was again sealed with a fresh adhesive sheet supplied in the package and it was allowed to incubate at 37^{o}C for 1 hour. After the incubation was complete, each well was aspirated and washed thrice by filling each well with 200 µL of wash buffer and providing a contact time of 120 seconds. 100 µL of horseradish peroxidase-avidin (1x) was introduced to each well and the plate was sealed with a fresh adhesive sheet and it was allowed to incubate at 37^{o}C for 1 hour. 90 µL of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was introduced to each well and the plate was lightly tapped to ensure complete mixing and removal of any air bubbles. It was incubated at 37^{o}C for 15-30 minutes in dark. 50 µL of stop solution was poured into each well and the OD of each well was recorded within 5 minutes at 450 nm and 540 nm using a microplate reader. The OD at 540 nm were subtracted from those at 450 nm to correct for optical imperfections in the plate. Individual values for each well were determined by using the standard plot (CUSABIO, 2022b)

4.2.3.3.4 Brain Derived Neurotrophic Factor (BDNF)

BDNF plays a pivotal role in the maintenance of adult cortical neurons the early dysfunction of which leads to the initial short-term memory deficits in AD. A deficiency of BDNF contributes to the shaping of neurodegeneration in AD. If any potential anti-AD agent can manipulate BDNF, it can be a valuable addition to the therapeutic approaches (Giuffrida et al., 2018; Lu et al., 2014).

All the reagents in the package were allowed to attain the room temperature and the assay was performed at room temperature. Serial dilutions were made by reconstituting the standard (12.8 ng/mL) with the supplied standard diluent. The plain standard diluent served as the zero standard (0 ng/mL). 40 µL sample was added to each well of the 96 well plate and then 10 µL of anti-beta secretase antibody was added to each sample well. The anti-beta secretase antibody was not added to the standard wells as the standard solution contained biotinylated antibody. Further, 50 µL of streptavidin-horseradish peroxidase was added to all wells except the blank control well. The plate was sealed and incubated at 37^{o}C for 60 minutes. Then the plate was unsealed and rinsed 5 times with the supplied wash buffer (0.35 mL of wash buffer with a contact time of 30-60 seconds). 50 µL of substrate solutions A and B were introduced to each well, sequentially. The plate was again sealed with a fresh supplied sealer and incubated in dark at 37^{o}C for 10 minutes. 50 µL of stop solution was poured into each well turning the blue colour into yellow instantly. OD of each well was recorded immediately (within 10 minutes of adding the stop solution) at 450 nm using a microplate reader. Individual values for each well were calculated by using the standard plot(Bioassay Technology Laboratory, 2022b).

4.2.3.4 Statistical analysis

The results have been reported as mean ± SEM. Locomotor activity and EL (Acquisition trials) were analysed using a two-way analysis of variance (ANOVA) test. All other behavioural tests, biochemical assays and results ELISA were analysed using a one-way ANOVA test. The statistical significance of the results was taken into consideration at $p<.001$, $p<.01$and $p<.05$ using Tukey's test. The statistical analyses were conducted with the help of GraphPad Prism v 9.4.1.

4.2.3.5 Histopathological analysis

The examination of the cerebral cortical and hippocampal regions of the mice's brain involved histopathological analysis, employing hematoxylin and eosin staining. TS of the cerebral cortex and hippocampus, each with a thickness ranging from 7 to 9 millimeters, were initially preserved in formal calcium and subsequently embedded in paraffin wax. Following this, the sections were sliced, subjected to dewaxing in xylene, and underwent a process of hydration involving decreasing concentrations of alcohol. They were then stained with hematoxylin, dehydrated through a gradual increase in alcohol concentration up to 70%, and finally stained with a 1% alcoholic eosin solution. Subsequently, the sections were differentiated in 90% alcohol and cleared in xylene. The stained transverse sections were later examined under a microscope (specifically, the Zeiss Primo star) for the purpose of conducting histopathological evaluations (Kumar et al., 2021).

CHAPTER-5

RESULTS AND DISCUSSION

5. RESULTS

***, ** and * have been used to represent p < .001, p < .01 and p < .05 in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify p < .001, p < .01 and p < .05, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent p < .001, p < .01 and p < .05, respectively.

5.1 Vanillin

5.1.1 Behavioural evaluation

5.1.1.1 Locomotor activity

Figure 5.1 shows how vanillin and other interventions had an impact on locomotor activity.

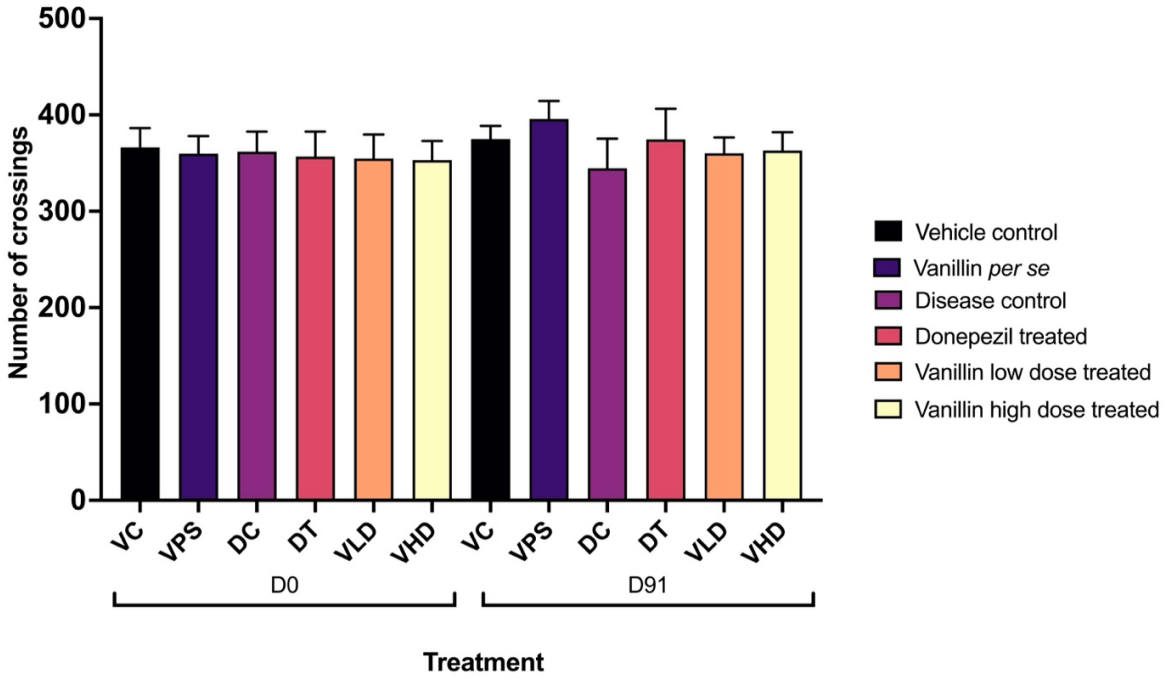


Fig. 5.1: Impact of vanillin and other interventions on locomotor activity expressed as mean ± SEM.

There was no consequential difference found between the number of crossings between all groups on both 0th day and 91st days of the protocol, respectively. Also, no consequential difference was found between the number of crossings between the groups on 91st day with the same groups on 0th day.

5.1.1.2 Transfer latency

Figure 5.2 illustrates how vanillin and other interventions influenced the transfer latency of animals in various groups, in the present study.

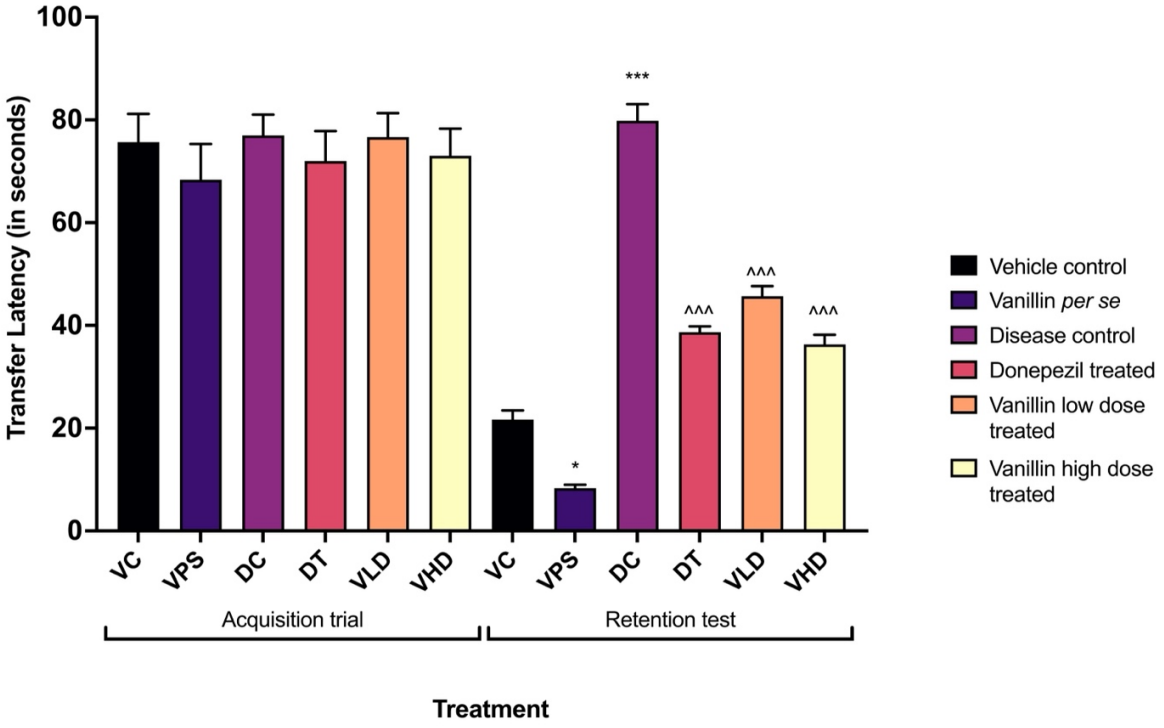


Fig.5.2: Impact of vanillin and other interventions on transfer latency expressed as mean ± SEM. ***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

Vanillin *per se* remarkably (p<.05) reduced the TL thereby indicating towards its nootropic activity. TL was remarkably (p<.001) higher in animals belonging to DC group contrasted with the VC group. All treatments i.e. donepezil, vanillin low dose and vanillin high dose remarkably (p<.001) decreased TL in comparison with the DC group. However, no consequential difference was found between donepezil treated group and either of the vanillin treated groups.

5.1.1.3 Investigation time (in NOR)

Figure 5.3 shows the effect that vanillin and other interventions had on the investigation time in NOR test.

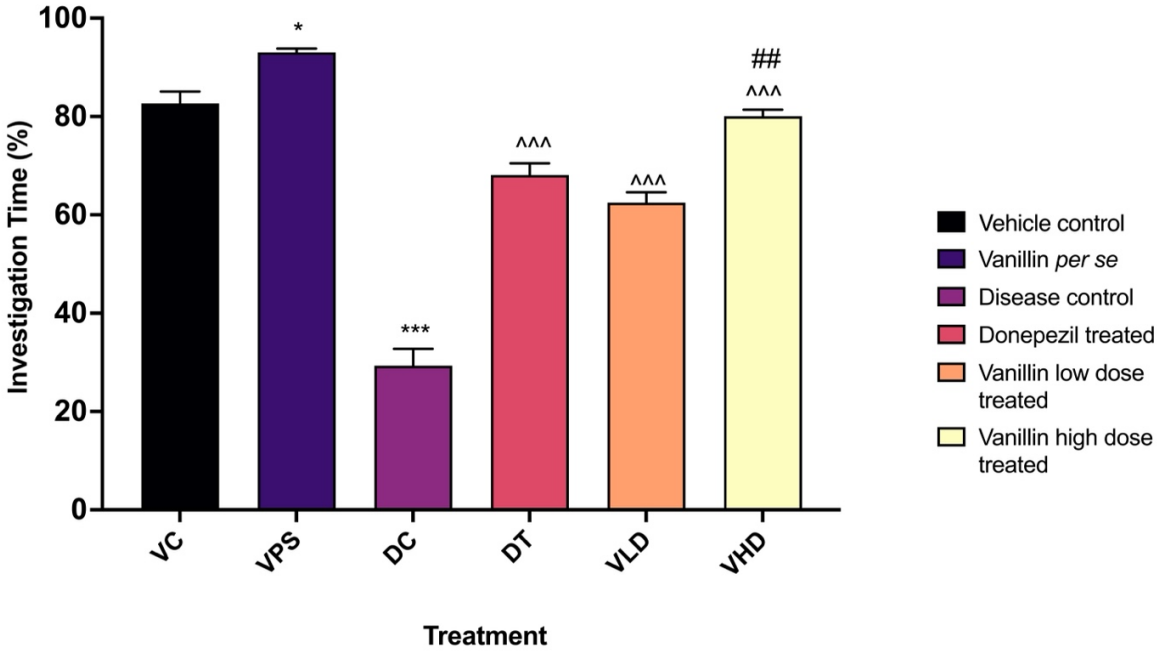


Fig.5.3: Impact of vanillin and other interventions on investigation time expressed as mean ± SEM. ***, ** and * have been used to represent p < .001, p < .01 and p < .05 in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify p < .001, p < .01 and p < .05, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent p < .001, p < .01 and p < .05, respectively.

In NOR paradigm, the vanillin *per se* group was found to remarkably ($p<.05$) increase the investigation time thereby indicating its nootropic effect. The investigation time was remarkably ($p<.001$) lesser for animals belonging to DC group in comparison with those in the VC group. All treatments i.e. donepezil, vanillin low dose and vanillin high dose remarkably ($p<.001$) increased investigation time in comparison with the DC group. Vanillin high dose treated group was found to have a remarkably ($p<.01$) increased investigation time in comparison with the donepezil treated group.

5.1.1.4 Escape latency

Impact of vanillin and other interventions on acquisition time in MWM paradigm has been illustrated in figure 5.4 and that in the probe trial is illustrated in figure 5.5.

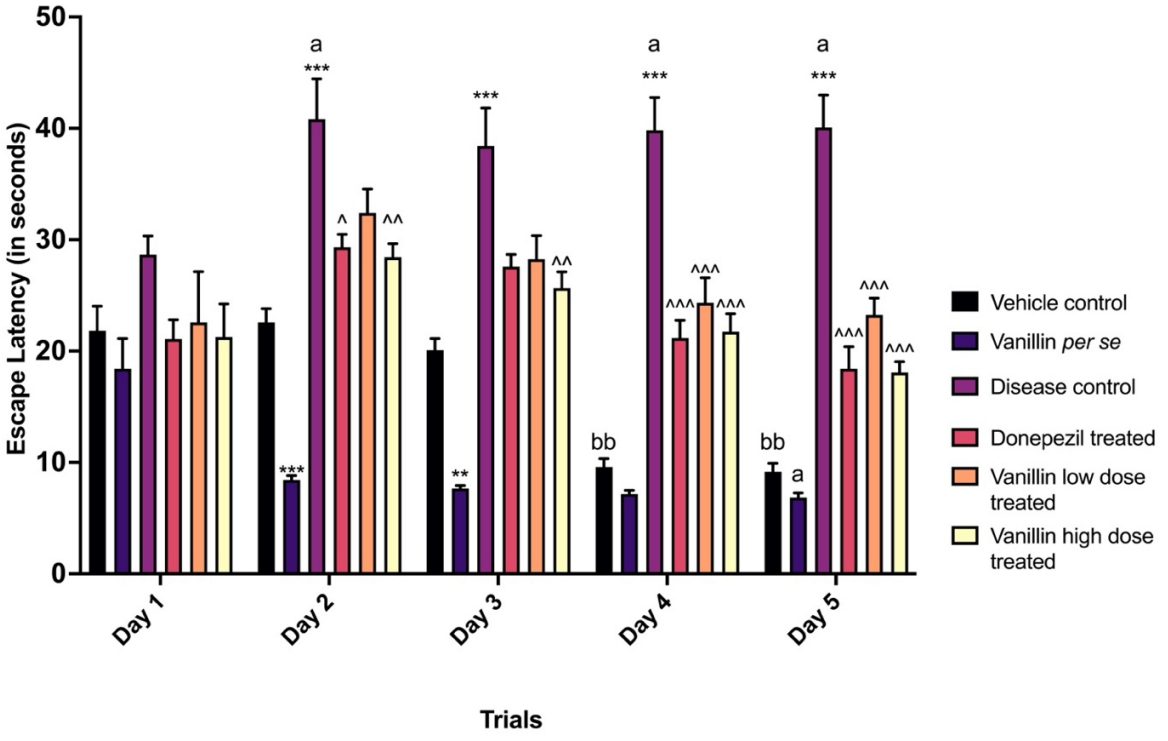


Fig. 5.4: Impact of vanillin and other interventions on escape latency during acquisition trials represented as mean ± SEM. ***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as

^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively. a represents $p < .05$ with respect to same group on day 1. bb represents $p < .01$ with respect to the same group on day 2.

On 1st day of the trials, there was no consequential difference between the EL of any group. On the 2nd day of the trials, vanillin *per se* group exhibited a remarkable ($p<.001$) reduction in EL in comparison with the group that received only the vehicle. The animals belonging to the DC group had a remarkably ($p<.001$) higher EL than those belonging to the VC group and a remarkably ($p < .05$) higher EL than the same group on 1st day. Donepezil treated and vanillin high dose treated groups exhibited a consequential ($p<.05$; $p<.01$) reduction in EL contrasted with the DC group. The group that received vanillin low dose demonstrated a reduction in the EL contrasted with the DC group but the difference was not statistically consequential. On the 3rd day of the trials, vanillin *per se* administration led to a remarkable ($p<.01$) reduction in EL in comparison with the VC group. The EL shown by DC group was remarkably ($p<.001$) higher than the group that received only the vehicle. Donepezil treatment and vanillin low dose treatment led to a reduction in the EL contrasted with the DC group, however, the difference was not statistically consequential. Vanillin high dose treatment group had a remarkable ($p<.01$) reduction in EL in comparison with the DC group. On the 4th day, the EL of the group which received only the vehicle remarkably ($p<.01$) decreased in comparison with the same group on 2nd day, however, it did not differ remarkably in comparison with the vanillin *per se* treatment on the same day. DC group had a remarkably ($p<.001$) higher EL than the group which received only the vehicle and a remarkably ($p<.05$) higher EL than its counterpart on 1st day. All treatments i.e. donepezil, vanillin low dose and vanillin high dose led to a remarkable ($p<.001$) reduction in the EL in comparison with the DC group. On 5th day, vanillin *per se* treated group exhibited a remarkably ($p<.05$) decreased EL than the same group on 1st day. Other results were similar to as that obtained on the 4th day.

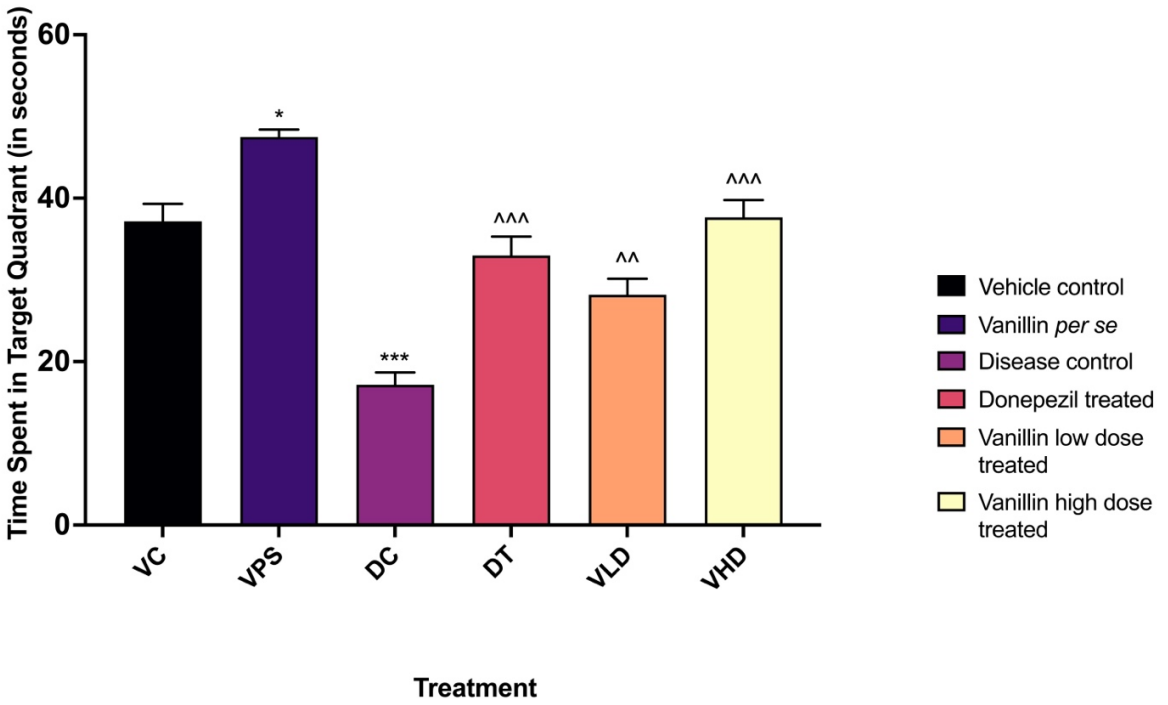


Fig. 5.5: Impact of vanillin and other interventions on the time spent in target quadrant (in the probe trial) expressed as mean ± SEM. ***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

Vanillin *per se* administration led to a remarkably ($p<.05$) higher time spent in the target quadrant than the VC group. DC group had a remarkably ($p<.001$) decreased time spent in the target quadrant than the VC group. Donepezil and vanillin high dose treatment, respectively, led to consequential ($p<.001$) increase in time spent in the target quadrant than the DC group. Vanillin low dose treatment group exhibited a remarkably ($p<.01$) higher in time spent in the target quadrant than the DC group. There was no statistically consequential difference found between time spent in the target quadrant for donepezil treated group and either of the vanillin treated groups.

5.1.2 Biochemical evaluation

5.1.2.1 TBARS

Figure 5.6 shows the levels of hippocampal and cortical TBARS in animals receiving vanillin and other interventions.

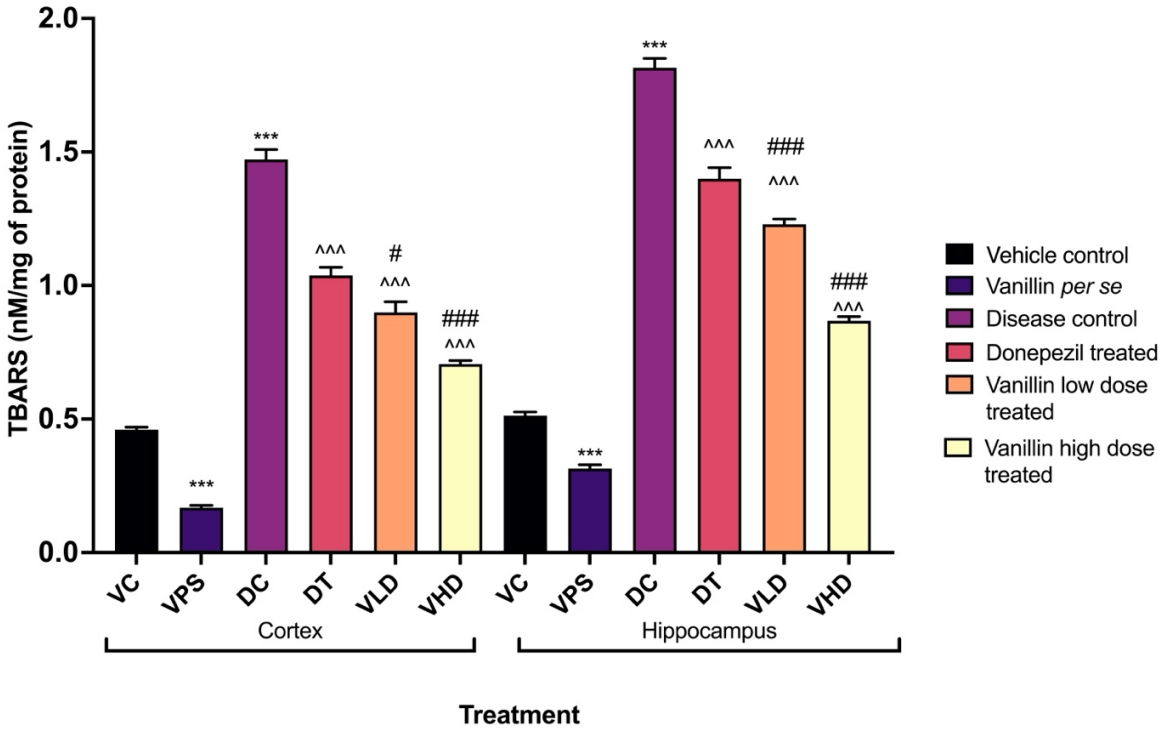


Fig. 5.6: Impact of vanillin and other interventions on TBARS expressed as mean ± SEM.

***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

In cortex, vanillin *per se* group exhibited a remarkable ($p<.001$) reduction in content of TBARS. DC group had a remarkably ($p<.001$) increased TBARS content in comparison with the VC group. All treatments i.e. donepezil, vanillin low dose and vanillin high dose exhibited a remarkable ($p<.001$) diminution in level of TBARS in comparison with the DC group. Vanillin low dose treatment remarkably ($p<.05$) decreased the TBARS level in comparison

with the donepezil treated group and vanillin high dose treated group exhibited a remarkable (p<.001) reduction in the content of TBARS in comparison with the donepezil treated group. Similar results were obtained in the hippocampus. However, in the hippocampal region, both vanillin low dose and high dose treated groups exhibited a remarkable (p<.001) reduction in the content of TBARS in comparison with the donepezil treated group.

5.1.2.2 GSH

Figure 5.7 illustrates the levels of GSH in cortex and hippocampus of test animals assigned to various treatment groups including vanillin treatment groups.

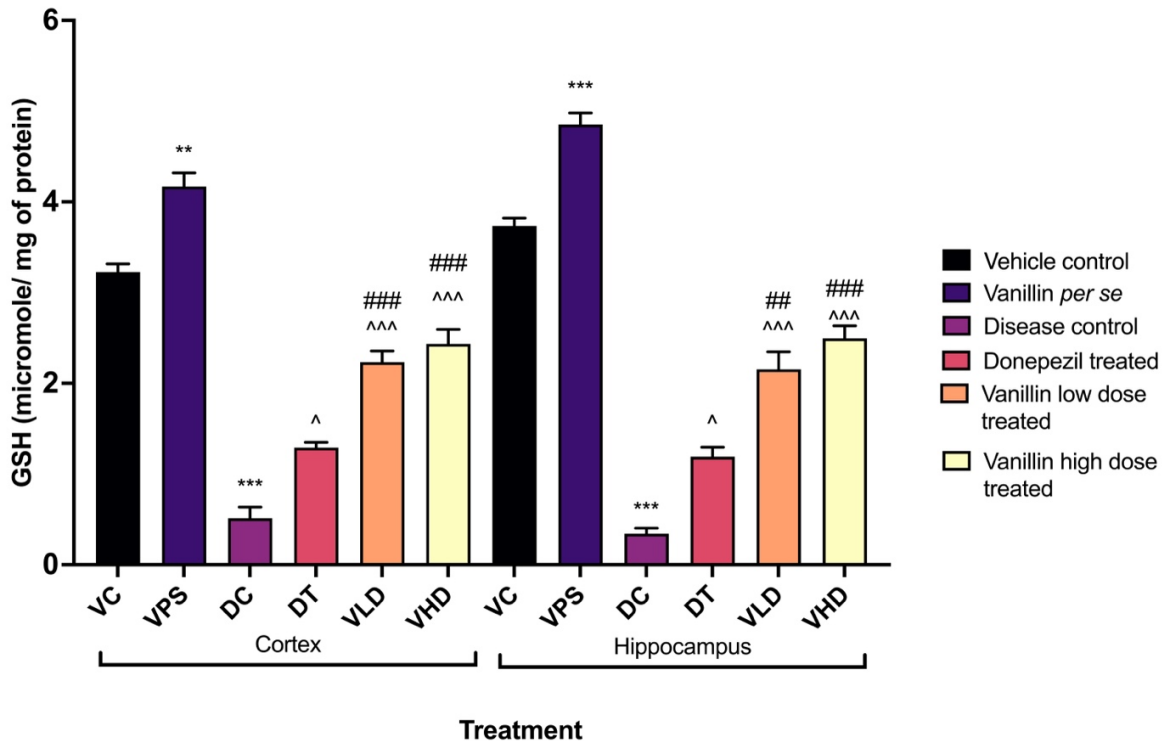


Fig. 5.7: Impact of vanillin and other interventions on GSH expressed as mean ± SEM.

***, ** and * have been used to represent p < .001, p < .01 and p < .05 in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify p < .001, p < .01 and p < .05, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent p < .001, p < .01 and p < .05, respectively.

In cortex, vanillin *per se* treatment exhibited a remarkably ($p<.01$) higher GSH in comparison with the VC group. For the DC group, a remarkable ($p<.001$) reduction in GSH was noted with respect to the VC group. While donepezil treatment remarkably ($p<.05$) increased GSH, both vanillin low dose and high dose treated groups exhibited a remarkable ($p<.001$) increase in GSH, in comparison with the DC group. The increase in GSH for both vanillin treated groups i.e. vanillin low dose and vanillin high dose groups was remarkably ($p<.001$) higher than observed in donepezil treated group. In hippocampus, vanillin *per se* treatment led to a remarkable ($p<.001$) increase in GSH in comparison with the VC group. The reduction in GSH for DC group was consequential ($p<.001$) contrasted with the VC group. Donepezil treatment increased the level of GSH remarkably ($p<.05$) than the DC group. Both vanillin low dose and high dose treated groups were observed to remarkably ($p<.001$) increase the levels of GSH, in comparison with the DC group. In comparison with the donepezil treated group, the rise in GSH was consequential for both vanillin low dose treatment group ($p<.01$) and vanillin high dose treatment group ($p<.001$).

5.1.2.3 CAT

Figure 5.8 shows CAT in cortical and hippocampal regions of brain in animals assigned to various treatment groups including vanillin treatment groups.

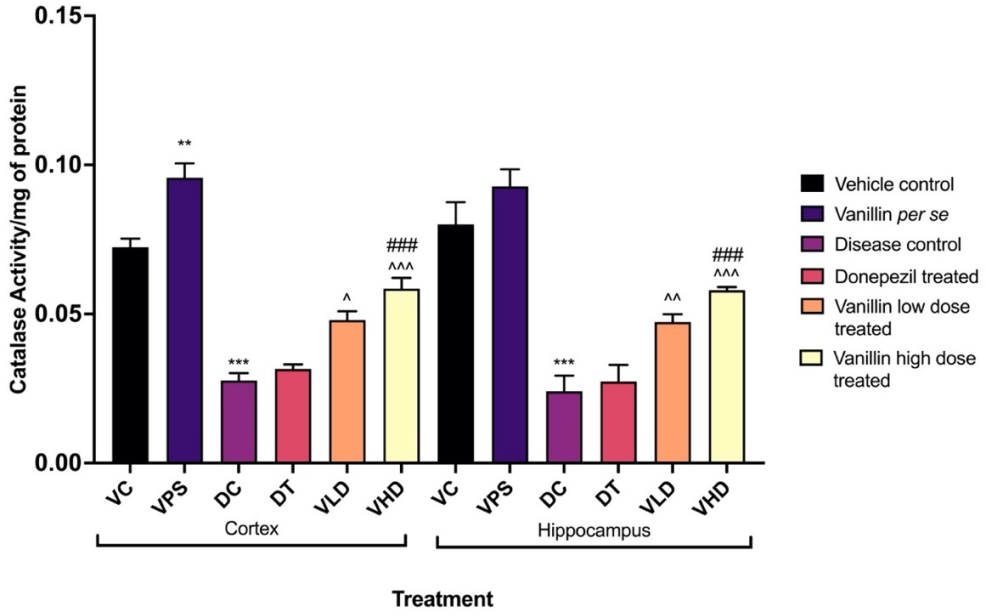


Fig. 5.8: Impact of vanillin and other interventions on CAT expressed as mean ± SEM.

***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

In cortex, there was a remarkable ($p<.01$) rise in the CAT for vanillin *per se* group in comparison with the group that received only the vehicle. The DC group had a remarkably ($p<.001$) lower CAT with respect to the group that received only the vehicle. Donepezil treatment increased the CAT contrasted with the DC group but it was not statistically consequential. Vanillin low dose treatment led to a remarkable ($p<.05$) rise in CAT while vanillin high dose led to a remarkable ($p<.001$) increase in CAT, in comparison with the DC group. Vanillin high dose treated group exhibited a remarkable ($p<.001$) rise in CAT with respect to the donepezil treated group. In hippocampus, vanillin *per se* treatment increased the

CAT but it was not statistically consequential. The CAT was remarkably (p<.001) lower in DC group than the VC group. Treatment with donepezil led to a statistically inconsequential increase in CAT when contrasted with the DC group. Treatment with low dose of vanillin elevated the CAT remarkably (p<.01) and vanillin high dose treatment led to a remarkable (p<.001) rise in the CAT, in comparison with the DC group. In comparison with the donepezil treated group, the vanillin high dose treated group was observed to have a remarkably (p<.001) higher CAT.

5.1.2.4 AChE

Figure 5.9 gives an illustration of cortical and hippocampal AChE activity in mice receiving vanillin and other interventions.

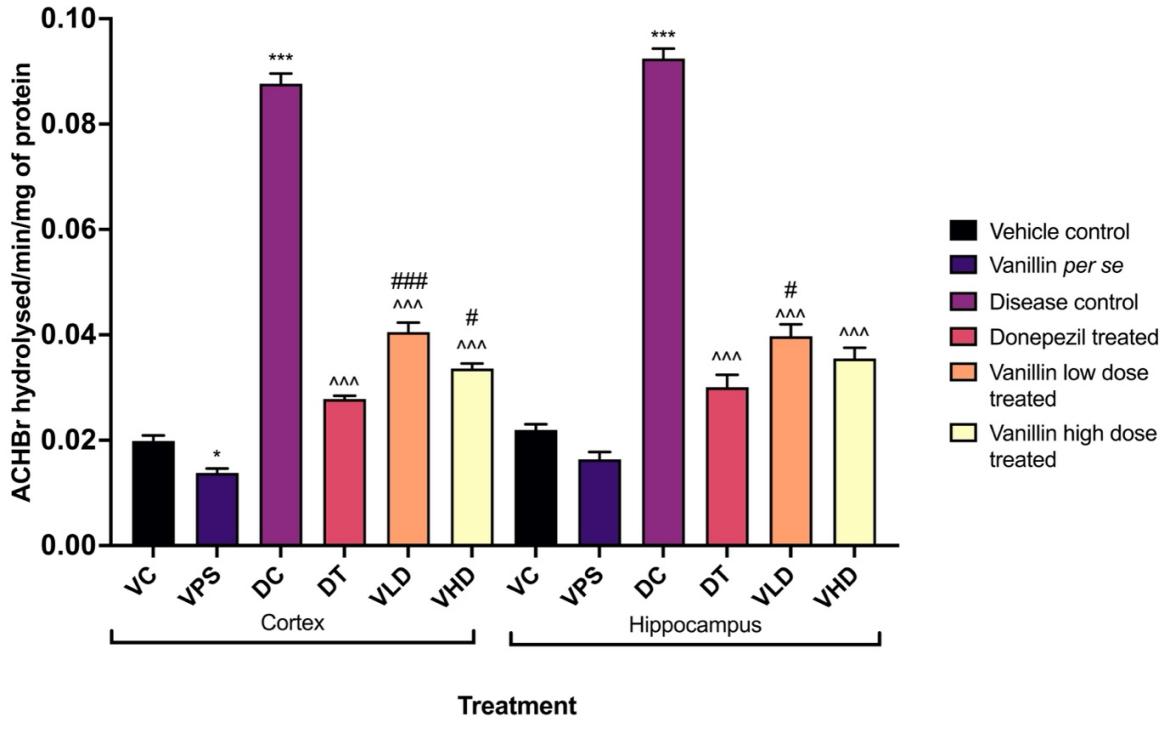


Fig. 5.9: Impact of vanillin and other interventions on AChE expressed as mean ± SEM.

***, ** and * have been used to represent p < .001, p < .01 and p < .05 in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify p < .001, p <

.01 and p < .05, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent p < .001, p < .01 and p < .05, respectively.

In cortex, vanillin *per se* treatment remarkably (p<.05) inhibited the activity of AChE in comparison with group that received only the vehicle. The AChE activity was remarkably (p<.001) higher in the DC group than the VC group. Donepezil treatment remarkably (p < .001) decreased the activity of AChE when contrasted with the DC group. Vanillin low dose treatment exhibited a remarkable (p<.001) reduction in activity of AChE in comparison with the DC group but it was remarkably (p<.001) higher than donepezil treated group. Vanillin high dose treatment led to a remarkable (p<.001) fall in activity of AChE than the DC group but it was remarkably (p<.05) higher than the donepezil treated group. In the hippocampus, vanillin *per se* exhibited a statistically inconsequential diminution in AChE activity than the VC group. DC group was observed to have a remarkable (p<.001) increase in AChE activity when compared with the VC group. All treatments i.e. donepezil, vanillin low dose and vanillin high dose remarkably (p<.001) decreased the activity of AChE when contrasted with the DC group. While vanillin low dose treatment group had a remarkably (p<.05) higher AChE activity than the donepezil treated group, vanillin high dose treatment had a comparable AChE activity with respect to the donepezil treated group.

5.1.3 ELISA

5.1.3.1 Beta secretase

Impact of vanillin and other interventions on beta secretase has been illustrated in figure 5.10.

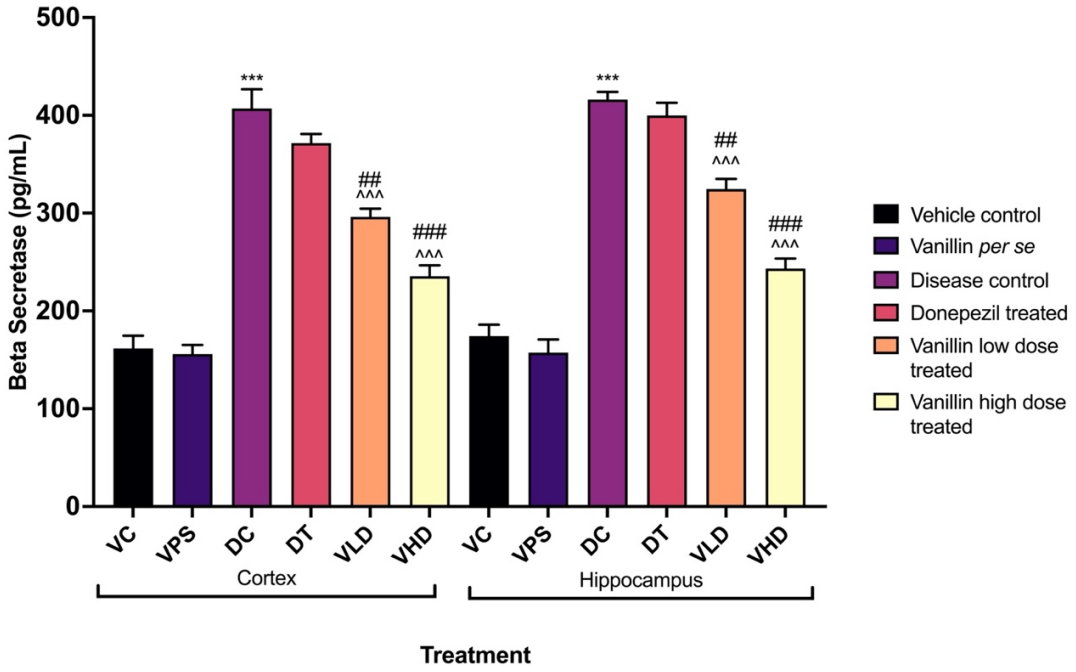


Fig. 5.10: Impact of vanillin and other interventions on beta secretase expressed as mean ± SEM. ***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

Similar results were obtained in cortex and hippocampus. Vanillin *per se* treatment exhibited a statistically inconsequential reduction in beta secretase activity. In DC group, beta secretase was remarkably ($p<.001$) higher than the group which received only the vehicle. While donepezil treatment was observed to have a statistically inconsequential reduction in beta secretase, both vanillin treated groups i.e. vanillin low dose treated and vanillin high dose treated groups exhibited a consequential ($p<.001$) reduction in beta secretase. Vanillin low dose treatment remarkably ($p<.01$) decreased beta secretase than the donepezil treated group.

Vanillin high dose treatment remarkably (p<.001) reduced the beta secretase in comparison with the donepezil treated group.

5.1.3.2 Caspase-3

Impact of vanillin and other interventions on caspase-3 has been illustrated in figure 5.11.

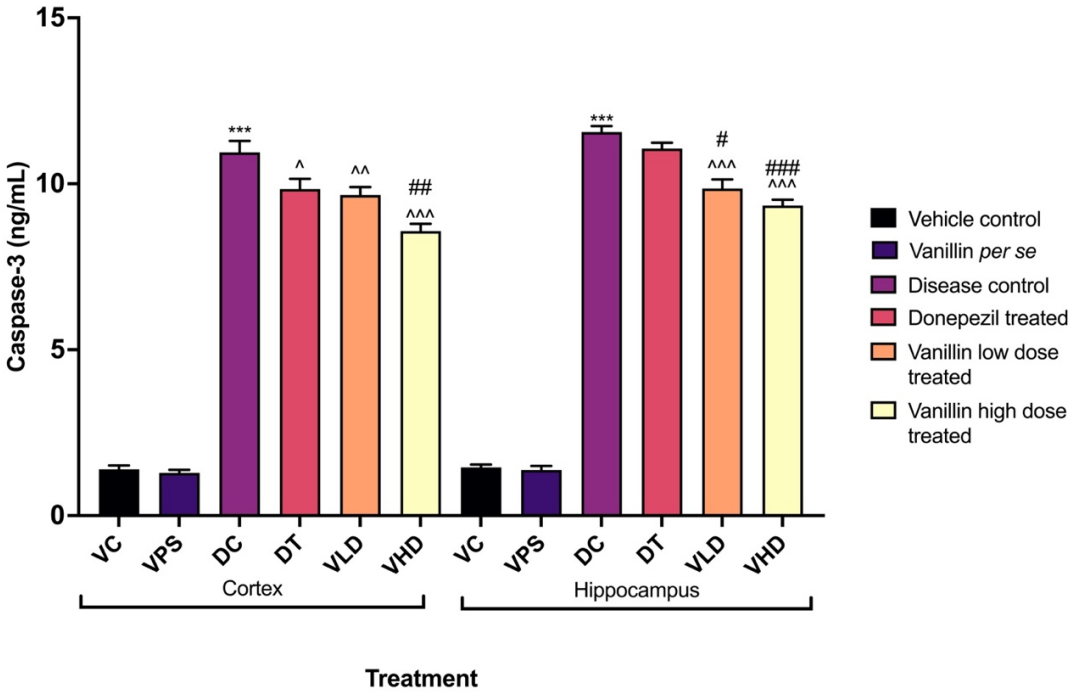


Fig. 5.11: Impact of vanillin and other interventions on caspase-3 expressed as mean ± SEM. ***, ** and * have been used to represent p < .001, p < .01 and p < .05 in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify p < .001, p < .01 and p < .05, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent p < .001, p < .01 and p < .05, respectively.

In cortex, vanillin *per se* treatment led to a statistically inconsequential reduction in caspase-3 level when contrasted with the VC group. The DC group exhibited a remarkably (p<.001) higher caspase-3 level than the VC group. Donepezil treatment led to a remarkable (p<.05) fall in the caspase-3 level with respect to the DC group. Vanillin low dose treatment was observed

to have a consequential (p<.01) diminishing effect on caspase-3 level while vanillin high dose treatment also exhibited a remarkable (p<.001) fall in caspase-3 level. In comparison with the donepezil treated group, the vanillin high dose treatment was able to reduction caspase-3 level remarkably (p<.01). In hippocampus, vanillin *per se* treatment decreased the level of caspase-3 in comparison with the VC group but it was not statistically consequential. Donepezil treatment led to a statistically inconsequential reduction in caspase-3 level. Both vanillin treated groups i.e. vanillin low dose treated and vanillin high dose treated groups exhibited a consequential (p<.001) reduction in level of caspase-3 contrasted with the DC group. In comparison with the donepezil treated group, vanillin low dose treatment remarkably (p<.05) reduced the level of caspase-3 while vanillin high dose treated group exhibited a remarkable (p<.001) fall in the level of caspase-3.

5.1.3.3 $A\beta_{1\text{-}42}$

Impact of vanillin and other interventions on $A\beta_{1\text{-}42}$ has been illustrated in figure 5.12.

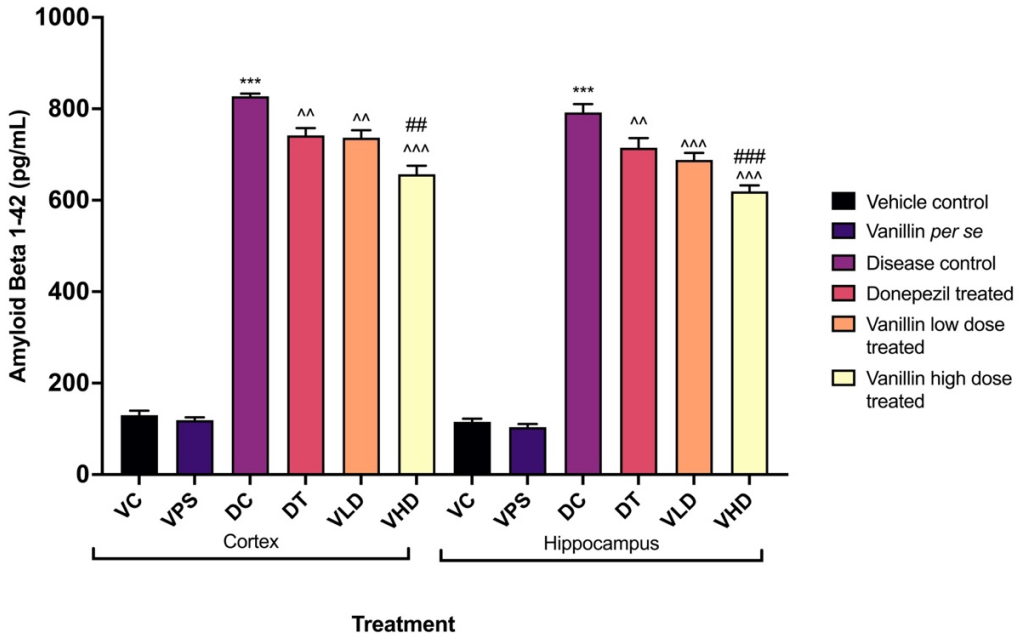


Fig. 5.12: Impact of vanillin and other interventions on $A\beta_{1\text{-}42}$ expressed as mean ± SEM.

***, ** and * have been used to represent p < .001, p < .01 and p < .05 in comparison with the VC group,

respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

In cortex, vanillin *per se* treatment reduced the $A\beta_{1-42}$ level when contrasted with the group which received only the vehicle but it was not statistically consequential. DC group exhibited a remarkable ($p<.001$) increase in the $A\beta_{1-42}$ level when contrasted with the group which received only the vehicle. Treatment with donepezil and vanillin low dose remarkably ($p<.01$) reduced the level of $A\beta_{1-42}$ when contrasted with the DC group, respectively. Vanillin high dose treatment reduced the level of $A\beta_{1-42}$ remarkably ($p<.001$when contrasted with the DC group. The level of $A\beta_{1-42}$ observed in vanillin high dose treated group was remarkably ($p<.01$) lower than the donepezil treated group. In hippocampus, vanillin *per se* treatment led to a statistically inconsequential reduction in level of $A\beta_{1-42}$ when contrasted with the group that received only the vehicle. $A\beta_{1-42}$was remarkably ($p<.001$) increased in the DC group than the VC group. Donepezil treatment led to a remarkable ($p<.01$) fall in the $A\beta_{1-42}$ level when contrasted with the DC group. Both vanillin treated groups i.e. vanillin low dose and vanillin high dose groups remarkably ($p<.001$) reduced the level of $A\beta_{1-42}$ when contrasted with the DC group, respectively. Vanillin high dose treatment was observed to reduce the level of $A\beta_{1-42}$remarkably ($p<.001$) in comparison with the donepezil treated group.

5.1.3.4 BDNF

Impact of vanillin and other interventions on BDNF has been illustrated in figure 5.13.

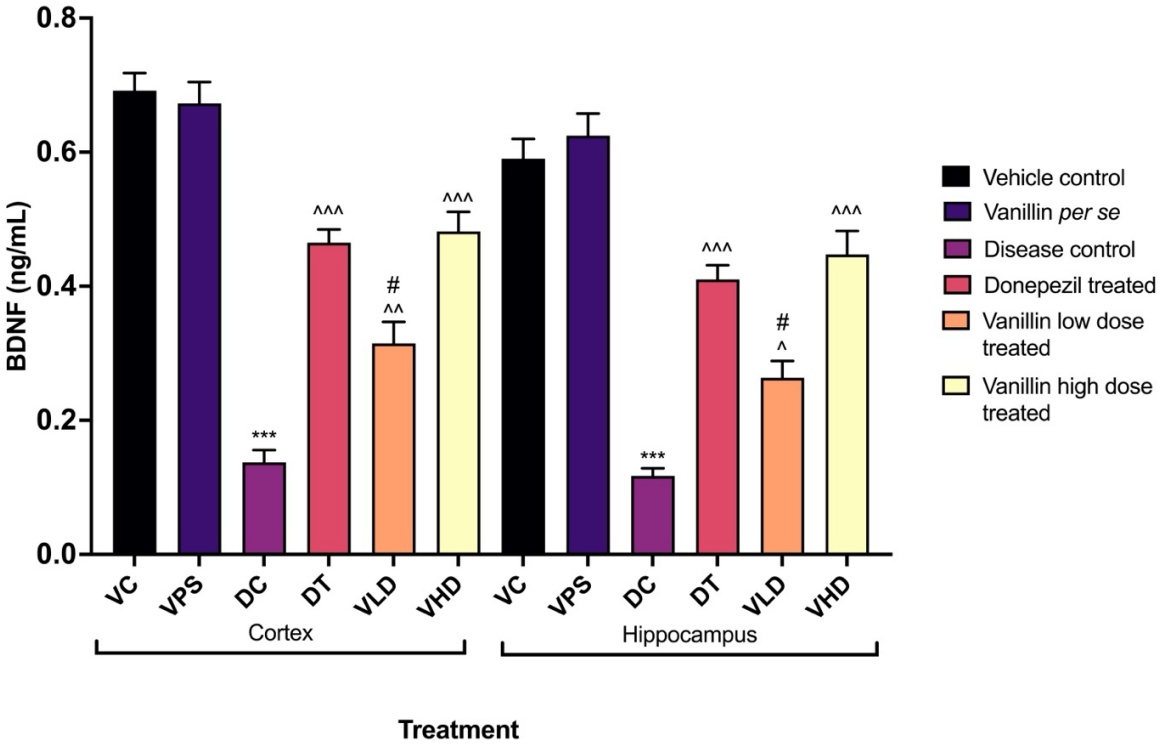


Fig. 5.13: Impact of vanillin and other interventions on BDNF expressed as mean ± SEM.

***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

In the cortex, treating with vanillin by itself did not result in a consequential change in BDNF levels contrasted with the control group that received a vehicle. However, the DC group showed a consequential ($p<.001$) reduction in BDNF levels contrasted with the VC group. Treatment with donepezil remarkably ($p<.001$) increased BDNF levels contrasted with the DC group. When treated with a low dose of vanillin, there was a consequential ($p<.01$) increase in BDNF levels contrasted with the DC group, but these levels were remarkably ($p<.05$) lower than those observed in the donepezil-treated group. The group treated with a high dose of vanillin exhibited BDNF levels remarkably ($p<.001$) higher than the DC group and similar to

the donepezil-treated group. In the hippocampus, treating with vanillin by itself resulted in a statistically inconsequential rise in BDNF levels contrasted with the control group that received a vehicle. In contrast, the DC group showed a consequential ($p<.001$) reduction in BDNF levels contrasted with the VC group. Both the donepezil-treated group and the high dose vanillin-treated group exhibited consequential ($p<.001$) increases in BDNF levels contrasted with the DC group. While treatment with a low dose of vanillin increased BDNF levels remarkably ($p<.05$) contrasted with the DC group, these levels were still remarkably ($p<.05$) lower than those observed in the donepezil-treated group.

5.1.4 Histopathological assessment

No signs of damage were seen in the histopathological analysis of cerebral cortex and hippocampus of VC and vanillin *per se* groups, respectively. The DC group showed shrunken nuclei and protoplasm in both regions i.e. cerebral cortex and hippocampus. The donepezil and vanillin treated groups, respectively, showed improvement in the features observed in the DC group. However, maximum protection was observed in vanillin high dose treated group (figure 5.14 a-b).

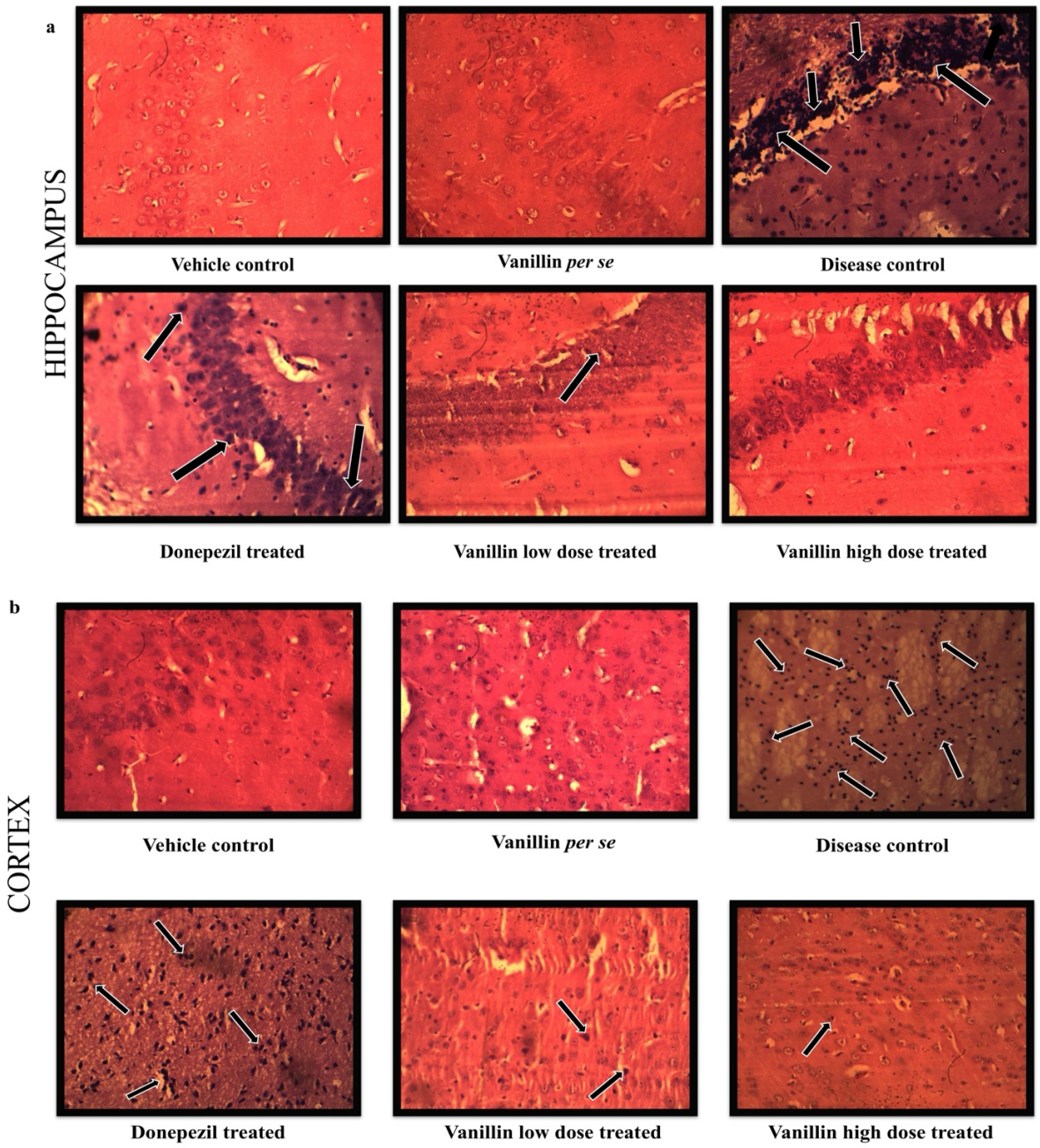


Fig. 5.14 (a-b): Histopathological analysis (40x) of TS of brain cortex and hippocampus using haematoxylin and eosin staining. The arrows mark the neuronal cells which have undergone degeneration as is evident from shrunken protoplasm and nuclei.

5.2 Magnolol

5.2.1 Behavioural evaluation

5.2.1.1 Locomotor activity

Impact of magnolol and other interventions on locomotor activity has been illustrated in figure 5.15.

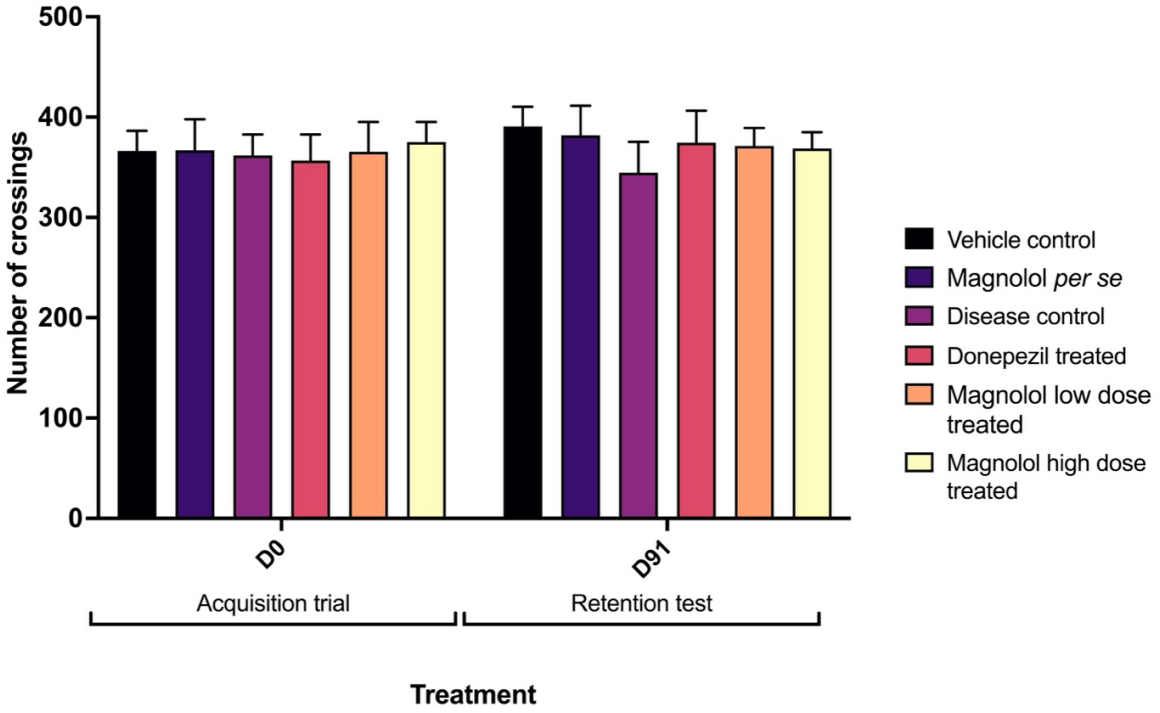


Fig. 5.15: Impact of magnolol and other interventions on locomotor activity expressed as mean ± SEM.

There was no consequential difference found between the number of crossings between all groups on both 0^{th} day and 91^{st} days of the protocol, respectively. Also, no consequential difference was found between the number of crossings between the groups on 91^{st} day with the same groups on 0^{th} day.

5.2.1.2 Transfer latency

Impact of magnolol and other interventions on TL has been illustrated in figure 5.16.

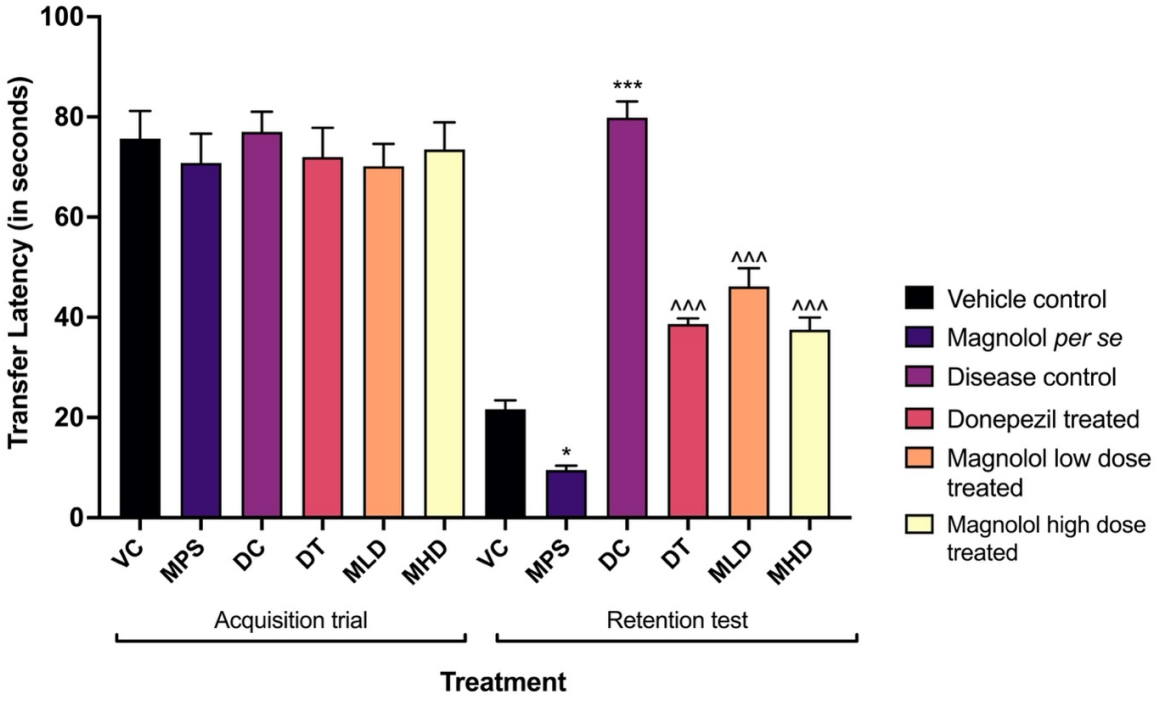


Fig.5.16: Impact of magnolol and other interventions on transfer latency expressed as mean ± SEM. ***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

Magnolol *per se* remarkably ($p<.05$) reduced the TL thereby indicating towards its nootropic activity. TL was remarkably ($p<.001$) higher for DC group contrasted with the VC group. All treatments i.e. donepezil, magnolol low dose and magnolol high dose remarkably ($p<.001$) decreased TL contrasted with the DC group. However, no consequential difference was found between donepezil treated group and either of the magnolol treated groups.

5.2.1.3 Investigation time (in NOR)

Impact of magnolol and other interventions on investigation time has been illustrated in figure 5.17.

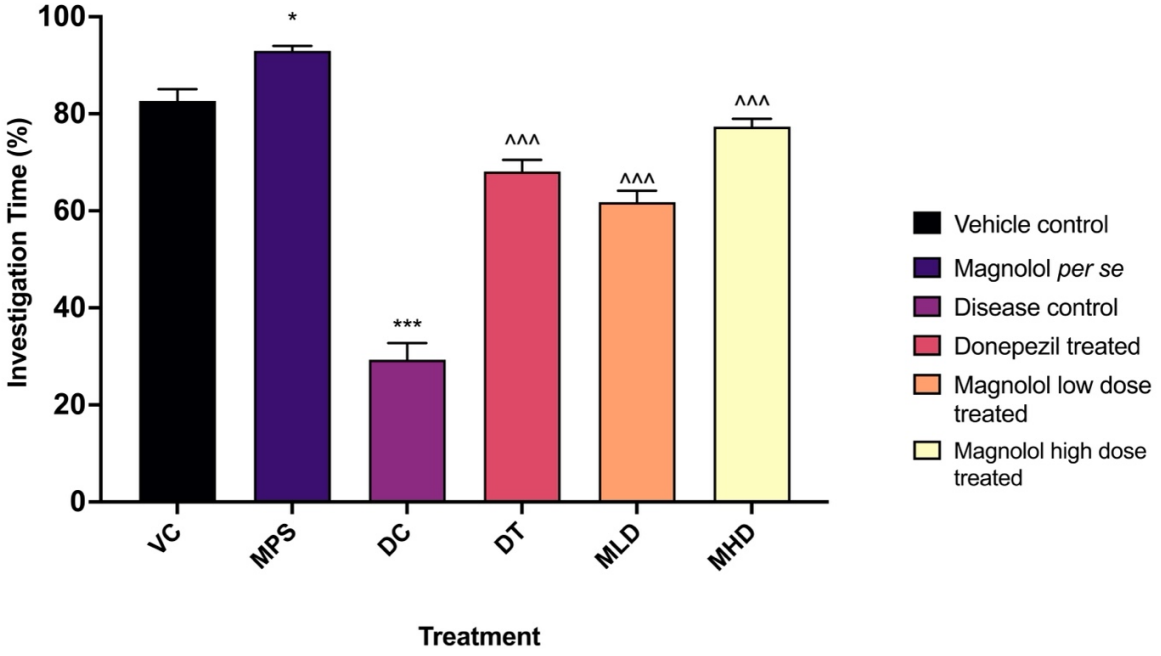


Fig.5.17: Impact of magnolol and other interventions on investigation time expressed as mean ± SEM. ***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

In NOR paradigm, the magnolol *per se* group was found to remarkably ($p<.05$) increase the investigation time thereby indicating its nootropic effect. The investigation time was remarkably ($p<.001$) lesser for DC group in comparison with the group that received only the vehicle. All treatments i.e. donepezil, magnolol low dose and magnolol high dose remarkably ($p<.001$) increased investigation time in comparison with the DC group.

5.2.1.4 Escape latency

Impact of magnolol and other interventions on acquisition time in MWM paradigm has been illustrated in figure 5.18 and that in probe trial has been illustrated in figure 5.19.

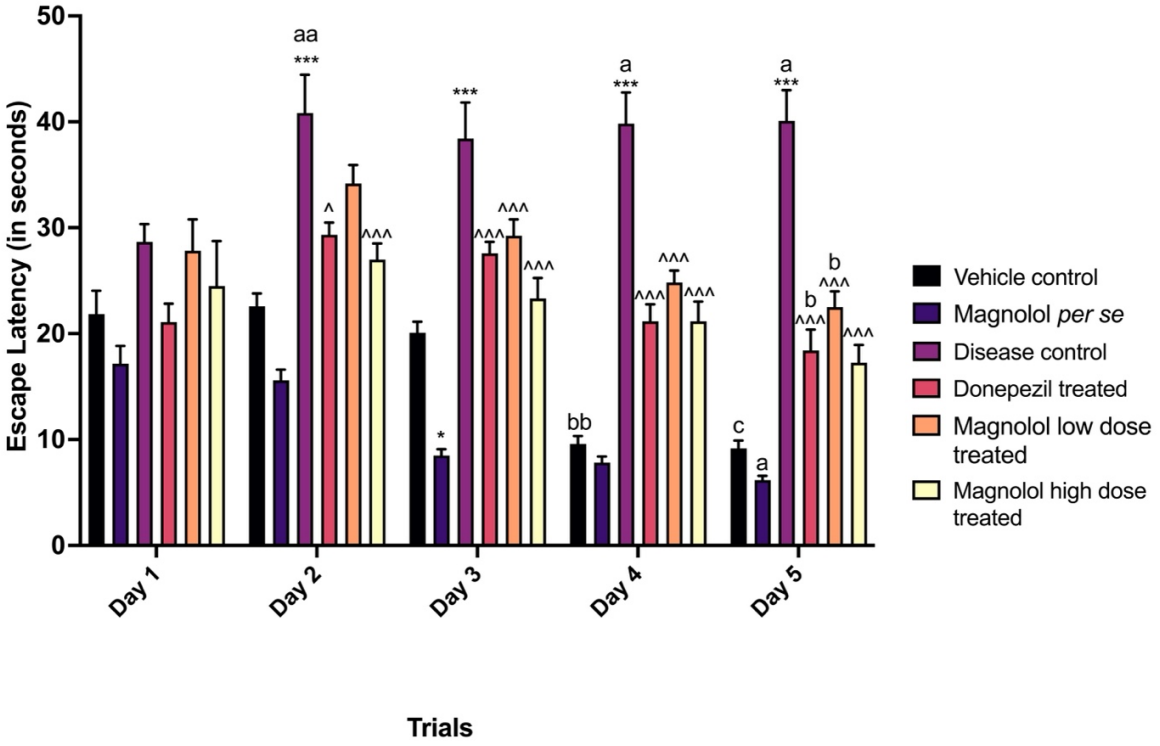


Fig. 5.18: Impact of magnolol and other interventions on escape latency during acquisition trials expressed as mean ± SEM. ***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively. **a indicates a significance level of p<.05 when contrasted with the corresponding group on day 1, while aa indicates a higher significance level of p<.01 for the same comparison on day 1. Similarly, b signifies a significance level of p<.05 when contrasted with the corresponding group on day 2, and bb signifies a higher significance level of p<.01 for the same comparison on day 2. Finally, c indicates a significance level of p<.05 when contrasted with the corresponding group on day 3.**

On 1st day, there was no consequential difference in the EL among all groups in the study. On the 2nd day, the DC group exhibited a remarkably ($p<.001$) higher EL than the VC group. It was also notably ($p<.01$) higher contrasted with the EL of same group on 1st day. Donepezil treated group had a remarkable ($p<.05$) reduction in EL than the DC group. Magnolol high dose treatment led to a remarkable ($p<.001$) reduction in EL than the DC group. On 3rd day, magnolol *per se* treatment remarkably ($p<.05$) decreased the EL than the group which received only the vehicle. The EL of DC group was remarkably ($p<.001$) higher than the VC group. All treatment groups i.e. donepezil, magnolol low dose and magnolol high dose groups, respectively, exhibited a remarkably ($p<.001$) lower EL than the DC group. On 4th day, the EL of VC group was remarkably ($p<.01$) lower than the same group on 2nd day. The EL of DC group was remarkably ($p<.001$) higher than the group which received only the vehicle and remarkably ($p<.05$) higher than the same group of 1st day. All treatment groups i.e. donepezil, magnolol low dose and magnolol high dose groups, respectively, exhibited a remarkably ($p<.001$) lower EL than the DC group. On 5th day, the EL of VC group was remarkably ($p<.05$) lower than the same group on 3rd day. The EL of DC group was remarkably ($p<.001$) higher than the group that received only the vehicle and remarkably ($p<.05$) higher than the same group of 1st day. All treatment groups i.e. donepezil, magnolol low dose and magnolol high dose groups, respectively, exhibited a remarkably ($p<.001$) lower EL than the DC group. The EL of donepezil treated and magnolol low dose treated groups, respectively, was remarkably ($p<.05$) lower than their counterparts on 2nd day.

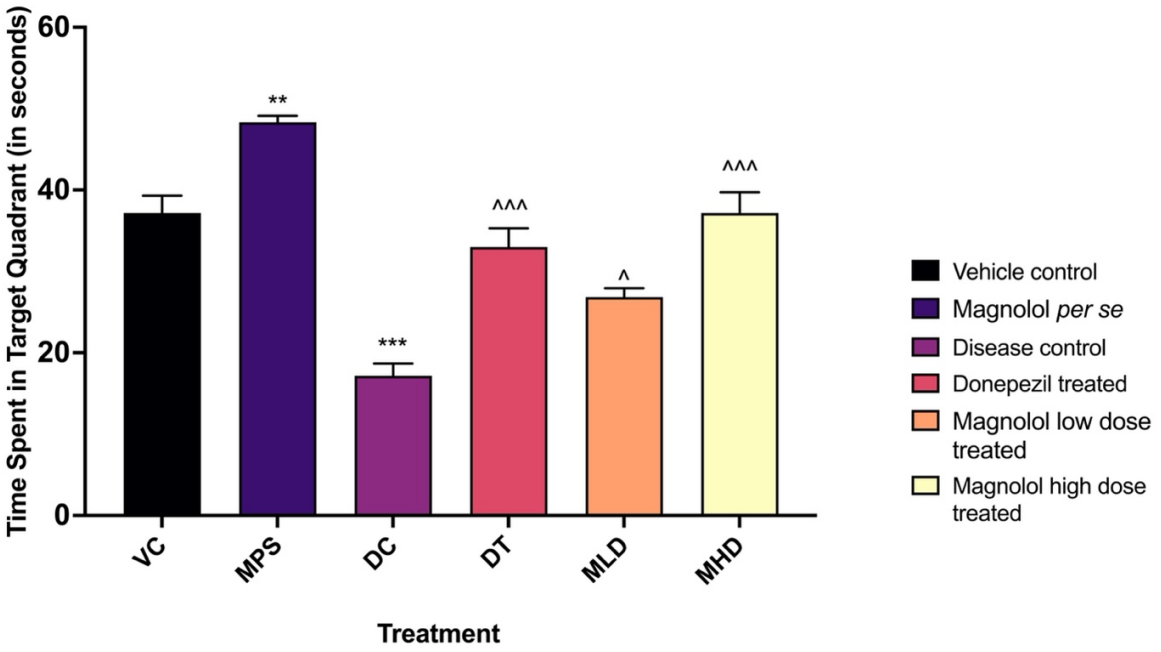


Fig. 5.19: Impact of magnolol and other interventions on time spent in target quadrant during probe trial expressed as mean ± SEM. ***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

The administration of magnolol by itself resulted in a notably higher amount of time spent in the target quadrant contrasted with the control group that received a vehicle ($p<.01$). In contrast, the DC group showed a consequential reduction in the time spent in the target quadrant contrasted with the VC group ($p<.001$). When treated with donepezil or a high dose of magnolol, there was a substantial increase in the time spent in the target quadrant contrasted with the DC group ($p<.001$ for both treatments). Likewise, the group treated with a low dose of magnolol exhibited a consequential increase in time spent in the target quadrant contrasted with the DC group ($p<.05$). Notably, there was no statistically consequential difference

observed in the time spent in the target quadrant between the group treated with donepezil and either of the groups treated with magnolol.

5.2.2 Biochemical evaluation

5.2.2.1 TBARS

Impact of magnolol and other interventions on TBARS has been illustrated in figure 5.20.

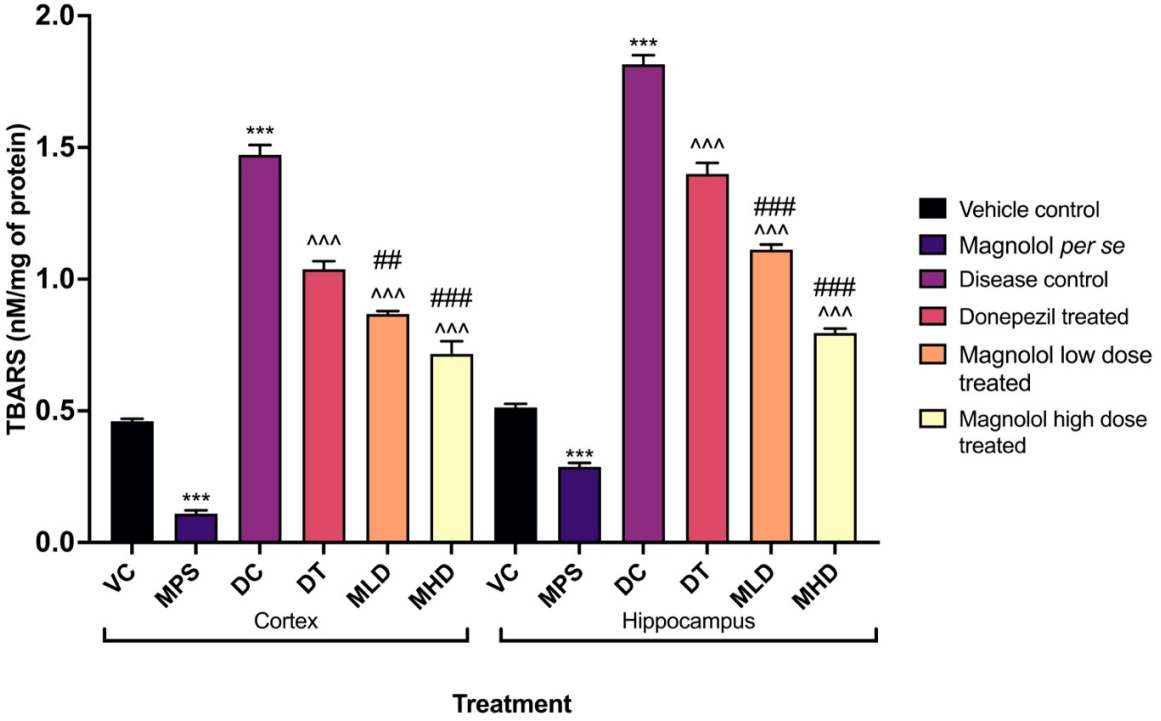


Fig. 5.20: Impact of magnolol and other interventions on TBARS expressed as mean ± SEM. ***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

In the cortex, the group treated with magnolol alone showed a consequential reduction in TBARS levels ($p<.001$) than the group which received only the vehicle. Conversely, the DC group displayed a consequential increase in TBARS levels contrasted with the VC group ($p<.001$). All treatments, including donepezil, low-dose magnolol, and high-dose magnolol,

resulted in a consequential reduction in TBARS levels contrasted with the DC group (p<.001 for all treatments). Specifically, the low-dose magnolol treatment led to a consequential reduction in TBARS levels contrasted with the group treated with donepezil (p<.01), and the high-dose magnolol treatment resulted in a consequential reduction in TBARS levels contrasted with the donepezil-treated group (p<.001). Similar results were observed in the hippocampus. However, in the hippocampal region, both the low-dose and high-dose magnolol-treated groups exhibited a consequential reduction in TBARS levels contrasted with the group treated with Donepezil (p<.001).

5.2.2.2 GSH

Impact of magnolol and other interventions on GSH has been illustrated in figure 5.21.

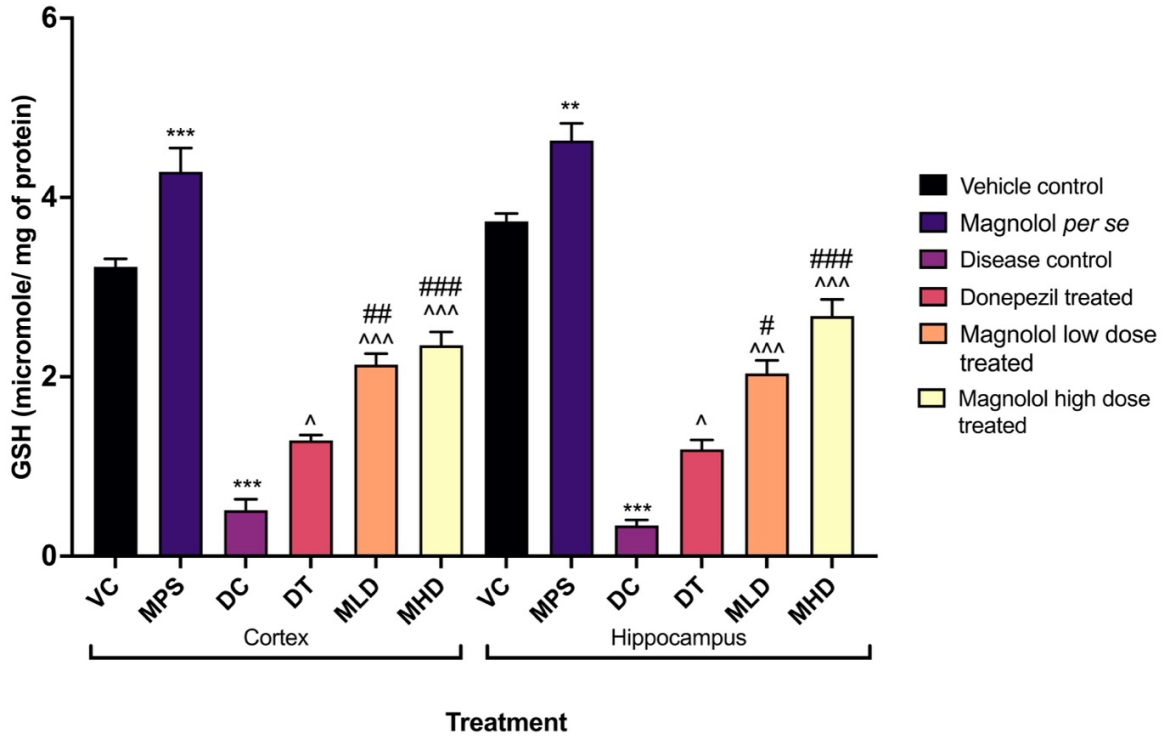


Fig. 5.21: Impact of magnolol and other interventions on GSH expressed as mean ± SEM.

***, ** and * have been used to represent p < .001, p < .01 and p < .05 in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify p < .001, p <

.01 and p < .05, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent p < .001, p < .01 and p < .05, respectively.

In the cortex, treatment with magnolol itself resulted in a consequential (p<.001) elevation in GSH levels when contrasted with the control group that received the vehicle. Conversely, the DC group exhibited a consequential (p<.001) reduction in GSH levels contrasted with the VC group. While donepezil treatment led to a consequential (p<.05) increase in GSH levels, both the low-dose and high-dose magnolol treatment groups showed a consequential (p<.001) increase in GSH levels contrasted with the DC group. Notably, the GSH levels in the low-dose magnolol-treated group were remarkably (p<.01) higher than those in the donepezil-treated group, and the high-dose magnolol treatment resulted in a consequential (p<.001) increase in GSH levels contrasted with the donepezil-treated group. In the hippocampus, treatment with magnolol itself led to a consequential (p<.01) increase in GSH levels contrasted with the VC group. The DC group, on the other hand, exhibited a consequential (p<.001) reduction in GSH levels contrasted with the VC group. Donepezil treatment remarkably (p<.05) increased GSH levels contrasted with the DC group. Both the low-dose and high-dose magnolol treatment groups showed a consequential (p<.001) increase in GSH levels contrasted with the DC group. When contrasted with the donepezil-treated group, the increase in GSH levels was consequential for both the low-dose magnolol-treated group (p<.05) and the high-dose magnolol-treated group (p<.001).

5.2.2.3 CAT

Impact of magnolol and other interventions on CAT has been illustrated in figure 5.22.

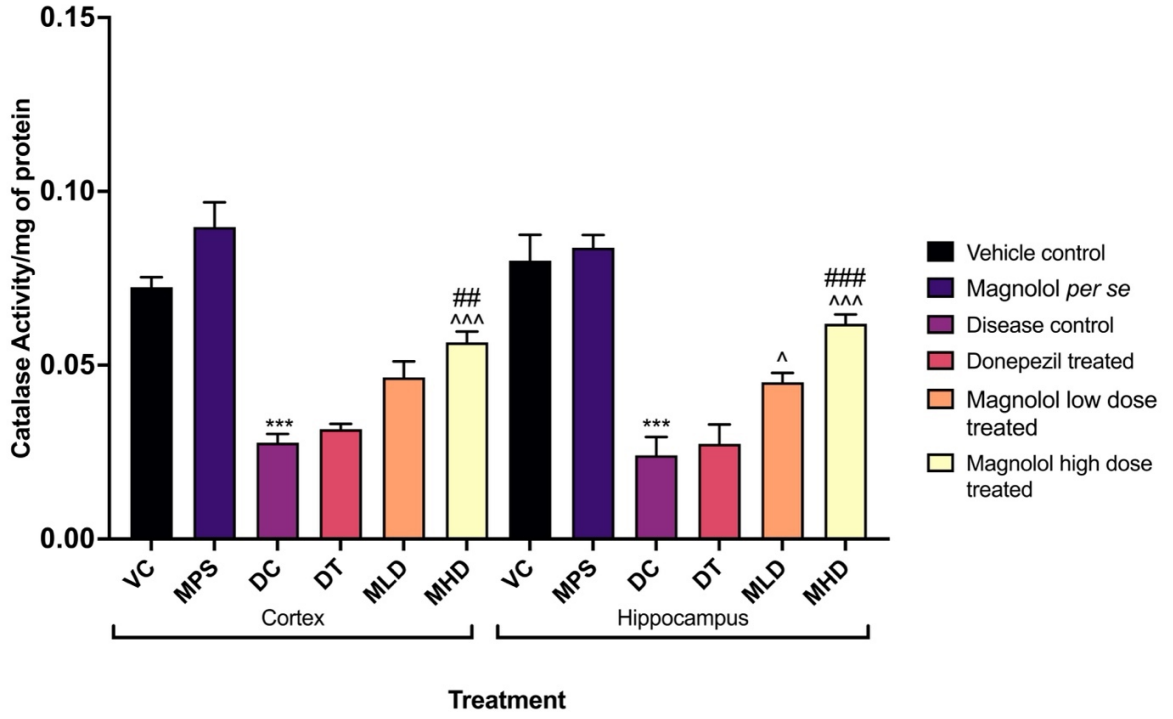


Fig. 5.22: Impact of magnolol and other interventions on CAT expressed as mean ± SEM.

***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

In the cortex, the CAT in the magnolol-treated group showed an inconsequential increase when contrasted with the VC group. Conversely, the DC group had a notably lower CAT ($p<.001$) contrasted with the VC group. While the CAT increased with donepezil treatment contrasted with the DC group, this increase did not reach statistical significance. In contrast, the low-dose magnolol treatment resulted in an inconsequential elevation in CAT, whereas the high-dose magnolol treatment led to a consequential ($p<.001$) increase in CAT contrasted with the DC group. Notably, the high-dose magnolol-treated group exhibited a consequential ($p<.01$) increase in CAT contrasted with the donepezil-treated group.

In the hippocampus, the CAT in the magnolol-treated group showed an inconsequential increase contrasted with the VC group. The DC group had a remarkably (p<.001) lower CAT contrasted with the VC group. Treatment with donepezil resulted in an inconsequential increase in CAT contrasted with the DC group. In contrast, the low-dose magnolol treatment led to a consequential (p<.05) increase in CAT, and the high-dose magnolol treatment resulted in a consequential (p<.001) increase in CAT contrasted with the DC group. When contrasted with the donepezil-treated group, the high-dose magnolol-treated group exhibited a remarkably (p<.001) higher CAT.

5.2.2.4 AChE

Impact of magnolol and other interventions on AChE has been illustrated in figure 5.23.

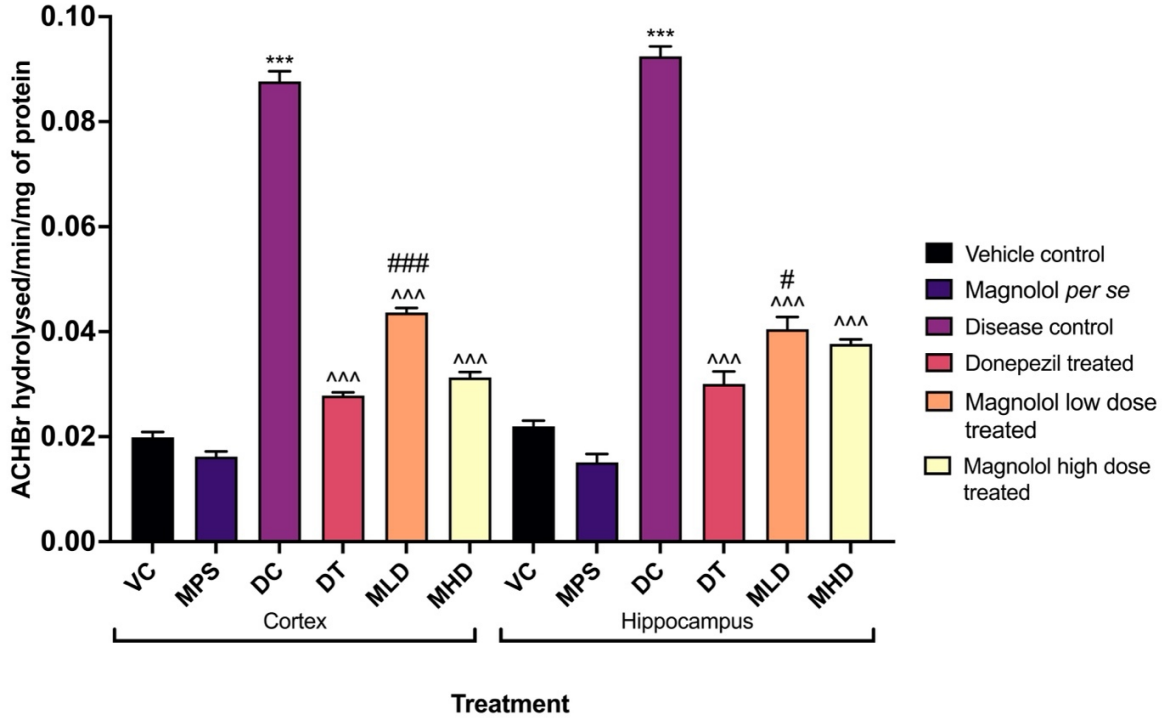


Fig. 5.23: Impact of magnolol and other interventions on AChE expressed as mean ± SEM. ***, ** and * have been used to represent p < .001, p < .01 and p < .05 in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify p < .001, p <

.01 and p < .05, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent p < .001, p < .01 and p < .05, respectively.

In the cortex, the administration of magnolol alone did not result in a consequential change in AChE activity when contrasted with the VC group. However, the DC group exhibited a consequential increase in AChE activity (p<.001) contrasted with the VC group. Treatment with donepezil remarkably decreased AChE activity (p<.001) contrasted with the DC group. The low-dose magnolol treatment led to a consequential reduction in AChE activity (p<.001) contrasted with the DC group, although it was remarkably higher than the AChE activity observed in the donepezil-treated group. On the other hand, the high-dose magnolol treatment resulted in a consequential reduction in AChE activity (p<.001) contrasted with the DC group, and it exhibited AChE activity comparable to that of the donepezil-treated group. In the hippocampus, the administration of magnolol alone showed an inconsequential reduction in AChE activity contrasted with the VC group. In contrast, the DC group had a consequential increase in AChE activity (p<.001) contrasted with the VC group. All treatments, including donepezil, low-dose magnolol, and high-dose magnolol, remarkably decreased AChE activity (p<.001) contrasted with the DC group. The low-dose magnolol treatment group had a remarkably higher AChE activity (p<.05) contrasted with the donepezil-treated group, whereas the high-dose magnolol treatment group exhibited AChE activity comparable to that of the donepezil-treated group.

5.2.3 ELISA

5.2.3.1 Beta secretase

Impact of magnolol and other interventions on beta secretase has been illustrated in figure 5.24.

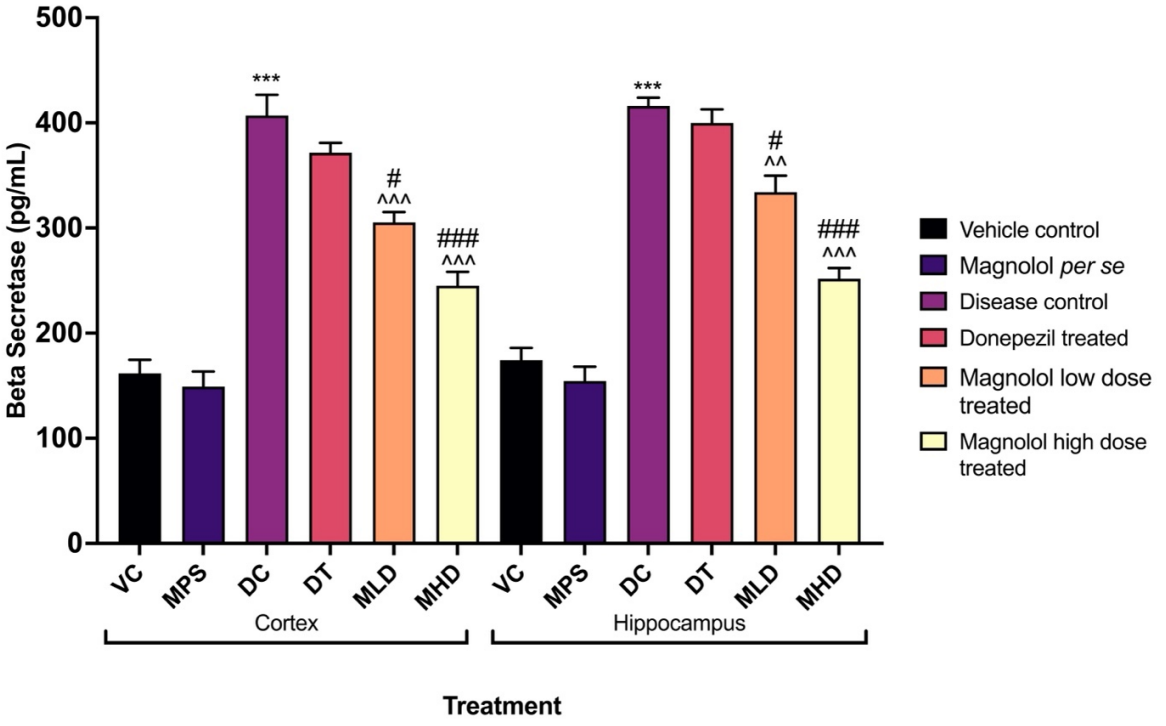


Fig. 5.24: Impact of magnolol and other interventions on beta secretase expressed as mean ± SEM. ***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

In cortex, there was no consequential inhibition of beta secretase activity observed in magnolol *per se* treated group with respect to the VC group. DC group had a remarkably ($p<.001$) accentuated beta secretase activity than the VC group. While donepezil treatment did not have any consequential reduction in beta secretase activity, both magnolol treated groups were observed to remarkably ($p<.001$) inhibit the enzyme's activity. Contrasted with the donepezil treated group, the beta secretase inhibition was remarkably ($p<.05$) lower in magnolol low dose treated group and remarkably ($p<.001$) lower in the magnolol high dose treated group. Similar

results were found in the hippocampus. However, magnolol low dose treatment caused a consequential (p<.01) reduction in the activity of beta secretase contrasted with the DC group.

5.2.3.2 Caspase-3

Impact of magnolol and other interventions on caspase-3 has been illustrated in figure 5.25.

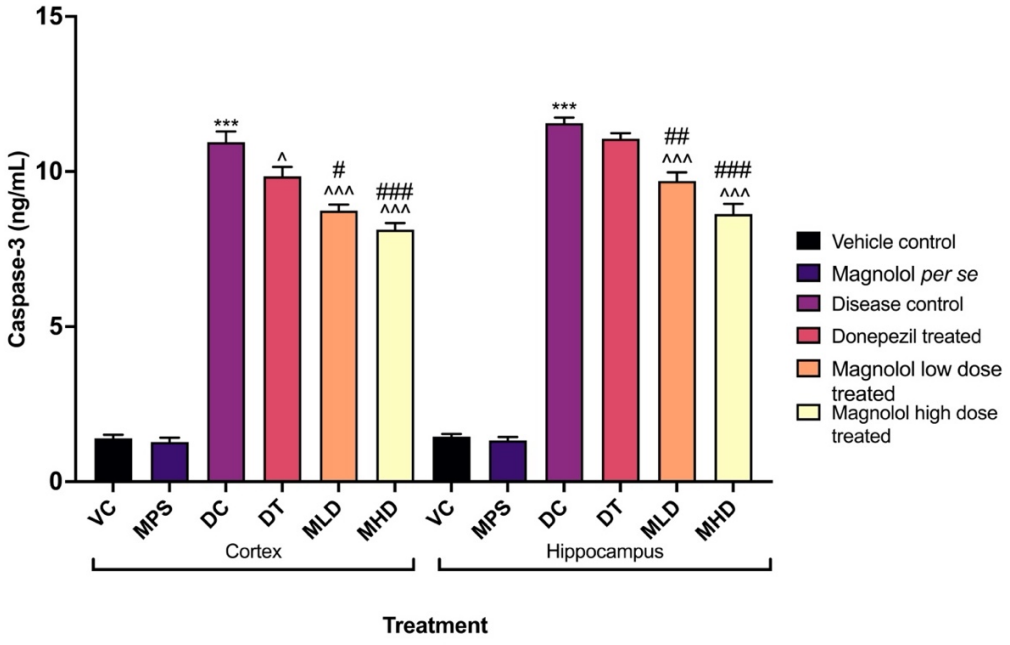


Fig. 5.25: Impact of magnolol and other interventions on caspase-3 expressed as mean ± SEM. ***, ** and * have been used to represent p < .001, p < .01 and p < .05 in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify p < .001, p < .01 and p < .05, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent p < .001, p < .01 and p < .05, respectively.

In cortex, magnolol *per se* treatment led to a statistically inconsequential reduction in caspase-3 level contrasted with the VC group. The DC group exhibited a remarkably (p<.001) higher caspase-3 level than the VC group. Donepezil treatment led to a consequential (p<.05) reduction in the caspase-3 level with respect to the DC group. Magnolol low dose and high dose treatments were observed to have a consequential (p<.001) diminishing effect on caspase-

3 level, respectively, contrasted with the DC group. Contrasted with the donepezil treated group, magnolol low dose treatment remarkably ($p<.05$) decreased the level of caspase-3 while the magnolol high dose treated group showed a consequential ($p<.001$) reduction in caspase-3 level. In hippocampus, magnolol *per se* treatment decreased the level of caspase-3 contrasted with the VC group but it was not statistically consequential. Donepezil treatment led to a statistically inconsequential reduction in caspase-3 level. Both magnolol treated groups i.e. magnolol low dose treated and magnolol high dose treated groups exhibited a consequential ($p<.001$) reduction in level of caspase-3. Contrasted with the donepezil treated group, magnolol low dose treatment remarkably ($p<.01$) reduced the level of caspase-3 while magnolol high dose treated group exhibited a consequential ($p<.001$) reduction in the level of caspase-3.

5.2.3.3 $A\beta_{1-42}$

Impact of magnolol and other interventions on $A\beta_{1-42}$ has been illustrated in figure 5.26.

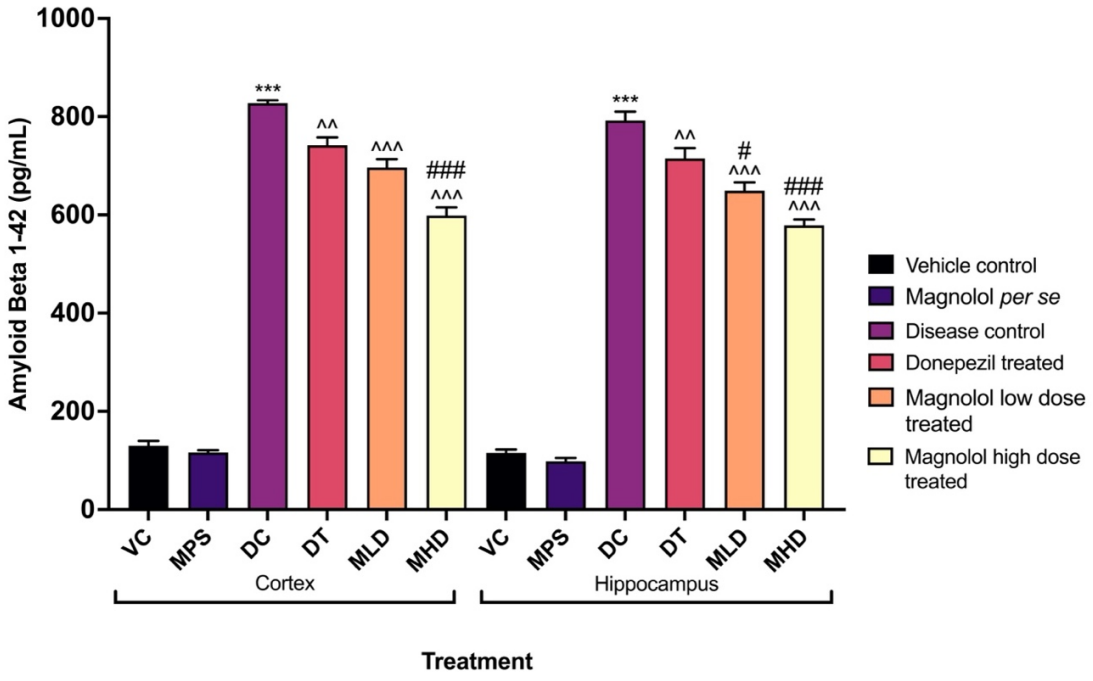


Fig. 5.26: Impact of magnolol and other interventions on $A\beta_{1-42}$ expressed as mean ± SEM.

***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group,

respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

In cortex, magnolol *per se* treatment reduced the $A\beta_{1-42}$ level than the group that received only the vehicle but it was not statistically consequential. DC group exhibited a remarkable ($p<.001$) increase in the $A\beta_{1-42}$ level contrasted with the group which received only the vehicle. Treatment with donepezil remarkably ($p<.01$) reduced the level of $A\beta_{1-42}$ than the DC group. Both magnolol treated groups reduced the level of $A\beta_{1-42}$ remarkably ($p<.001$) in comparison to the DC group. The level of $A\beta_{1-42}$ observed in magnolol high dose treated group was remarkably ($p<.001$) lower than the donepezil treated group. In hippocampus, similar results were obtained. However, the magnolol low dose treated group showed a remarkable ($p<.05$) diminution in the level of $A\beta_{1-42}$ than the donepezil treated group.

5.2.3.3 BDNF

Impact of magnolol and other interventions on BDNF has been illustrated in figure 5.27.

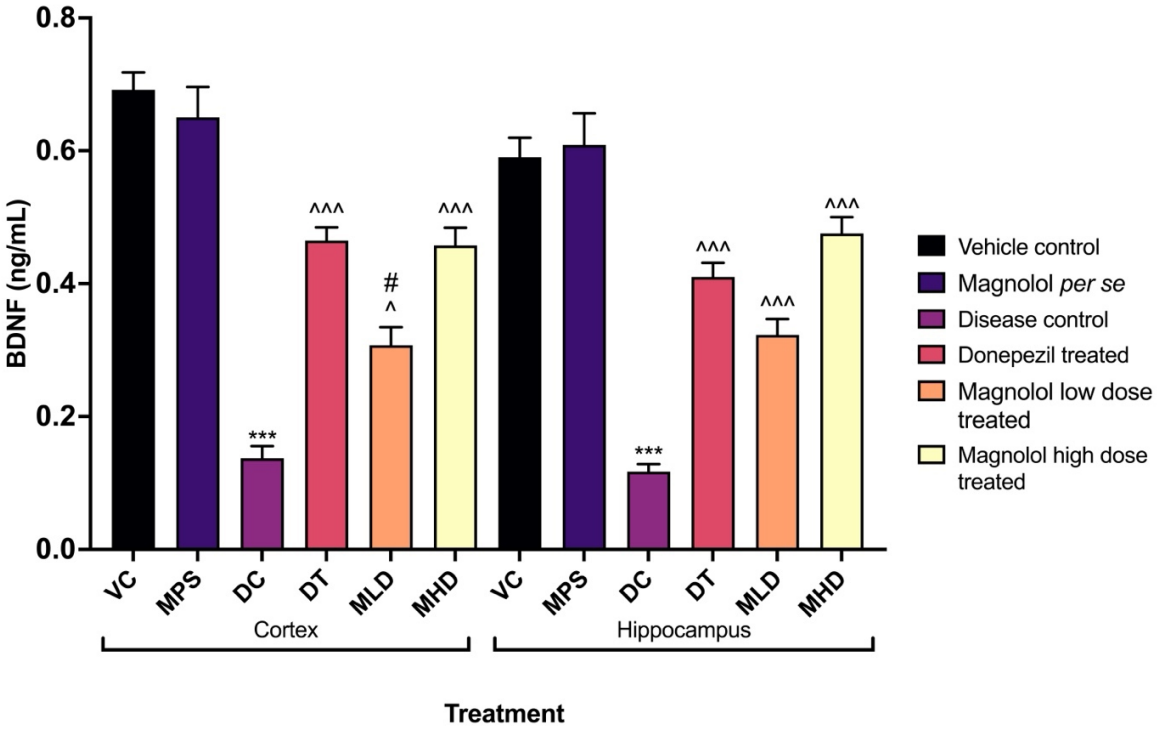


Fig. 5.27: Impact of magnolol and other interventions on BDNF expressed as mean ± SEM. ***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

In the cortex, the administration of magnolol alone did not lead to a consequential change in the level of BDNF contrasted with the group which received only the vehicle. However, the DC group exhibited a consequential reduction in BDNF levels ($p<.001$) contrasted with the group that received only the vehicle. Treatment with donepezil resulted in a consequential increase in BDNF levels ($p<.001$) contrasted with the DC group. The low-dose magnolol treatment was observed to remarkably elevate BDNF levels ($p<.05$) contrasted with the DC group. However, the BDNF levels in the low-dose magnolol-treated group were remarkably lower ($p<.05$) than those in the donepezil-treated group. On the other hand, the high-dose

magnolol-treated group exhibited BDNF levels that were remarkably higher ($p<.001$) than those in the DC group and comparable to those in the donepezil-treated group. In the hippocampus, the administration of magnolol alone resulted in a statistically inconsequential increase in BDNF levels contrasted with the VC group. In contrast, the DC group exhibited a consequential reduction in BDNF levels ($p<.001$) contrasted with the VC group. Treatment with donepezil led to a consequential increase in BDNF levels ($p<.001$) contrasted with the DC group, as did the low-dose and high-dose magnolol treatments.

5.2.4 Histopathological assessment

No signs of damage were seen in the histopathological analysis of cerebral cortex and hippocampus of VC and magnolol *per se* groups, respectively. The DC group showed shrunken nuclei and protoplasm in both regions i.e. cerebral cortex and hippocampus. The donepezil and magnolol treated groups, respectively, showed improvement in the features observed in the DC group. However, maximum protection was observed in magnolol high dose treated group (figure 5.28 a-b).

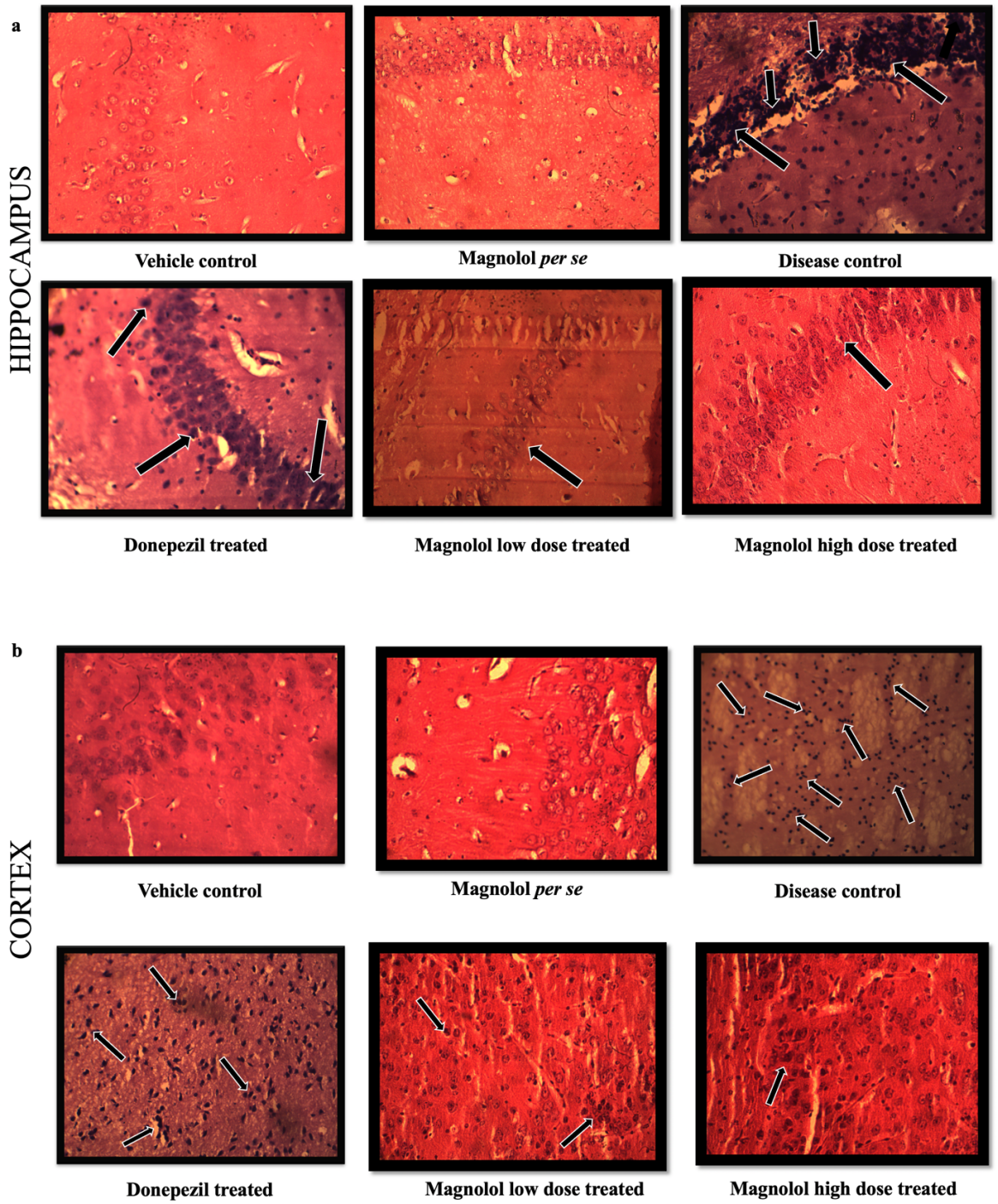


Fig. 5.28 (a-b): Histopathological analysis (40x) of TS of brain cortex and hippocampus using haematoxylin and eosin staining. The arrows mark the neuronal cells which have undergone degeneration as is evident from shrunken protoplasm and nuclei.

5.3 *L.rhamnosus* and various combinations of vanillin, magnolol and *L.rhamnosus*

5.3.1 Behavioural evaluation

5.3.1.1 Locomotor activity

Figure 5.29 represents the effects of various treatments on locomotor activity.

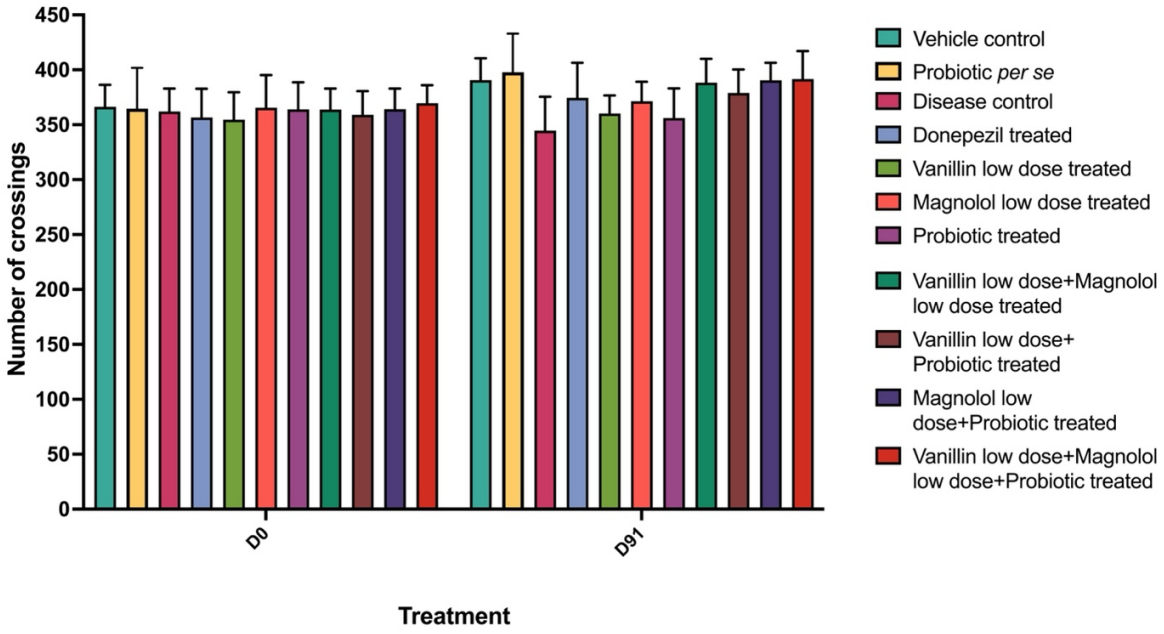


Fig. 5.29: Impact of various treatment interventions on locomotor activity expressed as mean ± SEM

The differences in locomotor activity of various groups within the trials conducted on 0^{th} day and 90^{th} day, respectively, were statistically inconsequential. Also, there was no statistically consequential difference in the locomotor activity of various groups on 91^{st} day contrasted with their counterparts on 0^{th} day.

5.3.1.2 Transfer latency

Figure 5.30 represents the effects of various treatments on TL.

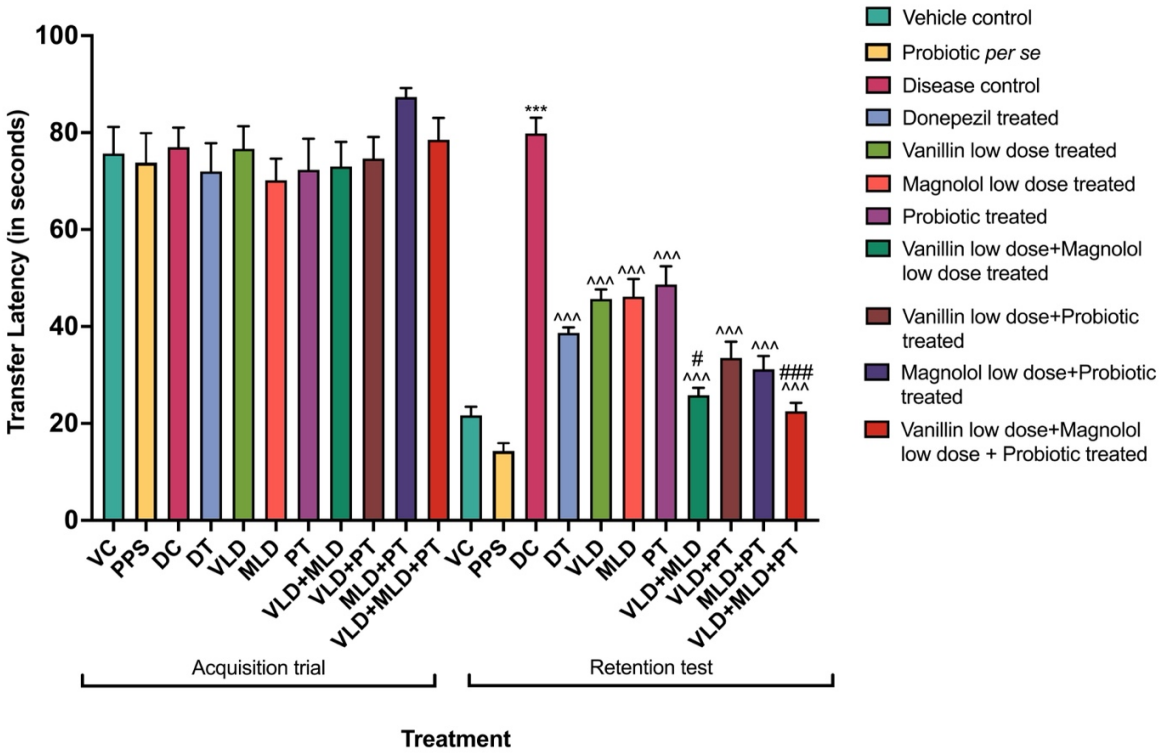


Fig. 5.30: Impact of various treatment interventions on TL expressed as mean ± SEM.

***, ** and * have been used to represent p < .001, p < .01 and p < .05 in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify p < .001, p < .01 and p < .05, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent p < .001, p < .01 and p < .05, respectively.

There was no statistically consequential difference in the TL of various groups during the acquisition trial. In the retention test, probiotic *per se* group showed a statistically inconsequential reduction in TL than the group that received only the vehicle. The DC group exhibited a notably (p<.001) higher TL contrasted with the group which received only the vehicle. All treatment interventions remarkably (p<.001) reduced the TL than the DC group. The combined treatment of vanillin and magnolol showed a notable (p<.05) reduction in TL

contrasted with the donepezil treated group. On the other hand, the reduction in TL than the donepezil treated group was consequential (p<.001) for the group that received the combination of vanillin, magnolol and probiotic.

5.3.1.3 Investigation time (in NOR)

Figure 5.31 represents the effects of various treatments on investigation time under the NOR paradigm.

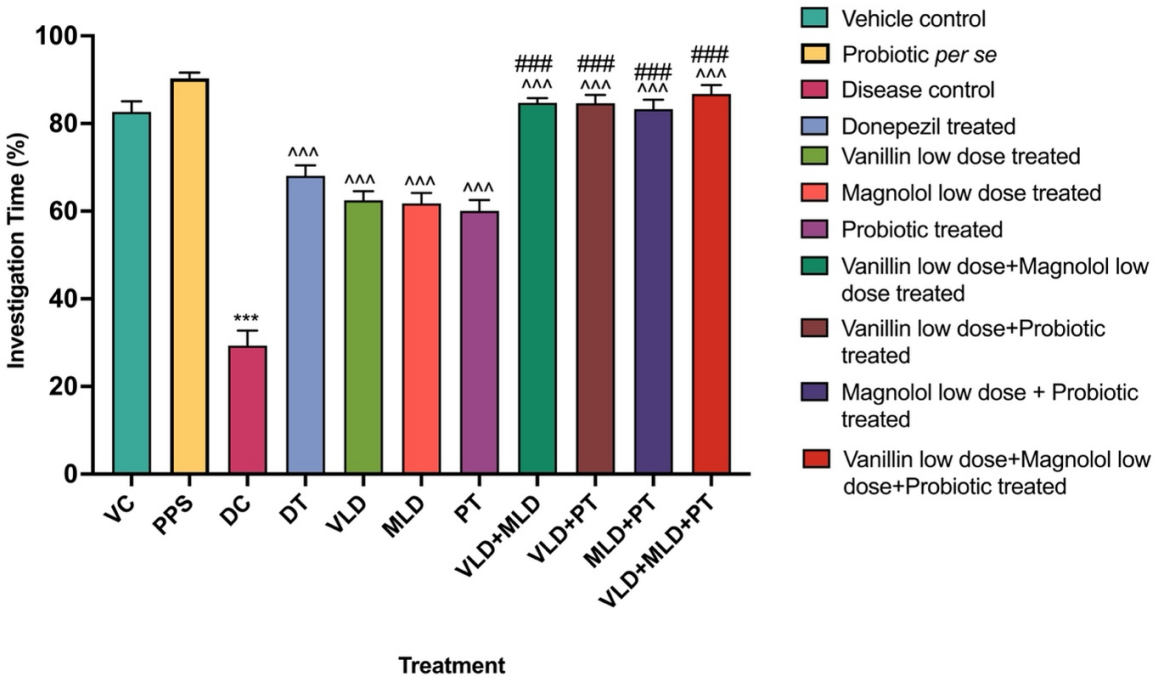


Fig. 5.31: Impact of various treatment interventions on investigation time expressed as mean ± SEM. ***, ** and * have been used to represent p < .001, p < .01 and p < .05 in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify p < .001, p < .01 and p < .05, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent p < .001, p < .01 and p < .05, respectively.

Probiotic *per se* group showed a statistically inconsequential increase in investigation time than the group that received only the vehicle. The DC group exhibited a remarkably (p<.001) lesser

investigation time in comparison to the VC group. All treatment interventions, respectively, led to a remarkably ($p<.001$) higher investigation time than the DC group. The groups that received various combinations i.e. vanillin + magnolol, vanillin + probiotic, magnolol + probiotic, and vanillin + magnolol + probiotic exhibited a remarkably ($p<.001$) higher investigation time, respectively, than the donepezil treated group

5.3.1.4 Escape latency

The effect of various treatments on EL during acquisition trials is shown in table 5.1. The effect of various treatments on the time spent in the target quadrant is shown in figure 5.32.

Table 5.1: Impact of various treatment interventions on EL (acquisition trials) represented as mean ± SEM.

Group	Day 1		Day 2		Day 3		Day 4		Day 5	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Vehicle control	21.83	2.21	22.58	1.22	20.08	1.04	9.58aa,bb	0.75	9.17aa,bbb	0.75
Probiotic per se	24.17	2.06	16.17	1.12	11.58aa	1.15	9.00aaa	0.90	7.08aaa	0.36
Disease control	28.67	1.67	40.83***,aa	3.61	38.42***,aa	3.40	39.83***,aa	2.94	40.08***,aa	2.91
Donepezil treated	21.08	1.73	29.33^	1.15	27.58^	1.09	21.17^^^	1.59	18.42^^^	1.97
Vanillin low dose treated	22.58	1.56	32.42	2.14	28.25	2.12	24.33^^^	2.25	23.25^^^	1.51
Magnolol low dose treated	27.83	2.95	34.17	1.76	29.25	1.54	24.83^^^	1.11	22.50^^^,b	1.50
Probiotic treated	23.67	2.28	36.00aa	1.82	30.83	1.59	27.58^^	2.09	24.42^^,b	1.83
Vanillin low dose + Magnolol low dose treated	25.17	2.41	19.67^^^	1.71	16.92^^^	1.06	16.75^^^	0.70	12.33^^^,aa	1.43

Vanillin low dose + Probiotic treated	29.08	2.67	23.17^^^	1.88	21.08^^^	1.90	18.25^^^	1.30	15.75^^^,aa	1.04
Magnolol low dose + Probiotic treated	26.50	2.31	23.25^^^	1.06	20.58^^^	1.29	17.92^^^	1.51	14.83^^^,a	1.17
Vanillin low dose + Magnolol low dose + Probiotic treated	26.33	2.13	17.25^^^,##	1.45	15.25^^^,##,a	1.07	10.42^^^,#,aaa	1.36	8.50^^^,aaa	0.48

***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively. a represents $p<.05$ with respect to same group on day 1, aa represents $p<.01$ with respect to same group on day 1, aaa represents $p<.001$ with respect to same group on day 1. b represents $p<.05$ with respect to the same group on day 2, bb represents $p<.01$ with respect to the same group on day 2.

During acquisition trials, there was no consequential difference in the EL between all group on 1st day of the trial. On the 2nd day, the EL of DC group was notably ($p<.001$) higher than the group that received only the vehicle on the same day and its own counterpart on day 1 ($p<.01$). When contrasted with the DC group, the reduction in EL on the 2nd day was consequential for the donepezil treated group ($p<.05$), and the groups receiving combined treatment i.e. groups that received various combinations i.e. vanillin + magnolol, vanillin + probiotic, magnolol + probiotic, and vanillin + magnolol + probiotic ($p<.001$). The group that received the combination of vanillin + magnolol + probiotic showed a remarkably ($p<.01$) lower EL than the donepezil treated group. On the 3rd day, the probiotic *per se* group showed a remarkable ($p<.01$) reduction in EL contrasted with its counterpart on day 2. the EL for DC group was notably ($p<.001$) more than that of the VC group and its own counterpart on day 1 ($p<.01$). In comparison to the DC group, the donepezil treated group exhibited a remarkable ($p<.05$) reduction in EL on the 2nd day. Furthermore, the groups that received combined treatments demonstrated a highly remarkable reduction in EL ($p<.001$). Among these combinations, the group that received the combination of vanillin + magnolol + probiotic exhibited a remarkably ($p<.01$) lower EL than the donepezil treated group and its own counterpart on day 1 ($p<.05$). On the 4th day, the reduction in EL for VC group was consequential ($p<.01$) contrasted with its own counterparts on days 1 and 2, respectively. The probiotic *per se* group showed a notable ($p<.001$) reduction in EL contrasted with its counterpart on day 2. The increase in EL for DC group was consequential ($p<.001$) than the VC group and its own counterpart on day 1 ($p<.01$). All the treatments led to a notable ($p<.001$) reduction in EL than to the DC group except for the probiotic treated group which showed a reduction in EL at a significance level of $p<.01$. The group that received vanillin + magnolol + probiotic demonstrated a remarkably ($p<.05$) lesser EL than the donepezil treated group and its own counterpart on day 1 ($p<.001$). On the

last day of the acquisition trials, the reduction in EL for VC group was consequential ($p<.01$) contrasted with its own counterpart on days 1 and consequential ($p<.001$) contrasted with its counterpart on day 2. The probiotic *per se* group showed a notable ($p<.001$) diminution in EL contrasted with its counterpart on day 2. All of the treatments resulted in a noteworthy reduction in EL contrasted with the DC group, with a consequential ($p<.001$) reduction observed except for the probiotic treated group, which exhibited a reduction in EL at a slightly lower significance level ($p<.01$). The magnolol low dose treated and the probiotic treated groups, respectively, demonstrated a remarkable ($p<.05$) fall in the EL contrasted with their own counterparts on day 2. The groups treated with a combination of vanillin + magnolol and vanillin + probiotic showed a notably ($p<.01$) lesser EL than their own counterparts on day 1. The reduction in EL for the group that received magnolol + probiotic was consequential ($p<.05$) contrasted with its counterpart on day 1. The reduction in EL was consequential ($p<.001$) for the group that received the combination of vanillin + magnolol + probiotic contrasted with its counterpart on day 1.

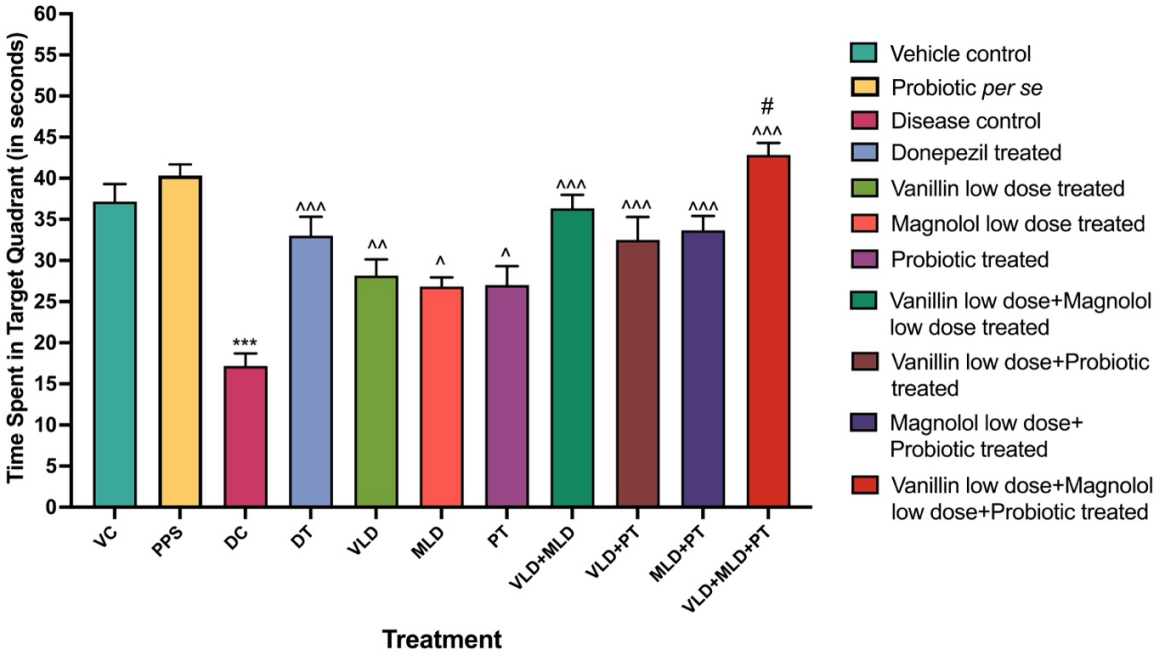


Fig. 5.32: Impact of various treatment interventions on time spent in the target quadrant expressed as mean ± SEM. ***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

The group that received the probiotic alone exhibited an inconsequential increase in the time spent in the target quadrant contrasted with the VC group. However, the animals in the DC group spent remarkably less time in the target quadrant ($p<.001$) than those in the VC group. Treatment with donepezil resulted in a consequential increase in the time spent by the animals in the target quadrant ($p<.001$) contrasted with the DC group. The group treated with vanillin showed a consequential increase in the time spent in the target quadrant contrasted with the DC group ($p<.01$). Similarly, both the magnolol-treated and probiotic-treated groups showed a consequential increase in this parameter ($p<.05$) contrasted with the DC group. The groups that received combinations of treatments displayed a highly consequential increase in the time spent in the target quadrant ($p<.001$) contrasted with the DC group. Among these, the group

that received vanillin + magnolol + probiotic showed a remarkably higher time spent in the target quadrant (p<.05) contrasted with the group treated with donepezil.

5.3.2 Biochemical evaluation

5.3.2.1 TBARS

Figure 5.33 illustrates the effects of various treatments on TBARS.

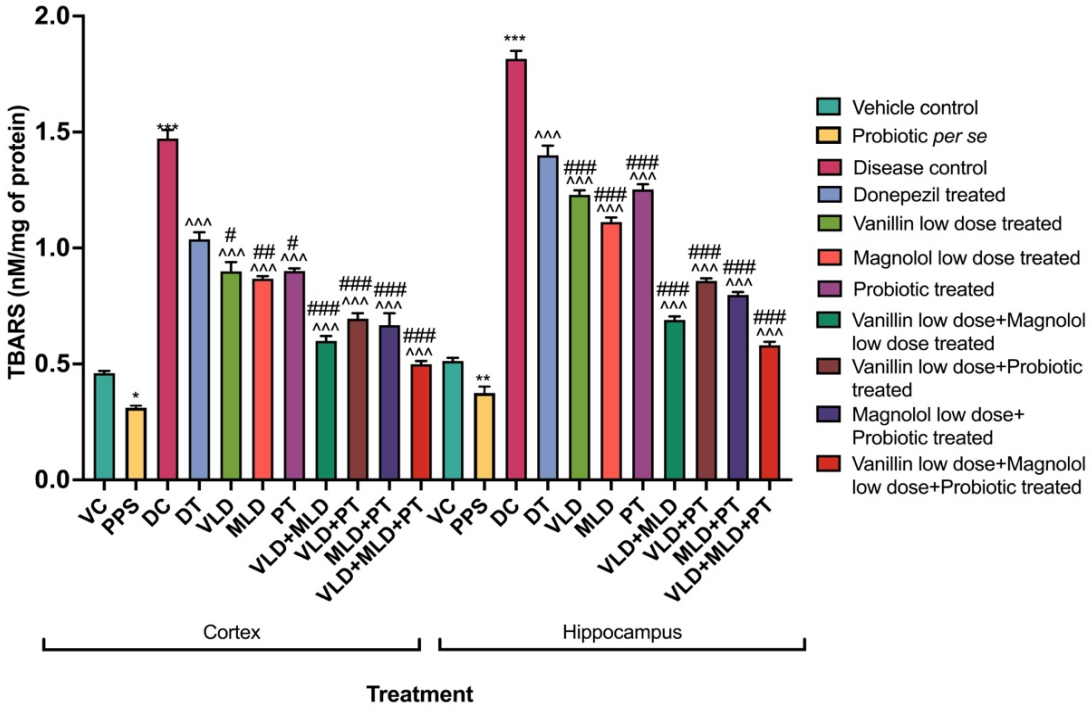


Fig.5.33: Impact of various treatment interventions on the levels of TBARS expressed as mean ± SEM. ***, ** and * have been used to represent p < .001, p < .01 and p < .05 in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify p < .001, p < .01 and p < .05, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent p < .001, p < .01 and p < .05, respectively.

The group that received the probiotic alone showed a consequential reduction in TBARS levels contrasted with the VC group (p<.05 in cortex and p<.01 in hippocampus). In both the cortex

and hippocampus, the DC group had remarkably higher TBARS levels contrasted with the VC group (p<.001). All treatment interventions resulted in a consequential reduction in TBARS levels contrasted with the DC group in both the cortex and hippocampus (p<.001). In the cortex, the reduction in TBARS levels was consequential for the groups that received vanillin (p<.05), magnolol (p<.01), probiotic (p<.05), and all the combination treatments (p<.001) contrasted with the donepezil-treated group. Similarly, in the hippocampus, the reduction in TBARS levels was consequential (p<.001) for the groups that received vanillin, magnolol, probiotic, and all the combination treatments contrasted with the donepezil-treated group.

5.3.2.2 GSH

Figure 5.34 shows the effects of various treatments on GSH.

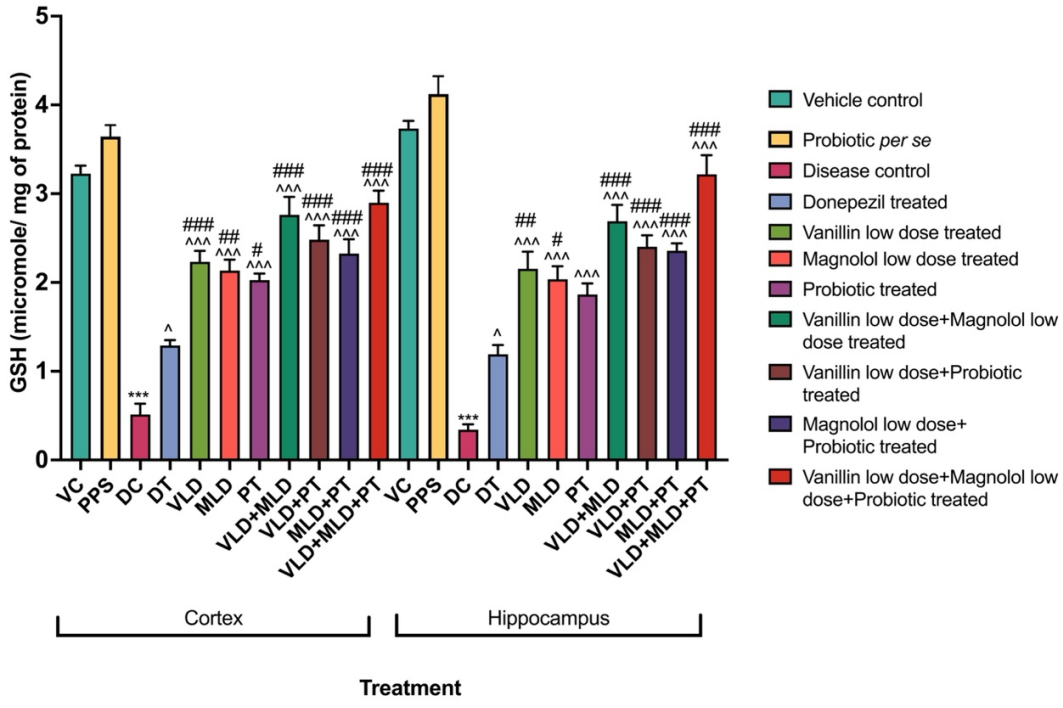


Fig. 5.34: Impact of various treatment interventions on the levels of GSH expressed as mean ± SEM. ***, ** and * have been used to represent p < .001, p < .01 and p < .05 in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify p

< .001, p < .01 and p < .05, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent p < .001, p < .01 and p < .05, respectively.

The group that received the probiotic alone showed an inconsequential increase in GSH levels contrasted with the group which received only the vehicle, both in the cortex and hippocampus. Conversely, in both the cortex and hippocampus, the DC group exhibited a consequential reduction in GSH levels ($p<.001$) contrasted with the VC group. Treatment with donepezil resulted in a consequential increase in GSH levels ($p<.05$) contrasted with the DC group, in both the cortex and hippocampus. There was a consequential increase in GSH levels ($p<.001$) for all treatment groups contrasted with the DC group, in both the cortex and hippocampus. Specifically, in the cortex, the increase in GSH levels was consequential for the groups that received vanillin ($p<.001$), magnolol ($p<.01$), probiotic ($p<.05$), and all combination treatments ($p<.001$) contrasted with the donepezil-treated group. In the hippocampal region, the increase in GSH levels was consequential for the groups that received vanillin ($p<.01$), magnolol ($p<.05$), and all combination treatments ($p<.001$) contrasted with the donepezil-treated group.

5.3.2.3 CAT

Figure 5.35 illustrates the effect of various treatments on the CAT.

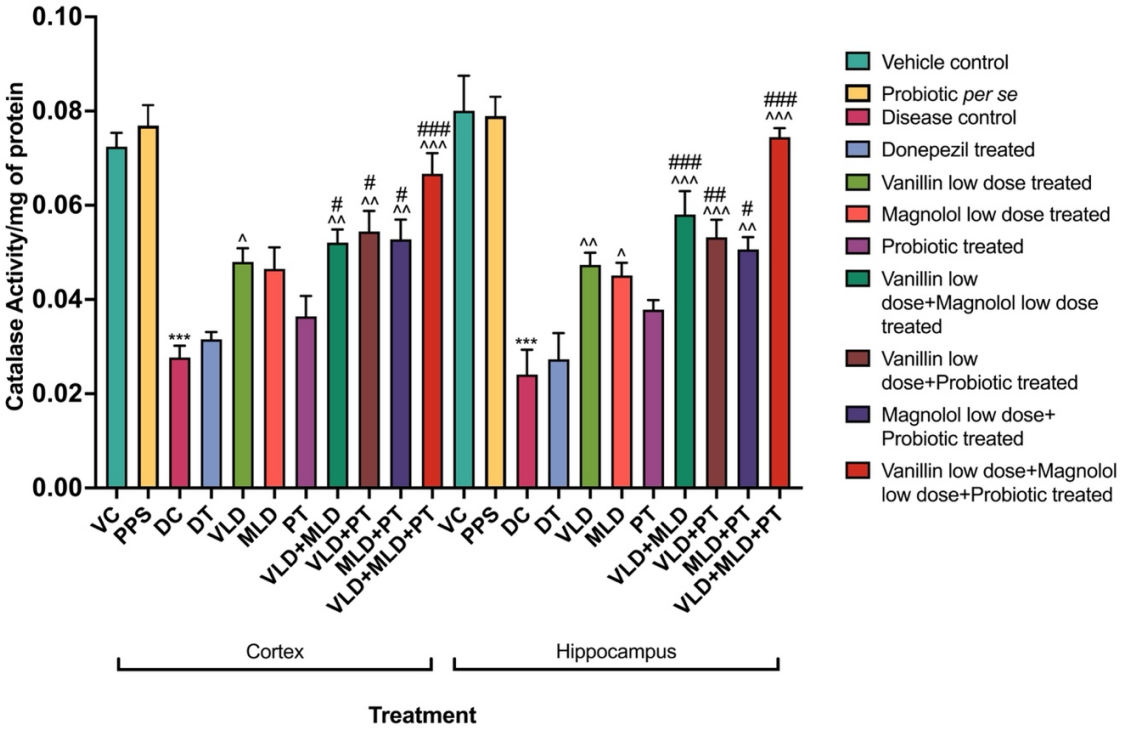


Fig. 5.35: Impact of various treatment interventions on CAT expressed as mean ± SEM.

***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

The group that received the probiotic alone showed no statistically consequential difference in CAT contrasted with the group that received only the vehicle, both in the cortex and hippocampus. In the cortex, the DC group exhibited a marked reduction in CAT ($p<.001$) contrasted with the group that received only the vehicle. In contrast, the vanillin-treated group showed a noteworthy increase in CAT ($p<.05$) contrasted with the DC group, while treatment with donepezil, magnolol, and probiotic led to an inconsequential increase in CAT. However,

the groups that received combinations of treatments, including vanillin + magnolol, vanillin + probiotic, and magnolol + probiotic, displayed a remarkably higher CAT ($p<.01$) than the DC group and a remarkably higher CAT ($p<.05$) than the donepezil-treated group. Notably, the group that received the combined treatment of vanillin + magnolol + probiotic exhibited a markedly higher CAT ($p<.001$) than both the DC group and the donepezil-treated group. In the hippocampus, the DC group exhibited a substantial reduction in CAT ($p<.001$) contrasted with the group that received only the vehicle. A consequential increase in CAT was observed in the vanillin-treated group ($p<.01$) and the magnolol-treated group ($p<.05$). Treatment with donepezil and probiotic resulted in an inconsequential increase in CAT contrasted with the DC group. However, the combination treatment groups showed a consequential increase in CAT contrasted with the DC group, with a significance level of $p<.001$ for the groups receiving vanillin + magnolol, vanillin + probiotic, and vanillin + magnolol + probiotic, and a significance level of $p<.01$ for the magnolol + probiotic treated group. When contrasted with the donepezil-treated group, the increase in CAT was consequential for all combination-treated groups, with a significance level of $p<.001$ for the vanillin + magnolol and vanillin + magnolol + probiotic treated groups, a significance level of $p<.01$ for the vanillin + probiotic treated group, and a significance level of $p<.05$ for the magnolol + probiotic treated group.

5.3.2.4 AChE

Figure 5.36 shows the effect of various treatments on AChE activity.

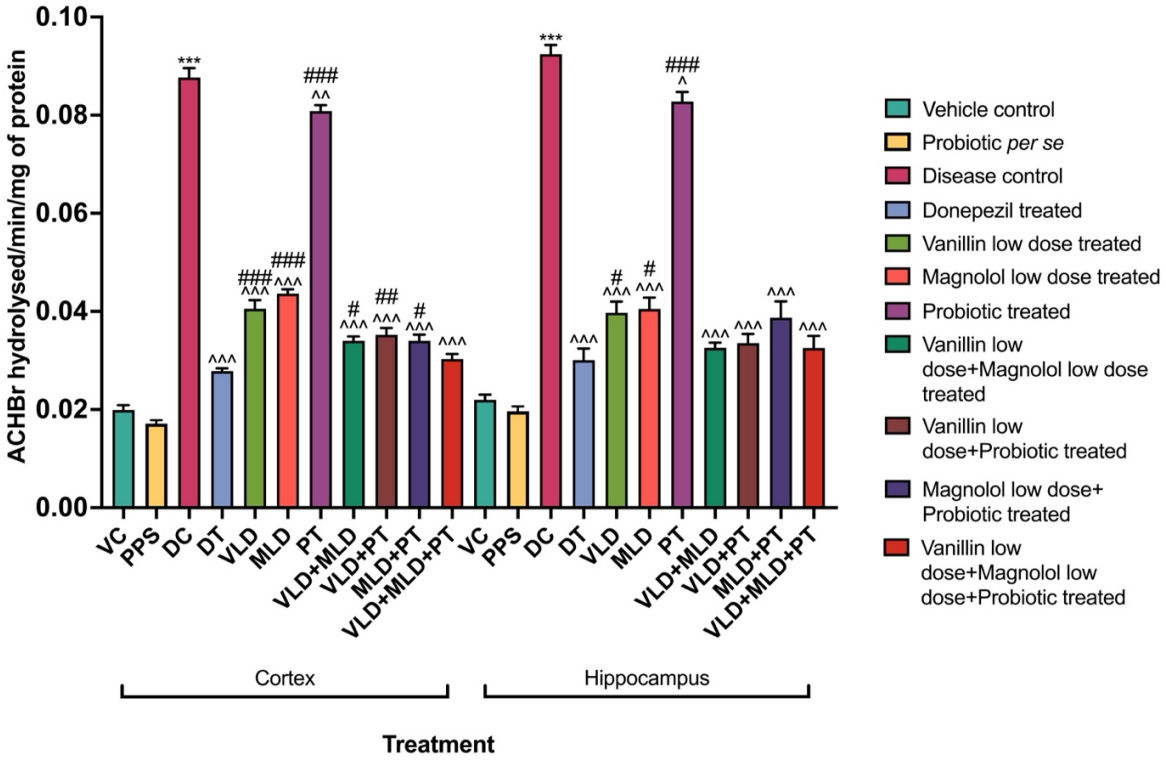


Fig. 5.36: Impact of various treatment interventions on AChE activity expressed as mean ± SEM. ***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

The group that received the probiotic alone showed an inconsequential reduction in AChE activity contrasted with the group that received only the vehicle, both in the cortex and hippocampus. In the cortex, the DC group exhibited remarkably higher AChE activity ($p<.001$) contrasted with the group that received only the vehicle. The lowest AChE activity among the treatment groups was observed in the donepezil-treated group, which was remarkably lower than the DC group ($p<.001$), in both hippocampus and cortex. The groups that received vanillin

and magnolol, respectively, displayed remarkably lower AChE activity ($p<.001$) than the DC group, but the activity was remarkably higher than the donepezil-treated group ($p<.001$). The probiotic-treated group showed a consequential reduction in AChE activity contrasted with the DC group ($p<.01$), but the activity was remarkably higher than the donepezil-treated group ($p<.001$). The group that received the combination of vanillin + magnolol exhibited remarkably lower AChE activity than the DC group ($p<.001$), but the activity was remarkably higher than the donepezil-treated group ($p<.05$). Similar results were observed for the group that received magnolol + probiotic. The group that received vanillin + probiotic demonstrated a consequential reduction in AChE activity contrasted with the DC group ($p<.001$), but it was remarkably higher than the donepezil-treated group ($p<.01$). The group that received the combination of vanillin + magnolol + probiotic showed a consequential reduction in AChE activity contrasted with the DC group ($p<.001$). In the hippocampus, the AChE activity of DC group was remarkably higher than the group that received only the vehicle ($p<.001$). The groups that received vanillin and magnolol, respectively, displayed remarkably lower AChE activity ($p<.001$) than the DC group, but the activity was remarkably higher than the donepezil-treated group ($p<.05$). The probiotic-treated group showed a consequential reduction in AChE activity contrasted with the DC group ($p<.05$), but the activity was remarkably higher than the donepezil-treated group ($p<.001$). All the combination-treated groups exhibited remarkably lower AChE activity contrasted with the DC group ($p<.001$).

5.3.3 ELISA

5.3.3.1 Beta secretase

Figure 5.37 illustrates the effect of various treatment approaches on beta secretase.

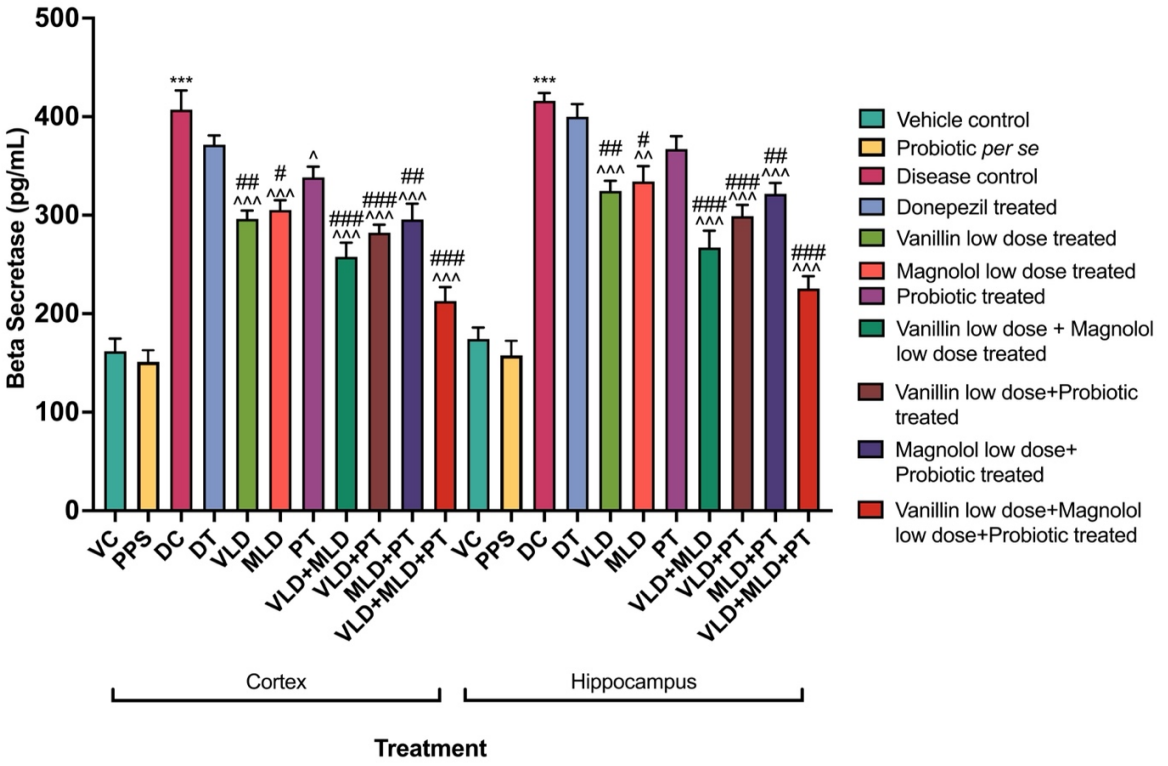


Fig. 5.37: Impact of various treatment interventions on beta secretase expressed as mean ± SEM. ***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

The group that received the probiotic alone showed an inconsequential reduction in beta secretase levels contrasted with the group that received only the vehicle, both in the cortex and hippocampus. In the cortex, the DC group exhibited remarkably higher levels of beta secretase ($p<.001$) contrasted with the group that received only the vehicle. The donepezil-treated group showed an inconsequential reduction in beta secretase levels contrasted with the DC group. Treatment with vanillin led to a consequential reduction in beta secretase levels ($p<.001$) contrasted with the DC group and a consequential reduction ($p<.01$) contrasted with the donepezil-treated group. The group that received magnolol exhibited a consequential reduction

in beta secretase levels contrasted with the DC group ($p<.001$) and the donepezil-treated group ($p<.05$). The probiotic-treated group showed remarkably lower levels of beta secretase than the DC group ($p<.05$). The combination-treated groups, including those receiving vanillin + magnolol, vanillin + probiotic, and vanillin + magnolol + probiotic, showed remarkably lower beta secretase levels ($p<.001$) contrasted with the DC group and the donepezil-treated group. The group that received magnolol + probiotic also exhibited remarkably lower beta secretase levels ($p<.001$) contrasted with the DC group and remarkably lower levels ($p<.01$) contrasted with the donepezil-treated group. In the hippocampus, the elevation of beta secretase levels was consequential contrasted with the group that received only the vehicle ($p<.001$). The donepezil-treated group and the probiotic-treated group exhibited inconsequential decreases in beta secretase levels contrasted with the DC group. The group that received vanillin showed a consequential reduction in beta secretase levels contrasted with the DC group ($p<.001$) and the donepezil-treated group ($p<.01$). The magnolol-treated group also exhibited a consequential reduction in beta secretase levels contrasted with the DC group ($p<.01$) and the donepezil-treated group ($p<.05$). The observations for the groups that received combination treatments were similar to those observed in the cortex.

5.3.3.2 Caspase-3

Figure 5.38 shows the effect of various treatments on the level of caspase-3.

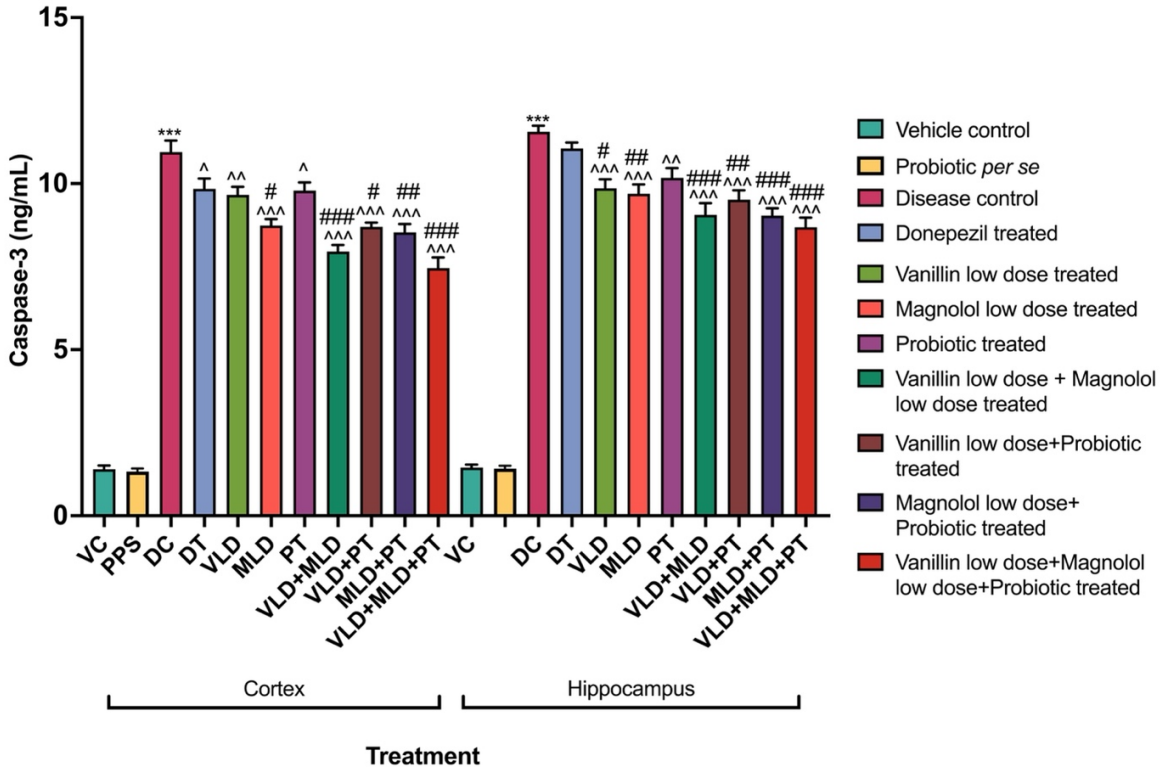


Fig. 5.38: Impact of various treatment interventions on caspase-3 expressed as mean ± SEM. ***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

The group that received the probiotic alone showed an inconsequential reduction in caspase-3 levels contrasted with the group that received only the vehicle, both in the cortex and hippocampus. In the cortex, the DC group exhibited remarkably higher caspase-3 levels ($p<.001$) contrasted with the group that received only the vehicle. The groups that received donepezil and probiotic, respectively, showed a consequential reduction in the level of caspase-

3 ($p<.05$) contrasted with the DC group. Treatment with vanillin resulted in a consequential reduction in the level of caspase-3 contrasted with the DC group ($p<.01$). The group that received magnolol showed a consequential reduction in the level of caspase-3 contrasted with the DC group ($p<.001$) and the donepezil-treated group ($p<.05$). The group that received the combination of vanillin and magnolol showed a consequential reduction in the level of caspase-3 contrasted with the DC group and the donepezil-treated group ($p<.001$). The group that received the combination of vanillin and probiotic showed a consequential reduction in the level of caspase-3 contrasted with the DC group ($p<.001$) and the donepezil-treated group ($p<.05$). The group that received the combination of magnolol and probiotic showed a consequential reduction in the level of caspase-3 contrasted with the DC group ($p<.001$) and the donepezil-treated group ($p<.01$). The group that received the combination of vanillin, magnolol, and probiotic showed a consequential reduction in the level of caspase-3 contrasted with the DC group and the donepezil-treated group ($p<.001$). In the hippocampus, the DC group exhibited remarkably higher caspase-3 levels ($p<.001$) contrasted with the group that received only the vehicle. Treatment with donepezil resulted in an inconsequential reduction in the level of caspase-3 contrasted with the DC group. Treatment with vanillin led to a consequential reduction in the level of caspase-3 contrasted with the DC group ($p<.001$) and the donepezil-treated group ($p<.05$). The group that received magnolol demonstrated a consequential reduction in the level of caspase-3 contrasted with the DC group ($p<.001$) and the donepezil-treated group ($p<.01$). Treatment with probiotic led to a consequential reduction in the level of caspase-3 ($p<.01$) contrasted with the DC group. The groups that received the combination treatments of vanillin + magnolol, magnolol + probiotic, and vanillin + magnolol + probiotic showed remarkably lower levels of caspase-3 contrasted with the DC group and the donepezil-treated group ($p<.001$). The group that received the combination of vanillin and

probiotic led to a consequential reduction in the level of caspase-3 contrasted with the DC group ($p<.001$) and the donepezil-treated group ($p<.01$).

5.3.3.3 $A\beta_{1-42}$

Figure 5.39 illustrates the effect of various treatment interventions on $A\beta_{1-42}$.

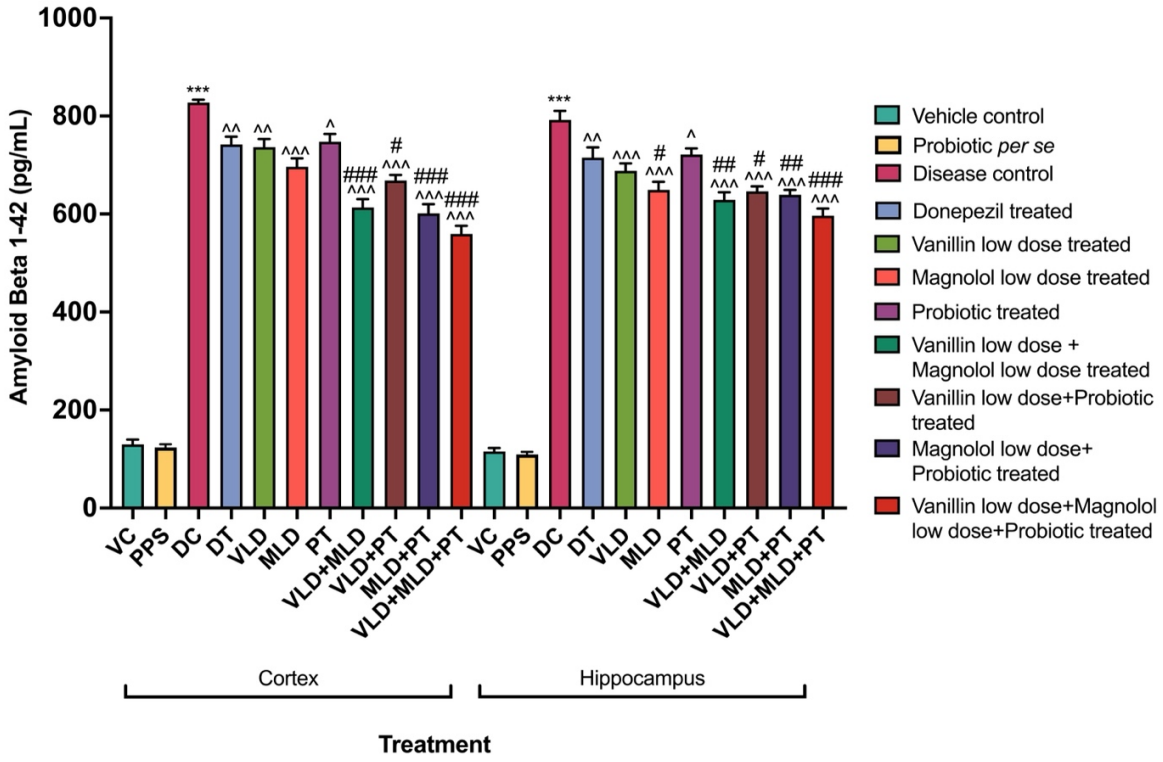


Fig. 5.39: Impact of various treatment interventions on $A\beta_{1-42}$ expressed as mean ± SEM.

***, ** and * have been used to represent $p < .001$, $p < .01$ and $p < .05$ in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify $p < .001$, $p < .01$ and $p < .05$, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent $p < .001$, $p < .01$ and $p < .05$, respectively.

Probiotic *per se* group demonstrated a statistically inconsequential diminution in the $A\beta_{1-42}$ levels than the VC group, in both, cortex and hippocampus. In cortex, the DC group demonstrated a remarkably ($p<.001$) higher level of $A\beta_{1-42}$ when contrasted with the group that

received only the vehicle. Treatment with donepezil and vanillin, respectively, led to a notable ($p<.01$) reduction in the $A\beta_{1-42}$ level contrasted with the DC group whereas the magnolol treated group demonstrated a remarkably ($p<.001$) lower level of $A\beta_{1-42}$ than the DC group. A consequential ($p<.05$) reduction in the level of $A\beta_{1-42}$ than the DC group was also observed in the probiotic treated group. The groups receiving the combinations of vanillin + magnolol, magnolol + probiotic and vanillin + magnolol + probiotic demonstrated a remarkable ($p<.001$) reduction in the level of $A\beta_{1-42}$ contrasted with the DC group and donepezil treated group, respectively. The group receiving the combined treatment of vanillin and probiotic showed a remarkably lower level of $A\beta_{1-42}$ contrasted with DC group ($p<.001$) and donepezil treated group ($p<.05$). In hippocampus, the level of $A\beta_{1-42}$ was notably ($p<.001$) higher than the group which received only the vehicle. Treatment with donepezil led to a consequential ($p<.01$) reduction in the level of $A\beta_{1-42}$ contrasted with the DC group. The groups that received vanillin and magnolol, respectively, showed a notably ($p<.001$) lower level of $A\beta_{1-42}$ contrasted with the DC group. Magnolol treated group showed a remarkable ($p<.05$) reduction in the level of $A\beta_{1-42}$ contrasted with the donepezil treated group. Treatment with probiotic led to a consequential ($p<.05$) reduction in the level of $A\beta_{1-42}$ contrasted with the DC group. The group that received a combination of vanillin and magnolol showed a noteworthy ($p<.001$) reduction in the level of $A\beta_{1-42}$ than the DC group and a consequential ($p<.01$) reduction in the level of $A\beta_{1-42}$ than the donepezil treated group. Similar result was observed for the group that received the combination of magnolol and probiotic. In the group that received a combination of vanillin and probiotic there was a consequential reduction in the level of $A\beta_{1-42}$ contrasted with the DC group ($p<.001$) and donepezil treated group ($p<.05$). The group receiving the combination of

vanillin + magnolol + probiotic demonstrated a noteworthy (p<.001) reduction in the level of $A\beta_{1-42}$ than the DC group and donepezil treated group, respectively.

5.3.3.4 BDNF

Figure 5.40 shows the effect of various treatments on the levels of BDNF.

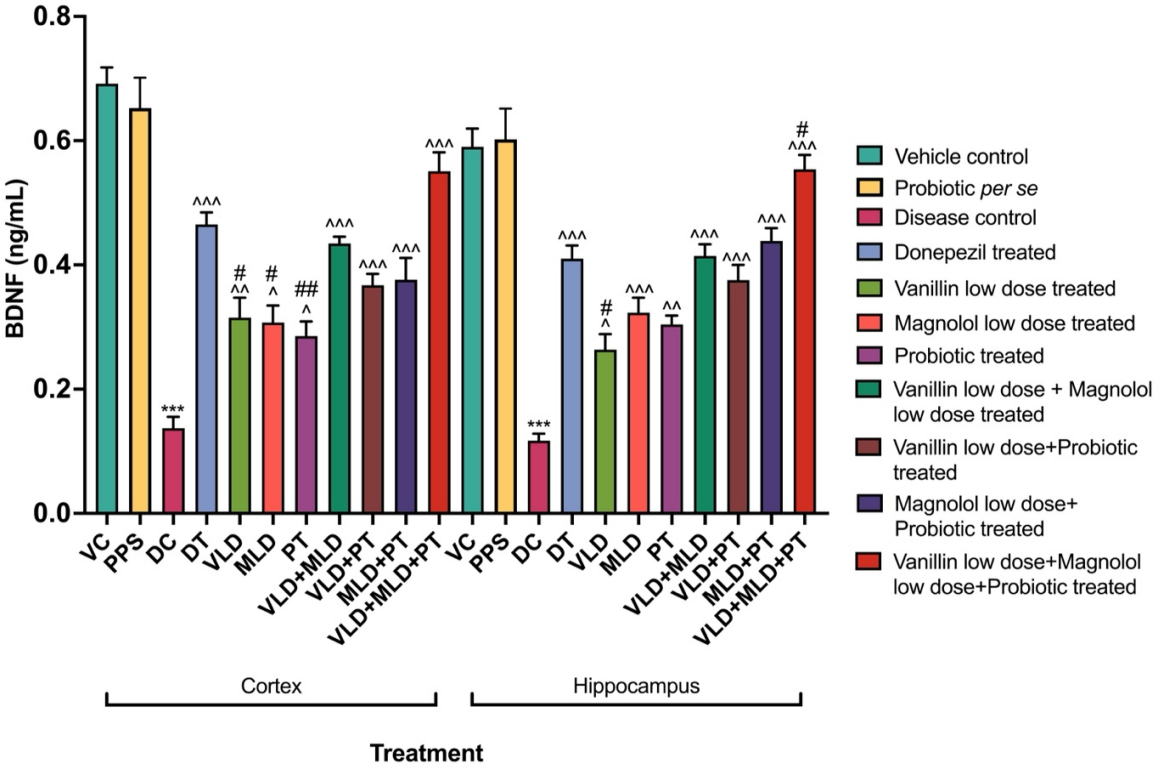


Fig. 5.40: Impact of various treatment interventions on BDNF expressed as mean ± SEM.

***, ** and * have been used to represent p < .001, p < .01 and p < .05 in comparison with the VC group, respectively. The comparisons with DC group have been represented as ^^^, ^^ and ^ which signify p < .001, p < .01 and p < .05, respectively. To represent comparisons with the donepezil treated group, ###, ## and # have been used to represent p < .001, p < .01 and p < .05, respectively.

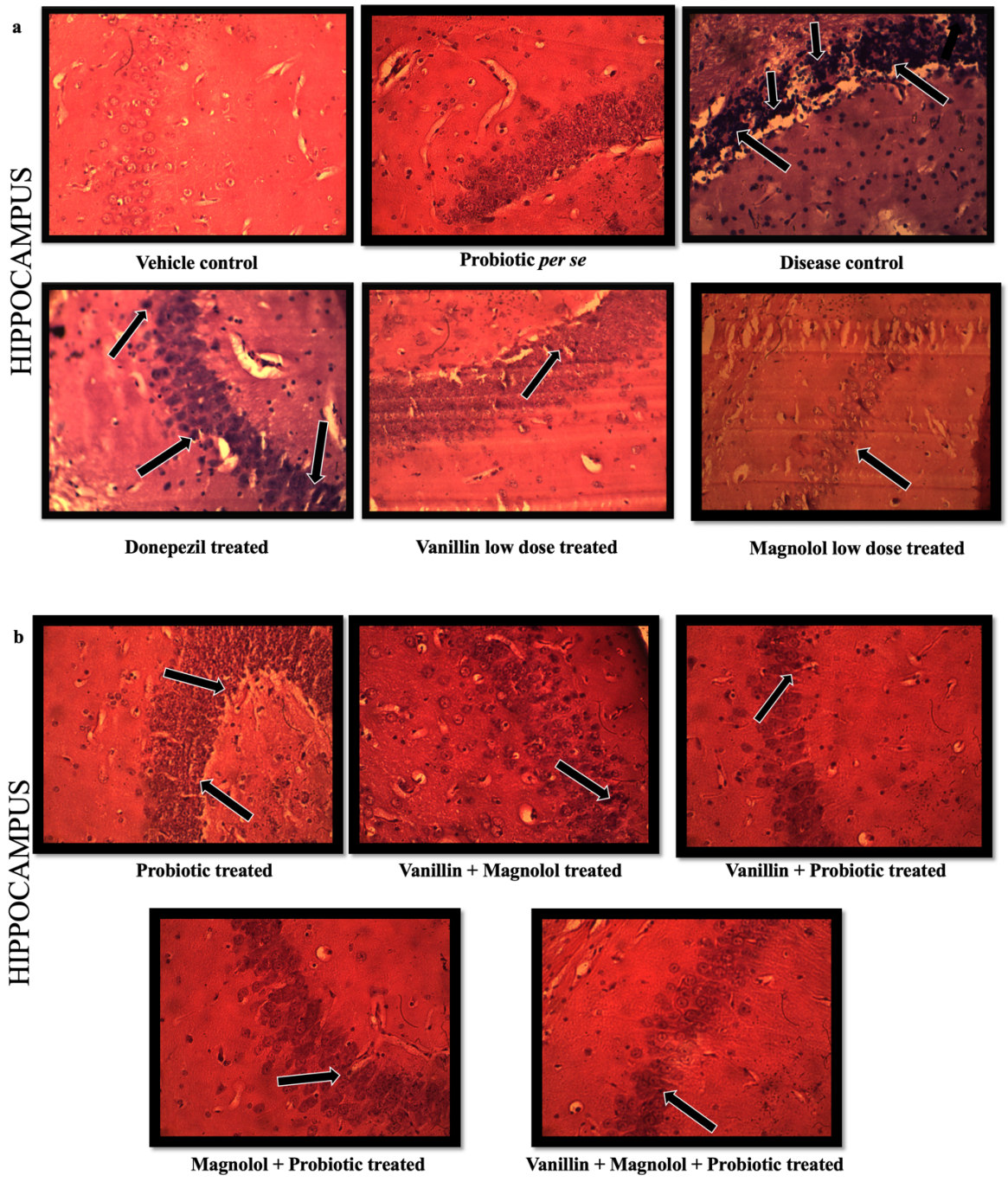
The group that received only the probiotic showed no statistically consequential difference in BDNF levels contrasted with the group that received only the vehicle, in both the cortex and hippocampus. In the cortex, the DC group exhibited remarkably lower BDNF levels (p<.001) contrasted with the group that received only the vehicle. Treatment with donepezil as well as

all the combination treatment groups resulted in a consequential increase in the level of BDNF contrasted with the DC group ($p<.001$). Vanillin treatment led to a notable increase in the level of BDNF contrasted with the DC group ($p<.01$), but the level was remarkably lower than that of the donepezil-treated group ($p<.05$). The group that received magnolol showed a consequential elevation in the level of BDNF contrasted with the DC group ($p<.05$), but the level was remarkably lower than that of the donepezil-treated group ($p<.05$). Probiotic treatment led to a consequential rise in the level of BDNF contrasted with the DC group ($p<.05$), but the level was remarkably lower than the donepezil-treated group ($p<.01$). In the hippocampus, the DC group exhibited remarkably lower levels of BDNF contrasted with the group that received only the vehicle ($p<.001$). Treatment with donepezil, magnolol, as well as various combinations (vanillin + magnolol, vanillin + probiotic, magnolol + probiotic, vanillin + magnolol + probiotic), led to a consequential increase in the level of BDNF contrasted with the DC group ($p<.001$). The group that received the combination of vanillin + magnolol + probiotic showed a remarkably higher level of BDNF contrasted with the donepezil-treated group ($p<.05$). Treatment with vanillin led to a consequential increase in the level of BDNF contrasted with the DC group ($p<.05$), but it was remarkably lower than the donepezil-treated group ($p<.05$). The group that received the probiotic showed a remarkably higher level of BDNF contrasted with the DC group ($p<.01$).

5.3.4 Histopathological assessment

No signs of damage were seen in the histopathological analysis of cerebral cortex and hippocampus of VC and magnolol *per se* groups, respectively. The DC group showed shrunken nuclei and protoplasm in both regions i.e. cerebral cortex and hippocampus. The donepezil, vanillin, magnolol, probiotic and combination treated groups, respectively, showed improvement in the features observed in the DC group. However, maximum protection was

observed in the group that received the combination of vanillin, magnolol and *L.rhamnosus* (figure 5.41 a-d).



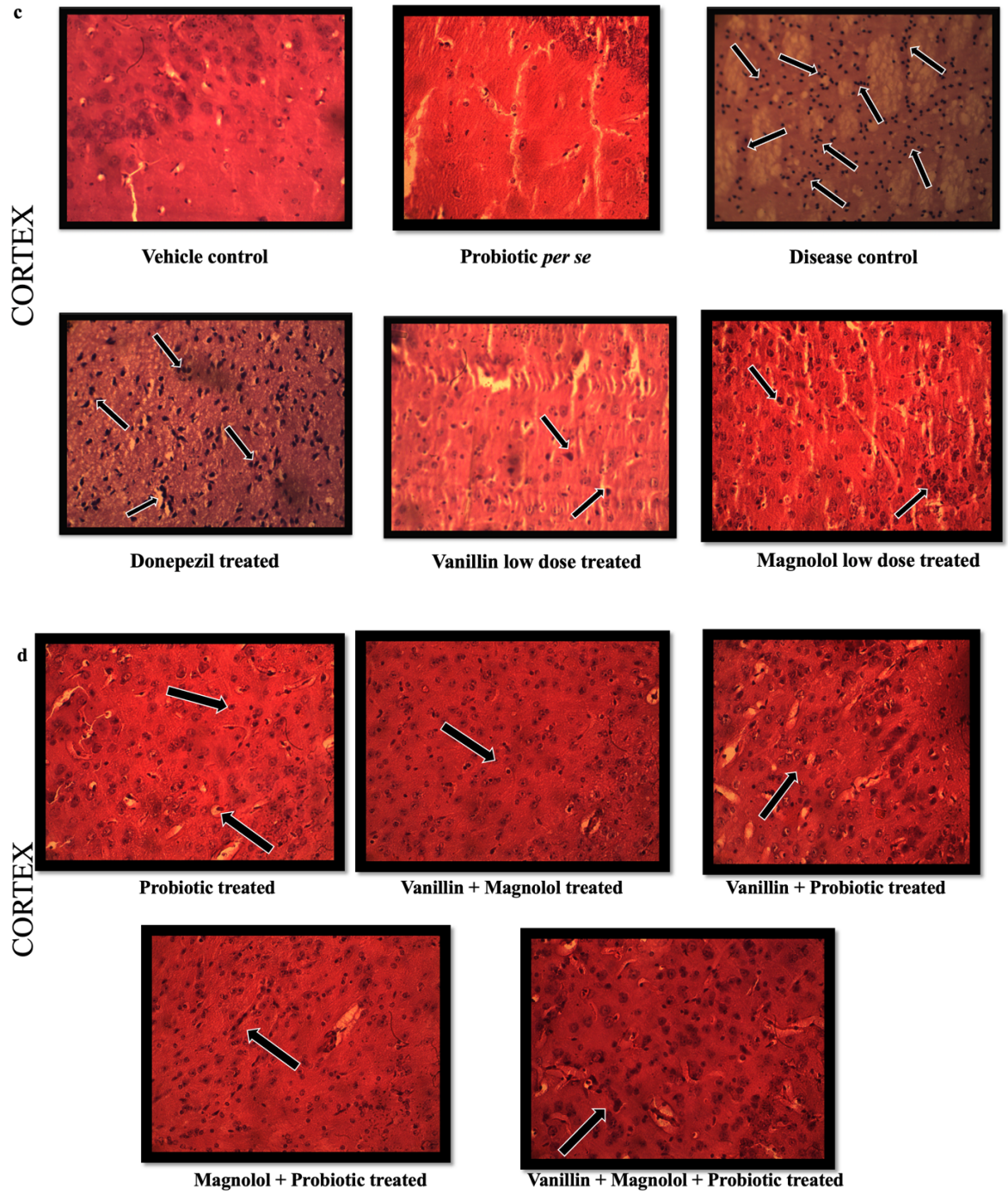


Fig. 5.41(a-d): Histopathological analysis (40x) of TS of brain cortex and hippocampus using haematoxylin and eosin staining. The arrows mark the neuronal cells which have undergone degeneration as is evident from shrunken protoplasm and nuclei.

5.4 Discussion

AD develops and progresses following multiple pathways. The major underlying pathological hallmarks are overexpression of AChE (leads to diminution in the level of acetylcholine), deposition of Aβ plaques (leads to mitochondrial dysfunction in neurons), increased level of caspase -3 (induces neuronal apoptosis) (Alzheimer's Association, 2017; Anand et al., 2017; Heneka et al., 2015). Oxidative stress significantly contributes to the initiation and advancement of AD (Betteridge, 2000; Huang et al., 2016). The current pharmacological interventions are unable to target the underlying pathological mechanisms and therefore provide nothing more than symptomatic relief (Molinuevo et al., 2005; Ogura et al., 2000). Antioxidant compounds have been gaining appreciable attention for their possible role in controlling the pathology of AD. One such antioxidant compound, which has been safely consumed by humans over the years is vanillin. It has been a part of some of our favourite baked goods, desserts, beverages and cosmetics. Not only does it provide a calming aroma, it also manipulates cellular mechanisms to elicit its ameliorative effect in several diseases (Anand et al., 2019; Dhanalakshmi et al., 2015; Gallage and Møller, 2015; Xu et al., 2015).

In a previous study, it was found that vanillin has an ameliorative effect in scopolamine induced dementia like cognitive impairment. Also, it was reported to have an *in vitro* acetylcholinesterase inhibitory activity which was also observed *in vivo*. It also has an appreciable antioxidant activity which is desirable to halt and/or delay the advancement of neurodegeneration (Anand et al., 2022). Vanillin was also reported to cause suppression of NOX-4 while simultaneously activating the Nrf2/HO-1 signalling pathway. Consequently, this dual action hindered both inflammation and apoptosis in cisplatin induced nephrotoxicity (Younis et al., 2020). This study has taken the assessment of potential usage of vanillin in anti-AD therapeutics a step further. Not only did vanillin remarkably reverse the behavioural

deficits (TL in elevated pus maze paradigm, EL in MWM paradigm, NOR test) in aluminium chloride and D-galactose inflicted AD but it was also seen to control certain key pathological hallmarks of the disease. It was found to remarkably reduce the level of beta secretase enzyme, caspase-3 enzyme and acetylcholinesterase enzyme. It is already reported that vanillin helps in disintegration of Aβ plaques *in vitro* (Song et al., 2016). *In vivo* Aβ disintegratory activity was observed in this study, confirming the previously reported *in vitro* effect. Vanillin treatment also remarkably increased the level of BDNF in diseased animals. In behavioural tests, it was also observed that vanillin is a good nootropic agent. The vanillin *per se* treatment exhibited consequential improvements in memory and cognitive capabilities in comparison with the VC counterparts.

This study has reported that vanillin ameliorates memory loss and cognitive deficits by targeting multiple pathological pathways of AD. Being a phenolic compound, its obvious antioxidant activity greatly aids in eliciting relief in AD. The free radical scavenging activity protects the neurons from undergoing degradation thereby maintain their integrity. Additionally, it has an inhibitory action on the acetylcholinesterase enzyme which elevates the cortical and hippocampal ACh levels. The protection that vanillin provides to neurons (including cholinergic neurons) owing to its antioxidant activity receives a value addition by improved levels of ACh. This results in betterment of memory and cognitive abilities. Also, vanillin has been shown to inhibit the enzyme beta secretase which plays a key role in cleaving the APP to neurotoxic $A\beta_{1-42}$ oligomers. The $A\beta_{1-42}$ oligomers in turn form aggregates that result in neuronal cell death. Vanillin, additionally, disintegrates the formed $A\beta_{1-42}$ plaques, which has already been proven *in vitro* previously (Song et al., 2016). These effects on the amyloid pathway provide a disease modifying relief in AD inflicted brain. Furthermore, BDNF levels were also found to be remarkably higher in vanillin treated groups. It indicates that

vanillin helps in restoring the BDNF mediated synaptic plasticity and promoting the survival of neuronal cells. Treatment with vanillin was also ensued a neuroprotective effect on cortical and hippocampal neurons as per the histopathological analysis.

Magnolol (as a part of Magnolia bark extract) has been used by humans since ages under Chinese and Japanese traditional medicine systems. Magnolol has been reported to have a neuroprotective effect *in vitro* by the virtue of its antioxidant and caspase-3 inhibitory activities (Hoi et al., 2010; Kuribara et al., 2000). In the present study, magnolol treatment has been shown to have a remarkable *in vivo* antioxidant activity. The expression of caspase-3 in cortical and hippocampal regions of mouse brain was also found to be reduced with magnolol treatment. Furthermore, magnolol has been reported to have an inhibitory effect on deposition of Aβ in a transgenic *C. elegans* (Xie et al., 2020). Magnolol has also been reported to suppress the M1 polarization and augment the M2 polarization through Nrf2/HO-1/NLRP3 signalling in depression like behaviour in mice (Tao et al., 2021). In our study, it was reported that magnolol decreases production of neurotoxic Aβ by inhibiting beta secretase enzyme and helps in disintegration of $A\beta_{1-42}$ oligomers. This study has reported that magnolol ameliorates the memory loss and cognitive deficits by targeting multiple pathological pathways of AD. Being a phenolic compound, its obvious antioxidant activity greatly aids in eliciting a relief in AD. The free radical scavenging activity protects the neurons from undergoing degradation thereby maintain their integrity. Additionally, it has an inhibitory action on the acetylcholinesterase enzyme which elevates the levels of ACh in cortex and hippocampus. The protection that magnolol provides to neurons (including cholinergic neurons) owing to its antioxidant activity is accentuated by improved levels of ACh. This results in betterment of memory and cognitive abilities. These effects on the amyloid pathway provide a disease modifying relief in AD inflicted brain. Furthermore, BDNF levels were also found to be remarkably higher in

magnolol treated groups. It indicates that magnolol helps in restoring the BDNF mediated synaptic plasticity and promoting the survival of neuronal cells.

Vanillin and magnolol, individually and in combination, further protect the neurons from undergoing apoptotic cell death by inhibiting caspase-3. However, the treatment with vanillin (both low dose and high dose) and similarly with magnolol could not reduce the level of $A\beta_{1-42}$ and caspase-3 to that observed in the VC group. It does not mean that the treatment with vanillin and/or magnolol was futile. The treatment produced an ameliorative effect which is shown by the consequential reduction in the level of these molecular markers contrasted with the DC group and donepezil treated group, respectively. As an adjunctive measure, vanillin and/or magnolol may delay the progression of amyloidosis and neurodegeneration even beyond the standard treatment and add some more quality years to the lives of the patients.

Probiotics have been reported to elicit potential anti-AD effects through the GBA. *L.rhamnosus*, among other probiotics, have been documented to have antioxidant and anti-inflammatory effects that make them worthy candidates to be screened for anti-AD effects (Anand et al., 2023; Capurso, 2019; Kowalski and Mulak, 2019; Segers and Lebeer, 2014). The probiotic was documented to exhibit a hepatoprotective impact by regulating the expression of PKC and the Nrf2/PGC-1α signalling pathway, thereby mitigating oxidative damage (Ahmed et al., 2022).

In our study, it has been found out that *L.rhamnosus* augments the potential anti-AD effects of vanillin and magnolol. The probiotic alone has been able to demonstrate good anti-AD activity as evident by its capability to ameliorate behavioural and biochemical deficits associated with the disease. Numerous clinical and laboratory experiments have underscored the significance of the microbiota in the progression of neurodegenerative disorders through

diverse microbial molecules that traverse from the gut to the brain via the GBA or nervous system. The aging process leads to a compromised BBB, potentially triggering cerebrovascular inflammation and CNS disorders. These molecules may exert direct influence on the BBB, affecting brain neurons, or alternatively, they can activate the immune and endocrine systems as a protective measure against neuroinflammation or neurodegeneration. Fascinatingly, the communities of microorganisms in the gut are linked to the mood and behaviour of the host, acting as contributing elements to cognitive decline. Research has indicated changes in the gut microbiota in AD (Zhang et al., 2022). The probiotic used in the current study was linked to enhanced cognitive performance in middle-aged and elderly individuals experiencing cognitive impairment (Sanborn et al., 2020). Another study reported that intervention with *L. rhamnosus* decreased the translocation of gut bacteria, reinstated the functions of the gut and BBB, and rebalanced gut bacteria in rats subjected to chronic noise. This protective effect against cognitive deficits and systemic inflammation was achieved by regulating the GBA. It stimulated the growth of advantageous bacteria, suppressed the proliferation of detrimental bacteria, ameliorated the dysregulation of bacteria producing SCFA and maintained the balance of SCFA levels (Li et al., 2023).

Vanillin and magnolol are phenolic compounds and thereby have an obvious antioxidant activity which has been augmented by the treatment of probiotic. The maintenance of neuronal integrity may be attributed to the free radical scavenging effect of vanillin, magnolol and *L.rhamnosus*, individually and in various combinations. The protection that these interventions provide to the cortical and hippocampal neurons is accentuated by the AChE inhibitory activity elicited by various treatments. These effects, when put together, contribute to the improvement of cognition and memory.

The inhibitory effect that these interventions have shown on the amyloid pathway suitably convey that vanillin, magnolol and *L.rhamnosus*, individually and in various combinations, may exert a disease altering influence in AD. The neuroprotective effect has been further supported by the capability of these interventions to control caspase-3 mediated neuronal apoptosis. Improvement in BDNF levels is an additional advantage that comes with the usage of vanillin, magnolol and *L.rhamnosus*, individually and in various combinations. It shows that these interventions can help in maintenance of synaptic plasticity that further aids in the survival of cortical and hippocampal neurons. Although the combination has not been demonstrated to completely restore various parameters like caspase-3 and $A\beta_{1-42}$ to the normal levels, the consequential effect on these markers is better than the standard treatment. The combination may not be a substitute for the standard treatment but it can be a potentially strong adjunctive measure.

L.rhamnosus, alone and in combination with natural phytoconstitutents i.e. vanillin and magnolol bear the weight of further research. The phytoconstituents, due to their negligible toxicity and wide acceptance, when combined with *L.rhamnosus* (a naturally occurring probiotic in human gut) may prove to be a safe, efficacious and cost-effective approach to manage AD.

CHAPTER-6

SUMMARY AND CONCLUSION

6. Summary and conclusion

AD is a highly prevalent and progressive neurodegenerative disorder that impacts millions of people worldwide. This debilitating ailment is characterized by a gradual decline in cognitive abilities and memory function, ultimately leading to severe disability and placing a substantial burden on both patients and their caregivers. While the precise cause of AD remains elusive, several theories have emerged to shed light on its underlying mechanisms. These theories encompass the amyloid beta hypothesis, which suggests that the accumulation of amyloid-beta plaques in the brain plays a central role in AD's development. These plaques are thought to disrupt normal neuronal function and trigger inflammation, contributing to cognitive deterioration. The cholinergic hypothesis emphasizes the depletion of acetylcholine, a vital neurotransmitter for cognitive function, as a key element in AD progression. The mitochondrial dysfunction theory posits that impaired energy production in the mitochondria of brain cells may contribute to neurodegeneration. Lastly, oxidative stress, arising from an imbalance between free radicals and antioxidants in the brain, has also been implicated in the advancement of AD.

Current pharmacotherapy for AD primarily involves drugs aimed at providing symptomatic relief, such as cholinesterase inhibitors (e.g. donepezil) and NMDA receptor antagonists (e.g. memantine). However, these medications are far from ideal, as they do not address the underlying causes of AD and are associated with various side effects. In light of these challenges, there is a dire need to develop safer, more effective, and disease-modifying treatments for AD. This study addresses this critical need by exploring potential therapeutic interventions in a mouse model of AD.

The experimental model used in the present study involved inducing AD-like pathology in Swiss Albino mice through the co-administration of aluminum chloride, a neurotoxin, and

d-galactose, a senescence accelerator. This approach aimed to mimic the pathological features and cognitive deficits observed in AD patients. The mice exhibited a range of AD-related symptoms, including behavioral and cognitive deficits, as well as cortical and hippocampal neurodegeneration. To identify potential therapeutic agents, the study focused on two natural phytoconstituents: vanillin and magnolol. Both compounds are known for their antioxidant properties due to their phenolic nature. It was hypothesized that these compounds might help counteract the oxidative stress associated with AD. Additionally, the probiotic *Lactobacillus rhamnosus* was evaluated for its potential anti-AD effects, given the emerging interest in the Gut-Brain Axis as a modulator of brain health.

The findings of the study were promising. Vanillin and magnolol, either alone or in combination, exhibited significant antioxidant activity by reducing the levels of TBARS and increasing levels of GSH and CAT. Moreover, these phytoconstituents demonstrated notable AChE inhibitory activity, which is essential for preserving cognitive function. Behavioral tests conducted on the mice further supported the potential therapeutic effects of vanillin and magnolol. These compounds ameliorated cognitive deficits, as evidenced by improved performance in tasks like the Elevated Plus Maze paradigm, Morris Water Maze test, and Novel Object Recognition (NOR) paradigm. Importantly, they also exhibited inhibitory effects on pathological markers associated with AD, including beta-secretase, caspase-3, and $A\beta_{1-42}$, suggesting their potential to modulate the disease process. Another noteworthy finding was the elevation of BDNF levels by vanillin and magnolol, both individually and in combination. BDNF is a neurotrophic factor crucial for neuronal survival and synaptic plasticity, and its upregulation is associated with improved cognitive function. *L. rhamnosus*, a commercially available probiotic, also showed promise as a potential anti-AD intervention. When administered alone, it exhibited beneficial effects in mitigating AD-like symptoms. However,

its effects were further enhanced when combined with vanillin and magnolol. This combination approach appeared to capitalize on the synergistic effects of these interventions, particularly their shared antioxidant properties.

The study's results suggest that vanillin, magnolol, and *L. rhamnosus*, both individually and in various combinations, warrant further investigation in pre-clinical and clinical research settings. These promising findings offer hope for the development of more efficacious, safer, and cost-effective approaches to the treatment and management of AD.

In conclusion, AD remains a challenging and devastating neurodegenerative disorder with a global impact. Despite numerous hypotheses regarding its pathology, there is still no definitive cure or disease-modifying treatment for AD. Current pharmacotherapies provide only symptomatic relief and are associated with side effects. The present study represents a significant step toward addressing this unmet medical need by exploring potential therapeutic interventions in a mouse-based model of AD. The use of vanillin and magnolol, natural phytoconstituents with antioxidant properties, offers a promising avenue for further research. These compounds not only demonstrated antioxidant activities but also exhibited acetylcholinesterase inhibitory effects and ameliorated behavioral deficits in the AD model. Importantly, they showed inhibitory effects on key pathological markers associated with AD and increased levels of BDNF, a neurotrophic factor crucial for neuronal health. Furthermore, the study introduces the potential role of probiotics, specifically *L. rhamnosus*, in AD management. Probiotics have a rich legacy of being used in treating various ailments, primarily related to gut health. The combination of *L. rhamnosus* with vanillin and magnolol highlighted the potential synergistic effects of these interventions, particularly their antioxidant properties.

The findings of this study undoubtedly offer promise in the ongoing quest to combat AD. However, it is crucial to interpret these results with a sense of caution, recognizing that

the research was conducted in a mouse model. While animal studies can provide valuable insights and serve as initial indicators of therapeutic potential, they do not necessarily guarantee success when applied to human subjects. As such, further stages of investigation, encompassing both pre-clinical and clinical studies involving human participants, are imperative to corroborate and translate these promising findings into practical therapies for AD. Moreover, the importance of safety cannot be overstated. Before any potential treatment can be considered for widespread use in humans, it must undergo rigorous evaluation to ascertain its safety profile. This entails thorough investigation into the potential short-term and long-term consequences of the treatments under consideration. AD is a complex and multifaceted condition, and the introduction of new therapies must be approached with the utmost caution to avoid unintended adverse effects. AD presents a significant and growing public health challenge, with millions of individuals and their families grappling with its devastating effects worldwide. Discovering effective treatments is an urgent priority, and the exploration of natural compounds and probiotics as potential therapies is a hopeful development. These novel approaches may offer innovative avenues to address AD, potentially complementing or enhancing existing treatments. Continued research in this direction holds the promise of delivering safer and more effective therapies for AD, ultimately improving the quality of life for those affected by the disease and alleviating the burden on their caregivers and loved ones.

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APPENDICES

APPENDIX-I

LOVELY PROFESSIONAL UNIVERSITY

Centre for
Research Degree Programmes

LPU/CRDP/PHD/EC/20200208/000594

Dated: 05 Mar 2019

Abhinav Anand
Registration Number: 41700258
Programme Name: Doctor of Philosophy (Pharmacology)

Subject: Letter of Candidacy for Ph.D.

Dear Candidate,

We are very pleased to inform you that the Department Doctoral Board has approved your candidacy for the Ph.D. Programme on 05 Mar 2019 by accepting your research proposal entitled: "Pharmacological Evaluation of Selected Phytoconstituents in combination with Probiotics for Alzheimer's disease using Animal Model :A Mechanistic study"

As a Ph.D. candidate you are required to abide by the conditions, rules and regulations laid down for Ph.D. Programme of the University, and amendments, if any, made from time to time.

We wish you the very best!!

In case you have any query related to your programme, please contact Centre of Research Degree Programmes.

Head

Centre for Research Degree Programmes

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APPENDIX-II

Publications related to Ph.D work

1. **Anand, A**., Khurana, N., Kaur, S., Ali, N., AlAsmari, A.F., Waseem, M., et al. (2023a). The multifactorial role of vanillin in amelioration of aluminium chloride and D-galactose induced Alzheimer's disease in mice. Eur J Pharmacol *954*: 175832.
2. **Anand, A**., Khurana, N., Kumar, R., and Sharma, N. (2023b). Food for the mind: The journey of probiotics from foods to anti-Alzheimer's disease therapeutics. Food Biosci *51*: 102323.
3. **Anand, A**., Khurana, R., Wahal, N., Mahajan, S., Mehta, M., Satija, S., et al. (2019). Vanillin: A comprehensive review of pharmacological activities.
4. **Anand, A**., Nuthan Kumar Babu, V., Giriyam, R., Turan, S., Sharma, N., Khurana, N., et al. (2021). The relationship of magnolol, an important phytoconstituent, with neurological disorders: An in silico evaluation. Plant Cell Biotechnol Mol Biol *22*: 389–397.
5. **Anand, A**., Sharma, N., Gulati, M., and Khurana, N. (2018). Amyloid Beta: The Foremost Protagonist in Alzheimer's Disease. Critical Examinations of Neurodegenerative Disorders. IGI Global, pp 235.
6. Kumar, R., Kumar, R., **Anand, A**., Sharma, N., and Khurana, N. (2019). Pharmacological Management of Alzheimer's Disease. Advances in Neuropharmacology Drugs and Therapeutics. Apple Academic Press and CRC Press (Taylor & Francis Group), pp 133.

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The multifactorial role of vanillin in amelioration of aluminium chloride and D-galactose induced Alzheimer's disease in mice

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ABSTRACT

The onset and progression of Alzheimer's disease (AD) are influenced by a variety of factors. These include oxidative stress, overexpression of acetylcholinesterase (AChE), depletion of acetylcholine levels, increased beta-secretase mediated conversion of Amyloid Precursor Protein (APP) to Amyloid Beta (Abeta), accumulation of Abeta oligomers, decrease in Brain Derived Neurotrophic factor (BDNF) and accelerated neuronal apoptosis due to elevated levels of caspase-3. The currently available therapeutic approaches are inadequate in affecting these pathological processes except maybe the overexpression of AChE (AChE inhibitors like donepezil, rivastigmine). There is an urgent need to develop disease modifying pharmacotherapeutic interventions which have appreciable safety and cost effectiveness. From previously reported *in vitro* studies and a preliminary assessment of neuroprotective effect in scopolamine induced dementia-like cognitive impairment in mice, vanillin has been used as the compound of interest in the present study. Vanillin, a phytoconstituent, has been used in humans, safely, in the form of a flavouring agent for various foods, beverages, and cosmetics. Owing to its chemical nature i.e. being a phenolic aldehyde, it has an additional antioxidant property that is congruent to the desirable characteristics that are sought in a suitable novel anti-AD agent. In our study, vanillin proved to have a nootropic effect in healthy Swiss albino mice as well as an ameliorative effect in aluminium chloride and D-galactose induced AD model in mice. Apart from tackling oxidative stress, vanillin was found to reduce the levels of AChE, beta secretase, caspase-3, enhance degradation of Abeta plaques and elevate the levels of BDNF, in cortical and hippocampal regions. Vanillin is a promising candidate for being incorporated into the search for safe and effective anti-AD molecules. However, further research might be needed to warrant its application clinically.

Credit author statement

Abhinav Anand: Conceptualization, contributed to the conception and design of the study, conducted the study under the supervision, Supervision, wrote the first draft of the manuscript, Writing – original draft. Navneet Khurana: contributed to the conception and design of the study, conducted the study under the supervision, Supervision. Satinder Kaur: Formal analysis, helped in the statistical analysis part of manuscript, wrote sections of the manuscript and proofread the manuscript. Nemat Ali: Formal analysis, helped in the statistical analysis part of manuscript, wrote sections of the manuscript and proofread the manuscript. Abdullah F. AlAsmari: Formal analysis, helped in the statistical analysis part of manuscript, wrote sections of the manuscript and proofread the manuscript. Mohammad Waseem: Formal analysis, helped in the statistical analysis part of manuscript, wrote sections of the manuscript and proofread the manuscript. Muzaffar Iqbal: Formal

Abbreviations: AD, Alzheimer's disease; Abeta, Amyloid Beta; AChE, Acetylcholinesterase; BDNF, Brain Derived Neurotrophic Factor; TBARS, Thiobarbituric Acid Reactive Substances; GSH, Reduced Glutathione; CAT, Catalase activity; TL, Transfer Latency; EL, Escape Latency; MWM, Morris Water Maze; ELISA, Enzyme Linked Immunosorbent Assay; NOR, Novel Object Recognition; ROS, Reactive Oxygen Species.

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Food for the mind: The journey of probiotics from foods to anti-Alzheimer's disease therapeutics

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disease which finds its place in the top five reasons for mortality across the globe. Till date, there is no disease modifying therapeutic approach for AD. The pharmacological treatment does not provide anything except mere symptomatic relief. In the recent years, research on the Gut Brain Axis (GBA) has garnered keen attention of researchers worldwide. It may be possible that a disease modifying approach for AD has been present in nature since ages waiting to be discovered. This comprehensive review aims at establishing a concrete link between the consumption of probiotics and neuroprotection in AD. Fermented foods are deeply seated in the culinary history of every civilization. The multiple benefits associated with the usage of probiotics have become more like a legacy that is handed down from generation to generation. The use of probiotics to achieve therapeutic aim in cases of AD via the GBA has become a highly researched area. Probiotics have anti-inflammatory and immunomodulatory effects. These have been found to hold significance in the field of food based anti-AD approaches. Probiotics have been reported to maintain the integrity of intestinal epithelium and the blood brain barrier. Also, the recently available knowledge about the molecular mechanisms behind the anti-inflammatory effect of probiotics in AD is promising. Adding more feathers to the hat, probiotics have been found to have remarkable antioxidant activity and cognition enhancing capability. All these factors make a very strong case in favour of probiotics to be explored more deeply as anti-AD therapeutics.

1. Introduction

While the process of fermentation is significantly older than the emergence of modern man, our ancestors have harnessed this microbe-based technology for production and preservation of foods for millennia. In several cases, some typical fermented foods serve as markers of cultural identity. Some archaeological studies have reported that some ancient civilisations were utilising these fermentation technologies quite fondly (Garbarino, 2021). Fig. 1 illustrates various fermentation milestones of human history (Farnworth, 2008).

The records show that intentional fermentation began in the Fertile Crescent as far back as 6000 BC. Since then, at least one fermented food has been an integral part of almost every civilisation (Densel, 2017). Some popular fermented foods have been listed in Table 1.

Some Eastern cultures have been using fermented foods for medicinal purposes, which form a therapeutic approach for the dietitians who believe in "food as medicine." Relationship between health and fermented foods has been found in records dating back to ancient Rome and China, and to date they exist as a field of immense interest for researchers worldwide (Foroutan, 2012).

Over the past few years, there has been an active research impetus on the relationship between functional foods and cognition. There have been several reports where consumption of certain foods has been able to halt or retard the progress of neurodegeneration (Romanenko, Kholin, Koliada, & Vaiserman, 2021). Nutraceuticals have been shown to manipulate various metabolic and biochemical processes which in turn proves to be beneficial in AD. It includes protection of neurons against attack of reactive oxygen species, mitochondrial damage, amyloid beta and tau dependent apoptotic cell death (Atlante, Amadoro, Bobba, & Latina, 2020). Post absorption and assimilation, nutraceuticals have been proven to behave like drugs to enhance the resistance of the body against certain offending agents (Helal et al., 2019). Probiotics are the nutraceuticals which contain live microbes intended to be used for imparting benefit(s) to the human body (Sharma, Amanjot, Sourav, Keerti, & Sujit, 2017). The crosstalk between the gut and brain (Ağagündüz et al., 2022) has been the ground for several studies

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VANILLIN: A COMPREHENSIVE REVIEW OF PHARMACOLOGICAL ACTIVITIES

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Abstract

Vanillin is the chief constituent of the vanilla bean extract obtained from the seed pods of *Vanillus planifolia* belonging to the family Orchidaceae. Nowadays, synthetic vanillin is being preferred as a flavouring agent in foods, beverages and pharmaceuticals. Chemically, it is a phenolic aldehyde with the molecular formula $C_8H_8O_3$. Over years it has been used as a flavor in sweet foods like chocolates & ice creams, in cosmetics like perfumes, and in pharmaceuticals to mask the unpleasant odor and tastes in medicines. Also, vanillin-HCl is extensively used a visualizing agent in Thin Layer Chromatography. Quite recently, vanillin has been studied by researchers all over the world for its pharmacological effects. The compound has exhibited remarkable effects in treatment and management of several pathological conditions. This review focuses on the different reported pharmacological activities of vanillin.

Keywords: Antioxidant, Alzheimer's disease, flavouring agent, neuroprotection, pharmacological effects, vanillin

Introduction

Recently, there has been increasing research in the field of natural foods, specifically with respect to the additives such as flavouring agents and preservatives. Amidst the variety of flavouring agents of natural origin being used today, vanilla encompasses a prominent place in the market. It has been extensively used for the preparation of cakes, soft drinks, ice creams, chocolates, liquors, perfumes, pharmaceuticals, and nutraceuticals (Ranadive, 1992). Natural vanilla is a concoction of components extracted from the cured pods of different Vanilla species: *Vanillus planifolia* and *Vanillus tahitensis* (Ramachandra Rao & Ravishankar, 2000). However, due to its pod quality and yield *V. planifolia* is valued the most (Sinha, Sharma, & Sharma, 2008).

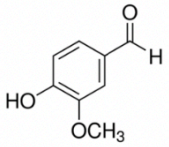

Fig. 1: Chemical structure of 4-Hydroxy-3-methoxybenzaldehyde (Vanillin) (CAS No. 121-33-5)

The flavour profile of vanilla comprises of more than 200 constituents, only 26 of which occur in amounts greater than 1 mg/kg. The aroma and flavour of vanilla extract is attributed mainly to presence of vanillin (Figure 1), which occurs in a concentration of 1-2% w/w in vanilla pods (Bettazzi, Palchetti, Sisalli, & Mascini, 2006; Sinha et al., 2008; Westcott, Cheetham, & Barraclough, 1993)

1.1 Physical and Chemical Properties

Table 1: Various properties of vanillin

Parameter	Description	Reference
Molecular weight	152.149 g/mol	(PubChem, 2017)
Appearance	White or very slightly yellow needles	(PubChem, 2017)
Odour	Pleasant aromatic vanilla odor	(The Merck Index Online, 2013)
Taste	Pleasant vanilla taste	(The Merck Index Online, 2013)
Boiling point	285 deg C	(The Merck Index Online, 2013)
Melting point	81-83 deg C	(Sigma-Aldrich, 2017)
Flash point	153 deg C	(Sigma-Aldrich, 2017)
Solubility	125 parts of water, 20 parts of glycerine, 2 parts of 95% alcohol, chloroform and ether	(PubChem, 2017)
Density	1.056	(The Merck Index Online, 2013)
Vapour pressure	1.18 X 10^{-4} mm Hg at 25 deg C	(PubChem, 2017)
Partition coefficient	log $K_{o/w}$ = 1.37	(PubChem, 2017)
Stability	Photosensitive, gradually oxidizes in presence of moisture	(The Merck Index Online, 2013)
Auto ignition	>400 deg C	(PubChem, 2017)
Decomposition	Emits acrid smoke and irritating fumes	(PubChem, 2017)
pH	Acidic to litmus	(The Merck Index Online, 2013)

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THE RELATIONSHIP OF MAGNOLOL, AN IMPORTANT PHYTOCONSTITUENT, WITH NEUROLOGICAL DISORDERS: An *In silico* EVALUATION

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ABSTRACT

Purpose of the work: While the world is going towards financially straining research on synthetic molecules, there are several phytochemicals that are still not explored to the fullest of their potential. Magnolol is a polyphenolic compound obtained from the root and stem bark of *Magnolia* species. The roots and bark of the plants have been in use for ages in Japanese and Chinese traditional medicine systems for the treatment of several neurological disorders. Magnolol can be explored for its beneficial effects in neurological disorders *in vivo*.

Techniques and methods used: This study aims to estimate the physicochemical properties, pharmacokinetics, toxicology of magnolol *in silico* with the help of online programs like SwissADME and admetSAR. Further, various possible biological activities have been predicted using another *in silico* model i.e., Prediction of Activity Spectra of Substances (PASS) online.

Major findings: Magnolol has been reported to be a slightly water soluble compound with high gastrointestinal absorption and a capability of permeating the Blood Brain Barrier. It has been predicted to have antioxidant activity along with membrane protecting capability. It has also been predicted to have beneficial effects in neurological disorders including but not limited to dementia. It has been estimated to belong to 'Category III' products for oral toxicity. Also, it has been estimated to be non-carcinogenic.

Conclusion: Magnolol has several desirable characteristics that make it an appropriate choice for *in vivo* evaluation for several neurological disorders like anxiety, depression, Parkinson's disease, Alzheimer's disease, to name a few.

Chapter 11
Amyloid Beta:
The Foremost Protagonist in Alzheimer's Disease

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ABSTRACT

Alzheimer's disease (AD), exhibiting accumulation of amyloid beta (Aβ) peptide as a foremost protagonist, is one of the top five causes of deaths. It is a neurodegenerative disorder (ND) that causes a progressive decline in memory and cognitive abilities. It is characterized by deposition of Aβ plaques and neurofibrillary tangles (NFTs) in the neurons, which in turn causes a decline in the brain acetylcholine levels. Aβ hypothesis is the most accepted hypothesis pertaining to the pathogenesis of AD. Amyloid Precursor Protein (APP) is constitutively present in brain and it is cleaved by three proteolytic enzymes (i.e., alpha, beta, and gamma secretases). Beta and gamma secretases cleave APP to form Aβ. Ubiquitin Proteasome System (UPS) is involved in the clearing of Aβ plaques. AD also involves impairment in UPS. The novel disease-modifying approaches involve inhibition of beta and gamma secretases. A number of clinical trials are going on worldwide with moieties targeting beta and gamma secretases. This chapter deals with an overview of APP and its enzymatic cleavage leading to AD.

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CHAPTER 5

Pharmacological Management of Alzheimer's Disease

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder, which is characterized by severe progressive loss of neurons in the central nervous system. AD is the basic type of dementia among the geriatrics that slowly decreases memory and alters the thought process, reduces understanding the simple tasks of the daily routine, and with passing time, it becomes more problematic. A neuronal destruction occurs in the AD with less number of viable neuron cells. As it is progressive in nature, it ultimately leads to shrinkage of the brain. The exact pathology of the AD is still unknown but it involves the accumulation of the amyloid-β (Aβ) and neurofibrillary tangles (NFTs) in the hippocampus and cortex region of the brain that both Aβ and NFTs lead degeneration and alteration function of the neurons. Various medicines are recommended for the treatment of the AD, but these medicines provide only symptomatic relief and are not able to treat the underline pathological cause. In the present chapter, various drugs which are used for the management of AD with their mechanism and side effects along with the drugs which are under investigation are discussed.

5.1 INTRODUCTION

The word dementia, obtained from the Latin word "de" and "mente" means "out (de) of one's mind (mente)." Dementia is a common term which is characterized by deterioration of cognitive function, involving loss of memory, impairment of language and disorientation as well as a degree of mental ability in the patient to interfere daily life (Boller and Forbes, 1998). Alzheimer's disease (AD) is the general type of dementia. AD accounts about 80% cases that are more than other all type of dementia such as 25% vascular dementia, 15% Lewy body dementia and frontotemporal (FT) dementia (Boller and Forbes, 1998; Alzheimer's Association, 2015).

AD was described first time by the neuropathologist and the German psychologist Alois Alzheimer in 1906. At the beginning of the 21st century, the highly pervasive dementia type in people older than 60 years was acknowledged (World Health Organization, 2012). In 2016, worldwide people suffering from dementia were estimated to be more than 47.5 million. By 2030, it is being estimated globally up to 75.6 million people with dementia (World Health

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Ameliorative effect of vanillin on scopolamine-induced dementia-like cognitive impairment in a mouse model

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Background: Alzheimer's disease (AD) is the most common form of dementia, which is among the top five causes of death in the United States. It is a neurodegenerative disorder that causes permanent loss of memory and cognition. The current pharmacotherapy for AD is based on providing symptomatic relief only and has many side effects. There is a need for a safer, disease-modifying drug for the treatment of AD.

Experimental approach: The PASS online software was used to screen phytoconstituents based on their predicted effects on various AD-related targets. Vanillin was selected as the compound of interest, as it has not been researched elaborately on any animal model of AD. The acetylcholinesterase inhibitory activity of vanillin was established *in vitro*. Thereafter, ameliorative effect of vanillin was evaluated using the exteroceptive memory model in scopolamine-induced cognitive impairment mice model.

Results: Vanillin showed an acetylcholinesterase inhibitory activity *in vitro*, and the IC_{50} value was calculated to be 0.033 mM. Vanillin significantly reversed the memory and behavioral deficits caused by scopolamine as demonstrated by significant improvement in memory in negative reinforcement, elevated plus maze, and spatial learning paradigms. Vanillin also proved to have a nootropic effect. Also, vanillin proved to have significantly better antioxidant and acetylcholinesterase inhibitory effects *in vivo* than donepezil hydrochloride. The potential anti-AD activity of vanillin was also confirmed by the reduction in IL-6 levels and TNF-α levels.

Conclusion: Our results suggest that vanillin is a safe and effective natural drug candidate having a great potential for the treatment of AD. However, more research is required to evaluate its effect on A beta plaques and Tau neurofibrillary tangles *in vivo*.

KEYWORDS

acetylcholinesterase, Alzheimer's disease, antioxidant, dementia, scopolamine, vanillin

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CHAPTER 10

Quantification of the SARS-CoV-2 RNA in Tissues by Quantitative Real Time-PCR

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Abstract: Severe Acute Respiratory Syndrome-coronavirus-2 (SARS-CoV-2) has become infamous recently due to its capability to cause a severe disease known as coronavirus disease-19 (COVID-19). Believed to have originated from Wuhan, Hubei province, China, SARS-CoV-2 has impacted a global scale, thereby making the World Health Organization (WHO) declare COVID-19 a pandemic. The range of R_0 values is between 2.43 and 3.10, showing the severity of the disease, highlighting this concern for health and public administration authorities. The major preventive steps from the governments and public health authorities are to conduct aggressive diagnostic tests and isolate the patients until the complete recovery of COVID-19. Out of various diagnostic methods, reverse transcription-polymerase chain reaction (RT-PCR) is an appropriate technique. The amplified RNA/DNA molecules can be quantified through quantitative real-time PCR (qRT-PCR). In this chapter, the basics of PCR shall be discussed along with the quantification of RNA from SARS-CoV-2 infected tissue samples.

Keywords: Coronavirus, COVID-19, Diagnosis, PCR, qPCR, RT-PCR.

INTRODUCTION

Human coronaviruses were first identified in the early 1960s, they have been associated with a significant incidence of infections in the upper respiratory tract. Since the beginning of the 21st century, at least five novel coronaviruses have been identified to cause severe human illnesses. It includes severe acute respiratory syndrome (SARS), Middle Eastern respiratory syndrome (MERS), and severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2), which caused

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CHAPTER 2

Different Cell Lines for SARS-CoV-2

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Abstract: The recent emergence of the novel *betacoronavirus*, pathogenic severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) with high nucleotide identity to SARS-CoV represents the causative agent of a potentially deadly disease coronavirus disease-19 (COVID-19) in Wuhan, China, and spreading across several countries globally pose a great global public health concern. Until a vaccine is available, effective therapy must be identified, and many clinical trialshave been executed worldwide. Various *in vitro* investigations are ongoing using different cell cultures to find alternative treatment options, allowing SARS-CoV-2 replications. Various cell lines are susceptible to the SARS-CoV-2 infection. In this chapter, literature regarding SARS-CoV-2 isolated in several cell lines commonly used for diagnostic or research purposes has been summarized. It also shows that SARS-CoV-2 can achieve high titers in various cell cultures derived from different species. In addition, these cell lines are extensively used in the diagnosis, to study pathophysiology, genome studies, and the finding of new targets for drug development and provide new ideas for the discovery of lead compounds with potential therapeutic agents against novel COVID-19.

Keywords: Anti-viral, Betacoronavirus, Cell lines, Clinical trials, Lead compounds, SARS-CoV-2.

INTRODUCTION

The first outbreak of the novel coronavirus occurred in Wuhan on December 12, 2019, in China, and abruptly spread to several other nations. The World Health Organization (WHO) declared a name for 2019-nCoV infectious disease coronavirus disease-19 (COVID-19) on February 11, 2020. The virus name was renamed Severe Acute Respiratory Syndrome-coronavirus-2 (SARS-CoV-2) by the International Committee of Taxonomy of Viruses, earlier called 2019-nCoV [1].

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Research Article

PREDICTION OF ANTI-PARKINSON POTENTIAL OF PHYTOCONSTITUENTS USING PREDICTION OF ACTIVITY SPECTRA OF SUBSTANCES SOFTWARE

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ABSTRACT

Objective: Neurodegenerative disorders are group of diseased conditions in which there is loss of neuron cells occur. The main objective of this study to find/search out the phytochemical with the help of prediction of activity spectra of substances (PASS), those show maximum activity over the selected targets of the Parkinson's disease (PD).

Methods: PASS is a valuable software which is used in this study, to predict the anti-Parkinson activity of different compounds. Canonical simplified molecular-input line-entry system is used for the prediction of anti-Parkinson activity which is obtained from PubChem website. The predicted activity also compared with marketed compound like levodopa.

Results: From the study, it was found that resveratrol was the only compound which has the activity on all the selected targets. On the other hand, stemazole and celastrol were found to have the least active compounds as both have the activity only on a single target.

Conclusion: In this research work, we tried to compile the information regarding the PASS predicted anti-Parkinson activity of some important phytoconstituents. We found that resveratrol can be a target for further investigation in the development of drug therapy for PD.

Keywords: Parkinson's disease, Prediction of activity spectra of substances, Levodopa, Postural instability.

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INTRODUCTION

Parkinson disease (PD) is the second most common, age-related chronic neurodegenerative disorder. Nowadays, PD is the most prevalent disease. Approximate 7–10 million people of the world surviving with the PD, of these 1 million lives in the U.S. men are at a greater risk of PD compared to woman. As the age increases, the incidence of PD also increases. Mostly the people with the age of 60 years or above are the targets of the PD. PD becomes the 14th leading cause of death in the U.S [1,2]. The key symptoms of PD are tremors, rigidity, bradykinesia, postural instability, cognitive dysfunction, and sleep disturbance. These symptoms appeared due to progressive loss of dopaminergic neurons in substantia nigra pars compacta of brain, which further lead to loss of dopamine (DA) [2,3]. Another pathological hallmark of PD is the presence of eosinophilic intracytoplasmic proteinaceous inclusion bodies that are mainly composed of fibrillar α-synuclein termed Lewy bodies and dystrophic neurites (Lewy neurites) presents in surviving neurons [4].

The etiology of PD is still unknown, but there are several factors such as environmental, genetics, age, and medication which can be responsible for the development of PD. The available drug therapy which includes levodopa and carbidopa provide only symptomatic relief but able to cure the underline cause of the PD. These drugs also produce certain side effects such as nausea, vomiting, hallucination, convulsions, and dry mouth (Fig. 1) [1,5].

Herbal medicines have a long history of safe and efficacious administration as therapeutic agent or dietary supplement in different parts of the world. Due to the presence of various type of phytochemical in them, these herb possesses protective and therapeutic effect in various indications. Nowadays, plant chemicals are of great interest for the development of medicine for various diseases [6]. Development of new safe and effective medicine is not an easy task. Various phytochemicals have been reported to be effective in *in vitro* and *in vivo* models of PD, but no one can enter into the mainstream of drug development due to lack of information.

Prediction of activity spectra for substances (PASS) is a computer-based software, which provides the information regarding the biological activities on different targets of the chemical compound on the bases of their chemical structure. The software predicts the activity of the compound in two probabilities; one is probable activity (Pa) and another one is probable inactivity (Pi). The value of Pa and Pi varies from 0.000 to 1.000. If Pa > Pi for any activity, only then that activity can be designated to the compound. If Pa value is more than 0.7 for any activity, then the probability of that action is high in pharmacological experiment. If Pa value is more than 0.5 but <0.7 for any activity, then the probability of that action is less in pharmacological experiment. If Pa value is <0.5, then the probability of observing that activity in pharmacological experiment is less [7-10].

METHODS

For the evaluation of the activity using PASS, first, we select certain phytoconstituents which have already reported to have pharmacological effect in the *in vivo* and *in vitro* models of PD, and one marketed standard drug compound for PD that is levodopa. For the prediction of activity in the different targets of PD, canonical simplified molecular-input line-entry system (SMILES) is used which is obtained from PubChem website shown in Table 1. These SMILES work as a molecular formula of the compound and are directly copied into the PASS software to predict the activity.

RESULTS

The selected phytochemical and marketed compound was predicted using PASS for nine activities in the PD shown in Table 2. These activities are as follows:

ORIGINAL ARTICLE

Evaluation of Nephroprotective Activity of Gallic Acid in Gentamicin-induced rat Model of Nephrotoxicity

Kunal Manshare[1], Abhinav Anand[1], Sanchit Mahajan[2], Saurabh Satija[1], Neha Sharma[1], Navneet Khurana[1], Navneet Duggal[1], Meenu Mehta[3]

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Abstract

Introduction: Renal damage due to gentamicin is associated with oxidative stress. Gallic acid is a phenolic compound that possesses antioxidant and anti-inflammatory properties. Hence, an attempt was made to explore the nephroprotective activity of gallic acid in gentamicin-induced nephrotoxicity. **Materials and Methods:** Wistar albino rats of either sex were used. Experimental nephrotoxicity was produced by intraperitoneal administration of gentamicin for 8 days. Rats were divided into five groups: Group I - normal control (normal Saline), Group II - gentamicin only treated group (100 mg/kg), Group III - gentamicin (100 mg/kg) + Vitamin C (200 mg/kg), Group IV (treatment group) - gentamicin (100 mg/kg) + gallic acid (200 mg/kg), and Group V - gentamicin (100 mg/kg) + gallic acid (400 mg/kg). The period of drug administration was of 8 days, in which animals were treated with Vitamin C and gallic acid. After the treatment for 8 days, the animals were sacrificed for the investigation of biochemical parameters and histopathological examination. **Results:** Gentamicin-induced nephrotoxicity was successfully reproduced. Concurrent administration of gallic acid along with gentamicin significantly prevented the rise in level of serum creatinine, serum urea, blood urea nitrogen, and total protein. Administration of gallic acid also leads to increase glutathione and superoxide dismutase level in the kidney. Therefore, gallic acid had significantly prevented nephrotoxicity as compared to the group receiving gentamicin drug alone. **Conclusion:** These results showed that gallic acid is effective as nephroprotective agent.

Key words: Gallic acid, gentamycin, nephrotoxicity

INTRODUCTION

Kidneys play a variety of important functions in the body. They help in maintaining the level of ions such as Na^+, K^+, Cl^-, Ca^{2+},and Mg^{2+} in blood plasma and excretion of nitrogenous waste products such as urea and creatinine from the body. Kidneys also play an important role in maintaining the acid-base balance in the body. Nephrotoxicity is defined as the renal damage and tubular injuries.[1] It produces harmful effects in our body and increases the level of ions, serum creatinine, serum urea, blood urea nitrogen (BUN), and serum total protein in the body.[2] Nephrotoxicity can be of various forms such as tubular necrosis, nephropathies, structural changes in kidney, and inflammation in renal cells.

Gentamicin is an aminoglycoside antibiotic that produces nephrotoxicity and 10–20% of people receiving aminoglycoside antibiotic gentamicin suffer from kidney disorders.[3] The mechanism of gentamicin-induced nephrotoxicity in rats is through generation of oxidative stress which is mainly due to the production of reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals, and hydrogen peroxide and inhibition of oxidative phosphorylation.[4,5]

ROS may result in the development of oxidative stress that may cause kidney damage.[6] Various inflammatory mediators responsible for inflammation may also result

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ORIGINAL ARTICLE

Identification of molecular targets of potential antidiabetic drugs using prediction of activity spectra for substances and molecular docking

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Abstract

Context: Diabetes mellitus is not a solitary sickness yet is a gathering of metabolic issue influencing countless on the planet. It is essentially described by incessant hyperglycemia because of deformities in insulin discharge or insulin activity. It is predicated that the quantity of diabetes individual on the planet could reach up to 366 million by the year 2030. Even though the instances of diabetes are expanding step by step, aside from insulin and oral hypoglycemic medications, no other method for treatment has been effectively grown up until now. **Objective:** In the present study, an initiative is tried to delineate the usefulness of prediction of activity spectra for substances (PASS) online software and molecular docking technique for providing new molecular ways of predicting new antidiabetic drug targets of potential phytoconstituents. **Materials and Methods:** In the study, important phytoconstituents having reported *in vitro* and *in vivo* antidiabetic activities have been reviewed. Among them, few phytoconstituents were selected for presenting to PASS online software. Pa and Pi value was predicted for these phytoconstituents on different antidiabetic target sites. Based on PASS prediction, five phytoconstituents were selected for molecular docking study using AutoDock Vina 4.0. Three target sites which were dipeptidyl peptidase-4 (DPP-4), glucagon-like peptide-1 (GLP-1), and α glucosidase were selected for prediction of probable affinities of these 5 selected phytoconstituents. **Result and Discussion:** Among these five constituents, diosmin showed best binding affinity with DPP-4, GLP-1, and α glucosidase that was −10.2 kcal/mol, −8.3 kcal/mol, and −9.7kcal/mol, followed by kaempferol. Results of the present study can be utilized for designing of further *in vitro* and *in vivo* antidiabetic studies for these phytoconstituents. **Conclusion:** This study suggested the usefulness of these software in predicting the probable antidiabetic targets sites of potential antidiabetic phytoconstituents.

Key words: Antidiabetic, hyperglycemia, hypoglycemia, molecular docking, phytoconstituents, prediction of activity spectra for substances

INTRODUCTION

Diabetes is the issue in which human body cannot make its own particular vitality.[1-12] Rather than getting transform into vitality, it gets changed over into sugar or supposed glucose. Pancreas is the organ which secretes the hormones called insulin which takes glucose into the phone into for vitality generation. If there should arise an occurrence of diabetes, the human system either do not secrete required insulin or is not able to utilize its own insulin extremely well. This outcome in the gathering of sugar in the blood that is the reason all called "sugar." This lack of insulin results in a condition Known as diabetes or in laymen dialect, it is known as "Sugar".[13-24] It might bring about different dangerous medical problems including heart issue, night visual deficiency, and renal dysfunctioning. This sickness is considered to be the seventh principal reason of death in The United States of America.[25]

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Publications in communication:

1. The Multifactorial Role of Magnolol in Amelioration of Aluminium Chloride and D-galactose Induced Alzheimer's disease in Mice.
2. *Lactobacillus rhamnosus*: A probiotic augmenting the ameliorative effects of vanillin and magnolol in aluminium chloride and D-galactose induced Alzheimer's disease in mice.
3. Classical and modified animal models for chemically induced Alzheimer's disease with assessment parameters: A review on applicability.
4. The potential role of vanillin in neurological disorders: An *in silico* evaluation

APPENDIX-III

Copyrights registered:

1. Kynurenine pathway and its role in neuroinflammation.
2. Potential implication of gut-brain-axis and toll like receptors in Alzheimer's disease.
3. Mechanism of aluminum-induced neurodegeneration.

Copyrights in communication:

1. Mechanism of D-Galactose induced senescence associated neurodegeneration.

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1. Electroconvulsiometer Apparatus
2. Cost effective liquid chromatogram.
3. IOT based agricultural field grooving machine.

Patent in communication

1. *Lactobacillus rhamnosus*: A probiotic augmenting the ameliorative effects of vanillin and magnolol in aluminium chloride and D-galactose induced Alzheimer's disease in mice.

Intellectual Property Office

Certificate of Registration for a UK Design

Design number: 6316607

Grant date: 10 October 2023

Registration date: 03 October 2023

This is to certify that,

in pursuance of and subject to the provision of Registered Designs Act 1949, the design of which a representation or specimen is attached, had been registered as of the date of registration shown above in the name of

Mr. Abhinav Anand, Mr. Suman Kumar Mekap, Dr. Sandhya Subhadra,

Kuchibhotla Phani Sarada Praneetha , Sabita Sahoo, Satyabrata Jena, Ms.

Neelam Kumari , Dr. Daisy Sharma

in respect of the application of such design to:

ELECTROCONVULSIOMETER APPARATUS

International Design Classification:
Version: 14-2023
Class: 24 MEDICAL AND LABORATORY EQUIPMENT
Subclass: 01 APPARATUS AND EQUIPMENT FOR DOCTORS, HOSPITALS AND LABORATORIES

Adam Williams
Comptroller-General of Patents, Designs and Trade Marks
Intellectual Property Office
The attention of the Proprietor(s) is drawn to the important notes overleaf.

ORIGINAL

मूल/No : 120449

सत्यमेव जयते

भारत सरकार
GOVERNMENT OF INDIA
पेटेंट कार्यालय
THE PATENT OFFICE

डिजाइन के पंजीकरण का प्रमाणपत्र
CERTIFICATE OF REGISTRATION OF DESIGN

डिजाइन सं. / Design No.	:	350952-001
तारीख / Date	:	07/10/2021
पारस्परिकता तारीख / Reciprocity Date*	:	
देश / Country	:	

प्रमाणित किया जाता है कि संलग्न प्रति में वर्णित डिजाइन जो **COST EFFECTIVE LIQUID CHROMATOGRAM** से संबंधित है, का पंजीकरण, श्रेणी **24-01** में 1.Dr. Vaneet Kumar 2. Dr. Anshul Chawla 3.Mr. Abhinav Anand 4.Ms. Shina Kalsi 5.Dr. S P Gautam 6.Ms. Dasiy 7.Prof. Ramesh Chandra Panda के नाम में उपर्युक्त संख्या और तारीख में कर लिया गया है।

Certified that the design of which a copy is annexed hereto has been registered as of the number and date given above in class **24-01** in respect of the application of such design to **COST EFFECTIVE LIQUID CHROMATOGRAM** in the name of 1.Dr. Vaneet Kumar 2. Dr. Anshul Chawla 3.Mr. Abhinav Anand 4.Ms. Shina Kalsi 5.Dr. S P Gautam 6.Ms. Dasiy 7.Prof. Ramesh Chandra Panda.

डिजाइन अधिनियम, 2000 तथा डिजाइन नियम, 2001 के अध्यधीन प्रावधानों के अनुसरण में।
In pursuance of and subject to the provisions of the Designs Act, 2000 and the Designs Rules, 2001.

INTELLECTUAL
PROPERTY **INDIA**
PATENTS | DESIGNS | TRADE MARKS
GEOGRAPHICAL INDICATIONS

निर्गमन की तारीख/Date of Issue : 16/12/2022

महानियंत्रक पेटेंट डिजाइन और व्यापार चिह्न
Controller General of Patents, Designs and Trade Marks

पारस्परिकता तारीख (यदि कोई हो) जिसकी अनुमति देश के नाम पर की गई है। डिजाइन का सत्त्वाधिकार पंजीकरण की तारीख से दस वर्षों के लिए होगा जिसका विस्तार, अधिनियम एवं नियम के निबंधनों के अधीन, पाँच वर्षों की अतिरिक्त अवधि के लिए किया जा सकेगा। इस प्रमाण पत्र का उपयोग विधिक कार्यवाहियों अथवा विदेश में पंजीकरण प्राप्त करने के लिए नहीं हो सकता है।

*The reciprocity date (if any) which has been allowed and the name of the country.Copyright in the design will subsist for ten years from the date of Registration, and may under the terms of the Act and Rules, be extended for a further period of five years.This Certificate is not for use in legal proceedings or for obtaining registration abroad.

ORIGINAL

मूल/No : 122810

सत्यमेव जयते

भारत सरकार
GOVERNMENT OF INDIA
पेटेंट कार्यालय
THE PATENT OFFICE

डिजाइन के पंजीकरण का प्रमाणपत्र
CERTIFICATE OF REGISTRATION OF DESIGN

डिजाइन सं. / Design No.	:	350954-001
तारीख / Date	:	07/10/2021
पारस्परिकता तारीख / Reciprocity Date*	:	
देश / Country	:	

प्रमाणित किया जाता है कि संलग्न प्रति में वर्णित डिजाइन जो **IOT BASED AGRICULTURAL FIELD GROOVING MACHINE** से संबंधित है, का पंजीकरण, श्रेणी **15-03** में 1.Dr. Anupamdeep Sharma 2. Dr. Neha 3.Dr. Vaneet Kumar 4.Dr. Sewa Singh 5.Mr. Abhinav Anand 6.Ms. Zeenat 7.Prof. Ramesh Chandra Panda के नाम में उपर्युक्त संख्या और तारीख में कर लिया गया है।

Certified that the design of which a copy is annexed hereto has been registered as of the number and date given above in class **15-03** in respect of the application of such design to **IOT BASED AGRICULTURAL FIELD GROOVING MACHINE** in the name of 1.Dr. Anupamdeep Sharma 2. Dr. Neha 3.Dr. Vaneet Kumar 4.Dr. Sewa Singh 5.Mr. Abhinav Anand 6.Ms. Zeenat 7.Prof. Ramesh Chandra Panda.

डिजाइन अधिनियम, 2000 तथा डिजाइन नियम, 2001 के अध्यधीन प्रावधानों के अनुसरण में।
In pursuance of and subject to the provisions of the Designs Act, 2000 and the Designs Rules, 2001.

निर्गमन की तारीख/Date of Issue : 29/12/2022

महानियंत्रक पेटेंट डिजाइन और व्यापार चिह्न
Controller General of Patents, Designs and Trade Marks

पारस्परिकता तारीख (यदि कोई हो) जिसकी अनुमति देश के नाम पर की गई है। डिजाइन का सत्वाधिकार पंजीकरण की तारीख से दस वर्षों के लिए होगा जिसका विस्तार, अधिनियम एवं नियम के निबंधनों के अधीन, पाँच वर्षों की अतिरिक्त अवधि के लिए किया जा सकेगा। इस प्रमाण पत्र का उपयोग विधिक कार्यवाहियों अथवा विदेश में पंजीकरण प्राप्त करने के लिए नहीं हो सकता है।

*The reciprocity date (if any) which has been allowed and the name of the country.Copyright in the design will subsist for ten years from the date of Registration, and may under the terms of the Act and Rules, be extended for a further period of five years.This Certificate is not for use in legal proceedings or for obtaining registration abroad.

APPENDIX-IV

Awards:

1. **Best oral presentation** for the presentation tiled "The multifactorial ameliorative effect of vanillin in a mouse model of Alzheimer's disease" at 3rd International Conference of Pharmacy held on November 09-10, 2022 at Lovely Professional University, Punjab.
2. **Third prize in oral presentation session** for the presentation titled "The multifactorial ameliorative effect of magnolol in a mouse model of Alzheimer's disease" at the International Conference on Recent Advances in Health Sciences held on April 14-15, 2023 at Lovely Professional University, Punjab.
3. **Best paper award** for the presentation titled "*Lactobacillus rhamnosus*: A probiotic augmenting the ameliorative effect of vanillin and magnolol in aluminium chloride and d-galactose induced Alzheimer's disease in mice" at the 6th International Multi-Track Conference on Sciences, Engineering, Management & Technical Innovation on July 17, 2023 in Hanoi, Vietnam organized by CT Group of Institutions in collaboration with CT University and City University Ajman.
4. **Research excellence award** by Institute of Scholars.

3ICP2022OBEST-02

Serial No. _____

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Certificate of Merit

This is to certify that Prof./Dr./Mr./Ms. ABHINAV ANAND has Presented Poster/ Oral Presentation on **The Multifactorial Ameliorative Effect of Vanillin in a Mouse Model of Alzheimer's Disease** and awarded for best poster/ oral presentation in the 3rd **International Conference of Pharmacy (ICP-2022)** on the **Theme of "Practice, Promotion & Publication of Innovation : A Way of Transforming Health"** held on **09th & 10th November 2022** organized by School of Pharmaceutical Sciences in a collaboration with Indian Pharmaceutical Association (IPA) at Lovely Professional University, Punjab.

Mr. Suresh Khanna
National Hon. Gen. Secretary, IPA

Dr. Bimlesh Kumar
Organizing Secretary

Dr. T.V. Naryana
National President IPA

Dr. Monica Gulati
LOC Chairperson

Sri Herbasia Biotech

Tishk International University, Iraq

ICRAHS
INTERNATIONAL CONFERENCE ON RECENT ADVANCES IN HEALTH SCIENCES

LOVELY PROFESSIONAL UNIVERSITY
Transforming Education Transforming India

KUST

Certificate No. 266028

Certificate of Merit

This is to certify that **Prof./Dr./Mr./Ms. Abhinav Anand** has participated in **Oral presentation session** on topic **The Multifactorial Ameliorative Effect of Magnolol in a Mouse Model of Alzheimer's Disease** awarded **Third prize** in the **International Conference on "Recent Advances in Health Sciences" (ICRAHS-2023)** on the Theme of "Interdisciplinary Research: A key to transform Health care." from 14th April, 2023 to 15th April, 2023 organized by School of Pharmaceutical Sciences in association with Komar University of Sciences and Technology at Lovely Professional University, Punjab.

Date of Issue : 01-05-2023
Place : Phagwara (Punjab), India

Prepared by
(Administrative Officer-Records)

Dr. M Vijay Kumar
General Chair

Prof. Dr. Kawis Aziz Faraj
Conference Co-Chair

Dr. Monica Gulati
Conference Chair

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IMSEMTI 2023
July 17, 2023

6th International Multi-Track Conference on
Sciences , Engineering, Management
& Technical Innovation

Certificate Of Merit

This to certify that **Mr. Abhinav Anand** of CT Institute of Pharmaceutical Sciences, Shahpur, Jalandhar, Punjab (Assistant Professor) and School of Pharmaceutical Sciences, Lovely Professional University, Punjab (Research Scholar) was awarded **BEST PAPER AWARD** for the presentation titled ***'Lactobacillus rhamnosus: A probiotic augmenting the ameliorative effects of vanillin and magnolol in aluminium chloride and D-galactose induced Alzheimer's disease in mice'*** in the 6th International Multi Track Conference on Sciences, Engineering, Management & Technical Innovation (IMSEMTI) held in Hanoi, Vietnam on July 17, 2023

Program Chair | Convener | Organizing Secretary

Certificate of Recognition

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UAM: KR-09-0001606 CIN: U80904KA2021OPC143961

Research Excellence Award 2023

awarded to

Abhinav Anand

Research Scholar
School of Pharmaceutical Sciences
Lovely Professional University, Phagwara (Punjab)

Assistant Professor
CT Institute of Pharmaceutical Sciences
CT Group of Institutions, South Campus, Shahpur, Jalandhar (Punjab)

For the work with the following details

Publication Type: Journal
Paper Title: Ameliorative Effect of Vanillin on Scopolamine-Induced Dementia-Like Cognitive Impairment in a Mouse Model
Journal Name: Frontiers in Neuroscience
Volume: 16
Month of Publication: November
Year: 2022, Page No: 1005972
ISSN: 1662-453X

Nanjesh Bennur
Chairman, InSc

20231REA7

Participation in conferences

CHANDIGARH GROUP OF COLLEGES
CGC LANDRAN
Building Careers. Transforming Lives.

Certificate of Participation

This is to certify that Prof./Dr./Mr./Ms. ABHINAV ANAND has participated in the Oral Presentation / Poster Presentation /Delegate / Member of LOC / Student Coordinator at the International Conference on "INDUSTRY DRIVEN PHARMACEUTICAL EDUCATION AND RESEARCH: CURRENT TRENDS AND FUTURE PROSPECTS" IDPER-2023 on 27th & 28th April, 2023.

Prof. (Dr.) Supriya Agnihotri
Organizing Secretary
HOD CCP

Prof. (Dr.) Saurabh Sharma
Convener
Director-Principal CCP

स्वास्थ्य एवं परिवार कल्याण मंत्रालय
MINISTRY OF HEALTH AND FAMILY WELFARE

Azadi Ka Amrit Mahotsav

PHARMA ANVESHAN-2023

National Pharmacy Education Day

A Tribute to Prof. M.L. Schroff

6th March 2023

Organized by

PHARMACY COUNCIL OF INDIA

A statutory body under Ministry of Health and Family Welfare, Government of India

Certificate of Appreciation

It is certified that

Dr./Mr./Ms. ..... **Abhinav Anand** .....

has participated as a Delegate/ Presenter in
Pharma Anveshan 2023 at Vigyan Bhawan, New Delhi
He / She presented a concept note.

Shri. Anil Mittal
(I/C) Registrar-cum- Secretary
Pharmacy Council of India

Dr. Montukumar M.Patel
President
Pharmacy Council of India

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Certificate of Paper Presentation

This certificate is presented to Abhinav Anand
from Lovely Professional University for
presenting a paper entitled The Relationship of Magnolol with Neurological Disorders
in the International E-Conference on Industry 4.0 to Pharm 4.0 : Futuristic Trends in Pharmaceutical Industry (FTPI-2021) organized by Centre of Excellence for Research & Innovation (CERI), CT Institute of Pharmaceutical Sciences in collaboration with NIGDE Turkey on July 20, 2021.

CHAIRMAN / CHANCELLOR
MANAGING DIRECTOR
CAMPUS DIRECTOR
CT GROUP OF INSTITUTIONS, SOUTH CAMPUS, SHAHPUR
CONVENER / DIRECTOR,
CT INSTITUTE OF PHARMACEUTICAL SCIENCES SOUTH CAMPUS, SHAHPUR

CT UNIVERSITY
PUNJAB · INDIA
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APPROVED BY UGC

Certificate

of Participation

September 26, 2020

This is to certify that

Abhinav Anand

has participated in Virtual International Conference

"Innovations in Pharmaceutical Sciences"

organized by

School of Pharmaceutical Sciences
CT University, Ludhiana, Punjab

Vice Chancellor
CT University

Chancellor
CT University

DNWH8S-CE000130

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ARIIA ATAL RANKING OF INSTITUTIONS ON INNOVATION ACHIEVEMENTS
Ministry of Education Government of India

Indian Pharmacy Graduates' Association

AMITY UNIVERSITY

IPGA Conclave 2020:
Next Generation Pharmacist

DIGITALIZATION | FUTURE JOBS | REGULATORY MECHANISMS | RESEARCH DIRECTIONS

CERTIFICATE

OF PARTICIPATION

This is to certify that Prof./Dr./Ms./Mr. **ABHINAV ANAND** has participated in the **IPGA Conclave 2020 : Next Generation Pharmacist** on the topic of ***"Digitalization, Future Jobs, Regulatory Mechanisms & Research Directions"***, held on **May 10, 2020**, organized by organized by Amity University Uttar Pradesh, Noida and Indian Pharmacy Graduates' Association, Delhi, India.

Sh. Atul Kr. Nasa
President - IPGA

Dr. Arun Garg
General Secretary

Dr. Vijay Bhalla
Treasurer

Dr. W. Selvamurthy
President - ASTIF

Dr. Satyendra Kr. Rajput
Director - AIISM

THE INDIAN SCIENCE CONGRESS ASSOCIATION

(Professional Body under Department of Science & Technology,
Ministry of Science & Technology, Government of India)

14, Dr. Biresh Guha Street, Kolkata – 700 017

Paper Presentation Certificate

This is to certify that Prof./Dr./Shri/Smt. Abhinav Anand
of Lovely Professional University
has presented a Paper (Oral/Poster) entitled "Ameliorative effect of vanillin ... dementia in mice" in the Section of Medical Sciences
including Physiology during
the 106th Indian Science Congress held at Lovely Professional University, Phagwara, Jalandhar from January 3 to 7, 2019.

His/Her Membership Number is.....

Date 06.01.2019

Prof. Prakash C. Dhara
Sectional President
Medical Sciences (Including Physiology)
Indian Science Congress Association

Office Seal

(Signature)
Sectional President

Online courses

VERIFIED
CERTIFICATE *of* ACHIEVEMENT

THE UNIVERSITY of TEXAS SYSTEM

George Perry
Dean of the College of Sciences and Semmes Foundation Distinguished University Chair in Neurobiology at The University of Texas at San Antonio

Rudolph J. Castellani
Professor of Pathology and Director of the Center for Neuropathology at Western Michigan University Homer Stryker MD School of Medicine

This is to certify that

Abhinav Anand

successfully completed and received a passing grade in

AD001x: Understanding Alzheimer's Disease: A Molecular and Genetic Approach

a course of study offered by UTSanAntonioX, an online learning initiative of University of Texas System through edX.

edX VERIFIED CERTIFICATE
Issued January 31, 2018

VALID CERTIFICATE ID
071d6f3b0680425b8d55872cd51ff287

udemy

Certificate no: UC-37d49c2a-6f24-47d4-968b-8d36ccd1f7a7
Certificate url: ude.my/UC-37d49c2a-6f24-47d4-968b-8d36ccd1f7a7
Reference Number: 0004

CERTIFICATE OF COMPLETION

Statistics/Data Analysis with SPSS: Descriptive Statistics

Instructors **Quantitative Specialists**

Abhinav Anand

Date **Sept. 27, 2021**

Yale
Center for Emotional Intelligence

COURSE CERTIFICATE

Oct 1, 2021

Abhinav Anand

has successfully completed

Managing Emotions in Times of Uncertainty & Stress

an online non-credit course authorized by Yale University and offered through Coursera

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Marc Brackett
Director
Yale Center for Emotional Intelligence

Verify at coursera.org/verify/FLE76DR7EP6Y
Coursera has confirmed the identity of this individual and their participation in the course.

Researcher Academy researcheracademy.com

ELSEVIER

Certificate of Excellence

This certifies that

Abhinav Anand

has successfully completed every module within

Certified Peer Reviewer Course

on Tuesday 22 September, 2020

Suzanne BeDell
Managing Director, Education
Reference & Continuity Books

Philippe Terheggen
Managing Director, Science,
Technology & Medical Journals

APPENDIX-V

SIGMA-ALDRICH

3050 Spruce Street, Saint Louis, MO 63103 USA
Email USA: techserv@sial.com Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:	Vanillin ReagentPlus™, 99 %
Product Number:	V1104
Batch Number:	BCCF3063
Brand:	Sigma-Aldrich
CAS Number:	121-33-5
Formula:	4-(HO)C_6H_3-3-(OCH_3)CHO
Formula Weight:	152.15
Quality Release Date:	21 JAN 2021

TEST	SPECIFICATION	RESULT
APPEARANCE (COLOR)	WHITE TO FAINT YELLOW	WHITE
APPEARANCE (FORM)	POWDER OR CRYSTALS	POWDER
PURITY (GC AREA %)	≥ 98.5 %	100.0 %
SOLUBILITY (COLOR)	COLORLESS TO VERY FAINT YELLOW	ALMOST COLORLESS
SOLUBILITY (TURBIDITY)	CLEAR	CLEAR
SOLUBILITY (METHOD)	C=50MG/ML(5%); ETHANOL	C=50MG/ML(5%); ETHANOL
CARBON CONTENT	62.2 - 64.1 %	63.4 %
INFRARED SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS
WAVELENGTH (1) (UV)	344 - 350 NM	348 NM
MOLAR ABSORBANCY INDEX (1)	≥ 24000	25162
WAVELENGTH (2) (UV)	245 - 251 NM	248 NM
MOLAR ABSORBANCY INDEX (2)	≥ 8000	8319
SOLVENT (UV)	C=0.005G/L; 0.01N NAOH (PH 12.3)	CONFORMS

Dr. Reinhold Schwenninger
Quality Assurance
Buchs, Switzerland

西安品诚生物科技有限公司
Xi'an Pincredit Bio-tech Co.,Ltd

CERTIFICATE OF ANALYSIS

产品名称 Product Name	厚朴提取物 Magnolia Bark Extract	**批量 Batch Size**	660kgs
产品规格 Product Spec	厚朴酚 Magnolol ≥98.0%	**批号 Batch Number**	20201201
植物名 Botanical Latin Name	*Magnolia officinalis Rehd.et Wils.*	**提取部位 Plant Part**	皮 Bark
生产日期 MFG. Date	Dec.19.2020	**有效日期 Retest Date**	Dec.18.2022
报告日期 Issue Date	Dec.29.2020		

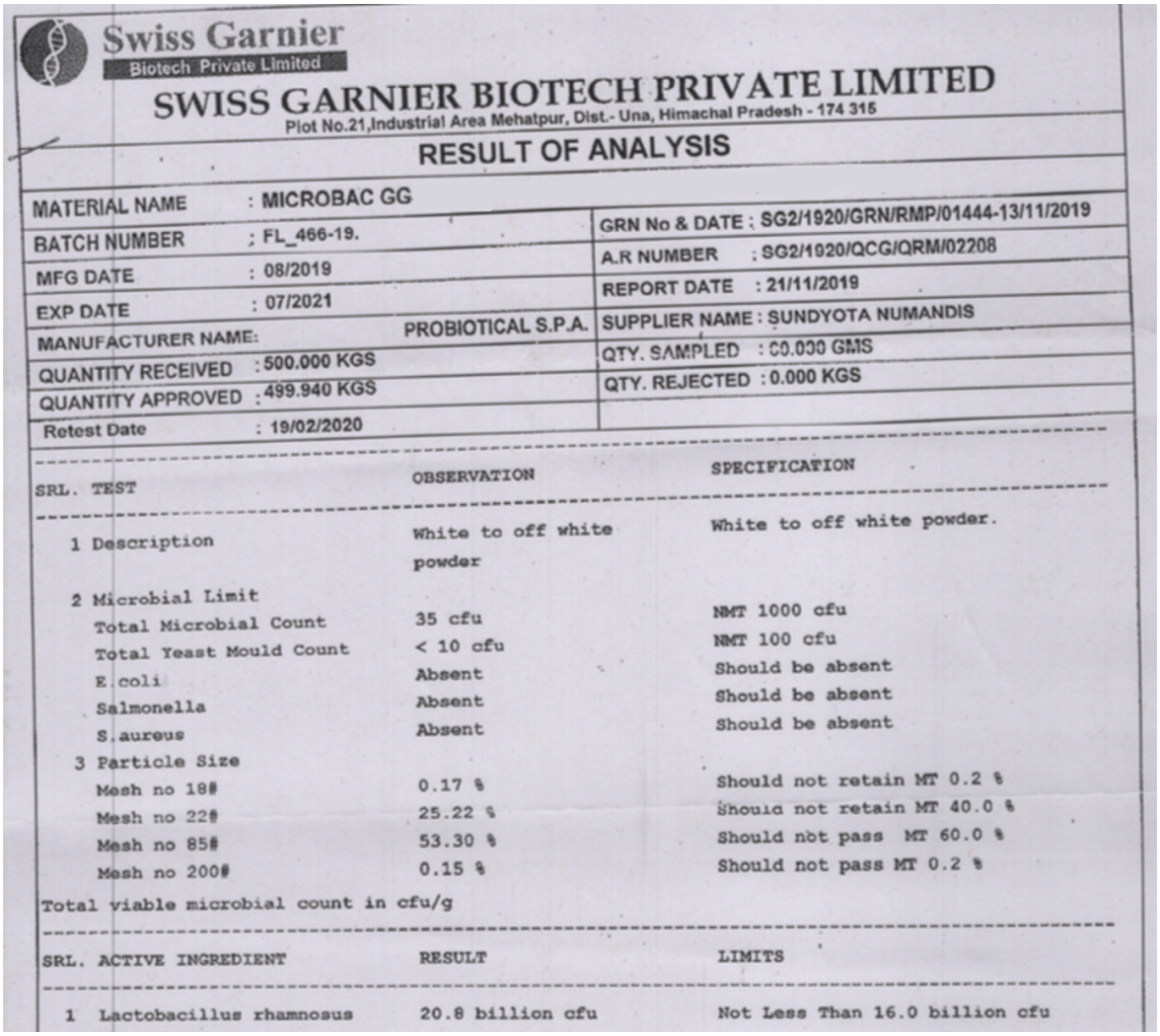
项目 ITEM	规格 SPECIFICATION	结果 RESULT	方法 TEST METHOD
物理描述 Physical Description			
外观 Appearance	白色粉末 White Powder	符合 Conforms	Visual
气味 Odor	厚朴特有气味 Unique smell of Magnolia Bark	符合 Conforms	Organoleptic
味道 Taste	厚朴特有味道 Unique taste of Magnolia Bark	符合 Conforms	Olfactory
粒径 Particle Size	80%-90%通过 80 目 80%-90% Through 80 Mesh	符合 Conforms	CP2015
化学检测 Chemical Tests			
厚朴酚含量 Magnolol	≥98.0%	98.19%	HPLC
水分 Water	≤1.0%	0.38%	CP2015 (40 oC, 4 h)
灰分 Ash	≤1.0 %	0.49%	CP2015
总重金属 Total Heavy Metals	<10 ppm	符合 Conforms	CP2015
微生物控制 Microbiology Control			
细菌 Aerobic Bacterial Count	≤1000 cfu/g	符合 Conforms	GB4789.2
酵母菌和霉菌 Total Yeast & Mold	≤100 cfu/g	符合 Conforms	GB4789.15
大肠杆菌 Escherichia Coli	不得检出 Negative	符合 Conforms	GB4789.38
沙门氏菌 Salmonella	不得检出 Negative	符合 Conforms	GB4789.4
金黄色葡萄球菌 Staphlococcus Aureus	不得检出 Negative	符合 Conforms	GB4789.10
结论 Conclusion	符合检测标准 Conforms to Specification		

存储条件 Storage: Store in highly sealed and preferably full containers in cool, dry and ventilated area.

保质期 Shelf Life: 24 months when properly stored. **Status:** Natural, Non-Irradiation.

检测人 Inspector: 复核人 Reviewer: 审核人 Auditor:

质量检测专用章



Swiss Garnier
Biotech Private Limited

SWISS GARNIER BIOTECH PRIVATE LIMITED
Plot No.21,Industrial Area Mehatpur, Dist.- Una, Himachal Pradesh - 174 315

RESULT OF ANALYSIS

MATERIAL NAME	: MICROBAC GG		
BATCH NUMBER	: FL_466-19.	GRN No & DATE	: SG2/1920/GRN/RMP/01444-13/11/2019
MFG DATE	: 08/2019	A.R NUMBER	: SG2/1920/QCG/QRM/02208
EXP DATE	: 07/2021	REPORT DATE	: 21/11/2019
MANUFACTURER NAME:	PROBIOTICAL S.P.A.	SUPPLIER NAME	: SUNDYOTA NUMANDIS
QUANTITY RECEIVED	: 500.000 KGS	QTY. SAMPLED	: 00.000 GMS
QUANTITY APPROVED	: 499.940 KGS	QTY. REJECTED	: 0.000 KGS
Retest Date	: 19/02/2020		

SRL.	TEST	OBSERVATION	SPECIFICATION
1	Description	White to off white powder	White to off white powder.
2	Microbial Limit		
	Total Microbial Count	35 cfu	NMT 1000 cfu
	Total Yeast Mould Count	< 10 cfu	NMT 100 cfu
	E.coli	Absent	Should be absent
	Salmonella	Absent	Should be absent
	S.aureus	Absent	Should be absent
3	Particle Size		
	Mesh no 18#	0.17 %	Should not retain MT 0.2 %
	Mesh no 22#	25.22 %	Should not retain MT 40.0 %
	Mesh no 85#	53.30 %	Should not pass MT 60.0 %
	Mesh no 200#	0.15 %	Should not pass MT 0.2 %

Total viable microbial count in cfu/g

SRL.	ACTIVE INGREDIENT	RESULT	LIMITS
1	Lactobacillus rhamnosus	20.8 billion cfu	Not Less Than 16.0 billion cfu

Manufacturing Site :
Sun Pharmaceutical Industries Ltd.
Vill. Toansa, P.O. Rail Majra,
Distt. SBS Nagar-144 533, Punjab, India

SUN PHARMA

CERTIFICATE OF ANALYSIS

Product	:	DONEPEZIL HYDROCHLORIDE			
Batch No.	:	3987412	**Report No.**	:	30001768653
Manufactured	:	November, 2018	**Sampling Date**	:	27.11.2018
Retest	:	October, 2021	**Quantity**	:	46.280 KG

Characteristics	Results	Specification
DESCRIPTION	Off-white crystalline powder.	White to off white crystalline powder.
SOLUBILITY	Soluble in chloroform,sparingly soluble in water,methanol and acetic acid.	Soluble in chloroform, sparingly soluble in water, methanol and acetic acid.
IDENTIFICATION		
- IR	The infrared absorption spectrum of the Potassium bromide dispersion of the sample is concordant with that of a similar preparation of Donepezil hydrochloride [Form-1] working standard.	The infrared absorption spectrum of the Potassium bromide dispersion of the sample must be concordant with that of a similar preparation of Donepezil hydrochloride (Form-1) working standard.
- Test For Chloride	Meets the requirement	Meets the requirement.
OPTICAL ROTATION (deg.)	+ 0.001	- 0.1 to + 0.1
WATER (% w/w)	5.58	4.0 to 7.0
SULPHATED ASH (% w/w)	0.05	NMT 0.1
HEAVY METALS (% w/w)	Less than 0.002	NMT 0.002
ASSAY (By HPLC) (% w/w, on anhydrous basis)	100.8	98.0 - 102.0
RELATED SUBSTANCES (By HPLC) (% w/w)		
- DNP-1	0.02	NMT 0.10

CONCLUSION : The batch complies with respect to the Specifications as above i.e. : DONEPEZIL HYDROCHLORIDE

Date of issue : 04.01.2019

Analyst 04-Jan-2019

Quality Assurance Manager 04-Jan-2019

APPENDIX-VI

ANIMAL HOUSE

Lovely Institute of Technology (Pharmacy), Lovely Professional University
Ludhiana- Jalandhar G.T. Road, Phagwara (Punjab), 144411
Registration Number -954/PO/ReRcBiBt/S/06/CPCSEA

CERTIFICATE

This is to certify that the project titled *"Pharmacological Evaluation of Vanillin, Magnolol and Probiotics Individually and in Combination with Each Other for Alzheimer's Disease using Animal Model: A Mechanistic Study" has* been approved by the IAEC.

Name of Principal Investigator: Dr. Navneet Khurana

IAEC approval number: LPU/IAEC/2020/75

Date of Approval: 25th October 2020

Animals approved: 101 Mice

Remarks if any: - NA

Dr. Monica Gulati	**Dr. Navneet Khurana**	**Dr. Bimlesh Kumar**
Biological Scientist, Chairperson IAEC	Scientist from different discipline	Scientist In-Charge of Animal House, Member Secretary IAEC