Chrysoeriol as a Potential Therapeutic Agent for Alzheimer's Disease: Behavioral and Molecular Insights from a Mouse Model.

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Supervisor: Dr. Aneeqa Noor

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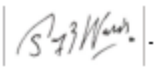
Signature (HOD): 

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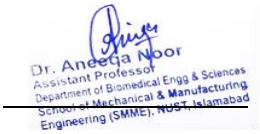
Supervisor Name: Dr. Aneeqa Noor Signature: 

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I dedicate my work to my incredible parents (Khalid Saeed and Samina Kosar) with all my respect, love, and gratitude. This work is a reflection of your belief in me, every whispered prayer and the boundless patience that has led me through every challenge. This thesis is not just a reflection of my journey, but a testament to your love, dedication, and endless support.

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LIST OF ABBREVIATIONS, AND SYMBOLS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer Disease
ADAM10	A Disinterring and Metalloproteinase Domain-Containing Protein 10
AKT	Protein Kinase B
ALA	Alanine
$AlCl_3$	Aluminum Chloride
ANOVA	Analysis of Variance
Apaf1	Apoptotic Protease Activating Factor 1
Apo E4	Apolipoprotein E4
APP	Amyloid Precursor Protein
ARG	Arginine
ASP	Aspartic Acid
Aß	Amyloid-Beta
BAK	BCL-2 Antagonist/Killer
BAX	BCL-2-Associated X Protein
BBB	Blood-Brain Barrier
BChE	Butyrylcholinesterase
BCL-2	B-Cell Lymphoma 2
CKD	Chronic Kidney Disease
CSF	Cerebrospinal Fluid
CT	Computed Tomography
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
DTT	Dithiothreitol
FDA	Food and Drug Administration
GI type	Gastrointestinal Type
GLYA	Glycine
GSK3B	Glycogen Synthase Kinase 3 Beta
H&E	Hematoxylin and Eosin
ILE	Isoleucine
LEU	Leucine
LOAD	Late-onset Alzheimer's Disease

LTP	Long-Term Potentiation
MCI	Mild Cognitive Impairment
MMSE	Mini-Mental State Examination
MoCA	Montreal Cognitive Assessment
MOMP	Mitochondrial Outer Membrane Permeabilization
MRI	Magnetic Resonance Imaging
NCBI	National Center for Biotechnology Information
NFTs	Neurofibrillary Tangles
NF-κB	Nuclear Factor Kappa B Cells.
NMDA	N-Methyl D-Aspartate
oligodts	Oligo(Deoxythymidines)
P110	Catalytic Subunit of Phosphoinositide 3-Kinase
P85	Regulatory Subunit of Phosphoinositide 3-Kinase
PBS	Phosphate-Buffered Saline
PD	Parkinson Disease
PDB	Protein Data Bank
PDK1	Phosphoinositide-dependent kinase-1
PET	Positron Emission Tomography
PFA	Paraformaldehyde
PHE	Phenylalanine
PI3K	Phosphoinositide 3-Kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate (PIP2)
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate (PIP3)
PLCG 2	Phospholipase C Gamma 2
PLD3	Phospholipase D3
PSEN1	Presenilin 1
PSEN2	Presenilin 2
PSP	Progressive Supranuclear Palsy
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-PCR	Real-Time Polymerase Chain Reaction
SEM	Standard Error of The Mean
SORL1	Sortilin-Related Receptor 1
STAT3	Signal Transducer And Activator Of Transcription 3
THRA	Threonine

TREM2	Myeloid Cells 2
TRP	Tryptophan
TRY	Tyrosine
VAL	Valine
WHO	World Health Organization

ABSTRACT

Alzheimer Disease (AD) is a progressive neurodegenerative disorder typically linked to dementia and other cognitive deterioration. As it progresses steadily and cannot be reversed, and till now unable to address the precise underlying pathologies of AD key contributors to the degeneration of neurons include apoptosis, mitochondrial dysfunction, and oxidative stress. Available therapies can aim to manage symptoms but are unable to address the underlying pathologies of AD. Therefore, investigating compounds that have therapeutic potential and can attenuate apoptotic neuronal loss, could lead to novel interventions and aim to mitigate the progression of neurodegenerative disorders. Chrysoeriol (3'-methoxy-4', 5, 7-trihydroxy flavone) is a flavonoid found in various plants, showing effective treatment results as anti-cancer, anti-inflammatory, anti-diabetic, and can reduce neuronal degradation. However, its application to AD models, especially the *in vivo* models has not been well investigated. Our research aims to investigate the efficacy of Chrysoeriol in the treatment of neurotoxicity associated with AD with the help of a well-established mouse model of AD. $AlCl_3$ has been used to mimic AD mice model and was divided into groups, control group, $AlCl_3$-induced group (20mg/kg, orally), and $AlCl_3$-treated with Chrysoeriol group (5mg/kg, intraperitoneally). Behavioral tests were performed after $AlCl_3$-induced and also after treated with Chrysoeriol including Morris Water test, Y-Maze Test, Novel Object Recognition, Open Field Test, and Evaluated-Plus Maze Test. Analysis reveals augmentation of exploratory activity and memory retention when the disease group treated with Chrysoeriol showed improved cognitive performance. Additionally, Chrysoeriol offered significant protection against Aβ-induced neuronal damage by enhancing the morphological and histological integrity of neurons within the hippocampus and cortex regions commonly impacted in AD. Furthermore, the results of the molecular analysis showed that Chrysoeriol plays a crucial role in the down-regulating of BAX while up-regulating of BCL-2 proteins in the hippocampus and cortex of AD mice. Our findings suggest that Chrysoeriol provides a protective effect against $AlCl_3$-induced apoptosis in mice models, primarily through activation of the PI3K/AKT pathway. These findings demonstrate that Chrysoeriol has therapeutic value and possible applicability for treating or halting AD pathologies while other neurodegenerative diseases should be explored further.

Keywords: Alzheimer's disease, Chrysoeriol, BAX, BCL.

CHAPTER 1: INTRODUCTION

1.1. Flavonoids

Flavonoids encompass a diverse group of over 6,000 natural compounds, categorized into six subgroups based on their structural chemistry: flavones, flavanols, flavonols, flavanones, isoflavones, and anthocyanins (Kim et al., 2019). These compounds are extensively researched for their potential in treating and preventing various disorders. Flavonoids have a variety of pharmacological characteristics including anti-inflammatory, anti-cancer, immunomodulatory, cardioprotective, and anti-infective effects (Sarfraz et al., 2020). Of all the polyphenols, flavonoids are the most diverse; they contain one heterocyclic ring and two phenyl rings. The orientation of the side chains and the number of hydroxyl groups are critical to influencing their chain terminators; they form a coordinating bond with the free radicals, the same as a metal catalyst. Phenolic antioxidants interact with free radicals and give hydrogen atoms which slow down oxidation (Mittal et al., 2023). Given that many flavonoids are derived from dietary sources, they are generally considered safer compared to other plant secondary metabolites. However, their safety profile and potential drug interactions in clinical settings still require comprehensive study and characterization.

1.2. Neuroprotection

Flavonoids can modulate various molecular pathways, reducing neuronal dysfunction and delaying the onset and progression of neurodegenerative disorders. They offer promising therapeutic alternatives for neurodegenerative conditions (Vauzour et al., 2008). Furthermore, flavonoids have been proven to alter synaptic plasticity and cognitive function, both of which are critical for brain health and preventing cognitive decline in AD. Additionally, it is known for its antioxidant, anti-diabetic, anti-inflammatory, anti-cancer, and neuroprotective properties (Shao et al., 2021). The research has indicated that flavonoids can enhance long-term potentiation, a cellular process that forms the basis of learning and memory and stimulate the production of brain-derived neurotrophic factor, a protein vital for creating new synapses and synaptic plasticity (Al Amin et al., 2024). These effects not only support cognitive function but also contribute to the resilience of neural networks against AD-related pathology. Overall, the multifaceted neuroprotective actions of flavonoids underscore their potential as therapeutic agents in the treatment and prevention of AD.

1.3 Chrysoeriol: chemical structure and sources

Chrysoeriol (3'-methoxy-4', 5, 7-trihydroxyflavone) is a flavonoid found in various plants, including celery, passionflower, and tarragon. Its chemical structure consists of a flavone backbone with hydroxyl groups at positions 5 and 7 and a methoxy group at position 3' as shown in figure 1.1. This structure contributes to its bioactivity and ability to interact with various molecular targets. Chrysoeriol and its compounds were found in a detailed analysis of 6 natural aromatic compounds isolated from methanolic extract of *Narthecium ossifragum (L.) Huds.* (Díaz et al., 2019). Chrysoeriol has been thoroughly studied within the genus *Cardiospermum*, especially in the species called *Cardiospermum halicacabum* (Baskaran et al., 2015). Other phenols are grouped into lignins, tannins, anthocyanins, and flavonols, which are all derived from the condensation of phenolic units. It has been recognized for its diverse biological activities, including anti-viral, anti-inflammatory, anti-bacterial, anti-proliferative, antioxidant, and enzyme-regulatory effects. These are generally linked to their antioxidant characteristics, particularly their ability to bind metal ions, neutralize free radicals, and inhibit radical-producing enzymes (Aboulaghras et al., 2022).

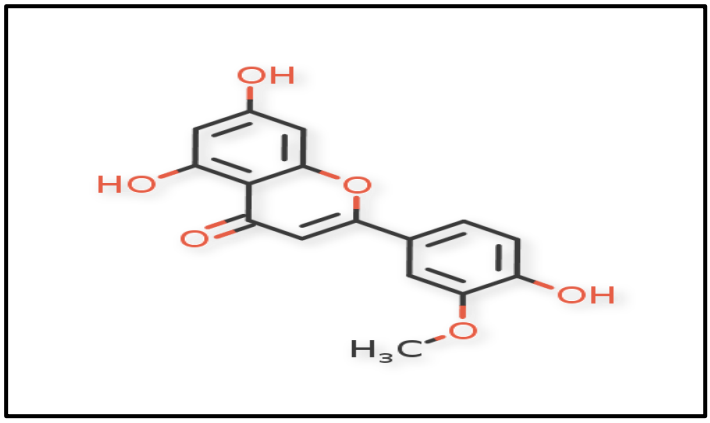


Figure 1.1. Chemical structure of Chrysoeriol: A flavonoid molecule has a planar structure because of conjugated double bonds inside the aromatic rings. The molecule has multiple hydroxyl groups attached to the rings, giving it a specific shape and properties. (CID: 5280666.).

1.4 Biological Properties of Chrysoeriol

Various studies have demonstrated that Chrysoeriol possesses a wide range of pharmacological activities, including anti-cancer, anti-diabetes, anti-inflammatory, and anti-microbial properties. Additionally, properties of antioxidant, anti-thrombotic, and anti-

arteritis, enlisted in figure 1.2. Attributed to its diverse cellular and molecular mechanisms of action. Some of them are as follows.

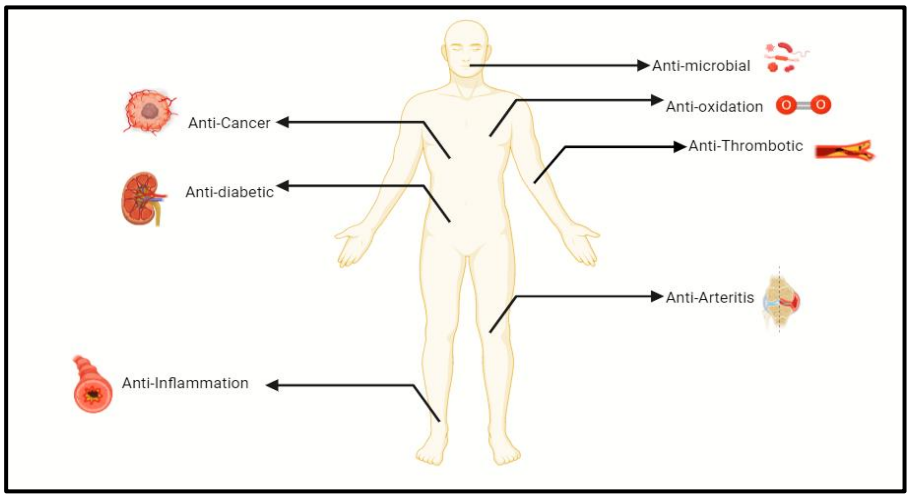


Figure 1.2. The various health benefits of Chrysoeriol: It highlights its potential to combat cancer, diabetes, inflammation, and microbial infections. Additionally, it the properties of antioxidant, anti-thrombotic, and anti-arteritis properties, suggesting its potential to support overall well-being.

1.4.1 Anti-cancer Properties

Chrysoeriol has demonstrated potent anticancer effects across a range of tumor cell lines, suggesting a novel strategy for cancer treatment. Chrysoeriol demonstrates potent anticancer effects across diverse tumor cell lines. A study evaluated its anticancer potential specifically on A549 human lung cancer cells, highlighting its promising efficacy in combating cancerous growth (Wei et al., 2019). Medicinal plant compounds with special reference to Chrysoeriol intervention have been carried out on human stomach cancer lines known as AGS cells. Besides, melanin when synthesized and accumulated may lead to skin cancer. Chrysoeriol has been proven to protect B16F10 cells successfully from melanogenesis. Studies indicate that Chrysoeriol significantly enhances the expression of melanogenic enzymes, including transcription factors TRP-1, TRP-2, TRY, and MITF (Yamabe et al., 2014).

1.4.2 Anti-Inflammatory Effect

Chrysoeriol exhibits anti-inflammatory properties by decreasing the presence of inflammatory mediators and proteins. It also is present in several dietary and medicinal plants, such as in *Lonicerae Japonicae Flos,* a dried flower bud or newly bloomed flower of

Lonicera Japonica Thunb. It can be observed that these herbs are widely used for complaints associated with inflammation. Chrysoeriol supplementation as found in herbal extracts has anti-inflammatory properties that work through the NF-kB pathway. Some of these extracts also inhibit the Stat3 signal transducers and activators of transcription in the cancer cells (Wu et al., 2020).

1.4.3 Anti-Diabetic Effect

Diabetes mellitus, currently ranked as the third leading cause of death globally following cardiovascular diseases, poses significant treatment challenges for medical professionals. Consequently, there has been a notable increase in exploring natural therapies for diabetes, particularly those derived from plant sources (Bolkent et al., 2000).

1.4.4 Antioxidant Effect

Another report has centered on the pharmacological effects of Chrysoeriol and other flavonoids extracted from *Tecoma stans (L.)* have been investigated utilizing *in vitro* models (Ramirez et al., 2016). Chrysoeriol has been researched for its ability to function as an antioxidant (Mishra et al., 2003).

1.4.5 Anti-Microbial Activity

Chrysoeriol and other flavonoids have been extracted from *S. palaestina* leaves. Out of these compounds, *cirsimaritin* showed appreciable antibacterial action against bacterial strains such as *Staphylococcus aureus, Escherichia coli, Staphylococcus epidermidis, Proteus vulgaris, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae*. In contrast, other compounds showed minimal or negligible activity against these bacterial strains (Aboulaghras et al., 2022).

1.4.6 Neuroprotective Activity

Based on clinical trial results, Chrysoeriol demonstrated efficacy in rats by promoting recovery from neurological deficits, mitigating neurological damage, shrinking ischemic areas, suppressing excessive production of pro-inflammatory cytokines, and balancing oxidative stress levels (Small et al., 1997).

1.5 Alzheimer's disease (AD)

AD is a progressive neurodegenerative condition typically linked to dementia and other cognitive deterioration. This becomes a considerable public health issue, driven by increased

life expectancy and a deeper understanding of its socio-economic impacts. AD progresses steadily and cannot be reversed; however, pharmacological treatments targeting cognitive impairment and both non-pharmacological and pharmacological interventions addressing behavioral issues associated with dementia can significantly improve the patient's quality of life (Bennett et al., 2021). AD involves degenerative changes across various neurotransmitter systems, including those that release glutamate, norepinephrine, serotonin, and certain neuropeptide systems. This condition is also marked by degeneration in specific brain regions including parietal and temporal lobes within the frontal cortex and hippocampus. These all contribute to challenging current Alzheimer's research, unable existing hypotheses to fully explain the cellular and regional distribution patterns characteristic of the disease's neuropathology (Li et al., 2016).

1.5.1 Epidemiology

This disease affects a substantial portion of the elderly population in many countries. It has become a public health challenge of this century. According to the WHO, nearly 47 million individuals were affected by this neurodegenerative disorder in 2015, another report states that in 2020, AD was one of the top three neurological causes of mortality around 20.3% globally (Feigin et al., 2020). Additionally, projections indicate that the number of people with dementia AD is expected to increase to 75 million by 2030 and reach 132 million by 2050 worldwide (Tay et al., 2024).

AD is the leading type of dementia, responsible for 60% to 80% of all cases. However, less than half of these are anticipated to be purely Alzheimer's, with the majority being a combination of different forms of dementia. It often has an extended asymptomatic, during which a cognitively normal person despite having symptoms. Moreover, it is hard to diagnose AD without accompanying neurodegenerative co-pathologies. It is so closely linked with aging that some speculate it might be a natural aspect. The point prevalence is said to be 10% in patients greater than 65 years and 40% in patients greater than 80 years. (DeTure et al., 2019). Age remains the strongest predictor of developing AD; the rate of all forms of dementia, including AD, doubles about every 6 years. The rate increases from 3.9 per thousand population in the 60-90 age groups to 104 years. Up to 8 persons per 1000 population of people with age more than 90 (Martin Prince et al., 2015).

1.5.2 Epidemiology in Pakistan

This disease is affecting a substantial portion of the elderly population in Pakistan, like many countries. The epidemiology of AD in Pakistan is an area of growing concern, reflecting its impact on public health (Ahmad et al., 2013). Research indicates that AD prevalence in Pakistan is increasingly significant, mirroring global trends where aging populations are more susceptible to neurodegenerative disorders. Several studies have highlighted the rising burden of AD in Pakistan, emphasizing the need for more comprehensive research and healthcare strategies to address this issue effectively (Adamson et al., 2020).

1.5.3 Etiology

Familiar AD may be caused by mutations in the genes coding PSEN1, PSEN2 & APP and thus presenting dominantly inherited AD. This genetic form of Alzheimer's affects less than 1% of all Alzheimer's patients, typically showing symptoms around 46.2 years of age, although onset can occur as early as 20 years old (Ryman et al., 2014). About 65% of patients with dementia are assumed to be affected at the age of 65 or later, and although genetic factors such as Apo E4 gene on chromosome 19 have been found in Late Onset AD, the majority of cases are considered to be sporadic. Factors contributing to the highest risk of AD include advancing age, a family history of the disease among first-degree relatives, and possession of the Apo E4 genotype. Additionally, research indicates that the Apo E4 allele significantly increases susceptibility to conditions like traumatic brain injuries, Down's syndrome, Lewy body dementia, and vascular dementia (Verghese et al., 2011).

Approximately 30 genes linked to LOAD have been identified through genome-wide association studies. Examples include Triggering receptors expressed on TREM2, PLD3, and ADAM10. These genes influence the processing of APP and tau proteins directly and play roles in regulating cholesterol metabolism, immune responses, and endocytosis. Their functions are well-documented in research on AD risk factors (Karch et al., 2015). Understanding the pathophysiological mechanisms of AD hinges on a comprehensive grasp of its current and emerging risk factors. AD is characterized by significant neuropathological features, notably senile plaques and NFTs. Senile plaques accumulate gradually in brain regions crucial for cognition, stemming from insoluble deposits of Aβ peptides derived from APP. These peptides, particularly Aβ42, are produced through proteolytic actions of β-secretase and γ-secretase enzymes. The initial deposition of Aβ42 may trigger cascades

leading to amyloid accumulation. While evidence suggests APP dysfunction and Aβ deposition contribute to AD, whether senile plaques directly cause the disease or result as a consequence remains unclear. Aβ peptides exhibit neurotoxicity, potentially damaging neurons through inflammation or oxidative stress. Concurrently, AD is characterized by the accumulation of NFTs, primarily composed of aberrantly phosphorylated tau protein. Tau, crucial for maintaining neuronal microtubule structure, undergoes pathological changes in AD, with tangles increasing in cortical regions as the disease advances. Recent research indicates that tau alterations often follow Aβ deposition in AD progression (Näslund et al., 2015).

1.5.4 Diagnosis

AD affects elderly people resulting in loss of cognitive and physical functions. The clinical signs of AD are memory impairment, aphasia, apraxia, disorientation, personality and behavioral changes, and cognitive loss especially the higher functions that involve planning, organizing, problem-solving, abstract thinking, and judgment. In the later stages of the disease affected people can have severe memory loss, poor decision-making, and poor coordination as well as difficulties in carrying out activities of daily living and need help with all their needs. Over the years, there has been an enhanced clinical appreciation of AD, and thus more specific diagnostic criteria have been developed, and diagnostic reliability is exhibited. Studies are being taken towards determining the primary indicators of the ailment and, in fact, its prodromal or asymptomatic phases. Diagnosis of AD is more an affair of raising exclusions, this can even be done formally using laid-down clinical criteria. However, despite these advances, finding means of combating AD is still out of sight (Desai et al., 2005).

On the onset, AD may manifest symptoms that are hard to distinguish from senile changes. Nowadays, diagnosing Alzheimer's involves interviewer and testing, but are cognitive ones. That requires checking the patient's medical history, and may recommend other causes of memory or confusion loss, excluding tests such as CT, MRI, or PET. It requires defining a different clinically ascertainable syndrome associated with AD and validating this with findings that confirm the Alzheimer's pathology, amyloid and tau proteins (Dubois et al., 2021). It is usually preceded by the portrayal of the clinical history and general assessment through physical examination, then by the usage of several

standardized mental status examination tools including the MMSE and MoCA. These tests assist in the assessment of different aspects of cognition that include memory, attention, language, and visuospatial functions.

Neuroimaging is involved in the diagnosis of AD with MRI and PET scans aiming at providing structural as well as functional images of the brain. MRI scans may reveal other features of the brains of people with AD, including the shrinking hippocampal and cortical thinning. Amyloid and tau can be done by PET scanning which is effective in identifying regions where abnormal proteins that characterize AD are present. Imaging also has applications in diagnosing AD; CSF analysis used some biomarkers of AD such as Aβ and tau proteins. Increased total tau and phosphorylated tau protein concentrations, and decreased Aβ42 concentrations in CSF are characteristic of AD. CSF analysis is also given priority as both Aβ and the tau domain are assessed concurrently and the cost is considerably less than amyloid PET, tau PET, or both. The Amyloid PET and Tau PET in clinical practice depend on the approval of the authorities and reimbursement from the payers in different countries. Prenatal diagnosis is also being worked out in the form of blood tests that would reveal certain issued biomarkers, more convenient than via invasive tissue sampling (Dubois et al., 2010)

1.5.5 Stages of AD

AD typically progresses through a series of stages and is clinically characterized by a gradual and progressive deterioration in cognitive abilities and functional skills, often accompanied by neuropsychiatric symptoms (Feldman et al., 2005) though the exact trajectory can vary for each individual. There is a visual change in the brain when differentiated from a healthy brain including shrinkage and neuronal loss as shown in figure 1.4. Understanding the stages of AD it can help healthcare providers and family members make informed care decisions.

1.5.5.1 Preclinical Stage

Neurological alterations may take place well before one notices the first sign of AD. At this stage disease process begins many years before the onset of clinical signs and symptoms also known as silent stage. At this stage physical symptoms have not yet appeared, but changings in brain occurs including the development of Aβ plaques and NFTs which linked to neuronal loss. (Prince et al., 1999). The International Working Group (IWG) has defined two different preclinical states: the pre-symptomatic and the asymptomatic at-risk state.

1) Pre-symptomatic AD understands that some individuals are preordained almost surely to progress to full clinical AD because they are known carriers of an autosomal dominant monogenic mutation.
2) The asymptomatic at-risk state is less clear-cut for the most part although its indecision stems from a different reason than that of an asymptomatic after infection state. Specifically, the asymptomatic at-risk participants, cannot have clinical manifestations of AD. This stage of AD has attracted much research attention since the field hypothesizes that early intervention may be the best approach to help (Dubois et al., 2021).

1.5.5.2 Mild (Early) Stage

Aβ peptides, a hallmark of AD, originate from APP and accumulate in the brain then are transported from the cortex to the dentate gyrus of the hippocampus which is crucial for cognitive function via the perforant pathway. APP-CTFs (C-terminal fragments) accumulate in the presynaptic site, where they are metabolized into Aβ peptides which disrupt normal neuronal communication. This accumulation also interferes with synaptic signaling, which further contributes to cognitive decline and neurodegeneration seen in the disease progression (Lazarov et al., 2002). As a result of this AD symptoms appear including.

- Remembering names.
- Recalling recent events.
- Locating valuable items.
- Planning and organizing.

As the disease progresses, individuals might:

Now and then call people by the wrong names, even if they are close relatives.

Become oblivious to the time of day or even days of the week.

- Need help in daily activities.
- Express unstable emotional responses or have changes in personality such as hallucinations, paranoia, or delusional thoughts.
- Suffer from physical changes of sleep disturbance and the likelihood of wandering away from home.

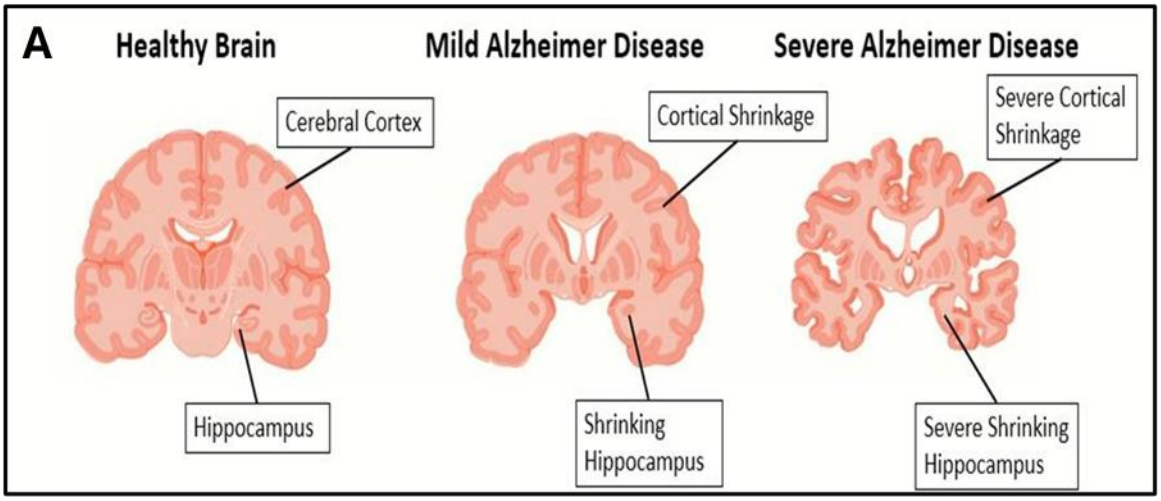
It is considered significant as it serves as an intermediate stage between typical aging and dementia, though not all individuals on this stage inevitably progress to AD (Angelucci et al.,

2010).

1.5.5.3 Severe (Late) Stage

At this stage, extensive deposition of amyloid-beta plaques in the brain disrupts cell communication and leads to neuroinflammation, ultimately contributing to neuronal death and synaptic lost. The aggregation of hyperphosphorylated tau protein forms neurofibrillary tangles, which are also prevalent in late-stage AD, spread throughout the brain, affecting areas critical for memory and cognition. Additionally, vascular damage, and chronic inflammation, and neuroplasticity loss lead to profound cognitive and functional impairments (Šimić et al., 2016; Parodi-Rullán et al., 2021; Brion et al., 1998)

- The loss of nearly all motor functions such as walking, sitting, and indeed swallowing.
- Incontinence of bowel and bladder.
- Restricted or very inadequate speaking skills, that is, an individual may be capable of speaking only a couple of words or phrases.
- The people in this stage undergo severe deterioration in physical as well as mental health.
- Hanging around and relying entirely on other people on all matters.
- Lack of awareness of recent events and the environment.
- They include increased predisposition to infections, particularly pneumonia (Dubois et al., 2016).



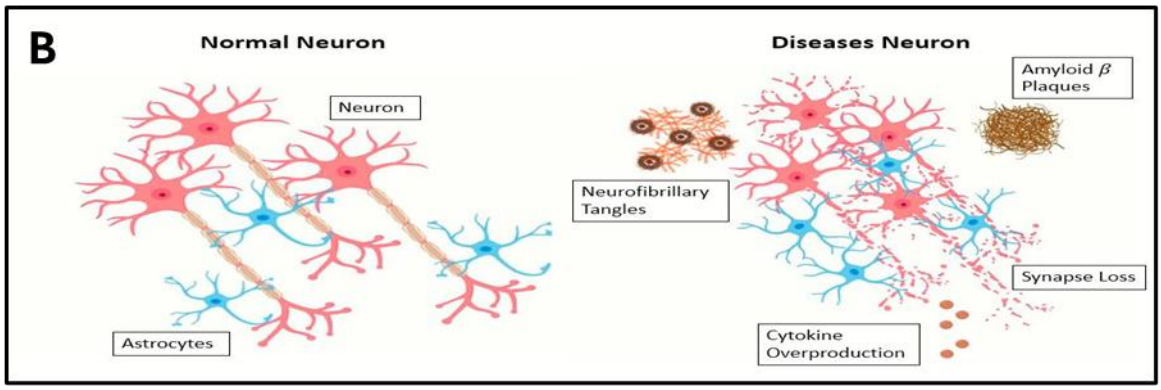


Figure 1.43. The progressive brain atrophy associated with AD: Figure A compares the appearance of a healthy brain to brains with mild and severe AD, focusing on the cerebral cortex and hippocampus. In a healthy brain, the cerebral cortex appears full and intact. With mild AD, cortical shrinkage is evident, and this becomes more pronounced in severe AD. The hippocampus, crucial for memory, shows similar atrophy. It is relatively normal in a healthy brain, shrinks in mild AD, and undergoes severe shrinkage in advanced stages. **Figure B** shows a comparison n between a healthy neuron to one affected by AD. The diseased neuron shows damage to its structure, including protein clumps and reduced connections.

1.5.6 Complications of AD

According to the CDC's National Center for Health Statistics 2023 study, AD is the sixth largest cause of death, killing over 120,000 people each year. AD primarily affects those over the age of 65, increasing their chance of developing a variety of problems that can have a significant impact on their health and quality of life. These difficulties might be classified into mental/behavioral and physical categories (Devanand et al., 1997).

1.5.6.1 Mental/Behavioral

1) Depression: There is a comorbidity in AD that has an impact on the disease and its management. The main clinical manifestations, which are typical for depression in Alzheimer's patients, include variability in mood, sleeping disorders, loss of interest in social interaction, and reduced attention.
2) Delirium and Agitation: Sun-downing is one of the late symptoms of AD and provokes severe difficulties for those who have the disease in their families. Hence, appropriate intervention to these symptoms is crucial if people with AD are to be protected and have their quality of life improved. However, the management of these conditions employing antipsychotic drugs has been aligned with elevated mortality and other effects.

3) Wandering: This is a behavior that is exhibited by most AD patients, and should always be dealt with appropriately to avoid serious consequences.

1.5.6.2 Physical

1) Fever and Infections: Elderly individuals with AD frequently suffer from infections, particularly respiratory and urinary tract infections. Difficulty in swallowing can lead to aspiration pneumonia etc.
2) Dehydration and Malnutrition: These conditions are common in AD patients due to difficulties with eating and drinking.
3) Falls: AD patients are at a higher risk of falls, which can lead to serious injuries.
4) Bladder and Bowel Problems: These issues are also common in individuals with AD, requiring ongoing management and care.

1.5.7 Genetics

1.5.7.1 Causative and Risk Genes

Analyzing the twin-fraternal data indicated that genetic influence is accountable for 60% to 80%

of the probability of AD development (Gatz et al., 2006). The usual Apo E4 variant implicates a considerable quantity of but not all AD heritability (Bellenguez et al., 2017). Further big genome-wide association studies have to be conducted to reveal new genetic bases of AD; the latest one to the present day included about 150000 patients with AD and over 300000 patients characterized by proxy phenotype AD - parental AD and unaffected controls without parental AD which added more than 40 new genetic risk factors for AD. Although it is crucial to note that the common risk allele for Apo E4, the total AD genetic risk derived in the present study ranges from 3 to 4 fold across different GWAS, other risk alleles implicated in AD have comparatively modest risk effects on total disease risk (Jansen et al., 2019).

Depending on the presence or absence of these risk alleles in the genome of an individual, scientists can calculate the polygenic risk score, the accuracy of which for the differentiation between patients with AD and healthy controls ranges from 75% to 85% (Escott-Price et al., 2017). Thus, 40 or so of these might primarily contribute to AD risk, the full preponderance of this accuracy can be attributed to the Apo E4 allele. The functional analysis of these risk loci showed that besides Aβ metabolism, immune response, cholesterol,

lipid dysfunction, endocytosis, and vascular factors might be involved in AD pathogenesis (Di Marco et al., 2015).

1.5.7.2 Protective Genes

Identifying the genetic variants that increase risk has enthused interest in the identification of protective genetic variants. The carriers of Apo E2 allele have an estimated 2 folds decrease of AD non-carriers (Holstege et al., 2017) which means that Apo E4 carriage compounds the lifetime risk of the ailment to an inconceivably low chance of having AD if one is a homozygous Apo E2 allele carriers (Reiman EM et al., 2020). It has to be found more frequent among a group of middle-aged participants when compared to other participants of the same or older age groups germline missense mutation in the PLCG2 gene, changed the amino acids and about a 1.9 fold lower risk of developing AD (Jonsson et al., 2012) further increasing the incidence of other types of dementia, and with a 2-3 times higher likelihood of attaining the age of one hundred cognitively health (Sims et al., 2017).

Genetic resilience was even reported in a pluralistic population, one which is made up of people with different origins, ethnicities, and from different parts of the world. Patient with PSEN1 gene mutation, and, basically, the individual's survival beyond the age of the onset of the symptoms typical for her family, caused by the rare protective variant caused by a homozygous gene, is situated in the Apo E3 allele (Arboleda-Velasquez et al., 2019). Klotho protein variants and longevity were also positively correlated with the similar effect of the produced gene, while the latter was negatively correlated with the increase of sweep (Belloy et al., 2020). Such protective thus, genetic variants offer great potential in AD research as they might uncover mechanistic characteristics of a phenomenon protecting cognitive health.

1.5.8 Comorbidity

In AD, comorbidity denotes the coexistence of one or more additional medical conditions alongside AD. These concurrent health issues can complicate the diagnosis, treatment, and overall management of Alzheimer's mentioned in figure 1.5. Below are some frequently observed comorbidities in Alzheimer's patients;

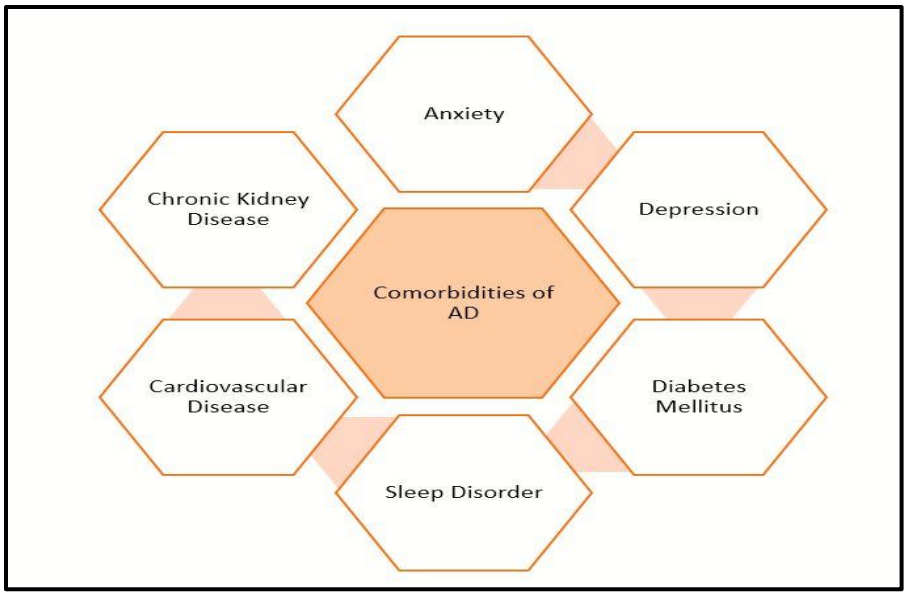


Figure 1.4. Comorbidities associated with AD: The diagram demonstrates the comorbidities of AD with other diseases that can complicate the diagnosis, treatment, and overall management of Alzheimer's.

1.5.8.1 Cardiovascular Diseases

Elevated blood pressure is a major risk factor for AD. The vascular damage resulting from hypertension can contribute to cognitive decline and the development of AD. Also, Coronary artery disease and heart failure are frequently observed in patients with AD. Additionally, individuals with AD have a higher incidence of both ischemic and hemorrhagic strokes (Gorelick et al., 2011).

1.5.8.2 Diabetes Mellitus

Type 2 diabetes is closely linked to a higher likelihood of developing AD. Factors such as insulin resistance and elevated blood sugar levels are thought to play a significant role in the development of Alzheimer's pathology (Arnold et al., 2018).

1.5.8.3 Depression and Anxiety

Depression and anxiety frequently co-occur with AD. These mood disorders can worsen cognitive impairment and significantly diminish the quality of life for those affected (Ownby et al., 2006).

1.5.8.4 Sleep Disorders

Patients with AD frequently experience sleep disturbances such as insomnia, sleep apnea, and fragmented sleep. These sleep issues can hasten the progression of AD (Bubu et al., 2017).

1.5.8.5 Chronic Kidney Disease (CKD)

Patients with CKD frequently experience cognitive impairments and are at an elevated risk for developing AD. This association is likely attributable to common risk factors and the widespread impact of CKD on brain function (Etgen et al., 2012).

1.5.9 Pathophysiology

AD is pathologically defined by the presence of neuritic plaques and neurofibrillary tangles in the brains of affected patients. These degenerative changes are connected with neuronal loss, which includes cholinergic neurons in the basal forebrain and neocortex. Unfortunately, due to a restrictive definition of 'mental illness', there is often little or no diagnosis of these factors in process culture organizations (Breijyeh et al., 2020). Pathophysiology related to AD are illustrated in diagram1.6.

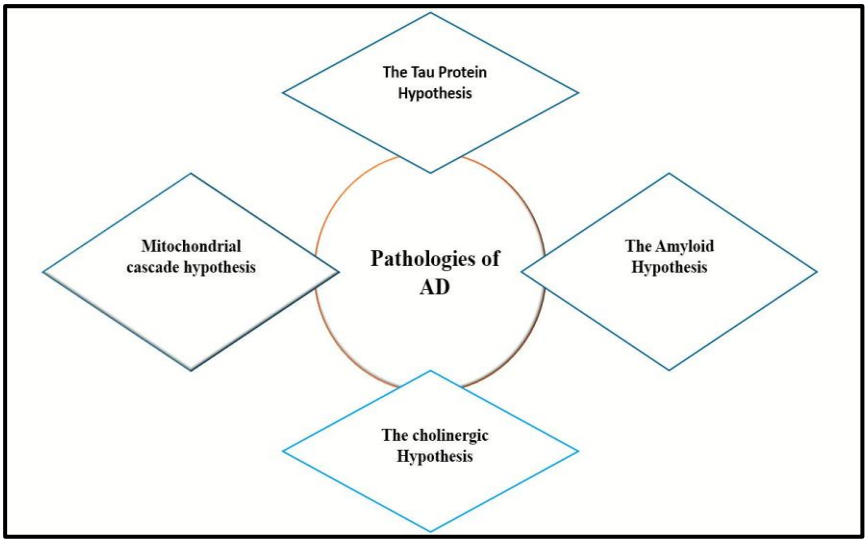


Figure 1.5. Pathologies related to AD: Four important pathophysiological explanations that play crucial roles in AD progression.

1.5.9.1 The cholinergic hypothesis

It claims that the neurotransmitter ACh is deficient in the brain due to neuronal loss in the cortex, and this is a major factor that triggers the development of AD. This hypothesis is due

to the acute loss of cholinergic neurons in AD, which suggests that ACh is significant to cognitive functions. Thus, it is thought that Aβ inhibits ACh function through cholinergic synapse and reduction in the release of Ach as shown in figure 1.7. They also cause clinically associated memory impairment in elderly patients (Hampel et al., 2018).

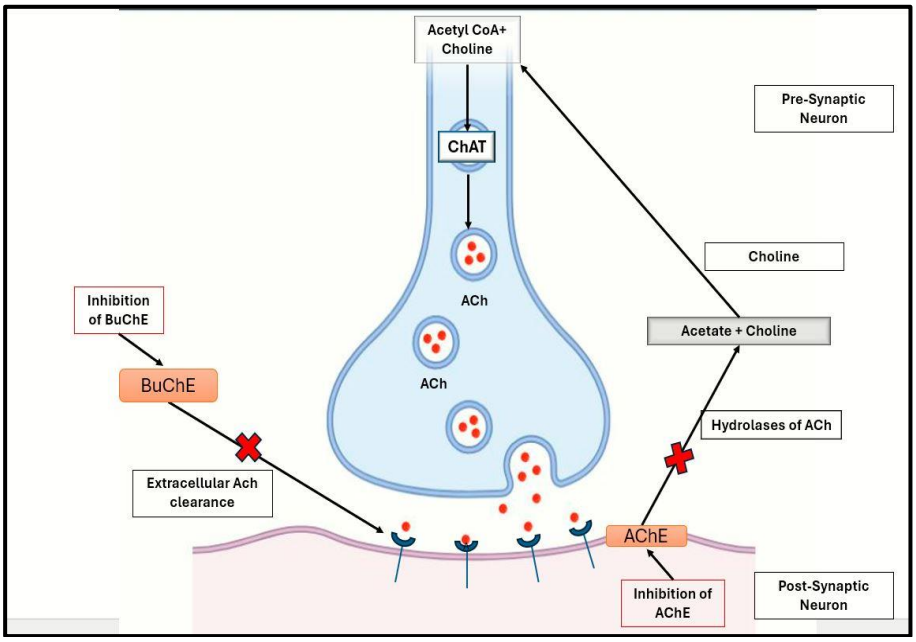


Figure 1.6. The cholinergic hypothesis: Shows the loss of cholinergic neurons causes AD.

1.5.9.2 The Amyloid Hypothesis

The pathophysiological mechanisms of AD have been widely investigated, and among them all the Amyloid Hypothesis is still the most accepted one, especially for inherited AD. Aβ hypothesis shown in figure 1.8, postulates that Aβ peptide is produced from APP through the activity of β-secretase and γ-secretases enzymes. Normally, APP is cleaved by either alpha or beta-secretase, the consequent products released by alpha and beta-secretase are not toxic to neurons but the products of beta-secretase followed by gamma-secretase are Aβ42. A rise in the levels of Aβ42 results in the precipitation of amyloid that is toxic to the neurons. Aβ 42 prefers the assembly of fibrillary amyloid protein rather than normal APP clearance (Paroni et al., 2019).

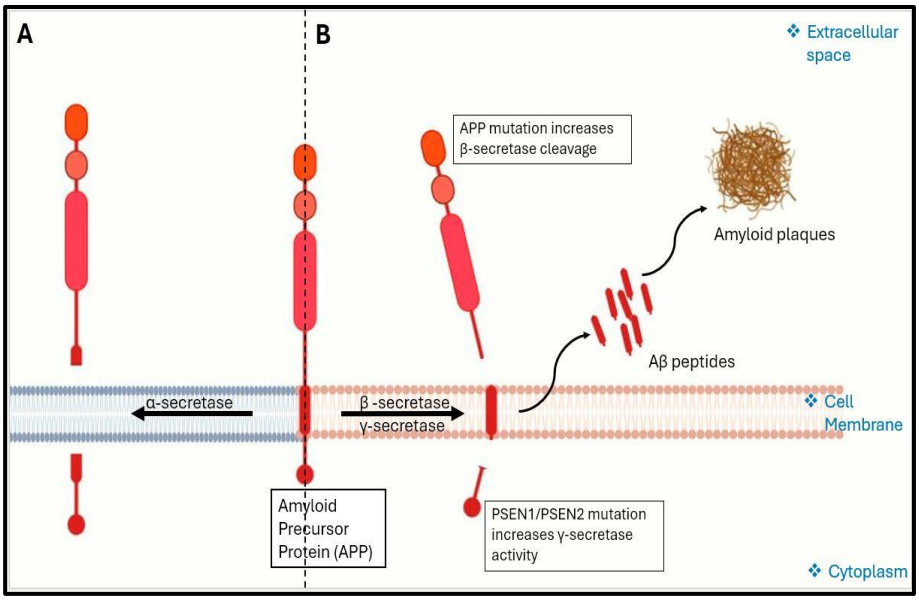


Figure 1.7. Amyloid Precursor Protein (APP): (A) Shows normal cleavage of APP, (B) Shows abnormal cleavage of APP leading to excess amyloid accumulation in AD.

1.5.9.3 *The Tau Protein Hypothesis*

The phosphorylated tau pathology proteins formed NFTs, help to stabilize microtubules, and are subject to several PTMs, such as phosphorylation. As shown in figure 1.9, when tau is hyperphosphorylated, it releases from microtubules and forms insoluble structures called PHFs and NFTs. The tau hypothesis suggests that tau tangle formation is followed by Aβ plaque formation and that hyperphosphorylation and aggregation of tau protein is the major factors for neuronal loss in AD (Lindwall et al., 1984).

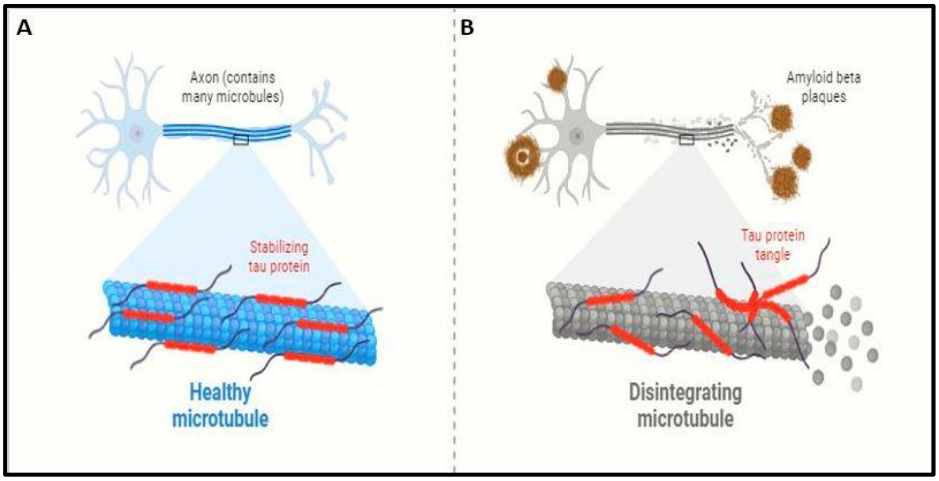


Figure 1.8. Tau Protein Tangles: (A) Shows stabilized Tau protein, (B) Shows disintegrated microtubules with Tau protein tangles in AD.

1.5.9.4 Mitochondrial cascade hypothesis

The mitochondrial cascade hypothesis describes that there is a baseline of some amount of mitochondrial function that then decays at a unique rate for each person. Mitochondrial dysfunction does, however, progressively decline to some critical point beyond which it begins to elicit the histopathological hallmarks of AD shown in figure 1.10. For instance, APP, or Aβ mutations in familial AD make mitochondrial damage worse and may turn upstream pathways as seen in LOAD. The hypothesis also proposes that genetic factors, as well as the environment, are determinants of the rate of change in the mitochondria. On the other hand, when the function of mitochondria is more sustainable then the aging of the brain slows down and when the function of the mitochondria is less sustainable, the aging of the brain increases. Lastly, the hypothesis states that AD is developed according to the baseline functional activity of mitochondria and the rate of their degeneration. Patients who at this baseline have low mitochondrial function and a rapid decline will manifest AD symptoms and histologic changes at a younger age. On the other hand, the person with better baseline function and slower decline rate will develop AD at a later stage. Intermediate outcomes are observed in two distinct cases; those with low baseline function and slow rates of mitochondrial decline, or those with high baseline function and fast rates of mitochondrial decline, will develop symptoms and AD histology changes at intermediate ages (Swerdlow et al., 2014).

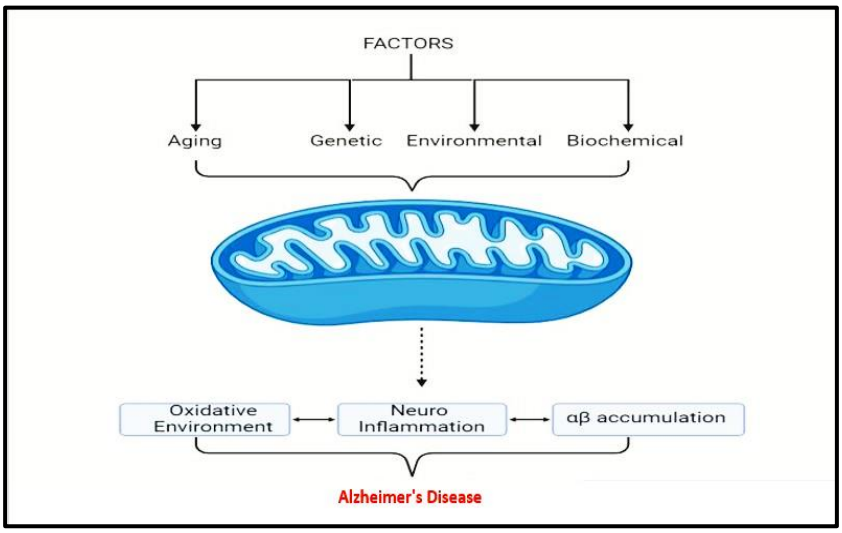


Figure 1.9. Mitochondrial cascade in AD: Mitochondrial dysfunction cause cognitive impairment in AD.

1.5.10 Histopathology

The usual histopathology of AD is characterized by the presence of three major features (Hussein et al., 2018):

1.5.10.1 Neuritic Plaques

Neuritic plaques are spherical media profile lesions containing extracellular Aβ in their center accompanied by swollen axons. These Aβ depositions are occasionally found to be distributed around meningeal and cerebral vessels in addition to the cortical gray matter of patients with AD. It is developed in gray matter is distributed in many sites and coalesces to form structures known as plaques. However, in some instances, the amyloid plaques of different illustrations have been discovered in the heads of some individuals who do not suffer from dementia. Other confirmed dementia patients who also completed one or more cerebral investigations were mentioned to be devoid of plaques.

1.5.10.2 Neurofibrillary Tangles

Neurofibrillary tangles are the fibrillary intracytoplasmic structures found in neurons and it is made up of a protein known as tau protein. Under normal conditions, tau protein is used to fasten microtubules at the extreme part of the neuron known as the axon. Protein traffic passes through the neuronal axons and it also forms a part of the intracellular transport. Tau achieves to participate in the dynamics of the microtubules; furthermore, it plays a role in the construction of microtubules along the neuronal axon.

In AD, due to the breakdown of the blood-brain barrier and enhanced procedure of Aβ formation in the extracellular matrix, tau acquires hyperphosphorylation. The type of phosphorylation in turn makes tau form aggregates inside the neurons and this leads to the issues of misfolded tau protein. These tau proteins deposit and transform into a structure called the neurofibrillary tangles which mainly consist of twisted paired helical filaments. AD starts with the neurofibrillary tangles manifesting in the hippocampus before progressing to the cerebral cortex of a person's brain. Instead, they accumulate in the neurons as Tau-aggregates.

1.5.10.3 Cortical Neuronal Degeneration

Cortical neuronal degeneration is the granulovacuolar degeneration of hippocampal pyramidal neurons and this is normally observed in AD patients, this cognition loss appears to be more linked to the reduction of the number of the pre-synaptic boutons that innervate

the afferent fibers which originate from pyramidal neurons in layer VI and, especially, lamina III-IV. And that is why the density's decrease can affect the processes in the sphere of thought to a greater extent than it seems by the number of plaques typical for AD.

1.5.11 Treatment / Management

Earlier there was no special treatment program for this disease, and now the practice indicates that symptomatic therapy is most often used in everyday work. Two categories of drugs are approved for treating AD: cholinesterase inhibitors, and partial NMDA antagonists, other categories of drugs also exist that have or may have antipsychotic effects (Leblhuber et al., 2018).

1.5.11.1 Cholinesterase Inhibitors

The cholinesterase inhibitors mechanism is based on increasing the concentrations of acetylcholine which is a neurotransmitter and participating in the conduction of nerve impulses, learning as well as in memory processes. Within this category, 3 drugs have received FDA approval for treating AD. It comprises of donepezil, rivastigmine and galantamine (Adlimoghaddam et al., 2018).

- Donepezil:
  - Medication of choice.
  - Used in the treatment of AD in stages with early mild dementia.
  - A fast, short-term, and easily reversible reaction in the environment that inhibits the enzyme acetylcholinesterase.
  - Once daily dosing with the tablet in the evening.
- Rivastigmine:
  - MCI and the early stage of dementia is addressed by these medicines.
  - A slowly hydrolyzing non-toxic, easily reversible, highly selective, and interacting with both AChE and BChE.
  - This product also exists in the oral and the transdermal form.
- Galantamine:
  - Recommended for MCI and early dementia at the present stage.
  - Modern, short-time, non-occupational reversible inhibitors of acetylcholinesterase.

- It is released in the market as a twice-daily tablet or once-daily extended-release capsule.
- They should not be prescribed to patients with end-stage renal disease, they are relatively contraindicated in cases of severe liver dysfunction.

Normally, cholinesterase inhibitors are associated with side effects of what is referred to as the GI type – these include vomiting, nausea, or diarrhea. These medications stem from the increase in vagal tone to cause cardiac conduction disorder, bradycardia, and syncope. It is best used in patients with moderate risk for cardiac conduction disorders and is relatively contraindicated in patients with severe cardiac conduction diseases.

1.5.11.2 Partial NMDA antagonists

- Memantine

- Memantine acts partially NMDA antagonist and reduces neural signaling by inhibiting NMDA receptors that increase the rate of intracellular accumulation of calcium.
- It is approved by FDA for the moderate to severe AD treatment. Weakness, Dizziness, constipation, and headache are examples of adverse effects of this drug.
- Add-on treatment with memantine is possible in patients with moderate to severe AD and relishing cholinesterase inhibitors like donepezil, rivastigmine, or galantamine (Khoury et al., 2018; Cummings et al., 2022).

1.5.11.3 Disease-Modifying Therapies for AD

Such drugs which in the past were used in managing AD have in the past relied solely in the symptomatic treatment of the condition. Many disorders of the brain are diagnosed at a stage when patients come to doctors, and by the time patients are diagnosed, pathological changes in the brain may have been present for more than 10 years. However, the new findings in the knowledge about the pathophysiology of AD and the rise in the diagnostic accuracy for imaging and biochemical markers currently allow us to identify preclinical and presymptomatic phases starting from MCI. Currently, there are novel disease-modifying therapies still under development; some of which have been approved recently.

- Aducanumab was granted accelerated FDA approval in June 2020, demonstrating a reduction in amyloid-beta plaques in the brain, though it did not meet the primary endpoint of clinical improvement in phase III trials (Padda et al., 2023).

- Lecanemab, approved in January 2023, has been shown to reduce amyloid-beta burden and slow disease progression by 27% in phase III trials (van Dyck CH et al., 2023).
- Donanemab, expected to receive FDA approval, has shown a 35% reduction in cognitive decline by decreasing the Aβ burden in the brain (Rashad A et al., 2022).

1.5.12 Other Management Strategies in AD

Taking care of AD involves handling other symptoms associated with the condition like anxiety, depression, and psychosis particularly in the later stages of the illness. Tricyclic antidepressants are contraindicated because of their side effects such as anticholinergic effects; worsening of cognitive function will occur. More specifically, antipsychotics should be used as a last resort and preferentially with acute agitation and only if non-pharmacological methods have been tried first, first-line safety being the patient and second-line safety being the caregiver. Before the administration of antipsychotics, one has a choice between SSRİ antidepressants (citalopram) and anticholinesterase inhibitors (donepezil).

Second-generation antipsychotic drugs are used in comparison with first generation antipsychotic drugs for the management of schizophrenia because of the absence of their extrapyramidal side effects (Grossberg et al., 2019). However, these treatments should get slightly better and the slight risks of stroke and raised mortality must therefore be balanced against this. With knowledge of the possible occurrence of extrapyramidal side effects, one has to give the smallest dose of an antipsychotic drug to sedate the patient. Benzodiazepines are also prohibited since evidence has also revealed that they are associated with worsening delirium and agitation. Among the blunt courses of action some of the blunt approaches as providing a familiar environment and addressing basic personal comfort needs as well as providing the child comforting things, shifting focus, removing all dangerous things such as the doorknob, and avoiding aggressive approaches to help control behavioral problems.

This is so because minor sleep disturbances must be controlled to reduce the amount of pressure that the caregivers have to bear and additionally improve the well-being of the patient. Some of the non-pharmacological approaches include ensuring adequate exposure to sunlight, increasing physical activity during the day, and adopting a nighttime sleep schedule that will help regulate the circadian sleep-wake ratios. If there is no improvement or if the patient develops side effects that cannot be borne, the treatment should be discontinued or

modified. Based on research, it has been deduced that aerobic exercise plays a role in preventing the progression of AD.

1.6 Aluminum Chloride ($AlCl_3$)

Aluminum is one of the most abundant metals on Earth and can be easily absorbed by the human body through sources like antacids, utensils, food additives, and water. However, it is a heavy metal that tends to accumulate in the body over time, potentially leading to toxic effects on various organs, including the brain, spleen, and liver (Wenk et al., 2003). Aluminum has been identified as a potential neurotoxin due to its ability to cross the blood-brain barrier (BBB), largely because of its strong affinity for transferrin receptors (Willhite et al., 2014). Once in the brain, it tends to accumulate, particularly in areas like the frontal cortex and hippocampus (Liaquat et al., 2019). Studies in both humans and animals have shown that aluminum buildup in the hippocampus can lead to abnormal accumulation of amyloid-beta (Aß), increased neuroinflammation, and eventually, neuronal death. Neuroinflammation plays a significant role in the development of neurological diseases, with microglial activation being a central event. This process can lead to deficits in hippocampus-dependent functions, such as learning and memory (Cheng et al., 2019).

1.6.1 Mechanism of action

The mechanism of action of $AlCl_3$ explained by (Sadek et al., 2019) is as follows;

- Entry of $AlCl_3$:
- Upon entering the body, $AlCl_3$ can cross the BBB, leading to its accumulation in the brain, where it particularly targets neuronal cells.
- Induction of Oxidative Stress:
- Exposure to $AlCl_3$ elevates the levels of ROS in neurons. This surge in oxidative stress inflicts damage on crucial cellular components, including lipids, proteins, and DNA.
- Mitochondrial Dysfunction:
- The increased ROS levels contribute to the dysfunction of mitochondria, resulting in a loss of mitochondrial membrane potential. This triggers mitochondrial outer membrane permeabilization (MOMP), during which pro-apoptotic proteins such as BAX, are activated and relocated to the mitochondrial membrane, facilitating MOMP.
- Release of Cytochrome c:

- Following MOMP, cytochrome-c is released from the space between the inner and outer mitochondrial membranes into the cytosol.
- Formation of the Apoptosome:
- In the cytosol, cytochrome-c binds to Apaf-1, leading to the assembly of the apoptosome. This complex then activates procaspase-9, which subsequently activates downstream effector caspases, predominantly caspase-3.
- Activation of the Caspase Cascade:
- Once activated, caspase-3 initiates the process of apoptosis by cleaving various substrates, resulting in:
- DNA Fragmentation: This leads to irreversible cellular damage.
- Cellular Disassembly: Characterized by cell shrinkage.
- Upregulation of Pro-Apoptotic Proteins:
- $AlCl_3$ can stimulate the increased expression of pro-apoptotic proteins like BAX while decreasing the levels of anti-apoptotic proteins such as BCL-2, which shifts the balance in favor of apoptosis.
- Increased GSK-3β Activity:
- $AlCl_3$ also elevated the levels of GSK-3β. This kinase plays a significant role in the hyperphosphorylation of tau proteins, a key feature in the pathology of AD. The result is the formation of neurofibrillary tangles, which further deteriorate neuronal function and lead to cell death.

1.7 Aims and Objectives

AD is one of the most commonly occurring neurodegenerative diseases, but current therapeutic interventions remain mainly symptomatic and do not significantly halt or reverse the disease process. Available therapies can aim to manage symptoms but are unable to address the underlying pathologies of AD. Chrysoeriol, having neuroprotective characteristics, which has demonstrated anti-oxidative and anti-inflammatory activities in different cell cultures, raises interest. However, its application to AD models, especially the *in vivo* models, has not been well investigated. Our research aims to investigate the efficacy of Chrysoeriol in the treatment of neurotoxicity associated with AD with the help of a well-established mouse model of AD. For this study, $AlCl_3$ has been used to mimic the *in vivo* AD mice model. The study will seek to establish whether Chrysoeriol has the potential to halt or

slow down the process of dementia, shielding the brain tissue, and altering the dysfunctional mitochondria. Furthermore, this study will assess the capability of the compound to influence other cellular activities that are keyed in AD including the reduction of Aβ plaques, by the downregulation of BAX and up-regulation of BCL-2 that has an important role in cell death and survival of neurons. These pre-clinical conclusions may lead to clinical testing of this strategy, helping to determine optimal dosing and potential synergism with current therapies. In the future, it may be possible to develop a new type of therapy that targets the underlying pathology of AD rather than just addressing its manifestations; thus, Chrysoeriol might become one of the promising therapeutic tools in the future.

1. Developing $AlCl_3$-induced mouse models of AD.
2. Treatment through Chrysoeriol in diseased mice model.
3. Behavioral analysis (Water Morris Test, Novel Object Recognition, Open Field Test, Y-Maze Test) to decisive neuroprotective efficacy of Chrysoeriol.
4. Enhanced Behavioral analysis shows that Chrysoeriol possesses reduced cognitive impairment and neurodegeneration and restores behavioral function.
5. Analyzing gene expression using RT-PCR to understand the molecular pathways that contribute to Chrysoeriol's neuroprotective properties.
6.

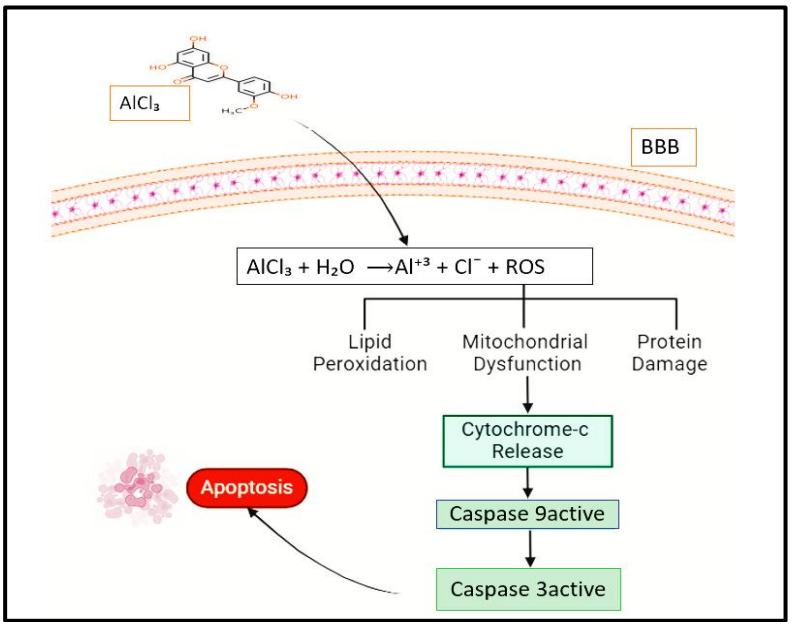


Figure 1.10. Mechanism of action of $AlCl_3$: $AlCl_3$ crossing BBB disrupting different pathways to cause AD.

CHAPTER 2: MATERIAL AND METHODOLOGY

2.1 Experimental Design

Eighteen male BALB/c mice, aged between 6 to 8 weeks, were acclimatized in the animal facility for one week. Experimental timeline listed in figure 2.1. They were housed in groups of three, with access to food and water during this acclimatization period. From day 8 to 50 mice were induced with $AlCl_3$ orally through an oral gauge. A behavior test was performed to ensure the effect of induction of disease in mice. Following the procedure, the treatment group received Chrysoeriol for 14 days, starting from day 53 and continuing until day 67.

Behavioral examinations were conducted from day 68 to day 77 of the study, encompassing tests such as the Water Morris Test, Novel Object Recognition, Y-maze exploration, open field assessment, and Evaluated-Plus Maze test. Tissue samples were collected on day 78. Onwards Histopathological procedures, including slide preparation, H&E staining, and slide analysis were taken, and Molecular analysis, specifically RT-PCR, was performed.

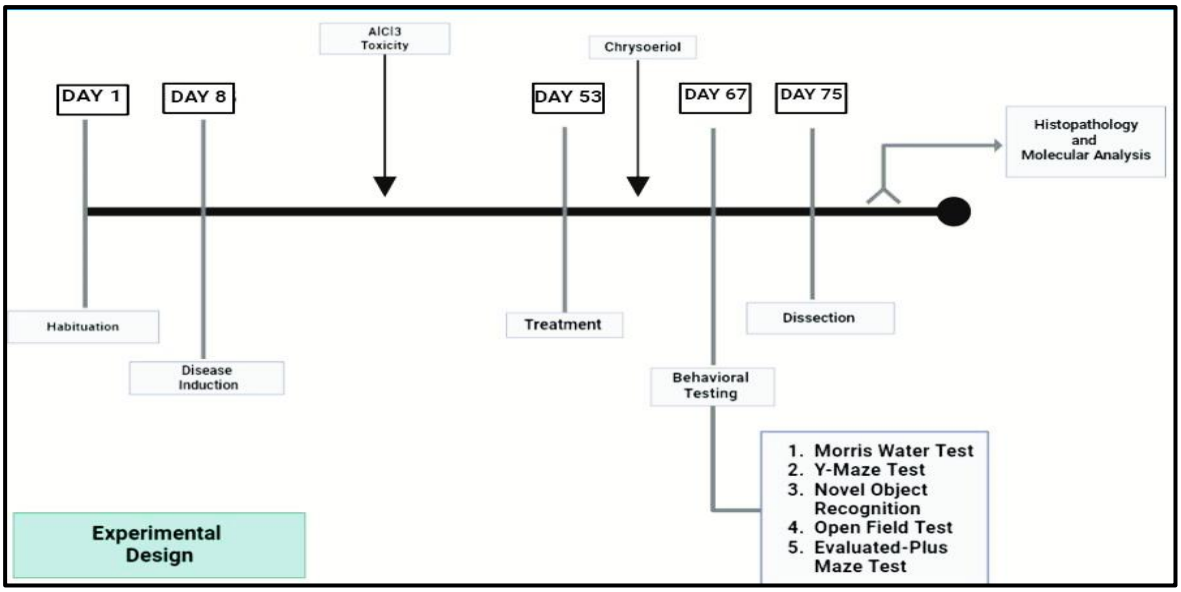


Figure 2.1. Experimental design. The experimental approach includes acclimating mice, inducing disease, administering treatment, analyzing behavioral data, sacrificing animals, histological inspection, PCR analysis, and statistical evaluation. The stages are presented sequentially to explain the research approach.

2.2 Ethical approval

Before initiating the *in vivo* study, Ethical approval was cleared by the NUST-IRB committee

of the National University of Science and Technology (NUST), Islamabad under (IRB number 05-2023-ASAB-02/02).

2.3 Animals housing and grouping

Male BALB/c mice were bred in NIH and were housed in the animal house of Atta-ur-Rahman School of Applied Biosciences (ASAB). They were used in the experiment. The mice were housed in cages maintained at a consistent temperature of 25 ±2 ℃ and subjected to a natural light-dark cycle of 12 hours. Mice were randomly assigned into three equal groups.

i. Control group (n=6).
ii. Diseased group (n=6) received $AlCl_3$ orally.
iii. Treatment group (n=6) received Chrysoeriol through IP.

2.4 Drugs and chemicals

All the chemicals and materials used in the study are listed in table 2.1, along with their sources, to ensure transparency and provide important reference information for repeatability.

Table 2.1. List of drugs, chemicals, and other materials along with their sources.

	Name	Source	Catalogs
1.	$AlCl_3$	Sigma Aldrich, USA	CAS-NO: 7784-13-6
2.	Chrysoeriol	Shaanxi Dideu Medichem Co. Ltd, China	CAS: 491-71-4
3.	Sodium Bicarbonate	Sigma Aldrich, USA	CAS: 144-55-8
4.	TRIzol isolation reagent	Fine Biotech Life Sciences, UK	Cat. No. FTR-100
5.	Reverse Transcriptase	Thermo Fisher Scientific, Lithuania	Cat. No. EP0441
6.	2% Agarose Gel	Sigma Aldrich, USA	CAS: 39346-81-1
7.	10X TBE buffer	Solarbio, China	Cat. No. T1050
8.	PCR master mix	Wizbio Solutions, South Korea	Cat. No. W1401-2

9.	SYBR green master mix	Wizbio Solutions, South Korea	Cat. No. W1711

2.5 *In Silico* Analysis

Before commencing the experimental phase, an initial *in silico* analysis was conducted using various software tools and computational methods. This step is pivotal in early-stage drug development and research, focusing on identifying potential therapeutic targets, validating safety, and evaluating repurposed medications. The study aimed to gain insights and make predictions based on hypotheses prior to experimental implementation. The three-dimensional structures of BAX (Q9D2C7) and BCL-2 (Q9DCS1) were retrieved from the AlphaFold PDB. Using the PubChem compound database, the chemical structure of Chrysoeriol (PubChem CID 5280666) was accessed. Subsequently, PyRx facilitated the docking process to elucidate the interaction between Chrysoeriol and BAX and BCL, assessing their binding affinity. This step allowed for understanding the structural basis and selectivity of Chrysoeriol towards BAX and BCL. Following docking simulations, Discovery Studio was employed to visualize the interaction patterns between Chrysoeriol and BAX, as well as BCL, focusing on the main molecular patterns of interactions.

2.5.1 Proteins

2.5.1.1 BAX

BAX (Q9D2C7) is a pro-apoptotic protein and is a part of the BCL-2 protein family. It tends to induce apoptosis by causing the outer membrane of the mitochondria to become permeable as well as to cause the release of cytochrome-c and result in the activation of caspases. It is found as an inactive protein located in the cytosol and is transported to the mitochondria on termination of the apoptotic signal. Elevated BAX is related to higher apoptosis, thus resulting in programmed cell death as reparation for stress or injury. BAX/BCL-2 mechanism of action is demonstrated in figure 2.2.

2.5.1.2 BCL-2

BCL-2 (Q9DCS1) is an anti-apoptotic protein that prevents cell death by inhibiting apoptosis and inhibits the release of cytochrome-c from mitochondria and consistent with this mechanism it inhibits the activation of caspase apoptosome. BCL-2 therefore counteracts

pro-apoptotic factors such as BAX and BAK by immobilizing them and thus maintaining the structural integrity of the mitochondria in the cell. Increased levels of BCL-2 protein are connected with diverse malignancies and its capacity to afford protection against apoptosis. It has been suggested that apoptotic imbalance is therapeutic and strategies that can modulate BCL-2 levels are used.

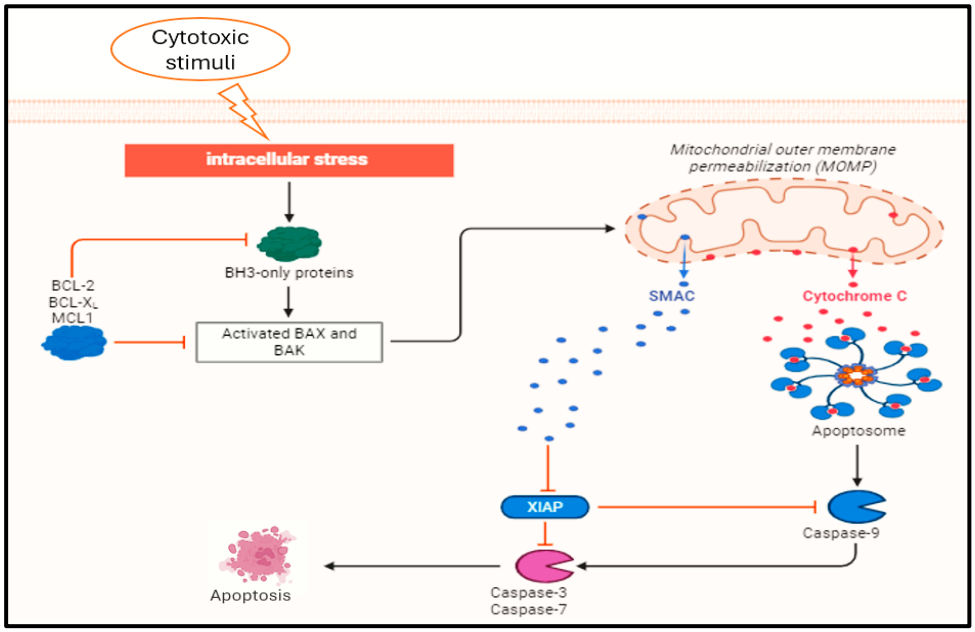


Figure 2.2. Apoptosis caused by BAX/BCL-2: Cytotoxic stimuli initiate intracellular stress signals which results in the releasing of pro-apoptotic proteins like SMAC and cytochrome-c, which activates caspase, resulting in cell death or apoptosis.

2.5.2 Software used for analysis

2.5.2.1 Alpha Folds

AlphaFold, created by DeepMind, is a revolutionary Al system designed to predict protein structures with exceptional precision using deep learning. By accurately forecasting the structures and interactions of proteins, DNA, RNA, ligands, and more. AlphaFold aims to revolutionize comprehension of biology and drug development. It provides valuable insights into protein folding and significantly aids in the understanding of biological processes and the advancement of drug discovery. The models are generated by supporting a wide range of scientific research endeavors.

2.5.2.2 PubChem

It is an extensive database maintained by the NCBI. It provides freely accessible information on the biological activities of small molecules, offering a valuable resource for researchers involved in drug discovery and other scientific inquiries. Users can search for chemicals by name, structure, or molecular formula, and the database includes data on chemical properties, biological activities, safety, and toxicity.

2.5.2.3 PyRx

PyRx is an open-source software application for computational drug discovery. It integrates several computational chemistry tools, enabling users to perform virtual screening, molecular docking, and other molecular modeling tasks. PyRx simplifies these processes by providing an easy-to-use graphical user interface, making it accessible to both experienced researchers and those new to the field.

2.5.2.4 Discovery Studio

It is a comprehensive suite of software tools designed for molecular modeling and simulation, developed by BIOVIA. It supports a wide range of activities, from protein modeling and simulation to ligand docking and pharmacophore modeling. Discovery Studio is widely used in the pharmaceutical industry for drug design and discovery, offering sophisticated tools and algorithms to aid researchers in understanding and predicting molecular behavior.

2.6 Induction Protocol

2.6.1 Dosing protocol of $AlCl_3$

The mice underwent to acclimatization for one week before $AlCl_3$ induction began. The day before the induction procedure started, they were weighed. $AlCl_3$ was given to the mice in accordance with the stated methodology, with special attention paid to the appropriate dosing schedule. Male mice aged eight weeks were received $AlCl_3$ orally at a concentration of 20mg/kg for 42 consecutive days shown in figure 2.3. On the 42 and 43 days, post-induction behavioral tests were conducted.

2.6.2 Dosing Protocol of Chrysoeriol

Chrysoeriol was given to the diseased mice at the concentration of 5mg/kg in 1% of DMSO solution once a day for 14 days intraperitoneally based on modifications from (Shao et al., 2021) shown in figure 2.4. The injection volumes were adjusted depending on the mice's body weight in grams. Behavioral assessments were carried out the day following the last Chrysoeriol dosage.

Figure 2.3. Administration of $AlCl_3$ orally: Mice received a daily dose of $AlCl_3$ solution at 20mg per kg for 42 days. $AlCl_3$ was meticulously dissolved in distilled water.

Figure 2.4. Administration of Chrysoeriol: Mice received a daily doseof Chrysoeriol at 5mg per kg for 14 days. Chrysoeriol was meticulously dissolved in 1% DMSO solution.

2.7 Behavioral testing

Behavioral tests were performed following both $AlCl_3$-induction and Chrysoeriol treatment. After inducing $AlCl_3$, mice underwent early behavioral evaluations to measure its baseline effects. The Open Field Test was performed to monitor for $AlCl_3$-induced motor and behavioral deficits. Following early examinations, Chrysoeriol treatment commenced and lasted 14 days. After Chrysoeriol treatment, additional behavioral tests were undertaken to assess any improvements in motor function and behavior. Animals were acclimated to the testing environment 10 minutes before the commencement of the tests. The evaluation included the Water Morris Test, The Y-Maze Test, The Novel Object Recognition, The Open Field Test, and The Elevated plus Maze Test.

2.7.1 Morris Water Test

The Morris Water Test was performed by following a previously established protocol to elevate hippocampal-dependent learning, including the acquisition and retention of spatial memory over the long term (Bromley-Brits et al., 2011) with slight modifications. A circular pool has a diameter of 150 cm and a depth of around 50 cm (a black pool should be used for white mice), while the optimized water temperature should be between 21-23 °C. Mice went in acquisition trails four times daily for five consecutive days, with at least a 10-minute interval between each trail. Each trail began with the mice being placed in the water at one of the four randomly selected starting positions. Mice that did not find the platform within the 60s were manually guided to it and allowed to remain for 10 seconds. The data were analyzed by measuring the time latency for all trials. A probe trial was conducted on the 6th day to evaluate spatial bias development. During this trial, the mice swam in the pool for 60 seconds with the platform removed. The time spent in each platform quadrant was recorded through a camera positioned above the center of the maze, allowing for later assessment.

2.7.2 Y-Maze Test

This assessment leverages mice's natural derive to explore novel objects, evaluating their short-term spatial recognition memory. Unlike radial maze, which necessitates food deprivation, or the light/dark cycle, or employs electrical foot shock, this test does not alter the animal’s motivational or emotional state. The Y-Maze Test has proven effective in

examining the impacts of various stressors on spatial memory performance. (Boon et al., 2012). The Y-Maze apparatus was painted with black color. It comprised three arms, forming a 120° angle with the others. Each arm was 8cm in width, 30 cm in length, and 15 cm in height. The arms were indistinguishable in appearance. Arm A and B were opened throughout 2 trials while arm C (novel arm), remained blocked during the first trial but opened during the second trial.

Figures 2.5. The Morris Water Maze: Utilized to evaluate spatial learning and memory in mice. This test involves a circular pool filled with water and a hidden platform. The time it takes for the mice to locate the platform is recorded. This assessment offers critical insights into cognitive function and the impact of experimental treatments or conditions on memory and learning abilities.

In the initial phase of the two-trail test, the mice were given the freedom to explore two arms, A and B: while the third arm was kept closed, for 10 minutes. During the second trial, the mice were allowed to explore all three arms for 10 minutes (Kraeuter et al., 2019). The video was recorded for the later assessments. Mice typically demonstrate exploratory behavior by frequently visiting new areas. In the Y-maze, it is expected that mice will alternate between the three arms, moving from the familiar arm to the novel arm, all while remembering which arms they have previously explored. However, under pathological conditions, mice struggle to alternate between the arms, indicating an inability to remember their previous explorations. The percentage of alternation is calculated using the formula provided by (Takeuchi et al., 2011; Kumar et al., 2011).

$$spontaneous\ altration\ = \frac{\text{number of alterations}}{\text{total number of entries}} \times 100$$

Figure 2.6. The Y Maze test: This assesses spatial memory and exploratory behavior in mice using a Y- shaped apparatus with three arms, 2 familiar and 1 novel. The pattern of arm entries and the time spent in each arm were recorded, providing insights into memory and cognitive function, as well as exploratory tendencies. This test is valuable for studying the effects of treatments or neurological conditions on cognition.

2.7.3 Novel Object Recognition

The Novel Object Recognition experiments were conducted following a previously established method with slight modifications (Radiske et al., 2017). These experiments took place in an open field box (40 x 40 x 40 cm). The process included four stages: habituation, training, and activation. During the test, and before each mouse's habituation and testing, the box was subsequently cleaned with 75% ethanol to eliminate any lingering scents. During habituation, No objects were placed in the open field box. The mice were individually introduced into the box and allowed to explore freely for 10 minutes. The second step is Training; two identical objects named AA were placed diagonally in the experimentation box. The mice were then introduced one by one and allowed to explore for 10 minutes. After familiarizing object A, the next step is Activation: the next day (retention time for 24 hours) where one of the familiar objects (A) is replaced with a novel object (B). The mice were reintroduced into the box to reactivate their memory of object A, exploring freely for 5 minutes. The video was recorded for later assessments.

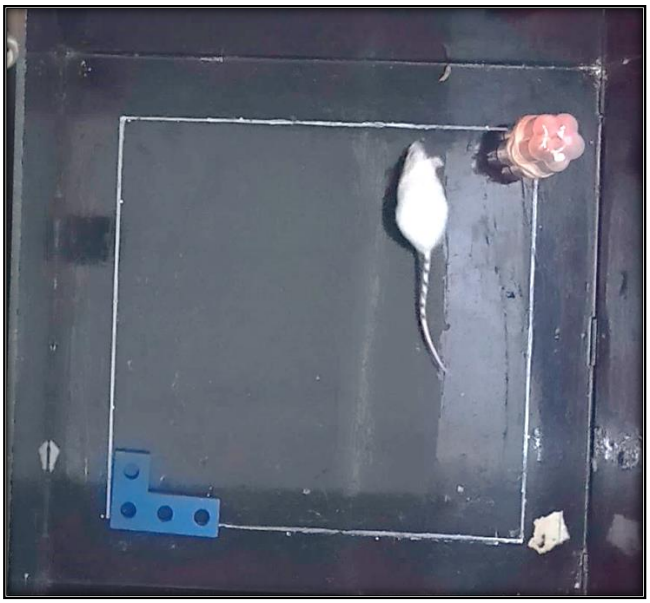


Figure 2.7. The Novel Object Recognition Test: Assesses cognitive processes by tracking a mouse's interactions with two objects: one familiar and one novel. The time spent exploring each object is measured, with a preference for the novel object indicating that the mouse remembers the familiar one and recognizes the novelty of the other. This test is instrumental in neuroscience for investigating cognitive functions like memory and attention in AD mouse models.

2.7.4 Open Field Test

The Open Field Test allows for straight forward and unbiased assessment of behavior without any need for prior training (Seibenhener et al., 2015), as it is extensively employed to evaluate exploratory and anxiety-related behaviors. The apparatus consists of a floor made up of hardwood, measuring 100 cm in width, 100 cm in depth, and 40 cm in height, which was divided into 16 squares using white lines in a 4x4 grid. The mice were placed in the center of the box, and their behavior was observed over 10 minutes. Behavior was recorded on the basis of entries in central and peripheral regions, with the entry defined as both forelimbs crossing a line. The entries into the central 4 squares and the outer 12 squares adjacent to the walls were counted separately (Takeuchi et al., 2011).

2.7.5 Elevated Plus-Maze Test

The elevated plus-maze is comprised of 4 arms of which 2 are open arms and 2 are enclosed arms of identical dimensions, and the surrounding is painted with black paint for white mice. There are different lengths of the apparatus for other animal models. The maze should be elevated approximately 30 cm above the ground, with each arm measuring about 30 cm in length and 5 cm in width (Mulder et al., 2004). Position the mouse on the central platform,

facing one of the open arms, and allow it to explore the maze. Use a video recorder for later assessment, the factors included in this test are; (1) the number of entries and (2) the duration spent in each arm. Clean the maze with 70% ethanol after each trial.

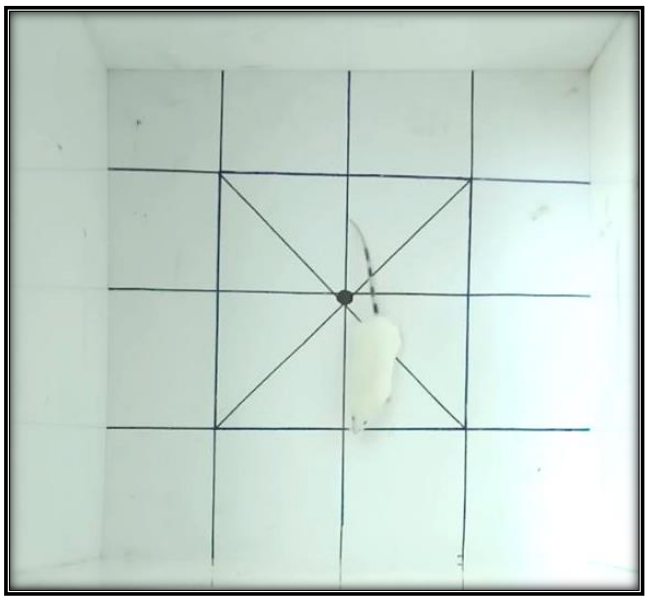


Figure 2.8. The Open Field Test: This test evaluates exploratory behavior and anxiety levels of a diseased model. This figure illustrates the mouse's movement across the squares. The time spent in the central region versus the peripheral region is recorded. These metrics are essential for understanding the mouse's exploratory tendencies and anxiety levels, both of which may be influenced by anxiety in AD.

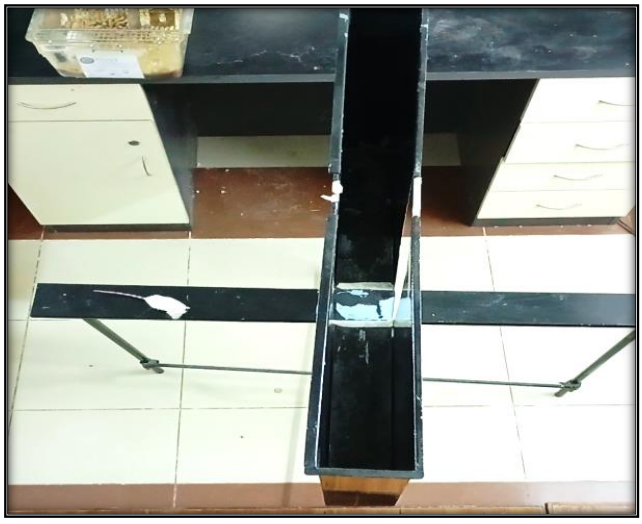


Figure 2.9. The Elevated Plus Maze: This test is conducted to assess anxiety-related behavior in mice. This test involves a plus-shaped apparatus with two open arms and two closed arms elevated above the ground. The time mice spend in the open arms versus the closed arms is measured. This assessment provides valuable insights into the effects of experimental treatments or conditions, such as stress or neurological disorders, on anxiety and exploratory behavior in mice.

2.8 Histopathological Analysis

Histopathology allows for the detailed microscopic examination of tissues, providing profound insights into the causes of diseases, the effectiveness of treatments, and the impact of experimental variables. By evaluating alterations in histological and morphological patterns, researchers can diagnose and classify diseases in animals, determine treatment efficacy. In toxicology studies, histopathology is crucial for assessing the effects of toxins and drugs on tissues. Additionally, it enhances the understanding of disease mechanisms, confirms the validity of animal models, and supports long-term investigative studies.

2.8.1 Dissection and Tissue Fixation

Tissue dissection and fixation were carried out the following day after the behavioral experiments. Mice were anesthetized intraperitoneally with 100 mg/kg of ketamine (17750-Pipelinepharma, Pakistan) for histopathological (David et al., 2022) in sterile saline by 120mg/kg (Overmyer et al., 2015). After anesthesia, an incision was made in the abdominal cavity to reveal the heart. The procedure involved inserting a needle into the right ventricle and creating an incision in the right atrium. The procedure involved infusing 80 ml of 0.9% saline and then 100 ml of a fixative solution comprising 4% paraformaldehyde (E-IR-R114, Elabscience, USA) in 0.1 M phosphate-buffered saline at pH 7.4. Following perfusion, the skull was bisected along the midline using scissors and a surgical knife to reveal the brain, which was then carefully extracted using fine forceps. To remove any excess fixative and blood from the sample, the tissues were rinsed with PBS (A9162.0100, Avantor, America). Brain tissue was then fixed with a 4% paraformaldehyde solution (Gage et al., 2012).

2.8.2 Hematoxylin and Eosin (H&E) Staining

H&E staining is a pivotal technique in histology that reveals intricate details about the cellular and structural makeup of tissues. This method employs two dyes, hematoxylin and eosin to differentiate cellular components when viewed under a light microscope. Hematoxylin, a basic dye, binds to acidic elements within the tissue, particularly nucleic acids in the cell nuclei, rendering them a purplish-blue hue. This specificity is due to hematoxylin forming complexes with acidic compounds, making the cell nuclei distinctly visible.

Figure 2.10. Mouse placement and needle insertion during transcardiac perfusion: Figure depicts the introduction of a needle into the heart for transcardial perfusion.

The slides were prepared by further processing the previously fixed tissue with 4% paraformaldehyde. To remove water from the tissue, it was first dehydrated by immersing it in increasing percentages of ethanol (70%, 95%, and 100%). Following dehydration, xylene (CAS No: 1330-20-70, Sigma Aldrich USA) used as a clearing agent to remove alcohol and make the tissue transparent. To rehydrate the tissue, microtome was used to cut thin slices of brain tissue (3-5µm) and immersed them in ethanol concentrations ranging from 100% to 70%. Then stained the rehydrated tissue slices with hematoxylin solution for a few minutes. After staining, the slides were washed to remove any excess pigment. Bluing was done by immersing the slides in a weak alkaline solution, such as ammonium water, to stabilize the hematoxylin stain, followed by another rinsing. After counterstaining with eosin solution, the slides were washed. Mounting media and cover slips were used to preserve tissue sections for further microscopic analysis (Feldman et al., 2014; Alturkistani et al., 2016). Steps are mentioned in figure 2.11.

2.8.3 Microscopic tissue analysis

The slides were examined using a light microscope (Optika B-150, Italy) at 10X and 40X magnifications to assess tissue structure, cell counts, and patterns. Photomicrographs were taken of the hippocampus, and cortex to compare differences between the three groups. The microscope aimed to investigate the effects of $AlCl_3$ and $AlCl_3$ -treated with Chrysoeriol on cell chemistry.

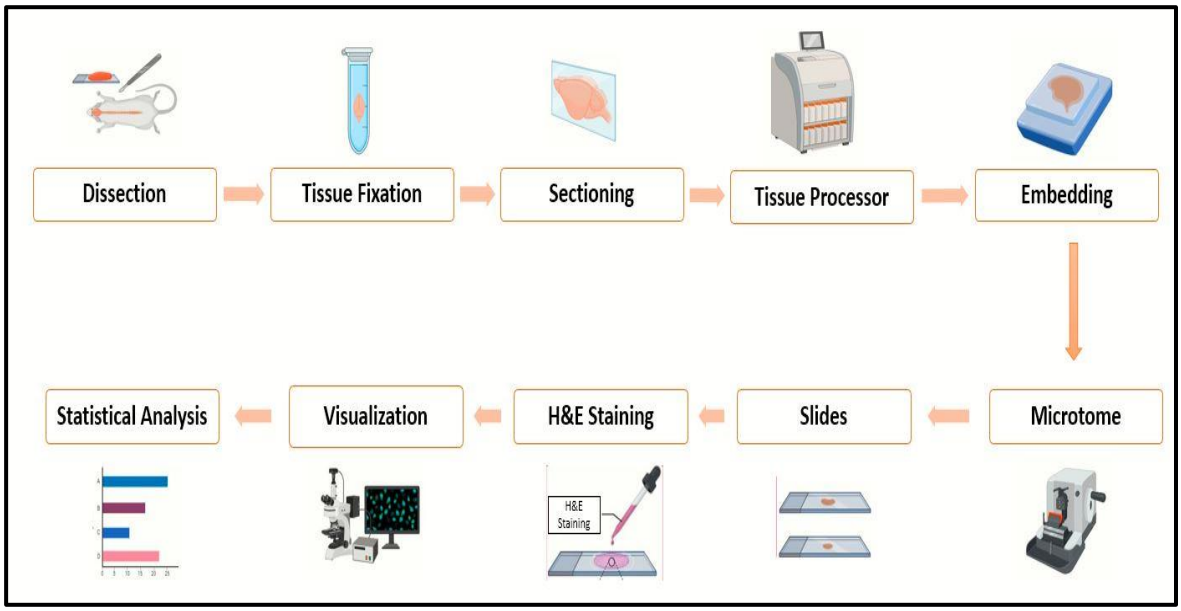


Figure 2.11. Steps for evaluating histological features: Histopathology reveals cellular stories through several phases, including tissue collection, fixation, embedding with a tissue processor, microtome sectioning for slides, staining, visualization, and analysis**.**

2.9 Analysis of Gene Expression

2.9.1 Tissue dissection

The mice were deeply anesthetized through chloroform inhalation. Following this, they were carefully decapitated with sharp scissors. The skull was then precisely incised along the central axis using scissors and a knife to expose the brain. The brain was gently removed from the skull with fine forceps. The sample was quickly frozen with dry ice and stored at -80 °C for future analysis.

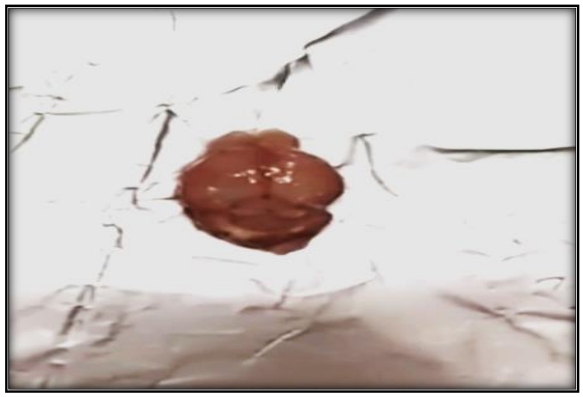


Figure 2.12. Mouse brain after dissection. The picture depicts the brain extracted from a mouse skull after dissection.

2.9.2 RNA Extraction

The extraction of RNA using TRIzol reagent (FTR 100, Fine Biotech Life Sciences, china) is a conventional and widely used method of isolating RNA in molecular biology and biochemistry. To begin with, 1000 µl of TRIzol solution was used to precipitate the sample by which the tubes were mixed through centrifugation at 12,000 rpm for 10 minutes at 4 °C. In the process of centrifugation, the contents of the test tube were split into two layers; the upper layer being the supernatant while the lower part was a sediment. After that, 200 µl of chloroform was applied to the sample, and the sample was shaken with moderate intensity for 30 seconds. The mixture was then subjected to centrifugation at 12, 000 rpm for 10 minutes at 4 °C for the second time and after that, the two phases were separated. As carefully as possible transfer the RNA-containing aqueous phase into a new tube. Subsequently, homogenize the contents of the tube by the addition of 500 µl of isopropanol. Let the mixture incubate at room temperature for ten minutes. Again spin the tube for 10 minutes at 4 °C, 12,000 rpm followed by discarding the supernatant. The resulting pellet is to be washed with 75% ethanol solution and the mixture requires to be centrifuged for 2 minutes at 4 °C at 12,000 rpm. Pour off the ethanol gently and leave the pellet to air-dry for 5-10 minutes. Resuspend the pellet in 20-50 µl Nuclease free water then store the samples

2.9.3 Assessment of extracted RNA Quality and Quantity

The extracted RNA was tested for concentration and purity using a Colibri NanoDrop spectrophotometer (TitertekBerthold, Germany). Both concentration and purity were within permissible limits. The acceptable RNA concentration, as evidenced by their absorbance is 260/280 nm and 260/230 nm. For RNA sample ratio optimal ranged from 1.8 to 2.2, 2 whereas below to the value of 1.80 are generally unsuitable as showing contamination that can cause false results in the process of qPCR.

2.9.4 Complementary DNA (cDNA) Synthesis

After the assessment of RNA quality and quantity of mice brain next step is to synthesize cDNA, the reaction mixture was prepared by using 2 µl of RNA, 6 µl of dNTPs, and 1.5 µl of oligo (dT's), 4 µl of RT buffer, 2 µl of DTT, 1 µl of reverse transcriptase, and 3.5 µl of

nuclease-free water. The reaction mixture was incubated in a thermal cycler at 42 °C for 60 minutes. After completion, cDNA was kept at 4 °C for future use in gradient and RT-PCR.

2.10 Polymerase Chain Reaction (PCR)

2.10.1 Designing of primer

The primers BAX and BCL-2, were chosen based on data from published papers (Cui et al., 2020), and purchased from Bionics, Islamabad, Pakistan. After that, by using the NCBI website ran Primer-BLAST to ensure that these primers gave the targets as mentioned in table 2.2. This step ensured that the primers were compatible with the PCR.

2.10.2 Gradient PCR

Gradient PCR (Multigene Optimax) was used to optimize the annealing temperature of the chosen primers. The cDNA was synthesized and included the components mentioned in Table 2.2. The PCR methodology involved an initial denaturation phase at 94 °C for 3 minutes, followed by 35 cycles at 94 °C for 30 seconds. At annealing step, the temperatures were evaluated to 58 °C for BAX and 64.5 °C for BCL-2. Lastly at extension step, the gradient temperature at 72 °C for 45 seconds, and final extension at 72 °C for 7 minutes. After PCR, gel electrophoresis was used to analysis of the presence of bands.

2.10.3 Reaction mixture

In the PCR tube, the total volume was made up to 25µl consisting of 12.5µl of PCR master mix (Wizbio Solutions, catalog no: W1401-2, South Korea) 8.5µl Nuclease-free water, 1µl of forward primer, 1µl of reverse primer, and 2µl of cDNA template

Table 2.2. List of all primers used. The following tables show the list of primers including their sequences, length, GC % content, and optimal temperatures

Name	Primer Sequence	Length	GC%	Temperature °C
β-actin	Forward	CATCCCCC AAAGATTC TAC	347	61.5 °C
β-actin	Reverse	CAAAGCCT TCATACAT C	347	61.5 °C
BAX	Forward	TGCTAGCA AACTGGTG CTCA	113	61.5 °C

BAX	Reverse	CTTGGATC CAGACAA GCAGC	113	61.5 °C
BCL-2	Forward	TTCTTTGA GTTCGGTG GGGTC	205	64.8 °C
BCL-2	Reverse	TCAGAGAC AGCCAGG AGAAATC	205	64.8 °C

2.10.4 Agarose Gel Electrophoresis

To ensure proper annealing at optimized temperatures, we used gel electrophoresis with 2% agarose (Sigma Aldrich, CAS: 39346-81-1, USA) compounds enlisted in table2.3, stained with ethidium bromide (Sigma Aldrich, catalog no. 39346, USA). The gel was run in TBE buffer (catalog no. T1051, Solarbio, China) for 45 minutes at 100V. To evaluate successful annealing, we matched band locations to a DNA ladder of 100 to 1500 bp (CAS: FNB500304, Fine Biotech Life Sciences, China). The gels were analyzed with a Benchtop 2UV transilluminator (LM-20, P/N 95044902, UVP Co., USA).

Table 2.3. Lists the components used in gradient PCR. This table lists all components used in gradient PCR, along with their amounts.

	Components	**Quantity**
1	cDNA templates	2.0µl
2	Forward primer	1.0µl
3	Reverse primer	1.0µl
4	PCR master mix	12.5µl
5	Nuclease free water	8.5µl

2.10.5 Real Time-PCR

BAX and BCL-2 expression levels in brain tissues were measured using Bio-Rad's real-time PCR detection system (C1000, BIO-RAD, USA). The 20µl of total reaction mixture was made with 4µl of qPCR Master (SYBR Green, Catalogue No: W2631, Wizbio, Korea), 1µl of

forward primer, 1µl of reverse primer, 1µl of cDNA templates, and 13µl of nuclease-free water components enlisted in table 2.4.

Table 2.4. List of ingredients used in qPCR. This table provides the list of components of the qPCR master mix along with their quantities.

	Components	Quantity
1	cDNA templates	1.0µl
2	Forward primer	1.0µl
3	Reverse primer	1.0µl
4	SYBR green master mix	4.0µl
5	Nuclease free water	13.0µl

2.10.6 Thermocycling parameters for RT-PCR

Figure 2.12 shows the Real-time PCR thermocycling parameters. PCR circumstances:

- First denaturation at 94 °C for 3 minutes, and second denaturation at 94 °C for 30 seconds.
- 35 cycles of annealing at 64.8 °C for BCL-2 and 58 °C for BAX for 30 seconds.
- Elongation at 72 °C for 45 seconds.
- Extension at 72 °C for 7 minutes, second extension at 4 °C for 4 minutes.

  The quality of the PCR product was assessed using amplification curves and agarose gel electrophoresis. The ΔCq values were used to analyze gene expression, normalized against the β-actin values.

2.11 Statistical analysis

Before performing any statistical analysis, the normality of all data sets was evaluated. The study compared differences among the control, $AlCl_3$-induced, and $AlCl_3$-treated with Chrysoeriol groups. To identify any significant differences between the groups, statistical tests such as the unpaired T-test and one-way ANOVA were performed, followed by Tukey's post-hoc comparison. Graphs were generated using Graph Pad Prism version 10.0, with statistical

significance determined at a threshold of $P < 0.05$. Data and results were expressed using the standard error of the mean (SEM).

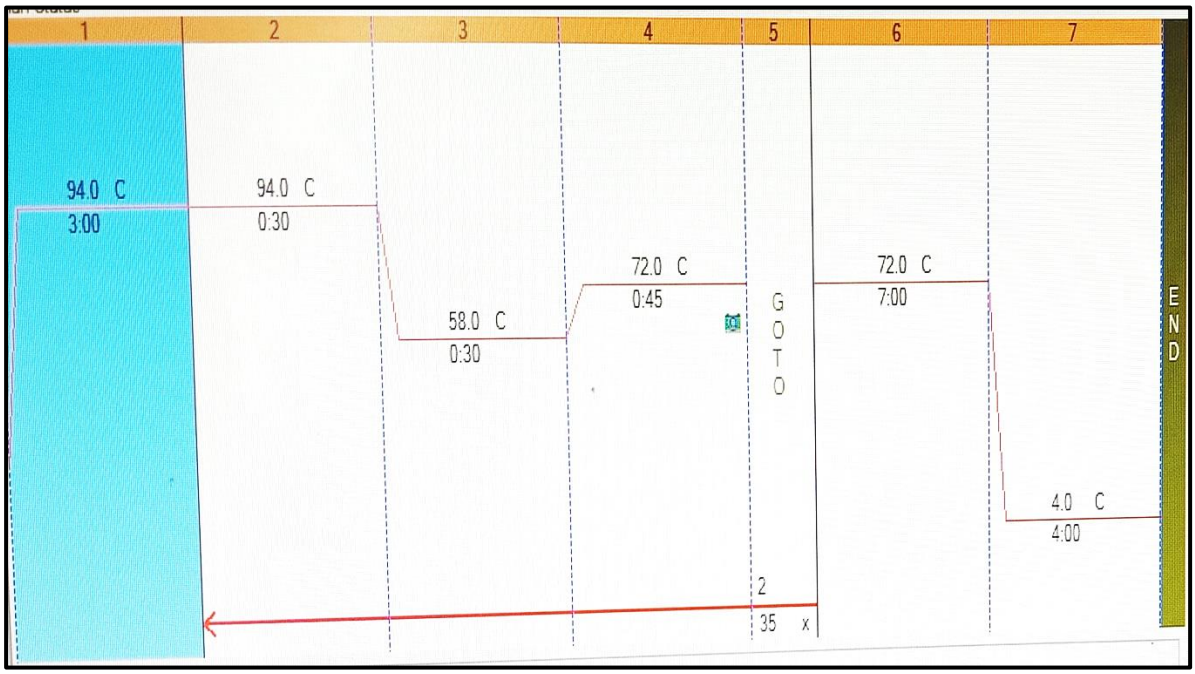


Figure 2.13. Cycling parameters for RT-PCR. The figure shows the thermocycling profile for both BAX at 58 °C and BCL-2 at 64.5 °C.

Chapter 3: RESULTS

3.1 Results of *In-Sillico* analysis

3.1.1 3D Structure of protein and ligands

Alpha fold's PDB format provides 3D structures of BAX and BCL-2 proteins. The chemical structure of the ligand, Chrysoeriol, is available from PubChem in SDF format, which includes information about its composition and confirmation. Figure 3.1 shows the structures of both the proteins and the ligands, which were subsequently polished and set to publication standard in Discovery Studio Visualizer. These structures are utilized for additional investigation, such as molecular docking.

3.1.2 Molecular docking analysis of Chrysoeriol with BCL-2 and BAX

The molecular docking of BAX and BCL-2 which are targets with the ligand Chrysoeriol were analyzed independently using a docking approach as shown in figure 3.2. The findings reveal important details regarding how BAX and BCL-2 interact with Chrysoeriol. This provides insight into Chrysoeriol's interaction with BAX (A) and BCL-2 (B), its involvement in neuroprotection, and potential future study areas.

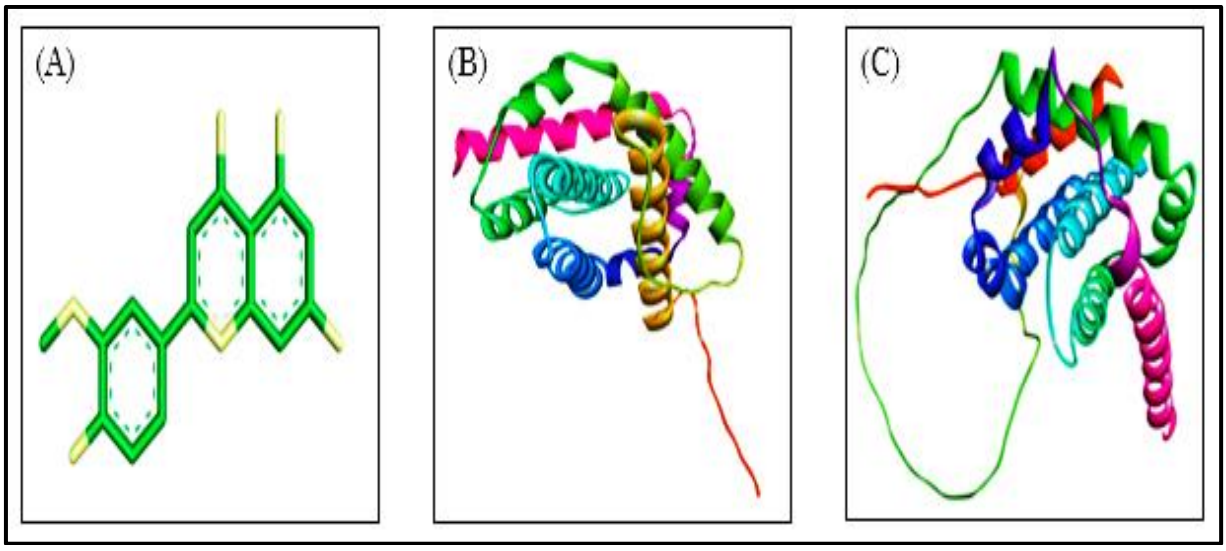


Figure 3.1. 3D structures of BAX, BCL-2, and Chrysoeriol. The figure presents the three-dimensional (3D) structure of ligand and proteins, (A) Chrysoeriol, (B) BAX, and (C) BCL-2.

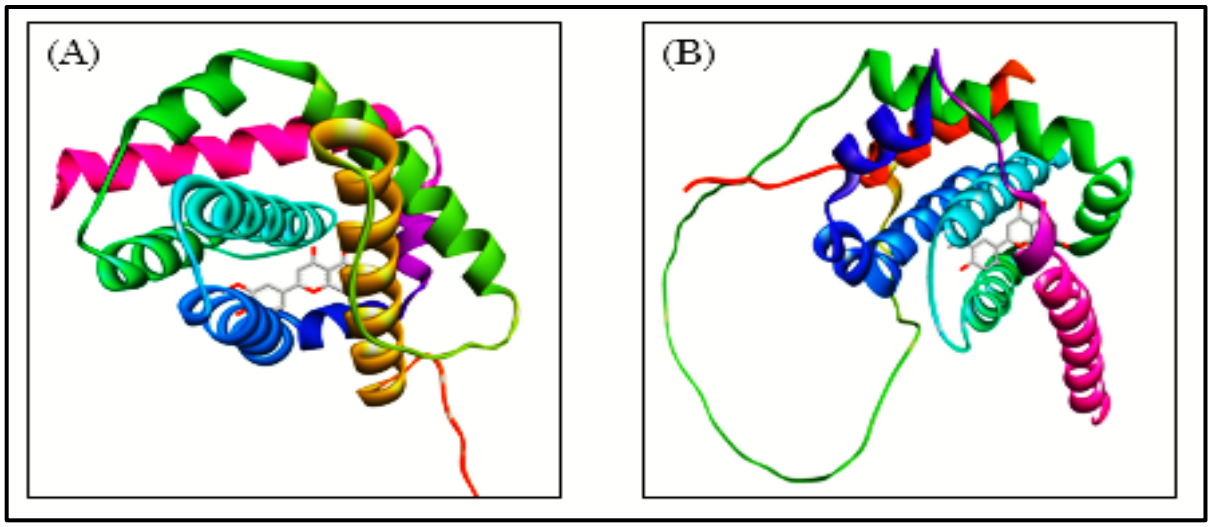


Figure 3.2 Visuals of docking interactions of Chrysoeriol, BAX and BCL-2. This is a computational representation of the interactions between (A) Chrysoeriol with BAX and (B) Chrysoeriol with BCL-2. The representation was derived using a docking analysis performed using Discovery Studio.

3.1.3 Binding Affinity

Figures 3.3 exhibit Chrysoeriol's effective interaction with target proteins BAX and BCL-2, as indicated by low binding energy graphs. The vertical axis depicts the binding affinities between Chrysoeriol and proteins, which are expressed in energy units (e.g., kcal/mol). Each point on both graphs represents a distinct computer simulation of docking or binding. Lower values on the y-axis imply that Chrysoeriol has a stronger binding affinity for the target proteins, indicating a favorable interaction. In contrast, greater values suggest a lesser binding affinity. This knowledge helps to better understand Chrysoeriol's potential as a ligand for BAX and BCL-2, as well as the stability of the ligand-protein complex and its biological significance for neuroprotection. It reveals that Chrysoeriol possesses most favorable binding affinity with BCL-2 with the average binding affinity of -6.5 kcal/mol and then with BAX with the average binding affinity of -6.2 kcal/mol shown in figure 3.3.

The binding of Chrysoeriol with BAX and BCL-2 active sites by using Discovery Studio Visualizer 3.0 as shown in figure 3.4. Figure 3.4 A shows the interaction of Chrysoeriol with BAX that possesses the amino acid residues TYR 115, VAL 111, LEU 161, LEU 63, GLYA 23, THRA 22, LEU 148, ARG 145, LEU 141, and ASP 142 engage in Van Der Waals interactions, while ALA 96, and LEU 144 forms an alkyl bond. Figure 3.4 B shows the interaction of Chrysoeriol with BCL-2 that possesses the amino acid residues amino acid residues TYR 177, ILE 144, PHE 148, PHE 101, ARG 143, VAL 153, VAL 130,

and VAL 131 of BCL-2 engage in Van Der Waals interactions, while LEU 182, and LEU 214 form an alkyl bond.

Figure 3.3. Binding energies from docking simulations. *In Silico* analysis shows (A) the interaction of Chrysoeriol with BAX, with binding energies that range between – 6 to – 6.8 kcal/mol and the average binding energy is **-6.2 kcal/mol.** (B) The interaction of Chrysoeriol with BCL-2, with binding energies that range between – 6.2 to – 7 kcal/mol and the average binding energy is **-6.5 kcal/mol**.

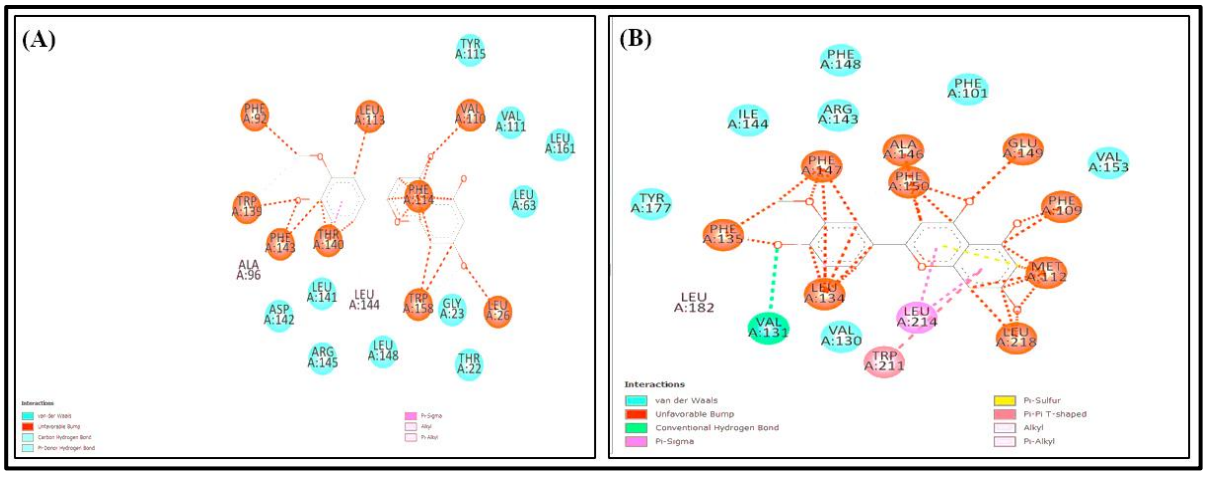


Figure 3.4. The possible locations where BAX and BCL-2 can bind. *In Silico* analysis shows (A) Shows the amino acid residues TYR 115, VAL 111, LEU 161, LEU 63, GLY 23, THR 22, LEU 148, ARG 145, LEU 141, and ASP 142 of BAX engage in Van Der Waals interactions, while ALA 96, and LEU 144 forms an alkyl bond. (B) Shows the amino acid residues TYR 177, ILE 144, PHE 148, PHE

101, ARG 143, VAL 153, VAL 130, and VAL 131 of BCL-2 engage in Van Der Waals interactions, while LEU 182, and LEU 214 form an alkyl bonds.

3.2 Behavioral assessment after disease induction with $AlCl_3$

3.2.1 Open Field Test

Figure 3.5 provides graphical illustrations of behavior affected by control and diseased groups in the open-field test. There were six mice in each group. The test examined the number of entries in the peripheral and central regions. Using the unpaired T-test, the results demonstrated a significant difference between the Control and $AlCl_3$-induced group, indicating that the diseased animals had fewer entries in the central region, however, the animals in the control group were more likely to spend time in the central region and had more entries to it.

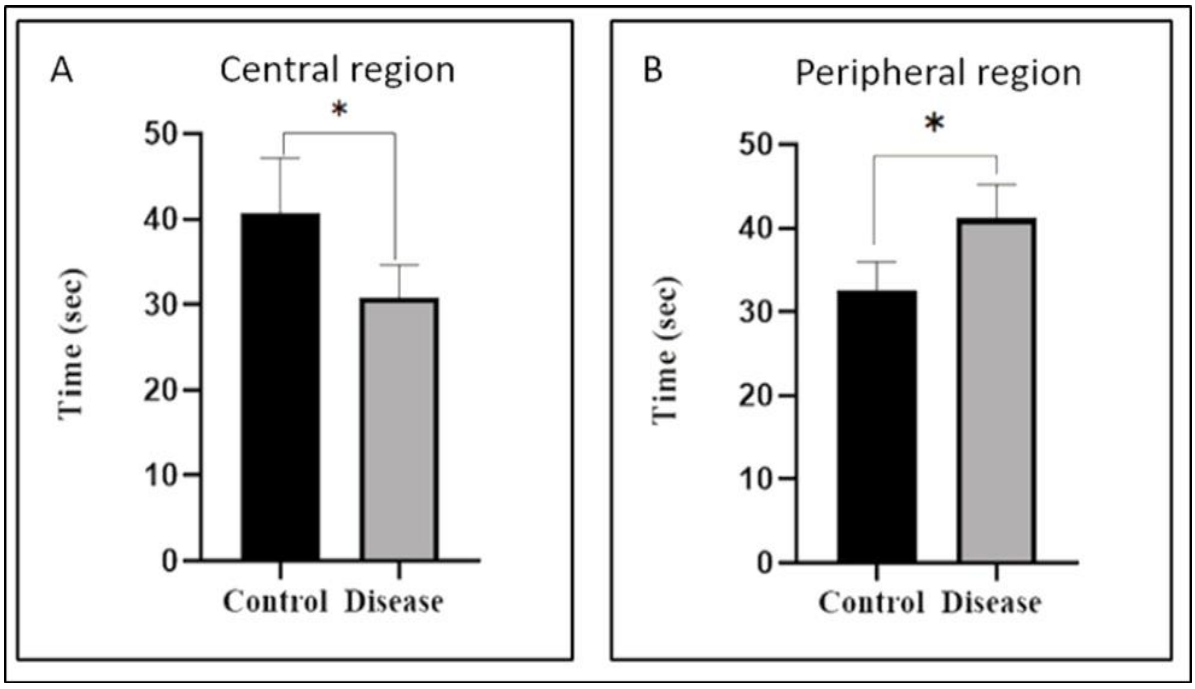


Figure 3.5 Open Field Test after disease induction with $AlCl_3$. Graphs A and B show no entries of control and $AlCl_3$-induced groups in central and peripheral regions. Comparison between the control and $AlCl_3$-induced groups was done by using T-test with a significant difference. Data is presented as mean ± SEM *$p<0.05$.

3.3 Behavioral assessment after treatment with Chrysoeriol

3.3.1 Morris Water Test

The Morris water test, measures escape latency, and the total number of quadrant crossings, to evaluate mice's spatial learning and memory. Figure 3.6, the graphs, demonstrates that the control group and the treatment group show the highest information related to the area that had probe in the trial periods, and the least amount of time taken to escape in the target quadrant.

However, the escape latencies of the diseased group were significantly longer and the entries in the target quadrant, showed that the $AlCl_3$-induced diseased mice hindered the learning and memory of the spatial task for the mice.

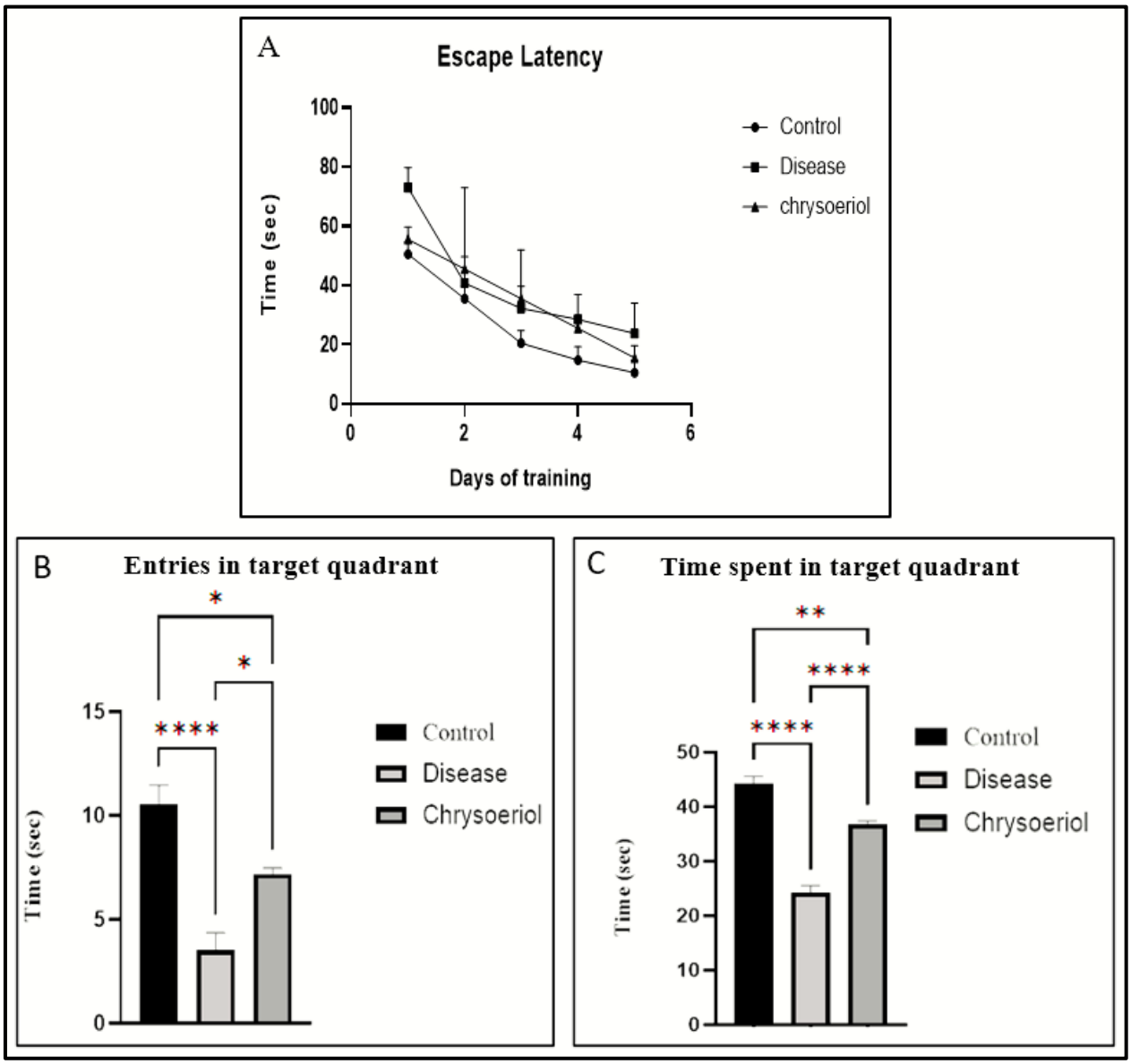


Figure 3.6 The Morris Water Test after treatment. Graph A shows the escape latency of six days of training. Figure B shows the total number of entries in the target quadrant. Figure C shows time spent in target quadrant. These three groups were compared using one-way ANOVA followed by Tukey's post-hoc test. Data is presented as mean ± SEM. *p<0.05, **p<0.01, ****p<0.0001.

3.3.2 Y-Maze Test

The control group shows a higher percentage of alternations, reflecting stronger spatial working memory due to intact cognitive function. In contrast, the untreated $AlCl_3$ group

performs poorly, while the Chrysoeriol-treated $AlCl_3$ group shows a significant improvement, indicating enhanced spatial working memory. The control group performs better in memory tasks, identifying the familiar arm more effectively. Although the Chrysoeriol-treated group doesn't match the control, it outperforms the untreated $AlCl_3$ group, highlighting the cognitive benefits of Chrysoeriol. Additionally, the control mice group showed a strong tendency to explore the novel arm, with the Chrysoeriol-treated $AlCl_3$ group also showing enhanced exploration compared to the untreated group.

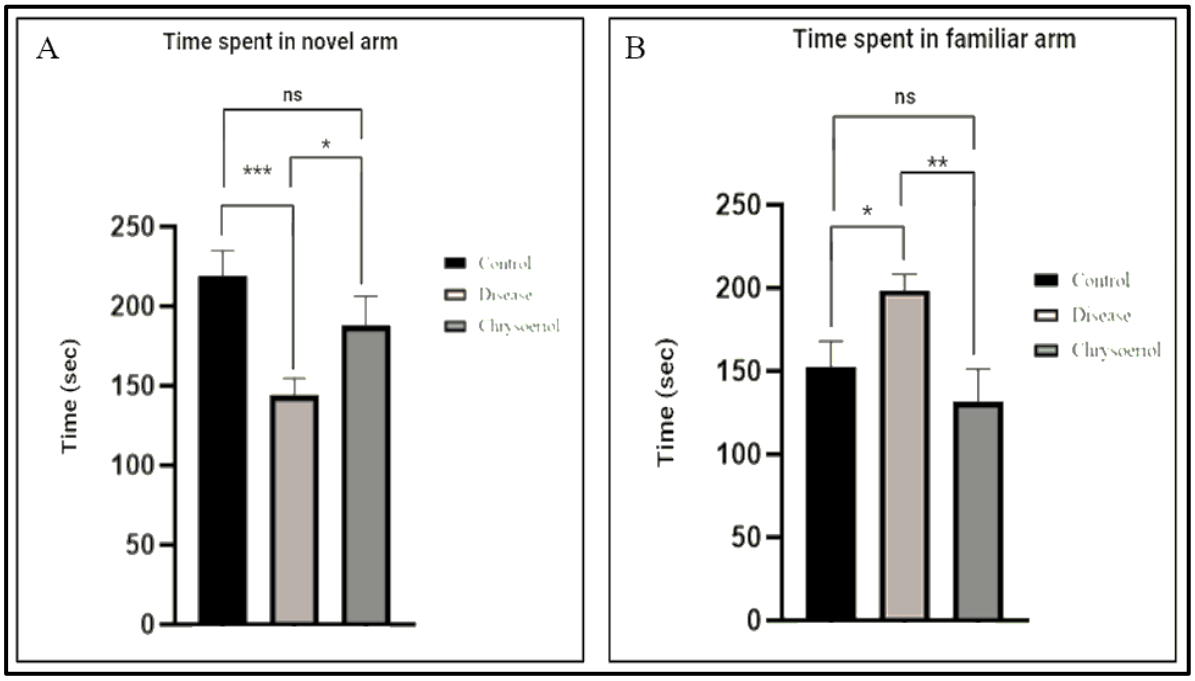


Figure 3.7. Y maze Test after the administration of Chrysoeriol. Graph A shows the percentage of alteration between the three arms. Graphs B and C show the number of entries in novel and familiar arms of control, $AlCl_3$, and Chrysoeriol-treated groups. These three groups were compared using one-way ANOVA followed by Tukey's post-hoc test. Data is presented as mean ± SEM. ns=non-significant, *p<0.05, **p<0.01 ***p<0.001.

3.3.3 Novel Object Recognition

In the Novel Object Recognition test, both the control and treated group exhibit a higher frequency of directing attention towards the novel object, indicating strong recognition memory and cognitive function. The increased exploration of the novel object reflects the mice's intact cognitive abilities. In contrast, the untreated group shows reduced interaction with the novel object, suggesting impaired recognition memory. The treated group demonstrates improved cognitive performance compared to the untreated group, with more

frequent and focused exploration of the novel object, underscoring the potential efficacy of the treatment in enhancing recognition memory.

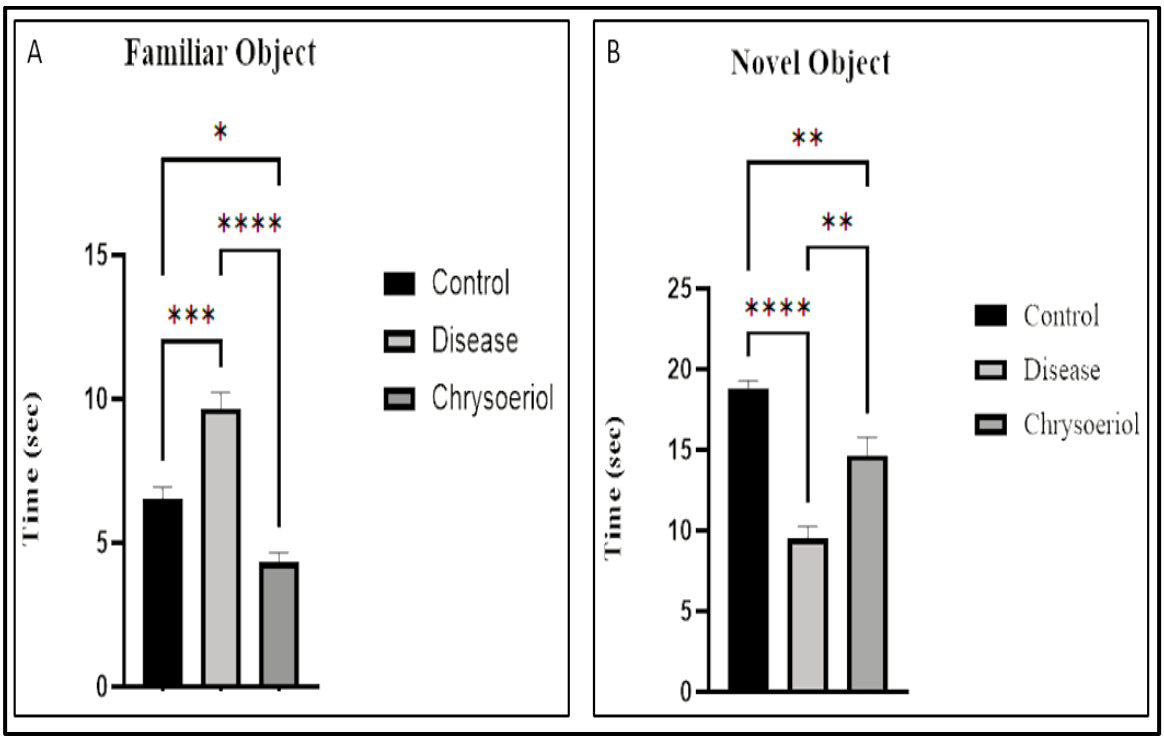


Figure 3.8. Novel Object Recognition after the administration of Chrysoeriol. Graph A and B demonstrate the attention of mice towards the novel and familiar object of control, $AlCl_3$, and $AlCl_3$-treated with Chrysoeriol groups. These three groups were compared using one-way ANOVA followed by Tukey's post-hoc test. Data is presented as mean ± SEM. *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001.

3.3.4 Open Field Test

The open-field test reveals significant differences in exploratory behavior among the experimental groups. The control group shows a higher average number of entries into the central region, indicating greater exploration and reduced anxiety. In contrast, the $AlCl_3$ group has the fewest central entries, suggesting neurological impairment and reduced exploration. Peripheral entries further highlight these differences, the control group has fewer, indicating centralized exploration, while the $AlCl_3$ group shows increased peripheral entries, reflecting heightened anxiety. Mice treated with Chrysoeriol fall between these extremes, suggesting that Chrysoeriol partially restores exploration patterns, modulating the effects of $AlCl_3$.

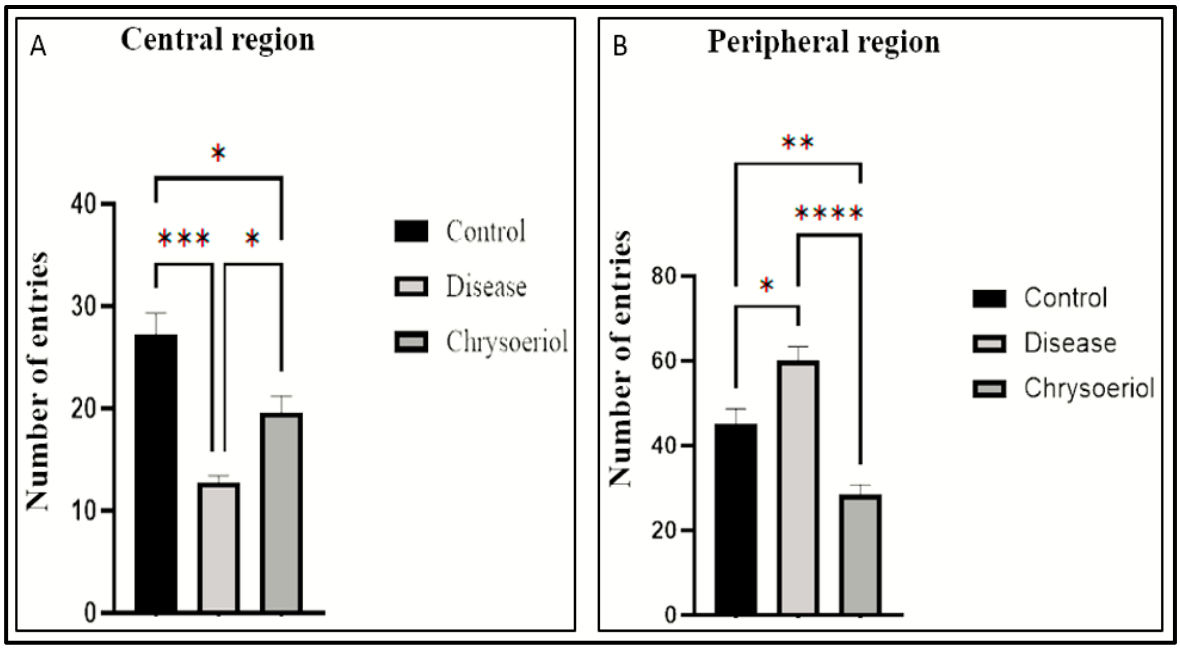


Figure 3.9. Open Field Test after the administration of Chrysoeriol: Graphs A and B show the number of entries in the central and peripheral regions of control, $AlCl_3$, and $AlCl_3$-treated with Chrysoeriol group. The comparison was done by using one-way ANOVA, followed by Tukey's multiple comparison tests. The error bars indicate the standard error of the mean ±SEM, *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001.

3.3.5 Elevated Plus-Maze Test

In the elevated plus maze test, the control and treated groups showed a higher number of entries and increased time spent in the open arms, indicating reduced anxiety and a greater willingness to explore. In contrast, the diseased group predominantly remains in the closed arms, suggesting heightened anxiety and reduced exploratory behavior. These results highlight the behavioral effects of the treatment, as the treated group exhibits more exploratory behavior similar to the control group, while the diseased group displays anxiety-like behavior by favoring closed arms.

3.4 Histopathological results

3.4.1 Effect of $AlCl_3$ and Chrysoeriol on Histopathology

The effect of Chrysoeriol on the mice subjected to IP was evaluated through histological staining using H&E staining. H&E staining allows for the visualization of cellular and tissue morphology. The findings, visually presented in Figures 3.11 and 3.12, focus on the cortex and hippocampus region stained with H&E which provides further insight related to the effect of Chrysoeriol on AD.

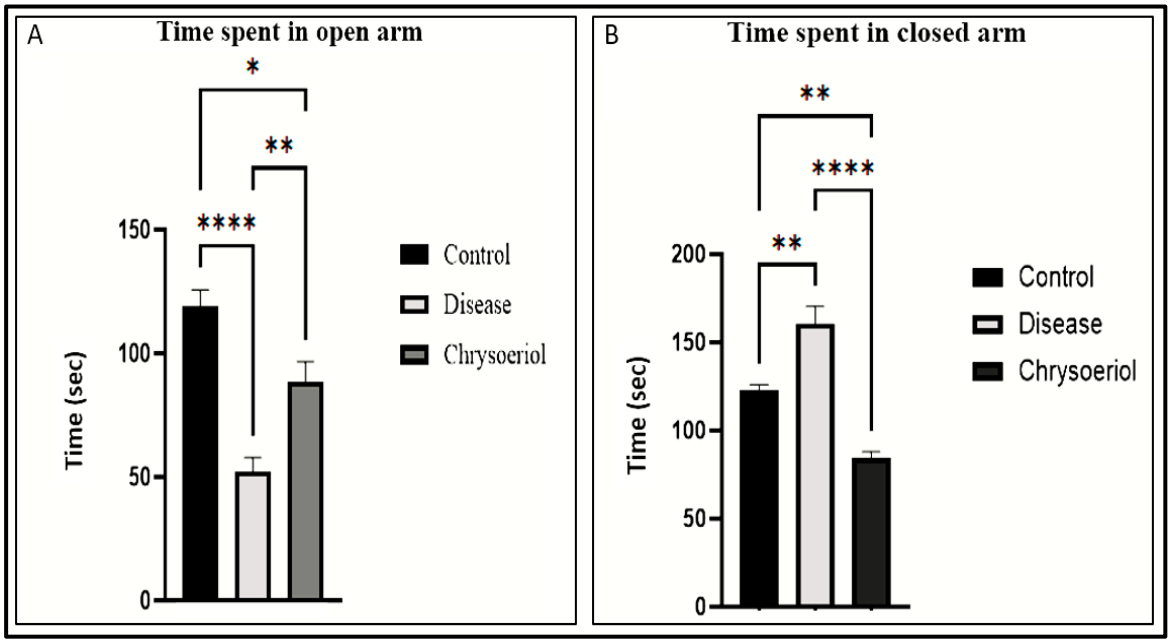


Figure 3.10. Elevated plus maze test after the administration of Chrysoeriol: Graphs A and B show the number of entries in open arms and closed arms. While, Graphs C and D show time spent in open arms and closed arms of control, $AlCl_3$, and $AlCl_3$+Chrysoeriol group. The comparison was done by using one-way ANOVA, followed by Tukey's multiple comparison tests. The error bars indicate the standard error of the mean (SEM) at a significance threshold is *p<0.05, **p<0.01, ****p<0.0001.

3.4.1.1 Cortex

Figure 3.11 shows the morphological analysis and the cell counts of cortex region of mice brain observed under 10X resolution In Figure A, the control group displays pyramidal cells with clearly visible nuclei, indicating healthy and intact neuronal cells. This serves as the baseline for normal prefrontal cortex histology without experimental manipulation. In contrast, Figure B illustrates the cortex of the $AlCl_3$ group, where histological staining shows deformed and disfigured neuronal cells. This altered morphology indicates the negative impact of AD, leading to structural damage to neurons. Figure C depicts the cortex of the $AlCl_3$ group treated with Chrysoeriol, showing different results. Treatment with Chrysoeriol appears to provide neuroprotection, as evidenced by the presence of pyramidal neuronal cells with distinct nuclei, similar to those in the control group. This preserved cellular architecture suggests that Chrysoeriol may mitigate the histopathological effects of $AlCl_3$. The neuroprotective effect is highlighted by green arrows indicating healthy neuronal cells, whereas red arrows point to degenerated neuronal cells, underscoring the contrast between

treated and untreated $AlCl_3$ groups. This histological analysis visually represents the protective impact of Chrysoeriol on prefrontal neurons. The noticeable improvement in cellular morphology, marked by the preservation of clear nuclei, suggests that Chrysoeriol may have a therapeutic role in reducing the histopathological damage due to AD.

Figure D shows results by using Image J software, cell counting was conducted on digital photomicrographs, and the results are presented in graph. The analysis reveals that the $AlCl_3$ group exhibited a decreased number of neurons in the cortex compared to both the control group and the $AlCl_3$ group treated with Chrysoeriol. This quantitative assessment highlights the detrimental impact of $AlCl_3$ on cortical neuronal populations. Additionally, it suggests a potential neuroprotective effect of Chrysoeriol, as indicated by the higher neuron count in the treated $AlCl_3$ group. These findings provide valuable quantitative evidence of Chrysoeriol's neuroprotective effects in the context of AD.

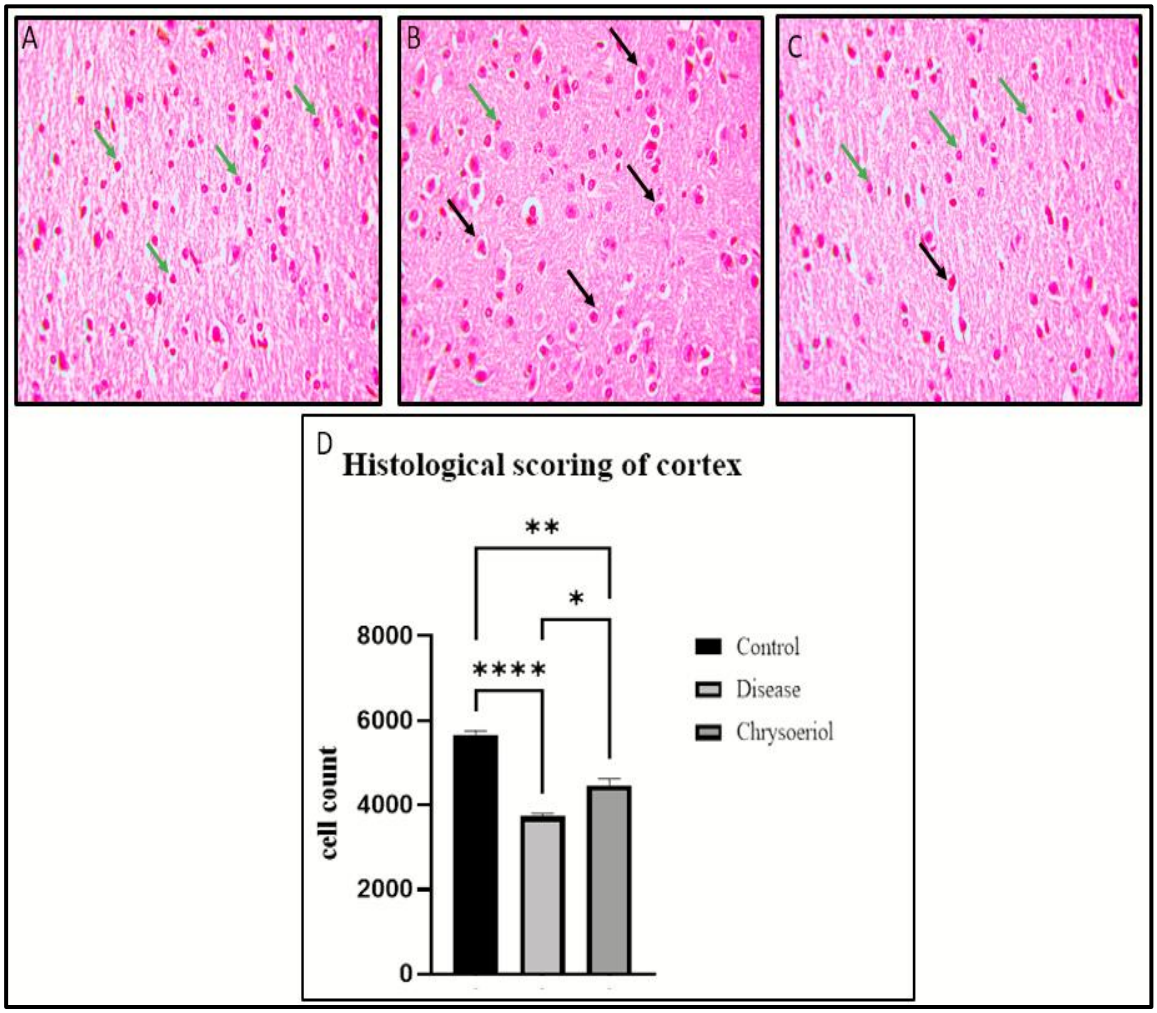


Figure 3.11. H&E Staining and cell counts of cortex at 40X: A) In the control mice, pyramidal cells with clearly visible nuclei were observed, indicating the presence of live neurons. B) The $AlCl_3$ group displayed deformed and distorted neuronal cells. C) The $AlCl_3$ group treated with Chrysoeriol showed signs of neuroprotection, evidenced by pyramidal neurons with distinct nuclei, suggesting the presence of viable neurons. Green arrows indicate healthy neural cells, while black arrows point to dead neuronal cells. D) Shows the comparison among these three groups Total cell count was done by using one-way ANOVA followed by Tukey's post-hoc test. Data is presented as mean ± SEM. ns=non-significant. ns=non-significant. *p<0.05, **p<0.01, ****p<0.0001.

3.4.1.2 Hippocampus

Figure 3.12 shows the morphological analysis and the cell counts of hippocampus region of mice brains observed under 40X resolution Figure A shows that the dentate gyrus of the hippocampal region from the control group display a well-organized, identifiable with well-defined structure. There is a high cell count. The cells appear uniformly distributed and exhibit typical morphology. There is minimal evidence of cell damage or loss. Figure B shows the dentate gyrus of the hippocampal region from the diseased group with significant structural alterations. The overall size of the hippocampus is reduced, indicating shrinkage due to disease progression. There is a noticeable decrease in cell density. Many regions exhibit sparse neuronal distribution. While the neurons in the diseased group display signs of degeneration including shrunken cells. These changes are indicative of neurodegeneration and cellular apoptosis commonly associated with Alzheimer's-like pathology induced by $AlCl_3$. Figure C shows the dentate gyrus of the hippocampal region from the treatment group with a partial restoration of the hippocampal structure. Although still smaller than the control group, the hippocampus in the treatment group is notably larger than in the diseased group. There is an increase in cell density compared to the diseased group, though it may not fully reach the levels observed in the control group. The distribution of neurons is more uniform, suggesting some recovery of cellular populations. There are fewer signs of cellular degeneration. This suggests that the treatment with Chrysoeriol has a neuroprotective effect, mitigating some of the damage induced by $AlCl_3$.

Figure D shows morphometric results by using Image J software, cell counting was conducted on digital photomicrographs, and the results are presented in graph. The analysis reveals that the $AlCl_3$ group exhibited a decreased number of neurons in the hippocampus as compared to both the control group and the $AlCl_3$ group treated with Chrysoeriol. This quantitative assessment highlights the detrimental impact of $AlCl_3$ on hippocampus neuronal populations. Additionally, it suggests a potential neuroprotective effect of Chrysoeriol, as

indicated by the higher neuron count in the treated $AlCl_3$ group. These findings provide valuable quantitative evidence of Chrysoeriol's neuroprotective effects in the context of AD.

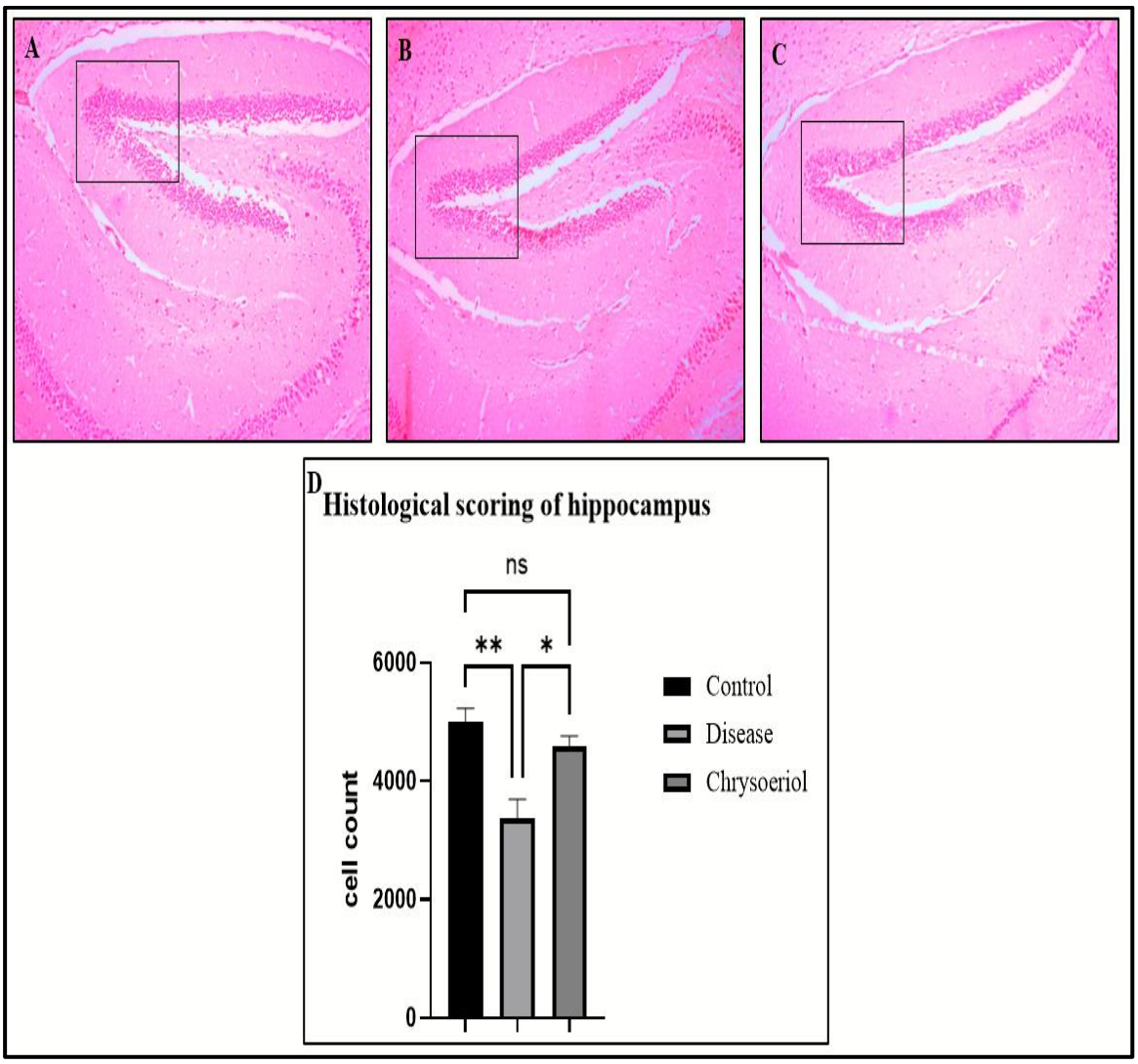


Figure 3.12. H&E Staining and cell counts of Hippocampus at 10X: The diagram displays H&E (Hematoxylin and Eosin) staining of dentate gyrus of hippocampal region from three groups: Control, Diseased, and Treatment. A) In the control mice, display a well-organized, identifiable with well-defined structure. There is a high cell count. B) The $AlCl_3$ group displayed that size of the hippocampus is reduced, indicating shrinkage due to disease progression. There is a noticeable decrease in cell density. C) The $AlCl_3$ group treated with Chrysoeriol showed signs of neuroprotection, evidenced by partial restoration of the hippocampal structure. Although still smaller than the control group, the hippocampus in the treatment group is notably larger than in the diseased group. There is an increase in cell density compared to the diseased group. D) Shows the comparison among these three groups Total cell count was done by using one-way ANOVA followed by Tukey's post-hoc test. Data is presented as mean ± SEM. ns=non-significant. *$p<0.05$, **$p<0.01$.

3.5 PCR Results

3.5.1 Gradient PCR results

Figures 3.13 and 3.14 shows the results of gel electrophoresis that shows the amplification for the BAX, and BCL-2 mRNA expression using gene-specific primers. Various distinct bands are visible, these conform the aligning to the predicted size of 113 base pairs for BAX, and 205 base pairs for BCL-2. Bands were seen at temperatures ranging from 60.0 °C to 68 °C for BCL-2, and 54 °C to 63.5 °C for BAX. This series of temperatures shows the PCR assay's sensitivity to different annealing temperatures. The best temperatures for BAX, and BCL-2 were 58 °C and 64.5 °C, respectively.

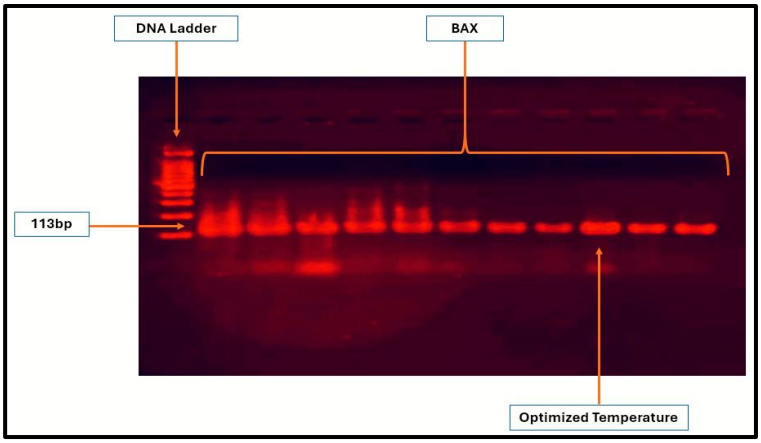


Figure 3.13: Optimization of BAX on gel electrophoresis. Shows the amplification of BAX using gene-specific primers on gel electrophoresis. The temperature of 58 °C for BAX shows promising results with a prominent band having 113 base pairs.

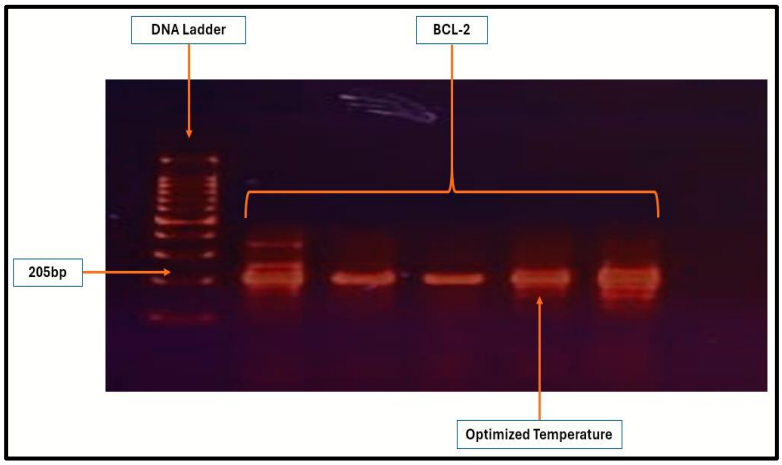


Figure 3.14: Optimization of BCL-2 on gel electrophoresis. Shows the amplification of BCL-2 using gene-specific primers on gel electrophoresis. At the temperature of 64.5 °C BAX shows promising results with a prominent band having 205 base pairs.

To investigate the alterations of the proteins associated with apoptosis in $AlCl_3$ treated mice with or without Chrysoeriol, the expression of BCL-2 protein, which inhibits the apoptosis by downregulating BAX protein, which promotes apoptosis analyzed using RT- PCR analysis. For this assessment quantitative RT-PCR was, ß-actin which acts as a housekeeping gene was used to normalize these genes. The results are summarized in Figure 3.13, which shows the expression patterns of BAX mRNA and its up-regulation in $AlCl_3$-induced mice, indicating decreased protein transcription under AD conditions resulting in neuronal loss. The present study observed that treatment with Chrysoeriol led to reduced BAX expression implying the possibility of Chrysoeriol in modulating the BAX down-regulation implicated in AD. On the other hand, figure 3.14 shows that the BCL-2 mRNA expression in the diseased group which was treated with Chrysoeriol revealed up-regulation of the genes, thus indicating an enhanced expression of this protective anti-apoptotic gene under neuronal damage induced in $AlCl_3$ group. These results suggested that Chrysoeriol has anti-apoptotic effect protection against $AlCl_3$-induced apoptosis in the brain tissue of mice.

3.5.2 RT-PCR results

To identify the cells undergoing apoptosis and further study the levels of specific molecules related to apoptosis in $AlCl_3$ treatment, either with or without Chrysoeriol, the mRNA levels of BCL-2 (an anti-apoptotic protein) and BAX (a pro-apoptotic protein) in mice were determined by using real-time PCR.

3.5.2.1 Chrysoeriol Down-regulates BAX mRNA Expression in AD mouse model

The expression level of BAX, a pro-apoptotic protein in mice models, was determined using real-time PCR as shown in Figure 3.15 (A). This was done by quantifying and standardizing the expression of these genes to ß-actin which was used as a housekeeping gene. The results show the inter-relationship between the control, $AlCl_3$-induced, and $AlCl_3$-treated with Chrysoeriol. In the $AlCl_3$-treated group, there was increased expression of BAX mRNA expression showing increased transcription of the protein gene that causes neuronal death under AD conditions. Additionally, treatment with Chrysoeriol showed that BAX was reduced resulting that Chrysoeriol may mimic the effect of lowering the over-activated BAX as implicated in the pathophysiology of AD.

3.5.2.2 Chrysoeriol Up-regulates BCL-2 mRNA Expression in AD mouse model

The expression level of BCL-2 which is an anti-apoptotic protein in mice models was determined by using real-time PCR as shown in figure 3.15 (B). This was done by quantifying and standardizing the expression of these genes to ß-actin which was used as a housekeeping gene. The results shows the inter-relationship between the three groups, the control, $AlCl_3$-induced, and $AlCl_3$-treated with Chrysoeriol. According to the findings the use of Chrysoeriol was found to have an anti-apoptotic property against $AlCl_3$- induced apoptosis in brain tissue by up-regulating the relative levels of BCL-2.

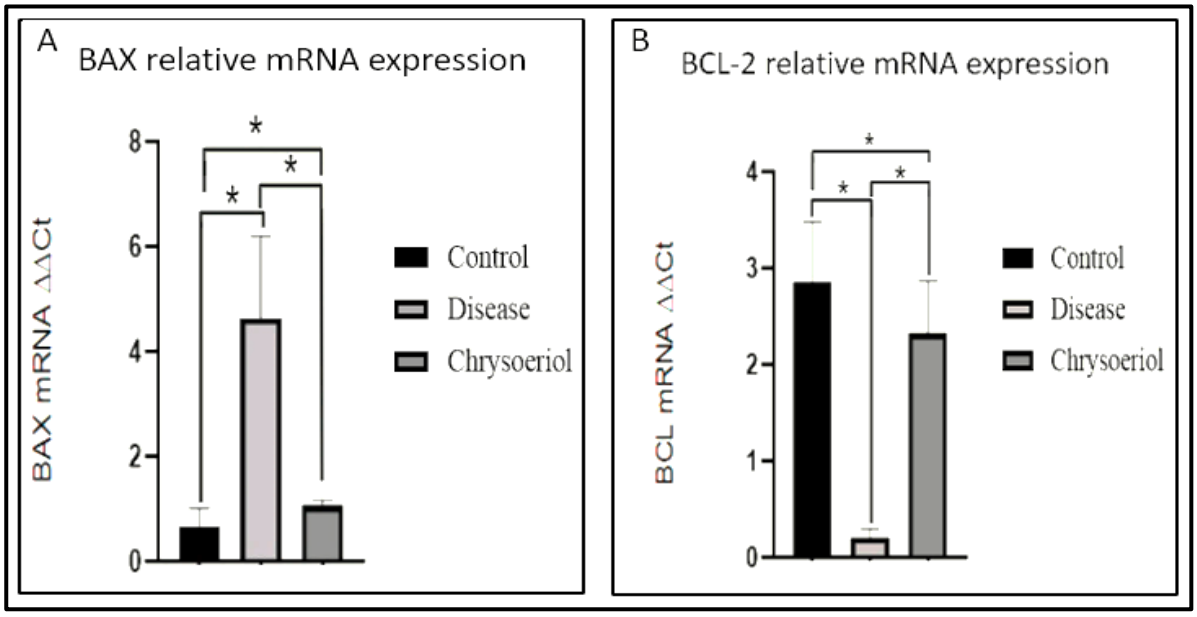


Figure 3.15: Relative expression result of BAX mRNA and BCL-2 mRNA (normalized to ß-actin). (A) Shows the up-regulation of BAX mRNA in the $AlCl_3$,-induced mice and the downregulation in the expression of BAX mRNA in the group that is treated with Chrysoeriol. (B) Shows the downregulation of BCL-2 mRNA in the $AlCl_3$,-induced mice, and the up-regulation in the expression of BCL-2 mRNA in the group that is treated with Chrysoeriol. Comparison among the three groups was done by using one-way ANOVA followed by Tukey's post-hoc test. Data is presented as mean ± SEM, *$p<0.05$.

CHAPTER 4: DISCUSSION

AD is a type of dementia that is chronic, and progressive and affects the neurons in the brain leading to cognitive impairment, memory loss, and behavioral changes. This becomes a considerable public health issue, driven by increased life expectancy and a deeper understanding of its socio-economic impacts. AD progresses steadily and cannot be reversed; however, pharmacological treatments targeting cognitive impairment and both non-pharmacological and pharmacological interventions addressing behavioral issues associated with dementia can significantly improve the patient's quality of life (Bennett et al., 2021). AD involves degenerative changes across various neurotransmitter systems, including those that release glutamate, norepinephrine, serotonin, and certain neuropeptide systems. This condition is also marked by degeneration in specific brain regions including parietal and temporal lobes within the frontal cortex and hippocampus. These all contribute to challenging current Alzheimer's research, unable existing hypotheses to fully explain the cellular and regional distribution patterns characteristic of the disease's neuropathology (Li et al., 2016).

The pathological hallmarks of AD are Aβ plaques, tau neurofibrillary tangles, and neuronal loss. These degenerative changes are connected with neuronal loss, which includes cholinergic neurons in the basal forebrain and neocortex. Even after years of research, no exact cure or treatment can completely stop the progression of AD. In the recent, natural compounds have been considered promising therapeutic agents because of their multiple targets and low side effects. Of these, Chrysoeriol, a flavonoid isolated from several medicinal plants and has been described to exhibit neuroprotective activity (Wenk et al., 2003), anti-microbial activity (Aboulaghras et al., 2022), antioxidant effect (Mishra et al., 2003), anti-diabetic effect (Bolkent et al., 2000), anti-inflammatory effect (Wu et al., 2020), and anti-cancer properties (Wei et al., 2019). The compound's antioxidant activity may help to neutralize free radicals and decrease oxidative stress which could lead to the activation of apoptotic pathways due to oxidative damage. Also, the anti-inflammatory property of Chrysoeriol may prevent the secretion of pro-inflammatory cytokines that trigger apoptosis in neurons in AD.

Our research aims to investigate the efficacy of Chrysoeriol in the treatment against neurotoxicity associated with AD with the help of a well-established mouse model of AD.

The study seeked to establish whether Chrysoeriol has the potential to halt or slow down the process of dementia, shielding the brain tissue, and altering the dysfunctional mitochondria. Before commencing the experimental phase, an initial *in silico* analysis was conducted using various software tools and computational methods. This step is pivotal in early-stage drug development and research, focusing on identifying potential therapeutic targets, validating safety, and evaluating repurposed medications. The study aimed to gain insights and make predictions based on hypotheses prior to experimental implementation. The three-dimensional structures of BAX (Q9D2C7) and BCL-2 (Q9DCS1) and the chemical structure of Chrysoeriol was accessed.

Molecular docking simulations indicated the binding interaction of Chrysoeriol with both BAX and BCL-2 in different ways, with different binding affinities. Different conformation and orientation of Chrysoeriol in the binding pocket of both proteins might be the reason for the differences in the binding affinities. According to the present findings, there exists a very large negative binding affinity between Chrysoeriol with BAX and BCL-2 by the most favorable binding location. The interaction of Chrysoeriol with BAX, with binding energies that range between –6 to –6.8 kcal/mol and the average binding energy is -6.2 kcal/mol and the interaction of Chrysoeriol with BCL-2, with binding energies that range between – 6.2 to – 7 kcal/mol and the average binding energy is -6.5 kcal/mol. The lowest binding affinity demonstrated the strongest interaction, indicating that Chrysoeriol has a strong binding affinity with BAX and BCL-2. This interaction can enhance the survival of neurons along with providing neuroprotection which are factors that the disease of Alzheimer's needs for the treatment.

In this study, the neuroprotective properties of Chrysoeriol were assessed by using the $AlCl_3$-induced mice models of AD. $AlCl_3$ is a well-established agent commonly used to induce Alzheimer-like symptoms and test the efficacy of neuroprotective compounds. Due to its highly lipophilic nature, $AlCl_3$ can cross the BBB (Willhite et al., 2014). For better understanding, the behavioral assessments were performed to analysis cognitive impairment, recovery mechanism, and behavioral symptoms of AD. AD is generally linked with learning deficits, memory dysfunction, and enhanced anxiety-like behaviors. In the present investigation, Chrysoeriol ameliorated the memory and learning ability in the AD-treated

mice as compared to the untreated AD mice. For this series of tests were performed including water-Morris, Y-maze, open field, Novel Object Recognition, and Evaluated-Plus Maze.

Our study revealed that Chrysoeriol evaluates spatial learning and working memory when comparison was done between the control, $AlCl_3$-induced and $AlCl_3$-treated group, Chrysoeriol treated group shows more entries in the target quadrant and spent more time in the target quadrant by remembering the place of the object and shorter escape latency showing improved spatial learning memory as ameliorating memory deficit commonly associated with AD. A different study has revealed that $AlCl_3$ degeneration results in significant disruptions to working memory (Bromley-Brits et al., 2011). Through the Novel Object Recognition, which is the most commonly used behavioral test for mice models, we successfully discovered that Chrysoeriol treated group shows enhanced results with increased exploration ability showing that treatment helps to restore the brain's ability to recognize different objects when compared with the $AlCl_3$-induced group that shows impaired performance indicating memory deficits which align with hippocampal dysfunction. The findings align with other study highlighting results that $AlCl_3$-treated group has a higher intensity of exploring behavior than the diseased group (Leger et al., 2013).

Furthermore, the Y-Maze Test indicated that treatment with Chrysoeriol shows greater exploratory behavior in the novel arm due to enhancing spatial working and restoring cognitive function. Another study showed the alignment with the current study that neuroprotective agents can enhance cognitive functions and spatial working in AD models. (Rebai et al., 2008). Behavioral analysis showed that Chrysoeriol improved locomotor activity. As data from the Open Field Test indicated that treated mice spent more time in the center area and less time in the peripheral, increased exploratory behavior as a result of the treatment, while the diseased group spent more time in the peripheral region and less time in the central region due to $AlCl_3$ exposure. Existing literature measured general motor function and anxiety levels (Seibenhener et al., 2015). Lastly, an evaluated-plus maze test was performed to assess anxiety-related behavior in mice, providing insight into the emotional aspect of the disease. Mice treated with Chrysoeriol show lower anxiety level by exploring the maze suggesting reduced neurodegeneration in the brain. These results highlight the diverse neuroprotective properties of Chrysoeriol in treating cognitive and behavioral impairments linked to AD Anxiety is a common physiological symptom in AD (Sarkar et al.,

2020) These behavioral improvements indicate that Chrysoeriol has neuroprotective properties against AD.

The next step Histopathological analysis was performed by using H&E staining which is a basic histochemical technique used in the study of tissue morphological changes that are evident in AD. Furthermore, in both the cortex and hippocampus affected by AD pathology, H&E staining enables identification of numerous microscale morphological changes including, neuronal shrinkage, loss of neurons, and astrogliosis (Nobakht et al., 2011). This technique enabled us to identify the effect of Chrysoeriol treatment on AD-induced mice models by preserving the structural integrity of the neurons, distinct pyramidal cells shape with an observable nucleus, unlike the neurons in the $AlCl_3$-induced group. Histomorphometric assessment was done by using Image-J software by counting cells revealed a higher density of cells within the Chrysoeriol-treated group implicating the possibility of neuron preservation than the diseased group. Furthermore, the Chrysoeriol-treated group replenished newly developed neurons more than the $AlCl_3$-induced group. Our findings indicated that Chrysoeriol has the potential to protect neurons and support cell growth by increasing cell numbers of neurons in the mouse brain; therefore, it may have the potential to counteract neurodegeneration in AD.

Previous research on AD has revealed a correlation between cell death and the changes in anti-apoptotic proteins in the human body, BCL-2 protein, which are known to exert an anti-apoptotic action due to the stabilization of transmembrane mitochondrial permeability and the prevention of the release of mitochondrial cytochrome-c into the cytoplasm (Zeng et al., 2007). PI3K/AKT signaling pathway is an important pathway to maintain homeostasis during the cell cycle. This pathway is activated when a variety of genes are on or off or when their regulation is disturbed and is associated with many diseases in humans. Further, overstimulation of the PI3K/AKT pathway associated with diseases including cancer and diabetes is documented whereas modulating their activity might concern cardiovascular diseases and primary neurological disorders like AD. Therefore, the molecular conformations of the upstream and downstream of PI3K / AKT signaling pathway are of great significance in many disease prevention and therapies (Gao et al., 2011; Chung et al., 2011). Another research has proven that in addition to the mTOR protein pathway of PI3K/AKT signaling is also effective in the formation of neuronal dendrites and dendritic spines. The enhancement

in synaptic plasticity and more specifically with Long-term potentiation (LTP) (Haibing et al., 2013; Figure 4.1).

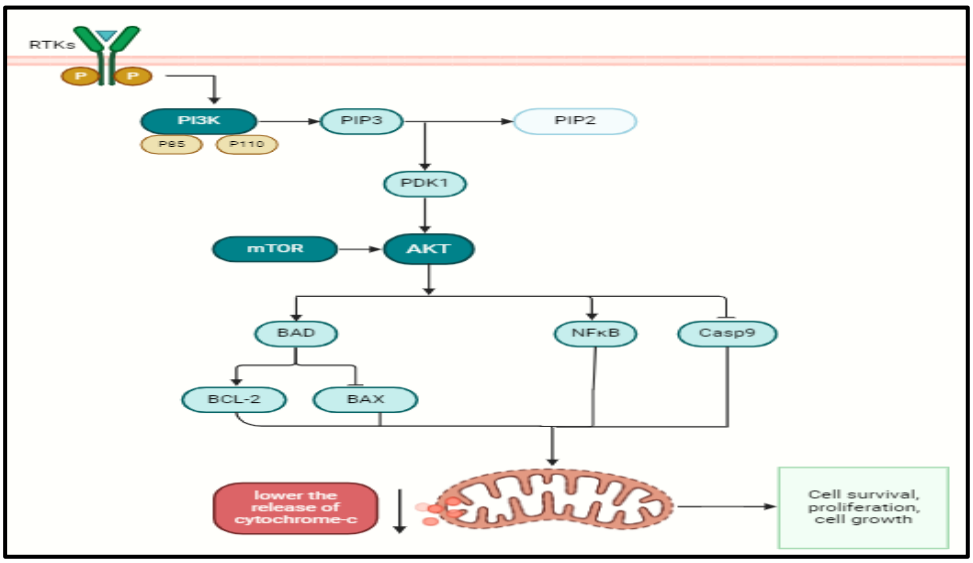


Figure 4.1. Neuroprotection through PI3K/AKT pathway followed by Chrysoeriol: This figure illustrates the effect of Chrysoeriol downregulated BAX expression while upregulated BCL-2 and NF-kB promoting cell survival. (Chung et al., 2011).

In the molecular analysis, qualitative RT-PCR was used to analyze the BAX and BCL-2 expression when normalized against β-actin as a housekeeping gene (Ruan & Lai, 2007). BAX induces apoptosis through the activation of caspases, helping to release cytochrome-c from the mitochondria. On the other hand, BCL-2 is an anti-apoptotic protein that prevents the apoptotic pathway by blocking the release of cytochrome-c and enhances cell survival. In the context of AD, an imbalance between BAX and BCL-2 which are pro-apoptotic and anti-apoptotic factors is involved in the process of neuronal death. The present investigation showed that Chrysoeriol treatment significantly downregulated BAX and upregulated BCL-2 in the hippocampus of AD mice through the pathway of PI3K/AKT. These changes in the BAX/BCL-2 ratio indicate that Chrysoeriol may prevent neurodegeneration through the suppression of apoptosis. The downregulation of BAX could inhibit the mitochondrial apoptotic pathway, and the upregulation of BCL-2 could promote the survival of neurons and their resistance to AD injury (Paradis et al., 1996; Chung et al., 2011). These molecular changes explain the behavioral enhancements, as the integrity and functionality of neurons

are essential for healthy cognition and affect. In addition, the anti-apoptotic effects of Chrysoeriol may be on the hippocampus and other regions of the brain as well.

Limitations:

Firstly, results from mouse models may not fully translate to humans due to species-specific differences in physiology and disease progression. Also, the exact molecular mechanisms by which Chrysoeriol exerts its effects are not fully understood, limiting the ability to predict and control its therapeutic outcomes. Secondly, Chrysoeriol might have off-target effects that were not anticipated or measured in the study, complicating the interpretation of its benefits. Moreover, the study focuses solely on AD, potentially overlooking how Chrysoeriol might interact with other neurodegenerative diseases or comorbidities.

CHAPTER 5: SUMMARY OF RESEARCH

AD is a progressive neurodegenerative disease that tends to be related to dementia and other diseases that affect cognition. This turned into a major public health concern through enhanced longevity and realization of the socioeconomic consequences. AD is inevitable and progressive; however, social and behavioral problems related to dementia and cognitive loss can be managed through different nonpharmacological and pharmacologic treatments that greatly enhance the patient's quality of life. This condition is also associated with the degeneration of some parts of the brain such as the parietal and temporal lobes, situated within the frontal cortex and the hippocampus. These all contribute to questioning present Alzheimer's research, new hypotheses cannot fully explain the cellular and regional distribution patterns seen in the neuropathology of the disease.

Our research aims to investigate the efficacy of Chrysoeriol in the treatment of neurotoxicity associated with AD with the help of a well-established mouse model of AD. For this study, $AlCl_3$ has been used to mimic the *in vivo* AD mice model, each mouse receive $AlCl_3$ at the dosage of 20mg/kg orally for 42 days to induce AD-like symptoms. After inducing disease AD-induced mice were treated with Chrysoeriol 5mg/kg intraperitoneally once daily for 14 consecutive days. The behavioral assessments were performed to analysis cognitive and behavioral symptoms of AD including Water Morris Test, Novel Object Recognition, Open Field Test, Y-Maze Test, and Evaluated-Plus Maze test. These behavioral improvements indicate that Chrysoeriol has neuroprotective properties, which may involve the regulation of synaptic plasticity and inflammation, which are important in AD development. Also improves spatial working ability, enhanced exploratory behavior, and motor coordination in mice models.

Histopathological insight using H&E staining which is a basic histochemical technique used in the study of tissue morphological changes that are evident in AD. Furthermore, both the cortex and hippocampus are affected by AD pathology. The diseased group displayed that the size is reduced, indicating shrinkage due to disease progression. There is a noticeable decrease in cell density which was revealed by using ImageJ software. The $AlCl_3$ group treated with Chrysoeriol showed signs of neuroprotection, evidenced by partial restoration. Although still smaller than the control group, the hippocampus in the treatment group is notably larger than in the diseased group and increased in the number of cells.

Additional molecular analysis provided more information that Chrysoeriol showed significant results. PI3K/AKT signaling pathway is an important pathway to maintain homeostasis during the cell cycle. In this study, $AlCl_3$-induced group shows up-regulation of BAX levels and lessened BCL-2 levels, leading to a decrease in the ratio of BCL-2/BAX and enhanced apoptotic activity. This study showed that Chrysoeriol treatment restored these changes, increasing the BCL-2/BAX ratio and decreasing neurotoxicity.

In general Chrysoeriol demonstrated moderate neuroprotective potential in AD-induced mice model, with significant enhancements in cognitive functions and minimal neuronal damage. Further, it affected major apoptotic signaling pathways, which may be useful in preventing cell death related to AD pathology. These results support the use of Chrysoeriol in the treatment of Alzheimer's and place it as a candidate that should be considered for further research alongside current treatments. However, more studies are needed to understand the underlying mechanism of its action.

CHAPTER 6: CONCLUSION AND FUTURE PERSPECTIVES

Our study based on *in vivo* analysis reveals the prospect of using Chrysoeriol to treat AD in mice with enhanced behavioral and molecular effects. Disease induced through $AlCl_3$ revels the damages in mice's neuronal functions, and effecting brain parts including cortex and hippocampus. Treatment with Chrysoeriol shows the augmentation of exploratory activity and memory retention due to reduced cognitive impairment. In terms of molecular changes, increased expression of the anti-apoptotic BCL-2 and decreased expression of the pro-apoptotic BAX indicate that Chrysoeriol possesses neuroprotection by enhanced cell survival and reduced neuronal apoptosis. Therefore, these results are in harmony with the idea that Chrysoeriol has therapeutic value for AD and the possible applicability of the flavonoid for treating or slowing Alzheimer's should be explored further.

Future perspective:

1. Identify the direct molecular targets of Chrysoeriol and elucidate how Chrysoeriol modulates these targets to produce neuroprotective effects, targeted at signaling pathways implicated in AD.
2. Continue the investigation of long-term consequences of Chrysoeriol on learning ability and neuron loss in the AD.
3. Determine other neuroprotective that can be used in conjunction with Chrysoeriol as well as cholinesterase inhibitors to further the therapeutic benefits of the compound.
4. To determine whether there are other proteins apart from BAX and BCL-2 that can qualify Chrysoeriol efficiency so that patients with AD can be managed according to their response to the drug.
5. Confirm the efficacy of Chrysoeriol in other animal models of AD to save for cross-species comparability.
6. As they do so, build on antecedent research that points to the mechanism of action of Chrysoeriol in AD, and develop then organize phase I and II clinical trials to assess Chrysoeriol's safety profile, tolerability, and effectivity on human Alzheimer's patients.
7. To establish whether Chrysoeriol has prevention features in models of early Alzheimer's, that is if it can slow down the development or advancement of the condition.
8. To evaluate the effect of Chrysoeriol on apoptosis genes the gene expression 'chip' assay should be done.

9. It may be as a confirmation of Chrysoeriol-induced neuroprotection, future studies might focus on finding other biomarkers of inflammation which might help in screening and later tracking of AD and other Neuro degenerative disorders.

REFERENCES

1. Aboulaghras, S., Sahib, N., Bakrim, S., Benali, T., Charfi, S., Guaouguaou, F. E., ... & Bouyahya, A. (2022). Health benefits and pharmacological aspects of Chrysoeriol. *Pharmaceuticals*, *15*(8), 973.
2. Adamson, M. M., Shakil, S., Sultana, T., Hasan, M. A., Mubarak, F., Enam, S. A., ... & Razi, A. (2020). Brain Injury and Dementia in Pakistan: Current Perspectives. Front. Neurol. 11: 299. doi: 10.3389/fneur. 2020.00299. *Brain Injury and Dementia in Pakistan: Current Perspectives Maheen M. Adamson*, *1*(2).
3. Adlimoghaddam, A., Neuendorff, M., Roy, B., & Albensi, B. C. (2018). A review of clinical treatment considerations of donepezil in severe Alzheimer's disease. *CNS neuroscience & therapeutics*, *24*(10), 876-888.
4. Ahmad, A., Owais, K., Siddiqui, M., Mamun, K., Rao, F., & Yousufzai, A. W. (2013). Dementia in Pakistan: national guidelines for clinicians. *Pakistan Journal of Neurological Sciences (PJNS)*, *8*(3), 17-27.
5. Al Amin, M., Dehbia, Z., Nafady, M. H., Zehravi, M., Kumar, K. P., Haque, M. A., ... & Emran, T. B. (2024). Flavonoids and Alzheimer's disease: reviewing the evidence for neuroprotective potential. *Molecular and Cellular Biochemistry*, 1-31.
6. Alturkistani, H. A., Tashkandi, F. M., & Mohammedsaleh, Z. M. (2016). Histological stains: a literature review and case study. *Global journal of health science*, *8*(3), 72.
7. *Angelucci, F., Spalletta, G., Iulio, F. D., Ciaramella, A., Salani, F., Varsi, A. E., ... & Bossu, P. (2010). Alzheimer's disease (AD) and Mild Cognitive Impairment (MCI) patients are characterized by increased BDNF serum levels. Current Alzheimer Research, 7(1), 15-20.*
8. Arboleda-Velasquez, J. F., Lopera, F., O'Hare, M., Delgado-Tirado, S., Marino, C., Chmielewska, N., ... & Quiroz, Y. T. (2019). Resistance to autosomal dominant Alzheimer's disease in an APOE3 Christchurch homozygote: a case report. *Nature medicine*, *25*(11), 1680-1683.
9. Arnold, S. E., Arvanitakis, Z., Macauley-Rambach, S. L., Koenig, A. M., Wang, H. Y., Ahima, R. S., ... & Nathan, D. M. (2018). Brain insulin resistance in type 2 diabetes and Alzheimer disease: concepts and conundrums. *Nature Reviews Neurology*, *14*(3), 168-181.

10. Baskaran, K., Pugalendi, K. V., & Saravanan, R. (2015). Antidiabetic and antihyperlipidemic activity of Chrysoeriol in diabetic rats, the role of HMG CoA reductase, LCAT, and LPL: in vivo and in silico approaches. *J. Pharm. Res*, *9*, 597-605.
11. Bellenguez, C., Charbonnier, C., Grenier-Boley, B., Quenez, O., Le Guennec, K., Nicolas, G., ... & Jurici, S. (2017). Contribution to Alzheimer's disease risk of rare variants in TREM2, SORL1, and ABCA7 in 1779 cases and 1273 controls. *Neurobiology of aging*, *59*, 220-e1.
12. Belloy, M. E., Napolioni, V., Han, S. S., Le Guen, Y., Greicius, M. D., & Alzheimer's Disease Neuroimaging Initiative. (2020). Association of Klotho-VS heterozygosity with risk of Alzheimer disease in individuals who carry APOE4. *JAMA neurology*, *77*(7), 849-862.
13. Bennett, S., Laver, K., MacAndrew, M., Beattie, E., Clemson, L., Runge, C., & Richardson, L. (2021). Implementation of evidence-based, non-pharmacological interventions addressing behavior and psychological symptoms of dementia: a systematic review focused on implementation strategies. *International Psychogeriatrics*, *33*(9), 947-975.
14. Bolkent, Ş., Yanardağ, R., Tabakoğlu-Oğuz, A., & Özsoy-Saçan, Ö. (2000). Effects of chard (Beta vulgaris L. var. cicla) extract on pancreatic B cells in streptozotocin-diabetic rats: a morphological and biochemical study. *Journal of ethnopharmacology*, *73*(1-2), 251-259.
15. Boon, W. C., & Simpson, E. R. (2012). Neuroendocrine inherited or induced aromatase enzyme deficits. In *Handbook of neuroendocrinology* (pp. 723-737). Academic Press.
16. Breijyeh, Z., & Karaman, R. (2020). Comprehensive Review on Alzheimer's Disease: Causes and Treatment. *Molecules (Basel, Switzerland)*, *25*(24), 5789.
17. Brion, J. P. (1998). Neurofibrillary tangles and Alzheimer's disease. *European neurology*, *40*(3), 130-140.
18. Bromley-Brits, K., Deng, Y., & Song, W. (2011). Morris water maze test for learning and memory deficits in Alzheimer's disease model mice. *JoVE (Journal of Visualized Experiments)*, (53), e2920.

19. Bubu, O. M., Brannick, M., Mortimer, J., Umasabor-Bubu, O., Sebastião, Y. V., Wen, Y., ... & Anderson, W. M. (2017). Sleep, cognitive impairment, and Alzheimer's disease: a systematic review and meta-analysis. *Sleep*, *40*(1), zsw032.
20. Cheng, X. J., Gu, J. X., Pang, Y. P., Liu, J., Xu, T., Li, X. R., ... & Liu, Y. (2019). Tacrine–hydrogen sulfide donor hybrid ameliorates cognitive impairment in the aluminum chloride mouse model of Alzheimer's disease. *ACS Chemical Neuroscience*, *10*(8), 3500-3509.
21. Cui, X., Li, M., He, Z., Hu, L., Liu, J., Yan, J., & Hua, L. (2020). MiR-302b-5p enhances the neuroprotective effect of IGF-1 in methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease by regulating inducible nitric-oxide synthase. *Cell biochemistry and function*, *38*(8), 1025–1035. https://doi.org/10.1002/cbf.3534
22. David, E. M., Pacharinsak, C., Jampachaisri, K., Hagan, L., & Marx, J. O. (2022). Use of ketamine or xylazine to provide balanced anesthesia with isoflurane in C57BL/6J mice. Journal of the American Association for Laboratory Animal Science, 61(5), 457-467.
23. Desai, A. K., & Grossberg, G. T. (2005). Diagnosis and treatment of Alzheimer's disease. *Neurology*, *64*(12_suppl_3), S34-S39.
24. DeTure, M. A., & Dickson, D. W. (2019). The neuropathological diagnosis of Alzheimer's disease. *Molecular neurodegeneration*, *14*(1), 32.
25. Devanand, D. P. (1997). Behavioral complications and their treatment in Alzheimer's disease. *Geriatrics (Basel, Switzerland)*, *52*, S37-9. . Burgio, L. (1996). Interventions for the behavioral complications of Alzheimer's disease: behavioral approaches. *International Psychogeriatrics*, *8*(S1), 45-52.
26. Devi, S., Kumar, V., Singh, S. K., Dubey, A. K., & Kim, J. J. (2021). Flavonoids: Potential candidates for the treatment of neurodegenerative disorders. Biomedicines, 9(2), 99.
27. Di Marco LY, Venneri A, Farkas E, Evans PC, Marzo A, Frangi AF. Vascular dysfunction in the pathogenesis of Alzheimer's disease—a review of endothelium-mediated mechanisms

28. Díaz, A. E. C., Herfindal, L., Rathe, B. A., Sletta, K. Y., Vedeler, A., Haavik, S., & Fossen, T. (2019). Cytotoxic saponins and other natural products from flowering tops of Narthecium ossifragum L. *Phytochemistry*, *164*, 67-77.
29. *Dubois, B., Feldman, H. H., Jacova, C., Cummings, J. L., DeKosky, S. T., Barberger-Gateau, P., ... & Scheltens, P. (2010). Revising the definition of Alzheimer's disease: a new lexicon. The Lancet Neurology, 9(11), 1118-1127.*
30. Dubois, B., Hampel, H., Feldman, H. H., Scheltens, P., Aisen, P., Andrieu, S., ... & Washington, D. C. (2016). Preclinical Alzheimer's disease: definition, natural history, and diagnostic criteria. *Alzheimer's & Dementia*, *12*(3), 292-323.
31. Dubois, B., Villain, N., Frisoni, G. B., Rabinovici, G. D., Sabbagh, M., Cappa, S., ... & Feldman, H. H. (2021). Clinical diagnosis of Alzheimer's disease: recommendations of the International Working Group. *The Lancet Neurology*, *20*(6), 484-496.
32. Escott-Price, V., Myers, A. J., Huentelman, M., & Hardy, J. (2017). Polygenic risk score analysis of pathologically confirmed Alzheimer disease. *Annals of neurology*, *82*(2), 311-314.
33. Etgen, T., Chonchol, M., Förstl, H., & Sander, D. (2012). Chronic kidney disease and cognitive impairment: a systematic review and meta-analysis. *American journal of nephrology*, *35*(5), 474-482.
34. Feigin, V. L., Vos, T., Nichols, E., Owolabi, M. O., Carroll, W. M., Dichgans, M., Deuschl, G., Parmar, P., Brainin, M., & Murray, C. (2020). The global burden of neurological disorders: translating evidence into policy. *The Lancet. Neurology*, *19*(3), 255–265. https://doi.org/10.1016/S1474-4422(19)30411-9.
35. Feldman, A. T., & Wolfe, D. (2014). Tissue processing and hematoxylin and eosin staining. Histopathology: methods and protocols, 31-43.
36. Feldman, H. H., & Woodward, M. (2005). The staging and assessment of moderate to severe Alzheimer disease. Neurology, 65(6_suppl_3), S10-S17.
37. Gage, G. J., Kipke, D. R., & Shain, W. (2012). Whole animal perfusion fixation for rodents. Journal of visualized experiments : JoVE, (65), 3564. https://doi.org/10.3791/3564

38. Gatz, M., Reynolds, C. A., Fratiglioni, L., Johansson, B., Mortimer, J. A., Berg, S., ... & Pedersen, N. L. (2006). Role of genes and environments for explaining Alzheimer disease. *Archives of general psychiatry*, *63*(2), 168-174.
39. Gorelick, P. B., Scuteri, A., Black, S. E., DeCarli, C., Greenberg, S. M., Iadecola, C., ... & Seshadri, S. (2011). Vascular contributions to cognitive impairment and dementia: a statement for healthcare professionals from the American Heart Association/American Stroke Association. *stroke*, *42*(9), 2672-2713.
40. Grossberg, G. T., Kohegyi, E., Mergel, V., Josiassen, M. K., Meulien, D., Hobart, M., Slomkowski, M., Baker, R. A., McQuade, R. D., & Cummings, J. L. (2020). Efficacy and Safety of Brexpiprazole for the Treatment of Agitation in Alzheimer's Dementia: Two 12-Week, Randomized, Double-Blind, Placebo-Controlled Trials. *The American journal of geriatric psychiatry : official journal of the American Association for Geriatric Psychiatry*, *28*(4), 383–400. https://doi.org/10.1016/j.jagp.2019.09.009
41. Hampel, H., Mesulam, M. M., Cuello, A. C., Farlow, M. R., Giacobini, E., Grossberg, G. T., ... & Khachaturian, Z. S. (2018). The cholinergic system in the pathophysiology and treatment of Alzheimer's disease. *Brain*, *141*(7), 1917-1933.
42. Holstege, H., Van Der Lee, S. J., Hulsman, M., Wong, T. H., Van Rooij, J. G., Weiss, M., ... & Scheltens, P. (2017). Characterization of pathogenic SORL1 genetic variants for association with Alzheimer's disease: a clinical interpretation strategy. *European Journal of Human Genetics*, *25*(8), 973-981.
43. Hussein, W., Sağlık, B. N., Levent, S., Korkut, B., Ilgın, S., Özkay, Y., & Kaplancıklı, Z. A. (2018). Synthesis and biological evaluation of new cholinesterase inhibitors for Alzheimer's disease. *Molecules*, *23*(8), 2033.
44. Jonsson, T., Atwal, J. K., Steinberg, S., Snaedal, J., Jonsson, P. V., Bjornsson, S., ... & Stefansson, K. (2012). A mutation in APP protects against Alzheimer's disease and age-related cognitive decline. *Nature*, *488*(7409), 96-99.
45. Karch, C. M., & Goate, A. M. (2015). Alzheimer's disease risk genes and mechanisms of disease pathogenesis. *Biological psychiatry*, *77*(1), 43-51.
46. Khoury, R., Grysman, N., Gold, J., Patel, K., & Grossberg, G. T. (2018). The role of 5 HT6-receptor antagonists in Alzheimer's disease: an update. *Expert opinion on investigational drugs*, *27*(6), 523-533.

47. Kim, D. H., Khan, H., Ullah, H., Hassan, S. T., Šmejkal, K., Efferth, T., ... & Rengasamy, K. R. (2019). MicroRNA targeting by quercetin in cancer treatment and chemoprotection. *Pharmacological Research*, *147*, 104346.
48. Kraeuter, A. K., Guest, P. C., & Sarnyai, Z. (2019). The Y-maze for assessment of spatial working and reference memory in mice. *Pre-clinical models: Techniques and protocols*, 105-111.
49. Kumar, T. P., Antony, S., Soman, S., Kuruvilla, K. P., George, N., & Paulose, C. S. (2011). Role of curcumin in the prevention of cholinergic mediated cortical dysfunctions in streptozotocin-induced diabetic rats. *Molecular and cellular endocrinology*, *331*(1), 1-10.
50. Lazarov, O., Lee, M., Peterson, D. A., & Sisodia, S. S. (2002). Evidence that synaptically released β-amyloid accumulates as extracellular deposits in the hippocampus of transgenic mice. *Journal of Neuroscience*, *22*(22), 9785-9793.
51. Leblhuber, F., Steiner, K., Schuetz, B., Fuchs, D., & Gostner, J. M. (2018). Probiotic supplementation in patients with Alzheimer's dementia-an explorative intervention study. *Current Alzheimer Research*, *15*(12), 1106-1113.
52. Leger, M., Quiedeville, A., Bouet, V., Haelewyn, B., Boulouard, M., Schumann-Bard, P., & Freret, T. (2013). Object recognition test in mice. *Nature protocols*, *8*(12), 2531-2537.
53. Li, H., Kang, T., Qi, B., Kong, L., Jiao, Y., Cao, Y., ... & Yang, J. (2016). Neuroprotective effects of ginseng protein on PI3K/Akt signaling pathway in the hippocampus of D-galactose/AlCl3 inducing rats model of Alzheimer's disease. *Journal of ethnopharmacology*, *179*, 162-169.
54. Liaquat, L., Sadir, S., Batool, Z., Tabassum, S., Shahzad, S., Afzal, A., & Haider, S. (2019). Acute aluminum chloride toxicity revisited: Study on DNA damage and histopathological, biochemical and neurochemical alterations in rat brain. *Life sciences*, *217*, 202-211
55. Lindwall, G., & Cole, R. D. (1984). The purification of tau protein and the occurrence of two phosphorylation states of tau in brain. *Journal of Biological Chemistry*, *259*(19), 12241-12245.

56. Martin Prince, A., Wimo, A., Guerchet, M., Gemma-Claire Ali, M., Wu, Y. T., Prina, M., ... & Xia, Z. (2015). World Alzheimer Report 2015. *The global impact of dementia. An analysis of prevalence, incidence, cost, and trends. London, UK: Alzheimer's Disease International*.
57. Mishra, B., Priyadarsini, K. I., Kumar, M. S., Unnikrishnan, M. K., & Mohan, H. (2003). Effect of O-glycosilation on the antioxidant activity and free radical reactions of a plant flavonoid, Chrysoeriol. *Bioorganic & medicinal chemistry*, *11*(13), 2677-2685
58. Mishra, B., Priyadarsini, K. I., Kumar, M. S., Unnikrishnan, M. K., & Mohan, H. (2003). Effect of O-glycosilation on the antioxidant activity and free radical reactions of a plant flavonoid, Chrysoeriol. *Bioorganic & medicinal chemistry*, *11*(13), 2677-2685.
59. Mittal, P., Garg, N., Kesari, N., & Gautam, S. (2023). Traditional Medicinal Plants as Hepatoprotective Agents: A Review
60. Mulder, G. B., & Pritchett, K. (2004). The elevated plus-maze. *Journal of the American Association for Laboratory Animal Science*, *43*(2), 39-40.
61. Näslund, J., Haroutunian, V., Mohs, R., Davis, K. L., Davies, P., Greengard, P., & Buxbaum, J. D. (2000). Correlation between elevated levels of amyloid β-peptide in the brain and cognitive decline. *Jama*, *283*(12), 1571-1577.
62. Nobakht, M., Hoseini, S. M., Mortazavi, P., Sohrabi, I., Esmailzade, B., Roosh, N. R., & Omidzahir, S. (2011). Neuropathological changes in brain cortex and hippocampus in a rat model of Alzheimer's disease. Iranian biomedical journal, 15(1-2), 51
63. Overmyer, K. A., Thonusin, C., Qi, N. R., Burant, C. F., & Evans, C. R. (2015). Impact of anesthesia and euthanasia on metabolomics of mammalian tissues: studies in a C57BL/6J mouse model. *PloS one*, *10*(2), e0117232.
64. Ownby, R. L., Crocco, E., Acevedo, A., John, V., & Loewenstein, D. (2006). Depression and risk for Alzheimer disease: systematic review, meta-analysis, and metaregression analysis. *Archives of general psychiatry*, *63*(5), 530-538.
65. Padda IS, Parmar M. StatPearls [Internet]. StatPearls Publishing; Treasure Island (FL): Jun 3, 2023. Aducanumab.

66. Paradis, E., Douillard, H., Koutroumanis, M., Goodyer, C., & LeBlanc, A. (1996). Amyloid β peptide of Alzheimer's disease downregulates Bcl-2 and upregulates Bax expression in human neurons. *Journal of Neuroscience*, *16*(23), 7533-7539.
67. Parodi-Rullán, R. M., Javadov, S., & Fossati, S. (2021). Dissecting the crosstalk between endothelial mitochondrial damage, vascular inflammation, and neurodegeneration in cerebral amyloid angiopathy and Alzheimer's disease. *Cells*, *10*(11), 2903.
68. Paroni, G., Bisceglia, P., & Seripa, D. (2019). Understanding the amyloid hypothesis in Alzheimer's disease. *Journal of Alzheimer's Disease*, *68*(2), 493-510.
69. Price, J. L., & Morris, J. C. (1999). Tangles and plaques in nondemented aging and "preclinical" Alzheimer's disease. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society*, *45*(3), 358-368.
70. Radiske, A., Rossato, J. I., Gonzalez, M. C., Köhler, C. A., Bevilaqua, L. R., & Cammarota, M. (2017). BDNF controls object recognition memory reconsolidation. *Neurobiology of learning and memory*, *142*, 79-84.
71. Ramirez, G., Zamilpa, A., Zavala, M., Perez, J., Morales, D., & Tortoriello, J. (2016). Chrysoeriol and other polyphenols from Tecoma stans with lipase inhibitory activity. *Journal of Ethnopharmacology*, *185*, 1-8.
72. Rashad A, Rasool A, Shaheryar M, Sarfraz A, Sarfraz Z, Robles-Velasco K, Cherrez-Ojeda I. Donanemab for Alzheimer's Disease: A Systematic Review of Clinical Trials. Healthcare (Basel). 2022 Dec 22;11(1)
73. Rebai, O., & Djebli, N. E. (2008). Chronic exposure to aluminum chloride in mice: exploratory behaviors and spatial learning. *Adv Biol Res*, *2*(1-2), 26-33.
74. Reiman EM, Arboleda-Velasquez JF, Quiroz YT, et al. Exceptionally low likelihood of Alzheimer's dementia in APOE2 homozygotes from a 5,000-person neuropathological study. Nat Commun 2020; 11: 667
75. Ryman, D. C., Acosta-Baena, N., Aisen, P. S., Bird, T., Danek, A., Fox, N. C., ... & Dominantly Inherited Alzheimer Network. (2014). Symptom onset in autosomal dominant Alzheimer disease: a systematic review and meta-analysis. *Neurology*, *83*(3), 253-260

76. Sadek, K. M., Lebda, M. A., & Abouzed, T. K. (2019). The possible neuroprotective effects of melatonin in aluminum chloride-induced neurotoxicity via antioxidant pathway and Nrf2 signaling apart from metal chelation. *Environmental Science and Pollution Research*, *26*(9), 9174-9183.
77. Sarfraz, A., Javeed, M., Shah, M. A., Hussain, G., Shafiq, N., Sarfraz, I., ... & Rasul, A. (2020). Biochanin A: A novel bioactive multifunctional compound from nature. *Science of the Total Environment*, *722*, 137907. Spagnuolo, C., Moccia, S., & Russo, G. L. (2018). Anti-inflammatory effects of flavonoids in neurodegenerative disorders. *European journal of medicinal chemistry*, *153*, 105-115.
78. Sarkar, D. (2020). A review of behavioral tests to evaluate different types of anxiety and anti-anxiety effects. *Clinical Psychopharmacology and Neuroscience*, *18*(3), 341.
79. Seibenhener, M. L., & Wooten, M. C. (2015). Use of the open field maze to measure locomotor and anxiety-like behavior in mice. *JoVE (Journal of Visualized Experiments)*, (96), e52434.
80. Shao, G., Chen, S., Sun, Y., Xu, H., & Ge, F. (2021). Chrysoeriol promotes functional neurological recovery in a rat model of cerebral ischemia. *Pharmacognosy Magazine*, *17*(76).
81. Šimić, G., Babić Leko, M., Wray, S., Harrington, C., Delalle, I., Jovanov-Milošević, N., ... & Hof, P. R. (2016). Tau protein hyperphosphorylation and aggregation in Alzheimer's disease and other tauopathies, and possible neuroprotective strategies. *Biomolecules*, *6*(1), 6
82. Sims, R., Van Der Lee, S. J., Naj, A. C., Bellenguez, C., Badarinarayan, N., Jakobsdottir, J., ... & Daniilidou, M. (2017). Rare coding variants in PLCG2, ABI3, and TREM2 implicate microglial-mediated innate immunity in Alzheimer's disease. *Nature genetics*, *49*(9), 1373-1384.
83. Small, G. W., Rabins, P. V., Barry, P. P., Buckholtz, N. S., DeKosky, S. T., Ferris, S. H., Finkel, S. I., Gwyther, L. P., Khachaturian, Z. S., Lebowitz, B. D., McRae, T. D., Morris, J. C., Oakley, F., Schneider, L. S., Streim, J. E., Sunderland, T., Teri, L. A., & Tune, L. E. (1997). Diagnosis and treatment of Alzheimer's disease and related disorders. Consensus statement of the American Association for Geriatric Psychiatry,

the Alzheimer's Association, and the American Geriatrics Society. *JAMA*, *278*(16), 1363–1371

84. Swerdlow, R. H., Burns, J. M., & Khan, S. M. (2014). The Alzheimer's disease mitochondrial cascade hypothesis: progress and perspectives. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1842*(8), 1219-1231.
85. Takeuchi, H., Iba, M., Inoue, H., Higuchi, M., Takao, K., Tsukita, K., ... & Takahashi, R. (2011). P301S mutant human tau transgenic mice manifest early symptoms of human tauopathies with dementia and altered sensorimotor gating. *PloS one*, *6*(6), e21050.
86. Tay, L. X., Ong, S. C., Tay, L. J., Ng, T., & Parumasivam, T. (2024). Economic burden of Alzheimer's disease: A systematic review. *Value in Health Regional Issues*, *40*, 1-12.
87. van Dyck CH, Swanson CJ, Aisen P, Bateman RJ, Chen C, Gee M, Kanekiyo M, Li D, Reyderman L, Cohen S, Froelich L, Katayama S, Sabbagh M, Vellas B, Watson D, Dhadda S, Irizarry M, Kramer LD, Iwatsubo T. Lecanemab in Early Alzheimer's Disease. N Engl J Med. 2023 Jan 05;388(1):9-21.
88. Vauzour, D., Vafeiadou, K., Rodriguez-Mateos, A., Rendeiro, C., & Spencer, J. P. (2008). The neuroprotective potential of flavonoids: a multiplicity of effects. *Genes & nutrition*, *3*, 115-126.
89. Verghese, P. B., Castellano, J. M., & Holtzman, D. M. (2011). Apolipoprotein E in Alzheimer's disease and other neurological disorders. *The Lancet Neurology*, *10*(3), 241-252.
90. Wei, W., He, J., Ruan, H., & Wang, Y. (2019). In vitro and in vivo cytotoxic effects of Chrysoeriol in human lung carcinoma are facilitated through activation of autophagy, sub-G1 cell cycle arrest, cell migration and invasion inhibition, and modulation of MAPK/ERK signaling pathway. *J BUON*, *24*(3), 936-942.
91. Wenk, G. L. (2003). Neuropathologic changes in Alzheimer's disease. *Journal of Clinical Psychiatry*, *64*, 7-10.
92. Willhite, C. C., Karyakina, N. A., Yokel, R. A., Yenugadhati, N., Wisniewski, T. M., Arnold, I. M., ... & Krewski, D. (2014). Systematic review of potential health risks posed by pharmaceutical, occupational and consumer exposures to metallic and

nanoscale aluminum, aluminum oxides, aluminum hydroxide and its soluble salts. *Critical reviews in toxicology*, *44*(sup4), 1-80.

93. Wu, J. Y., Chen, Y. J., Bai, L., Liu, Y. X., Fu, X. Q., Zhu, P. L., ... & Yu, Z. L. (2020). Chrysoeriol ameliorates TPA-induced acute skin inflammation in mice and inhibits NF-κB and STAT3 pathways. *Phytomedicine*, *68*, 153173
94. Wu, J. Y., Chen, Y. J., Bai, L., Liu, Y. X., Fu, X. Q., Zhu, P. L., ... & Yu, Z. L. (2020). Chrysoeriol ameliorates TPA-induced acute skin inflammation in mice and inhibits NF-κB and STAT3 pathways. *Phytomedicine*, *68*, 153173
95. Yamabe, N., Kang, K. S., Lee, A. Y., Lee, D., Choi, J. M., Lee, S., ... & Lee, S. (2014). Identification of anti-cancer active components of Taraxacum coreanum on human gastric cancer AGS cells. *Journal of the Korean Society for Applied Biological Chemistry*, *57*, 187-190
96. Zeng, Z., Sarbassov, D. D., Samudio, I. J., Yee, K. W., Munsell, M. F., Ellen Jackson, C., ... & Konopleva, M. (2007). Rapamycin derivatives reduce mTORC2 signaling and inhibit AKT activation in AML. Blood, 109(8), 3509-3512