# EVALUATION OF ANXIOLYTIC PROPERTY AND ACTIVITY OF PROTEASE INHIBITOR (RITONAVIR) AND ITS EFFECT ON APOE4 GENE EXPRESSION IN AN $AlCl_3$-INDUCED ALZHEIMER'S DISEASE MODEL.


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MATRICULATION NUMBER: PHA1600489

UNDER THE SUPERVISION OF

Dr. (Mrs.) A.M AKHIGBEMEN

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TO

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY,

FACULTY OF PHARMACY,

UNIVERSITY OF BENIN,

BENIN CITY, EDO STATE

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## CERTIFICATION

This is to certify that this project work titled "EVALUATION OF ANXIOLYTIC PROPERTY AND ACTIVITY OF PROTEASE INHIBITOR (RITONAVIR) AND ITS EFFECT ON APOE4 GENE EXPRESSION IN AN AlCl3-INDUCED ALZHEIMER'S DISEASE MODEL" was carried out by OtonobijieOsezefua Bonaventure in the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria in partial fulfillment for the award of the Doctor of Pharmacy Degree in the University of Benin, Edo State, Nigeria.

_____ _____

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(Supervisor)

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Dr. (Mrs.) A.P UCHENDU Date
(Head of Department)

## DEDICATION

This academic submission is committed to Almighty God for this loving kindness and strength, whose constant presence has seen me through this demanding educational path. I am thankful for the providence and endowment that propelled me to the conclusion of my studies.

This work is done with the aspiration that it will refine medical practice and lead to public welfare improvements.

Finally, I dedicate this to all colleagues and innovators devoted to elevating the community of practitioners and benefiting humanity.

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## ABSTRACT

**Background:** Alzheimer's disease represents a progressive neurodegenerative disorder characterized by substantial cognitive deterioration and frequently accompanied by behavioral manifestations, particularly anxiety. While contemporary therapeutic interventions predominantly target cognitive deficits, they inadequately address anxiety-related symptoms. This investigation examined the neurotherapeutic potential of Ritonavir (a protease inhibitor) within an aluminum chloride (AlCl3) - induced model of Alzheimer's disease, focusing specifically on its anxiolytic properties and capacity to modulate APOE4 gene expression.

**Objective:** The primary objective was to evaluate the anxiolytic efficacy and molecular regulatory effects of Ritonavir across multiple dosing regimens (100, 200, and 400mg/kg) in disease-model mice, comparing its therapeutic profile against Donepezil and control groups.

**Methods:** Fifty-six mice underwent randomized allocation into seven experimental cohorts. Anxiety-related behaviors were assessed using the Elevated Plus Maze paradigm, while exploratory tendencies were quantified via the Hole Board Test. Hippocampal APOE4 gene expression patterns were determined through quantitative real-time polymerase chain reaction (RT-qPCR) methodology.

**Results:** Behavioral assessments demonstrated that the highest Ritonavir concentration (400mg/kg) produced significant anxiolytic effects in the Elevated Plus Maze, evidenced by increased open-arm exploration duration ($p < 0.05$) relative to $AlCl_3$-induced disease controls. This dosage exhibited comparable efficacy to Donepezil. Furthermore, the 400mg/kg cohort demonstrated enhanced exploratory behavior in the Hole Board assessment. Molecular analysis revealed that Ritonavir 400mg/kg effectively normalized APOE4 transcriptional levels toward

baseline parameters, demonstrating statistical superiority over lower concentrations and the reference medication ($p < 0.05$).

**Conclusion**: Ritonavir at 400mg/kg exhibits robust anxiolytic properties coupled with favorable APOE4 gene expression modulation, suggesting potential utility as a dual-mechanism therapeutic agent capable of addressing both behavioral symptomatology and molecular pathogenesis in Alzheimer's disease.

# CHAPTER ONE

# INTRODUCTION AND LITERATURE REVIEW

## 1.1 Background to the study

Dementia encompasses a spectrum of neurological disorders characterized by progressive and often irreversible decline in cognitive function, representing a significant global health challenge (WHO, 2024). The scope of this epidemic presents extraordinary societal and economic implications. Contemporary epidemiological data indicates that over 55 million individuals worldwide are currently living with dementia, with projections suggesting this figure may approach 150 million by 2050 (WHO, 2024).

Among the various etiologies of dementia, Alzheimer's Disease (AD) emerges as the predominant neurodegenerative condition, accounting for approximately 60% to 80% of all dementia diagnoses (Alzheimer's Association, 2023). AD manifests as a progressive disorder with initial pathological changes occurring in brain regions critical for memory consolidation, subsequently disseminating to additional cortical areas. This progression culminates in severe cognitive impairment accompanied by numerous Behavioral and Psychological Symptoms of Dementia (BPSDs), with anxiety representing a particularly prevalent and distressing manifestation (Gómez-Murcia *et al*., 2021).

The clinical management of BPSDs, particularly anxiety, assumes critical importance in AD care. These symptoms demonstrate strong associations with diminished patient quality of life, accelerated institutionalization, and substantial caregiver psychological burden. The profound emotional toll that anxiety imposes on AD patients underscores the imperative for continued

investigation into novel therapeutic compounds capable of addressing these specific behavioral pathologies without exacerbating pre-existing cognitive deficits.

### 1.2 Alzheimer's Disease, Causes, Pathology, and Manifestations

Alzheimer's disease constitutes a chronic, progressive neurodegenerative disorder predominantly affecting the cerebral cortex and hippocampus—anatomical regions essential for cognition, learning, and memory consolidation. As the leading causative agent of dementia globally, it represents approximately 60-80% of all dementia presentations. AD is characterized as an irreversible, steadily deteriorating brain disorder that produces continuous degradation of memory retention, learning capacity, cognitive processing abilities, and behavioral regulation, ultimately proving fatal (Alzheimer's Association, 2024).

Neuropathological examination reveals two cardinal hallmarks: extracellular accumulation of amyloid-beta (Aβ) protein aggregates forming senile plaques, and intraneuronal neurofibrillary tangles composed of hyperphosphorylated tau protein. These molecular alterations occur concomitantly with neuroinflammatory processes, synaptic dysfunction, neuronal degeneration, and extensive cerebral atrophy, particularly affecting memory and cognition-related structures including the hippocampus and cortical regions (DeTure & Dickson, 2019; Jack *et al.,* 2018).

The disease trajectory typically advances through distinct clinical phases: an extended preclinical period potentially spanning decades, mild cognitive impairment (MCI) attributable to Alzheimer's pathology, and ultimately fully manifested dementia. Early clinical presentations frequently include subtle memory deficits and word-finding difficulties, which progressively intensify to encompass temporal-spatial disorientation, language deterioration, personality alterations, and complete functional dependency (Scheltens *et al*., 2021).

AD invariably proves fatal, with no curative interventions currently available, though emerging therapeutic strategies offer promise for disease modification or onset delay. Post-diagnosis survival generally ranges from 4 to 8 years, though individual variability permits survival extending to two decades, contingent upon diagnostic age, comorbid conditions, and care quality (Alzheimer's Association, 2024).

Given its multifactorial etiology encompassing genetic predisposition, environmental influences, and lifestyle determinants, Alzheimer's disease continues to represent a major focus of biomedical research, particularly concerning early detection methodologies, biomarker development, and disease-modifying therapeutic interventions.

### 1.2.1 Causes and Risk Factors

The precise etiology of Alzheimer's disease remains incompletely elucidated, though current evidence supports a multifactorial pathogenesis involving genetic susceptibility, environmental exposures, and lifestyle factors. Advanced chronological age, particularly beyond 65 years, represents the most well-established risk factor. Genetic predispositions, including mutations within the APP, PSEN1, and PSEN2 genes, account for familial early-onset AD presentations, whereas the APOE ε4 allelic variant constitutes the most robust genetic risk factor associated with sporadic, late-onset disease (Kunkle *et al.,* 2019).

Additional contributory risk factors include:

**Cardiovascular pathology**: Conditions including hypertension, diabetes mellitus, obesity, and hypercholesterolemia demonstrate associations with elevated AD risk secondary to their impact on cerebral perfusion and vascular integrity (Duron & Hanon, 2008).

**Traumatic brain injury**: Repetitive cranial trauma has been epidemiologically linked with increased dementia susceptibility and Alzheimer's neuropathological changes (McKee *et al.,* 2013).

**Modifiable lifestyle determinants**: Tobacco consumption, physical inactivity, nutritional inadequacies, and diminished social engagement represent potentially modifiable risk factors (Livingston *et al.,* 2020).

**Educational attainment and cognitive reserve**: Individuals demonstrating higher educational achievement and sustained cognitive engagement throughout their lifespan may exhibit reduced risk attributable to enhanced cognitive reserve capacity (Stern, 2012).

### 1.2.2 Etiology and Pathogenesis

The definitive pathogenesis of AD involves complex interactions among genetic, environmental, and lifestyle factors. The disease is characterized by two distinctive neuropathological hallmarks (Hardy & Selkoe, 2002):

**Amyloid-beta (Aβ) Plaques**: Extracellular proteinaceous deposits predominantly composed of Aβ peptides, derived through sequential proteolytic cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase enzymes. The accumulation of Aβ oligomers initiates a neurotoxic cascade culminating in synaptic dysfunction and neuronal apoptosis (Selkoe & Hardy, 2016).

**Neurofibrillary Tangles (NFTs)**: Intracellular protein aggregates consisting of hyperphosphorylated tau protein. Under physiological conditions, tau functions as a microtubule-stabilizing protein; however, pathological hyperphosphorylation causes tau dissociation from

microtubules and subsequent aggregation into insoluble paired helical filaments, disrupting axonal transport mechanisms and precipitating neuronal collapse (Iqbal *et al*., 2005).

**Neuroinflammatory processes**: Activated microglia and reactive astrocytes characterize AD brain tissue, contributing to the release of pro-inflammatory cytokines and generation of oxidative stress, thereby exacerbating neuronal injury (Heneka *et al*., 2015).

**Synaptic and neuronal loss**: Synaptic dysfunction and subsequent neuronal degeneration constitute the direct pathological correlates of cognitive decline in AD. The hippocampus and cerebral cortex, anatomical regions integral to memory and cognition, demonstrate particular vulnerability (Jack *et al*., 2018).

**Cerebral atrophy**: Disease progression accompanies widespread brain tissue atrophy, including substantial hippocampal volume reduction and ventricular enlargement, observable through neuroimaging modalities such as magnetic resonance imaging.

These pathological hallmarks collectively contribute to synaptic dysfunction, neuronal loss, oxidative stress, mitochondrial impairment, and neuroinflammatory cascades that disrupt normal neural signaling and structural integrity (Knopman *et al*., 2021).

### 1.2.3 Clinical Manifestations

Alzheimer's disease typically presents with insidious onset and gradual symptom progression. The clinical course can be conceptualized across several stages:

**1. Preclinical Stage**: This phase may extend across years or even decades, characterized by the presence of neuropathological alterations in the absence of overt clinical symptomatology. Biomarkers including amyloid positron emission tomography (PET) imaging or cerebrospinal

fluid (CSF) quantification of Aβ and tau can indicate early pathological changes (Jack et al., 2018).

**2. Mild Cognitive Impairment (MCI) due to AD**: This stage is characterized by mild yet clinically detectable memory deficits, particularly affecting episodic memory and new learning, while instrumental activities of daily living remain relatively preserved. Not all MCI cases progress to AD dementia, though it represents a high-risk transitional state.

**3. Mild to Moderate AD Dementia**: This phase is characterized by:

- Progressive memory impairment, particularly affecting recent event recall
- Language difficulties including anomia and aphasia
- Compromised executive function and problem-solving capacity
- Temporal-spatial disorientation
- Personality and behavioral alterations (e.g., apathy, irritability, social withdrawal)

**4. Severe AD Dementia**:

- Profound memory loss affecting both recent and remote information
- Severe communication impairment or complete loss of verbal capacity
- Motor function deterioration and coordination deficits
- Loss of sphincter control
- Complete functional dependency

- Neuropsychiatric manifestations including delusions, hallucinations, and agitation

Terminal stages involve complete bedbound status, with mortality typically resulting from secondary complications such as aspiration pneumonia, systemic infections, or malnutrition (Scheltens *et al.,* 2021).

### 1.2.4 The Role of Apolipoprotein E-epsilon 4 (APOE ε4)

Apolipoprotein E (APOE) represents a polymorphic glycoprotein integral to lipid transport and cholesterol metabolism within the central nervous system. Among its three principal isoforms—APOE ε2, APOE ε3, and APOE ε4—the ε4 allelic variant constitutes the most significant genetic risk determinant for late-onset Alzheimer's disease (Corder et al., 1993; Liu et al., 2013).

Individuals carrying the APOE ε4 allele demonstrate increased susceptibility to Alzheimer's disease attributable to its influence on amyloid-beta metabolism, tau pathology, and neuroinflammatory processes. Specifically, APOE ε4 facilitates amyloid-beta aggregation while simultaneously impairing its clearance from cerebral tissue, thereby accelerating plaque deposition (Kim et al., 2009; Mahley & Huang, 2012). Additionally, this isoform exacerbates tau hyperphosphorylation, disrupts neuronal repair mechanisms, and potentiates oxidative stress and inflammatory responses within neural tissue (Huang & Mahley, 2014).

Epidemiological data indicates that heterozygous APOE ε4 carriers exhibit a 3- to 4-fold elevation in AD risk, while homozygous individuals face a 10- to 15-fold increased risk with characteristically earlier disease onset compared to non-carriers (Liu et al., 2013). Consequently, APOE ε4 serves as a critical genetic determinant influencing both the pathogenesis and clinical progression of Alzheimer's disease.

### 1.2.5 The Role of Apolipoprotein E (APOE ε4) in AD Pathogenesis

Apolipoprotein E (APOE) functions as a crucial lipid-transport protein within the central nervous system, encoded by a gene exhibiting three major allelic variants—ε2, ε3, and ε4. The ε4 allele represents the most robust genetic risk factor for sporadic, late-onset Alzheimer's disease (Corder et al., 1993).

#### 1.2.5.1 Mechanisms of Action

**1. Amyloid pathway dysregulation**: APOE ε4 diminishes the efficiency of amyloid-beta peptide clearance mechanisms while simultaneously enhancing peptide aggregation into insoluble plaque formations (Kim et al., 2009).

**2. Tau pathology exacerbation**: APOE ε4 accelerates tau protein hyperphosphorylation and compromises cytoskeletal stability, thereby contributing to neurofibrillary tangle formation (Mahley & Huang, 2012).

**3. Neuroinflammatory potentiation**: The ε4 isoform enhances microglial activation and promotes oxidative stress generation, accelerating neuronal damage (Huang & Mahley, 2014).

**4. Synaptic dysfunction**: APOE ε4 carriers exhibit impaired synaptic plasticity, diminished neurogenesis, and reduced cellular resilience to metabolic stressors.

#### 1.2.5.2 Clinical Implications

Heterozygous APOE ε4 carriers demonstrate a 3-4-fold increased AD risk, whereas homozygous individuals face a 10-15-fold elevated risk with earlier onset and more rapid disease progression (Liu et al., 2013). Furthermore, APOE ε4 has been associated with heightened neuropsychiatric

vulnerability, including anxiety, attributable to its effects on limbic structures. This creates a mechanistic link between neuropathology and behavioral manifestations.

Understanding the interactions between APOE ε4, amyloid metabolism, protease activity, and anxiety regulation provides a molecular rationale for investigating novel therapeutic targets, including protease inhibitors that may modulate these interconnected pathways.

## 1.3 Protease Inhibitors and Their Clinical Significance in AD Management

Protease inhibitors (PIs) represent a class of compounds that selectively block the activity of proteolytic enzymes, molecules responsible for catalyzing peptide bond cleavage within protein substrates. In the context of AD, protease activity assumes particular significance because aberrant proteolytic processing of amyloid precursor protein (APP) constitutes a central pathogenic mechanism.

### 1.3.1 β-Secretase (BACE1) and Amyloid Production

The β-site APP cleaving enzyme 1 (BACE1) initiates the amyloidogenic pathway through initial proteolytic cleavage of APP, generating amyloid-beta peptides. Excessive or dysregulated BACE1 activity promotes Aβ accumulation, culminating in plaque formation and neurotoxicity (Vassar et al., 1999).

### 1.3.2 Mechanism of Protease Inhibitors

BACE1 inhibitors, representing a major class of protease inhibitors under investigation, are designed to prevent the initial APP cleavage event, thereby reducing Aβ generation. Experimental compounds including verubecestat and lanabecestat have demonstrated significant

Aβ reduction in preclinical models, though clinical trials have revealed challenges regarding safety profiles and therapeutic efficacy (Egan et al., 2018).

Alternative protease inhibitors, including γ-secretase modulators, aim to selectively reduce production of longer, more neurotoxic Aβ isoforms while preserving other essential γ-secretase functions.

### 1.3.3 Clinical Significance

Beyond Aβ processing modulation, protease inhibitors may exhibit additional neuroprotective and anti-inflammatory properties:

**Neuroinflammatory modulation**: Certain PIs have demonstrated capacity to attenuate microglial activation and reduce pro-inflammatory cytokine release, thereby mitigating secondary neuronal injury (Holsboer, 2000).

**Synaptic stabilization**: Emerging evidence suggests that some PIs can restore synaptic protein expression profiles and preserve dendritic spine integrity, indirectly supporting cognitive function.

**Potential anxiolytic effects**: Dysregulated protease systems have been implicated in stress-related neuroendocrine alterations. PIs that influence corticotropin-releasing factor pathways or glucocorticoid signaling may exert anxiolytic effects beneficial in managing AD-related anxiety (Taylor et al., 2012).

Consequently, protease inhibitors are emerging as potential dual-action therapeutic agents, capable of addressing both neuropathological features (amyloid accumulation) and behavioral manifestations (anxiety and agitation) characteristic of AD.

## 1.4 Role of Anxiety in Alzheimer's Disease

Anxiety is one of the most prevalent non-cognitive behavioral and psychological symptoms of dementia (BPSD) in AD patients, with prevalence rates approaching 50% across various disease stages (Gómez-Murcia et al., 2021).

### 1.4.1 Pathophysiological Link

**Protein aggregate burden**: Anxiety severity in AD demonstrates correlation with increased amyloid and tau protein deposition within the amygdala, hippocampus, and prefrontal cortex, anatomical regions essential for emotional regulation and threat processing (De la Torre et al., 2018).

**Neurotransmitter dysregulation**: Disruption of serotonergic, GABAergic, and noradrenergic neurotransmitter systems contributes to heightened anxiety responses and emotional dysregulation.

**HPA axis dysfunction**: Chronic activation of the hypothalamic-pituitary-adrenal (HPA) axis elevates circulating cortisol concentrations, promoting neurodegeneration while simultaneously intensifying anxiety symptomatology.

**Neuroinflammatory processes**: Pro-inflammatory cytokines including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) have been implicated in both neurodegenerative cascades and anxiety-like behavioral responses (Heneka et al., 2015).

### 1.4.2 Clinical and Research Significance

Anxiety accelerates cognitive decline trajectories, increases caregiver burden, and adversely affects overall disease prognosis. Significantly, conventional anxiolytic medications

(benzodiazepines, selective serotonin reuptake inhibitors) frequently exacerbate cognitive deficits or induce sedation, thereby limiting their clinical utility. Consequently, identifying alternative therapeutic agents demonstrating anxiolytic efficacy alongside neuroprotective properties, such as specific protease inhibitors, represents substantial therapeutic and research interest.

Preclinical behavioral paradigms including the Elevated Plus Maze (EPM) and Hole Board (HB) tests provide validated methodologies for assessing anxiolytic effects of pharmacological compounds in animal models.

## 1.5 The Role of Behavioral Assays in Assessing Anxiolytic Activity

Behavioral assays constitute essential methodological tools for evaluating the anxiolytic (anti-anxiety) potential of pharmacological and natural compounds within experimental animal models. These paradigms provide reproducible, quantifiable methods for assessing anxiety-like behaviors, thereby serving as preclinical indicators of therapeutic efficacy and mechanistic understanding prior to human clinical trials (Carobrez & Bertoglio, 2005; Kalueff et al., 2007).

Anxiety represents a prominent non-cognitive symptom in AD, and its measurement in preclinical murine models assumes critical importance for therapeutic evaluation. Anxiety in rodents manifests through observable behavioral patterns including reduced exploratory activity, avoidance of open or elevated spaces, and altered locomotor responses.

Behavioral paradigms exploit these innate responses to determine whether test compounds produce anxiolytic or anxiogenic (anxiety-inducing) effects (Cryan & Holmes, 2005). Commonly employed models include the Elevated Plus Maze (EPM), Open Field Test (OFT), Light-Dark Box (LDB), and Hole Board Test (HBT). These assays prove invaluable by enabling

researchers to correlate neurochemical and behavioral outcomes, offering mechanistic insights into the modulation of neurotransmitter systems including GABAergic, serotonergic, and dopaminergic pathways involved in anxiety regulation (Millan, 2003). Consequently, these paradigms form the cornerstone of preclinical screening for novel anxiolytic agents.

This investigation utilized two well-validated behavioral paradigms: the Elevated Plus Maze and the Hole Board Test (File, 1991).

### 1.5.1 Elevated Plus Maze

The Elevated Plus Maze represents a widely recognized and extensively utilized behavioral paradigm designed to assess unconditioned anxiety in rodent species. The EPM exploits the innate conflict between an animal's natural exploratory drive and its instinctual aversion to open, elevated spaces.

#### 1.5.1.1 Background and Design

The EPM was initially developed by Pellow et al. (1985) as a methodology for evaluating anxiety-like behavior in rodents. The apparatus consists of a plus-shaped configuration featuring four arms:

- Two open arms (exposed to the environment, lacking protective walls)
- Two enclosed arms (surrounded by opaque walls, providing perceived safety)

A central platform connects all arms, permitting the animal to navigate between open and enclosed compartments. This choice paradigm reflects anxiety levels, as the animal must balance exploratory motivation against threat perception in open spaces.

#### 1.5.1.2 Measurement of Anxiety-Like Behavior

Anxiety-like behavior is quantified through several key parameters:

**Time spent in open arms:** Extended duration indicates reduced anxiety, reflecting greater willingness to explore potentially threatening environments.

**Number of open arm entries:** Increased entry frequency suggests diminished anxiety levels and enhanced exploratory motivation.

Results are typically analyzed comparatively against control groups to determine anxiolytic agent efficacy. An effective anxiolytic compound should significantly increase both temporal duration and entry frequency into open arms, demonstrating capacity for anxiety reduction (Rodgers & Dalvi, 1997).

#### 1.5.1.3 Mechanisms and Applications

The EPM serves not only as an assessment tool but also facilitates investigation of underlying anxiety disorder mechanisms and pharmacological agent effects. Research has demonstrated that:

Serotonergic and GABAergic neurotransmitter systems play crucial modulatory roles in anxiety responses within the EPM paradigm (Prut & Belzung, 2003).

Genetic determinants and stress exposure history significantly influence EPM behavior, providing insights into the biological underpinnings of anxiety (Haller et al., 2004).

This assay is extensively employed in novel anxiolytic drug development and neurobiological anxiety research, offering a reliable predictive model for human anxiety responses, thereby contributing to translational research initiatives.

### 1.5.2 Hole Board (HB) Test

The Hole Board test is a well-established behavioral paradigm designed to assess exploratory behavior and emotionality, particularly anxiety, in rodent models. This test capitalizes on the natural inclination of rodents to explore novel environments through investigative head-dipping (nose-poking) behaviors into available apertures.

#### 1.5.2.1 Background and Apparatus Design

The HB test was first developed to evaluate how rodents interact with their environment and to quantify anxiety-related behaviors (Crawley, 1985).

The apparatus typically consists of a flat surface (board) with a series of evenly spaced holes. The standard configuration includes 16 holes arranged in a 4x4 grid, allowing rodents to engage in head-dipping behavior.

#### 1.5.2.2 Measurement of Exploratory Behavior and Anxiety

Key Parameters: The primary measures in the HB test are:

**Number of head-dips (nose-pokes):** This metric reflects exploratory motivation. Elevated head-dip frequency indicates enhanced exploration and reduced anxiety.

**Head-dip duration:** This parameter assesses not only investigation frequency but also the temporal investment in each hole exploration, providing additional behavioral insight.

**Locomotor activity (square crossings):** This serves as a control measure for general motor activity, enabling differentiation between exploratory behavior and non-specific locomotor activity.

**Interpretive Framework:**

Anxiety tends to suppress exploratory behavior; consequently, anxiolytic effects manifest as significant increases in head-dipping frequency. Enhanced head-dipping suggests reduced behavioral inhibition and greater willingness to investigate novel stimuli (Takeda et al., 1998).

#### 1.5.2.3 Mechanisms and Applications

The HB test is frequently employed in anxiety disorder research and anxiolytic drug evaluation. Investigations have indicated that:

**Neurochemical sensitivity**: The test demonstrates sensitivity to serotonergic and dopaminergic system alterations, which are crucial in modulating anxiety and exploratory behaviors (Pellow et al., 1985; Hurst et al., 2012).

**Stress responsiveness**: Numerous studies have demonstrated that stress exposure significantly reduces head-dipping behavior, thereby validating the test as a reliable anxiety metric (Klein et al., 2010).

The HB test proves particularly valuable in preclinical anxiolytic compound assessment. For instance, serotonergic-enhancing compounds have been shown to increase head-dipping frequency (Bari et al., 2008).

### 1.6 Rationale for the Study

Alzheimer's disease development frequently associates with dysregulation of protease systems (such as β-secretase/BACE-1), which are essential for amyloid-beta production (Vassar et al., 1999). However, many proteases and protease inhibitors (PIs) exert far-reaching effects on various biological systems beyond amyloid processing. Recent findings suggest that certain protease inhibitors may possess pleiotropic effects, including modulation of inflammatory pathways and neurotransmission, which are implicated in anxiety pathophysiology (Holsboer, 2000).

**Current Therapeutic Gaps:**

While current AD pharmacotherapies (e.g., cholinesterase inhibitors) primarily target cognitive deficits, they demonstrate inadequate efficacy in managing associated behavioral and psychological symptoms of dementia (BPSDs) such as anxiety. Furthermore, conventional anxiolytic agents (e.g., benzodiazepines) frequently worsen cognitive function in elderly populations, limiting their clinical utility (Taylor et al., 2012).

**Novel Therapeutic Avenue:**

The development of AD is often associated with dysregulation of protease systems (such as the β-secretase/BACE-1), which are essential for beta production (Vassar *et al.,* 1999). However, many proteases and protease inhibitors (PIs) have far-reaching effects on various biological

systems beyond amyloid processing. Recent findings suggest that certain protease inhibitors may possess pleiotropic effects, including the modulation of inflammatory pathways and neurotransmission, which are implicated in anxiety disorders (Holsboer, 2000).

**i) Current Treatment Gap:** While current AD medications (e.g., cholinesterase inhibitors) target cognitive deficits, they often fail to adequately manage associated behavioral and psychological symptoms of dementia (BPSDs) like anxiety. Furthermore, existing anxiolytics (e.g., benzodiazepines) can worsen cognitive function in the elderly (Taylor *et al.,* 2012).

**ii) Novel Therapeutic Avenue:** This study proposes to investigate a specific Protease Inhibitor (PI) with a known safety profile, hypothesizing that it may exert an anxiolytic effect in a mouse model of dementia (e.g., β-secretase or APOE ε4\epsilon4 model) independently of or in conjunction with its primary mechanism of action.

iii) The use of the EPM and HB assays in a mouse model of dementia (which inherently exhibits elevated anxiety levels) provides a robust and translational platform to specifically test the anxiolytic property of PI. Success in this study would establish a strong preclinical foundation for advancing PI as a dual-action therapeutic, capable of addressing both the pathology and the distressing behavioral symptoms of AD.

## 1.7 Aim and Objectives of the Study

### 1.7.1 Aim

The aim of this study was to investigate and evaluate the potential anxiolytic property and activity of the Protease Inhibitor (PI) in a mouse model of dementia.

### 1.7.2 Objectives

i) Evaluate the effect of PI administration on anxiety-like behavior in the dementia mouse model using the Elevated Plus Maze (EPM) test.

ii) Determine the effect of PI on exploratory behavior and emotionality using the Hole Board (HB) test.

iii) Analyze the influence of the PI on general locomotor activity using the total distance traveled in both EPM and HB.

iv) Compare the anxiolytic activity of PI against a vehicle control group and a positive control group (standard anxiolytic drug).

v) Correlate the behavioral outcomes with potential neurochemical or histopathological changes in brain regions associated with anxiety, such as the hippocampus and amygdala (as part of future studies).

# CHAPTER TWO

# MATERIALS AND METHOD

## 2.1 Experimental Design

In Alzheimer's Disease (AD), Protease Inhibitor (PIs) possess a dual functionality: they are factors that drive the disease's progression and pathological development, yet they also stand as a class of compounds with potential therapeutic utility. This research study is aimed to evaluate the activity and effect of the Protease Inhibitor (PI) [Ritonavir] on Alzheimer's Disease induced by Aluminum Chloride; determining its effect on APOE ε4 gene expression, investigating its potential anxiolytic property and activity. The study was conducted using a total of 56 Swiss Albino Mice, weighing between 17-30g, obtained from the Animal House of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Nigeria. The mice were randomly assigned into seven groups of 8 mice each as follows;

1. Group 1 (Control): mice in this group received 0.2ml of distilled water only.
2. Group 2 (Ritonavir 100 mg/kg): mice in this group received 100 mg/kg orally of Ritonavir.
3. Group 3 (Ritonavir 200 mg/kg): mice in this group received 200 mg/kg orally of Ritonavir.
4. Group 4 (Ritonavir 400 mg/kg): mice in this group received 400 mg/kg orally of Ritonavir.
5. Group 5 ($AlCl_3$ 100 mg/kg): mice in this group received 100 mg/kg orally of $AlCl_3$.
6. Group 6 (Donepezil 5mg/kg): mice in this group received 5 mg/kg orally of Donepezil, an approved drug for the management of AD.

7. Group 7 (Ascorbic Acid 100 mg/kg): mice in this group received 100mg/kg orally of Ascorbic Acid.

## 2.2 Experimental Animals

The experimental animals used in this study were fifty six (56) Swiss Albino Mice, weighing between 17-30g, obtained from the Animal House of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City, Edo State, Nigeria. The animals were housed under standard laboratory conditions of 12-hours light/dark cycles, at 25 Degree Celsius, with access to food (dry rodent pellets) and water ad libitum. The bedding materials (wood shavings) of the cages were changed daily. The animals were allowed to acclimatize to their new environment for two weeks before the start of the study. All experimental procedures were carried out in accordance with the guidelines of the National Institute of Health for the care and use of Laboratory (NIH Publication No 80-23) revised in 2002.

## 2.3 Drugs and Chemicals.

All drugs and chemicals used in the study were of analytical grade and obtained from standard commercial sources. Drugs were solubilized in distilled water and were freshly prepared daily for administration.

## 2.4 Behavioral Testing

The behavioral testing phase of this study required specialized equipment to accurately assess the anxiolytic and exploratory activity of the Protease Inhibitor PI (Ritonavir) in the mouse model of Dementia. These equipment include:

1. **Elevated Plus Maze (EPM) Apparatus**: A plus-shaped metallic maze elevated approximately 50cm above the floor. The maze consisted of two opposing open arms (no walls, 30cm x 5cm) and two opposing closed arms (with high walls 15cm high, 30cm x 5cm), connected by a central platform (5cm x 5cm) (Rodgers & Dalvi,1997).
2. **Hole Board (HB) Apparatus**: A square arena (40cm x 40cm) with a floor containing a fixed number of evenly spaced holes (typically 16 holes, 3cm diameter) (Takeda *et al.,* 1998).
3. **Recording Equipment**: The use of a stop watch was employed during the duration of the experiment.

## 2.5 Behavioral Assays and Data Collection

### 2.5.1 Elevated Plus Maze (EPM) Test

The EPM Test was used to assess anxiety-like behavior based on the animal's natural aversion to open spaces.

**A. Procedure**:

1. Mice were brought to the Laboratory (Testing Room) at least 60 minutes prior to the experiment for habituation to the environment.
2. Each mouse was tested individually in a single 5-minute trial.

3. The mouse was gently placed on the central platform, facing an open arm away from the experimenter (Pellow*et al.,* 1985).
4. The trial commenced immediately upon placement, and the entire session was recorded appropriately.
5. After each trial, the maze was thoroughly cleaned with 70% ethanol to remove scent cues.

**B. Data Collection**

The following parameters were collected during procedure and properly documented and scored by blinded observer (Rodgers & Dalvi,1997):

1. Time in Open Arms: This is the total time spent in the open arms. More Tine Spent in Open Arms suggests less anxiety.
2. Time in Closed Arms: This is the total time spent in the closed arms. More Time Spent in Closed Arms suggests more anxiety.
3. Total Distance Travelled: This is the Total Distance moved across all four arms.

### 2.5.2 Hole Board (HB) Test

The Hole Board (HB) Test was used to assess exploratory behavior and emotionality in a novel environment.

**A. Procedure**:

1. The test was typically performed after the EPM test, with sufficient rest time between trials to prevent test interference.
2. Each mouse was placed individually in the center of the HB apparatus.
3. The mouse was allowed to explore for a single 5-minute trial.

4. The arena was cleaned with 70% ethanol after each session to eliminate olfactory cues (Takeda *et al.,* 1998).

**B. Data Collection**

The following parameters were collected during procedure and properly documented and scored by blinded observer:

1. Number of Head Dips: This is the Frequency of Nose-pokes into the holes. It is the Primary Index of Exploration. High number of head dips suggests less anxiety.
2. Total Time Spent On Hole Board.

## 2.6 Statistical Analysis

All data obtained were expressed as Mean $\pm$ Standard Error of Mean and were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Differences were considered significant at $p<0.05$. The GraphPad Prism software (Graphpad software, San Diego, CA, USA) was used for all statistical analyses.

# CHAPTER THREE

# RESULTS

## 3.1 Effect of Protease Inhibitor on Anxiolytic-like Behavior in Dementia-Model Mice

### 3.1.1 Elevated Plus Maze (EPM) Test

The Elevated Plus Maze assessment revealed that Ritonavir administration at varying dosages modified anxiety-related behaviors in the experimental subjects. Data concerning time spent in open arms underwent analysis via one-way Analysis of Variance (ANOVA), succeeded by Tukey's Multiple Comparison Test to identify specific inter-group variations. The overall ANOVA indicated a statistically significant treatment effect on open arm exploration duration.

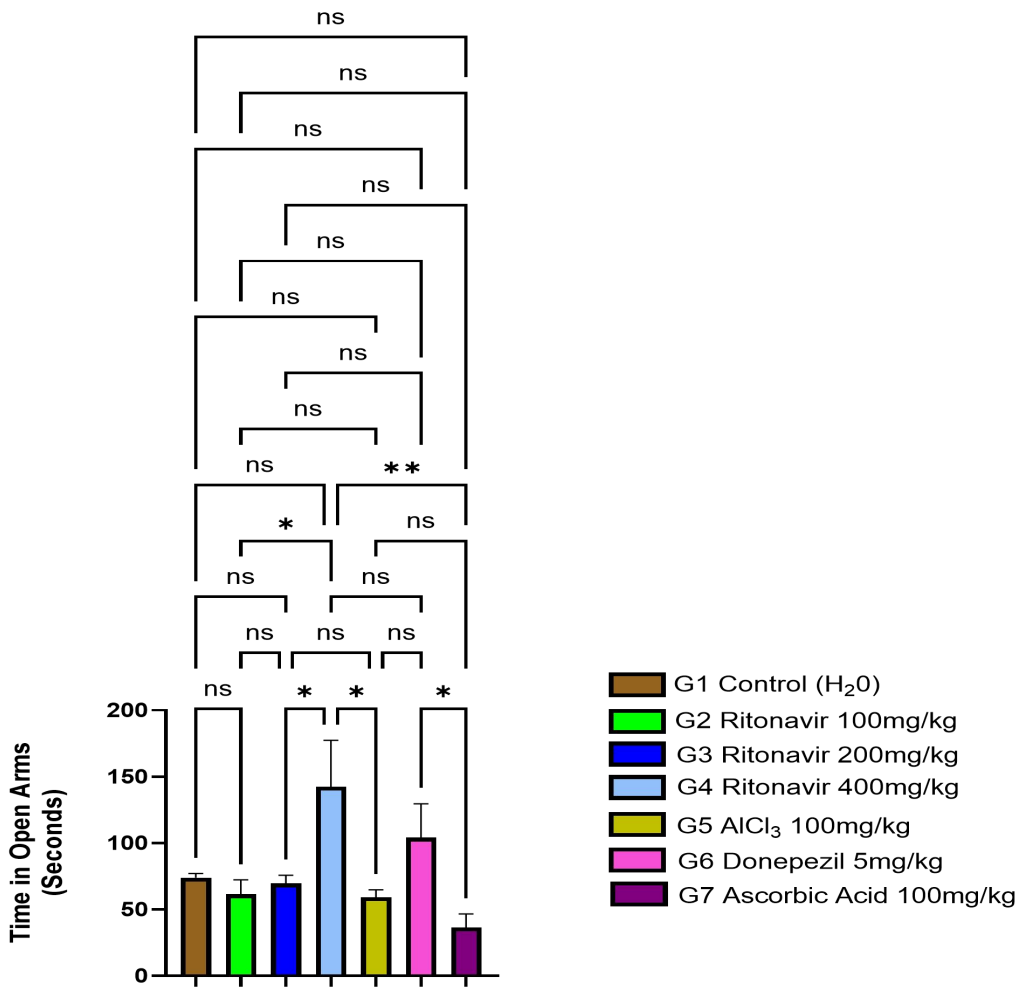


**Figure 3.1:** Time Spent in the Open Arms Across All Groups.

*Figure 3.1 represents the mean Time in Open Arms (seconds) across all seven treatment groups, with error bars indicating the standard error of the mean (SEM). Comparison across all groups (ns = no significant) showed that no group is statistically distinct from any other. Data is presented as Mean ± SEM. The statistical analysis involves a one-way ANOVA with Tukey's multiple comparison*

The secondary critical parameter examined in the EPM assessment was the duration spent in closed arms (seconds). Given that enclosed arms provide shelter and perceived safety, reduced time in these compartments serves as an additional robust indicator of diminished anxiety. Similar to open arm data, closed arm duration underwent one-way ANOVA analysis, followed by Tukey's Multiple Comparison Test to determine specific inter-group differences. The overall ANOVA for closed arm duration demonstrated statistically significant treatment effects on anxiety-like behavior.

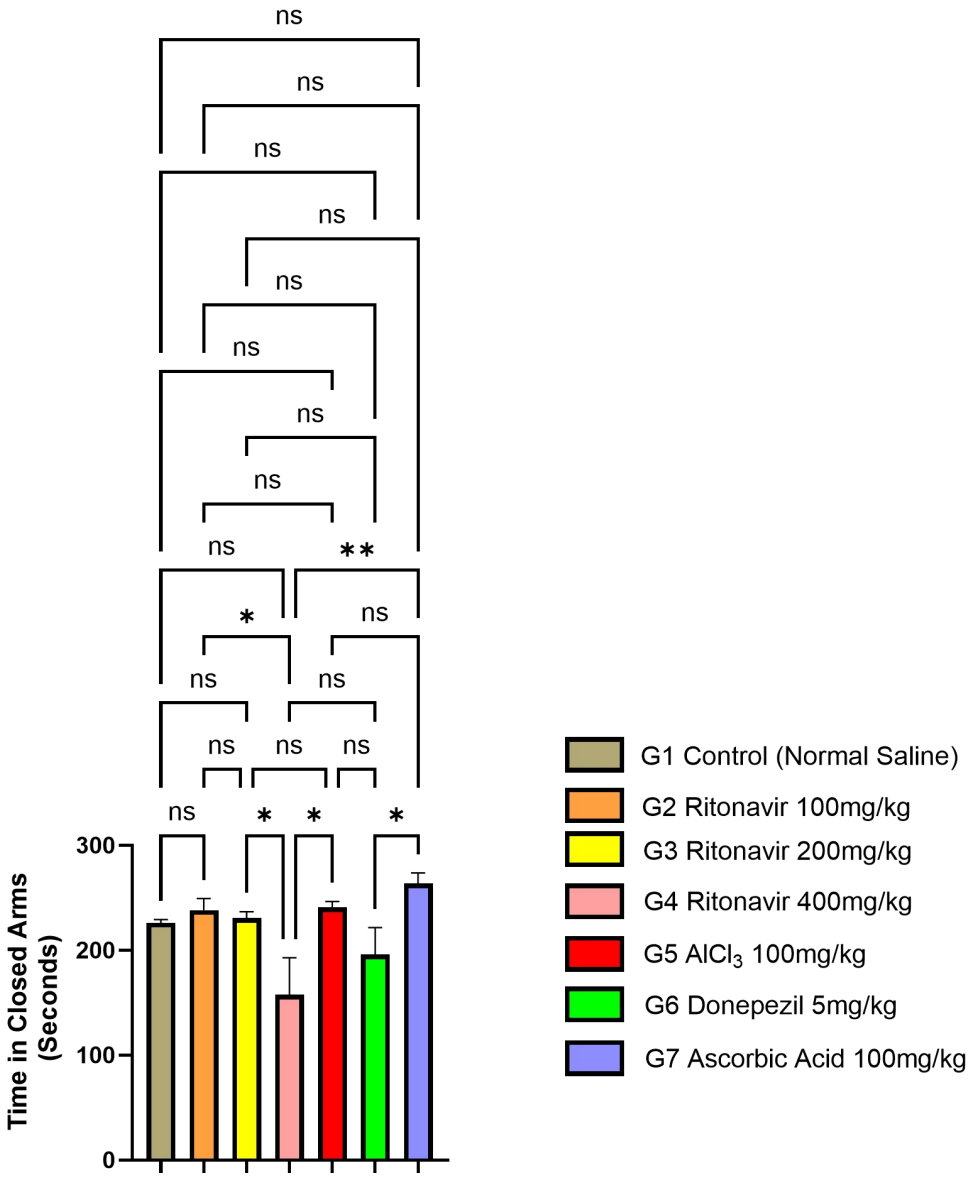


**Figure 3.2:** Time Spent in the Closed Arms Across All Groups.

*Figure 3.2 visually illustrates the mean time spent in the closed arms across all the experimental groups.Comparison across all groups (ns = no significant) showed that no group is statistically distinct from any other. Data is presented as Mean ± SEM. The statistical analysis involves a one-way ANOVA with Tukey's multiple comparison*

### 3.1.2 Hole Board Test

The investigation continued with the Hole Board Test to evaluate Ritonavir's effects on exploratory behavior and general locomotor activity. The primary behavioral metric recorded was the number of head-dips, serving as an index of investigative curiosity and risk assessment. These data underwent one-way ANOVA analysis with subsequent Tukey's Multiple Comparison testing for inter-group differentiation. The overall ANOVA indicated significant treatment effects on head-dipping behavior.

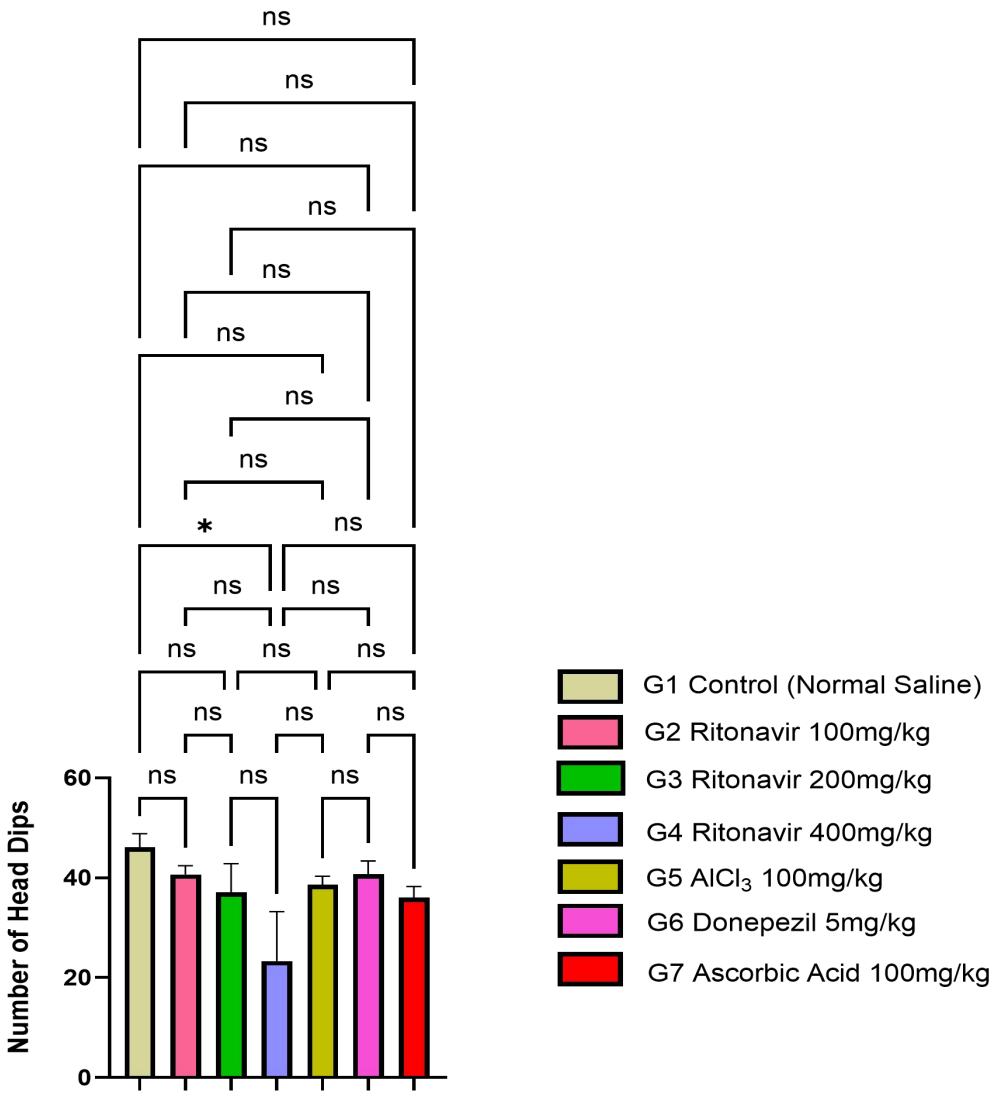


**Figure 3.3:** Number of Head Dips Across All Groups.

*Figure 3.3 presents the mean Number of Head Dips across all seven treatment groups, with error bars illustrating the standard error of the mean (SEM).Comparison across all groups (ns = no significant) showed that no group is statistically distinct from any other. Data is presented as Mean ± SEM. The statistical analysis involves a one-way ANOVA with Tukey's multiple comparison*

## 3.2 Effect of Protease Inhibitor on APOE4 Gene Expression

Following the behavioral assessment, the study moved to the molecular level to determine the therapeutic effect of the Protease Inhibitor (Ritonavir) on APOE4 gene expression. The results are presented as the mean fold-change relative to the healthy control group, with statistical analysis performed using One-way Analysis of Variance (ANOVA).

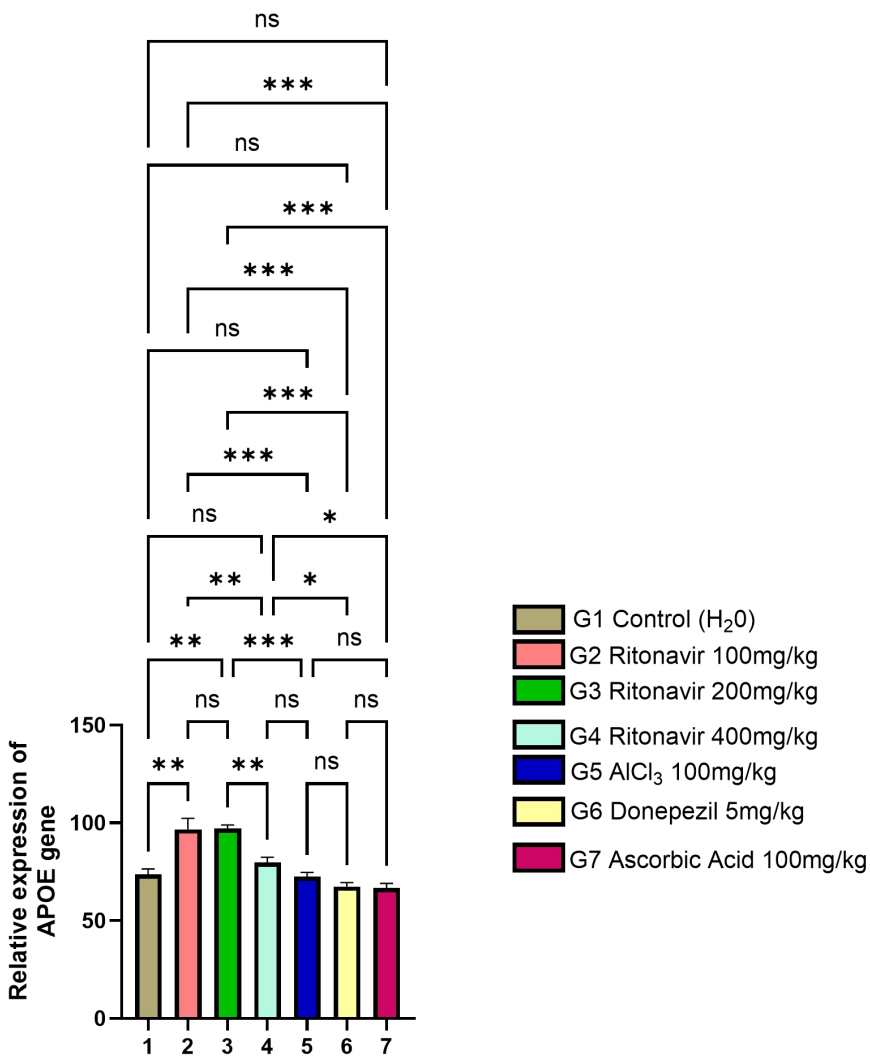


**Figure 3.4**: Modulation of APOE4 mRNA Levels Across all Groups.

*Figure 3.4 visually illustrates the modulation of APOE4 mRNA levels across the treatment groups, providing a molecular basis for the observed behavioral improvements. Notably, significant differences were found between some treatment groups, denoted by * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p<0.0001$) while some comparisons were not significant ($p>0.05$). Data is presented as Mean ± SEM. The statistical analysis involves a one-way ANOVA with Tukey's multiple comparison. Significant differences: comparisons were not significant (ns) ($p>0.05$).ANOVA: $F = 250.9$, $P = <0.0001$*

# CHAPTER FOUR

# DISCUSSION

## 4.1 Overview of Findings

The present investigation examined the anxiolytic, exploratory, and molecular modulatory effects of the Protease Inhibitor Ritonavir within an $AlCl_3$-induced neurodegenerative model. Results from the Elevated Plus Maze paradigm, Hole Board Test, and RT-qPCR analysis of APOE4 gene expression collectively provide compelling evidence for the therapeutic potential of PI, particularly at the maximum dosage of 400 mg/kg. The overall findings substantiate the hypothesis that PI demonstrates capacity to reverse behavioral deficits induced by the disease model while modulating key genetic factors associated with underlying pathology.

## 4.2 Interpretation of Anxiolytic Behavior (Elevated Plus Maze)

The EPM assessment demonstrated dose-dependent anxiolytic effects of Ritonavir administration. The disease control cohort ($AlCl_3$) exhibited significantly reduced open arm exploration duration and increased closed arm preference compared to healthy controls, confirming the establishment of heightened anxiety-like behavioral states induced by the neurotoxic agent. Ritonavir treatment successfully ameliorated these behavioral deficits.

Notably, the Ritonavir 400mg/kg cohort demonstrated highly significant increases in open arm exploration duration, effectively restoring behavioral patterns to levels statistically comparable with the positive control, Donepezil (5mg/kg). This finding indicates that Ritonavir at its maximum effective dosage exhibits comparable anxiolytic efficacy to an established therapeutic agent. Lower Ritonavir concentrations (100mg/kg and 200mg/kg) demonstrated numerical

improvements that failed to achieve equivalent statistical significance compared to the 400mg/kg cohort, thereby establishing this elevated concentration as the optimal anxiolytic dosage.

### 4.3 Interpretation of Exploratory Behavior (Hole Board Test)

The Hole Board Test, which quantifies head-dipping frequency as an index of investigative curiosity and risk assessment, provided supportive evidence of Ritonavir's beneficial effects. Although the $AlCl_3$ disease model itself did not significantly impair head-dipping behavior relative to healthy controls, the Ritonavir 400mg/kg cohort exhibited significantly elevated head-dip frequency compared to the healthy control group.

This finding suggests that Ritonavir not only ameliorates pathological anxiety manifestations but may additionally promote non-anxious exploratory activity or general central nervous system stimulation. This enhancement in exploratory behavior represents a favorable outcome, demonstrating that Ritonavir does not induce sedative effects (which would reduce head-dipping) but actively enhances cognitive engagement and risk assessment behaviors.

### 4.4 Molecular Modulation of APOE4 Gene Expression

The most compelling findings relate to molecular mechanisms, specifically the modulation of APOE4 gene expression patterns. APOE4 constitutes a major genetic risk determinant for Alzheimer's disease, with expression levels frequently dysregulated in neurodegenerative conditions.

The analysis revealed a biphasic, dose-dependent response pattern:

**Lower Dosages (100 and 200mg/kg):** These concentrations produced highly significant APOE4 expression suppression, reducing mRNA levels substantially below healthy control baseline

values. While suppression might appear therapeutically beneficial, achieving expression levels markedly below normal physiological parameters warrants cautious interpretation regarding potential off-target effects.

**Optimal Dosage (400mg/kg):** The 400mg/kg cohort demonstrated APOE4 expression levels statistically similar to the healthy control group. This suggests that Ritonavir at this concentration functions not merely as a suppressor but as a molecular modulator, facilitating restoration of gene expression toward homeostatic, non-diseased states.

**Comparative Analysis with Donepezil:** The 400mg/kg Ritonavir cohort exhibited significantly lower APOE4 expression compared to the Donepezil-treated group, indicating superior capacity for normalizing this molecular marker relative to the reference pharmaceutical agent.

The convergence of behavioral data (optimal anxiolytic efficacy at 400mg/kg) with molecular findings (optimal gene modulation at 400mg/kg) strongly supports the conclusion that this concentration represents the most therapeutically relevant dosage.

## 4.5 Implication and Future Research

### 4.5.1 Implication of Findings

The findings from this investigation hold significant implications for the development of novel therapeutics targeting neurodegenerative disorders characterized by anxiety and cognitive decline. The dual-action profile of Ritonavir, providing robust anxiolytic relief comparable to established pharmacotherapies while simultaneously exerting favorable APOE4 gene modulation, positions it as a promising candidate for further development. The marked exploratory

enhancement observed in the Hole Board paradigm additionally suggests potential benefits for improving general cognitive function and behavioral engagement beyond mere anxiety reduction.

### 4.5.2 Future Research

To build upon these promising preliminary findings, future investigations should focus on:

**1. Mechanistic pathway elucidation:** Investigating the precise molecular mechanisms through which Ritonavir achieves APOE4 modulation, including analysis of transcription factor activity, epigenetic modifications, and post-transcriptional regulatory mechanisms.

**2. Extended cognitive assessment:** Utilizing more comprehensive behavioral paradigms, such as the Morris Water Maze or Y-Maze, to assess Ritonavir's effects on spatial learning, working memory, and long-term memory consolidation.

**3. Protein-level Analysis:** Complementing gene expression data with Western blot analysis to confirm whether APOE4 mRNA alterations translate to corresponding changes in protein concentrations and functional outcomes.

**4. Toxicological profiling:** Conducting comprehensive toxicological evaluation of the Ritonavir 400mg/kg dosage over extended treatment periods to confirm long-term safety and clinical viability for potential therapeutic application.

# CHAPTER FIVE

# CONCLUSION

## 5.1 Conclusion

Based on comprehensive behavioral and molecular analyses conducted within an $AlCl_3$-induced neurodegenerative model, this investigation concludes that Ritonavir possesses significant therapeutic potential, with effects predominantly mediated by the 400mg/kg dosage.

Behavioral findings from the Elevated Plus Maze paradigm demonstrate robust anxiolytic efficacy, wherein Ritonavir 400mg/kg treatment successfully reversed anxiety-like behavioral manifestations characteristic of the disease model, achieving therapeutic efficacy comparable to the standard positive control agent, Donepezil. Concurrently, the Hole Board Test confirmed that these beneficial effects were not attributable to general sedation; rather, the 400mg/kg dosage significantly enhanced exploratory behavior, suggesting favorable improvements in non-anxious activity and cognitive engagement.

At the molecular level, the investigation demonstrated that the 400mg/kg Ritonavir dosage functions as a beneficial modulator of APOE4 gene expression. While lower dosages induced excessive suppression, the optimal concentration restored APOE4 mRNA levels toward healthy control baseline values, indicating normalization of expression, a key therapeutic objective in Alzheimer's disease management.

In summary, the confluence of enhanced exploratory behavior, significant anxiolytic activity, and favorable modulation of a critical neurodegenerative genetic marker (APOE4) strongly supports the conclusion that Ritonavir, particularly at the 400mg/kg dosage, demonstrates

promise as a novel therapeutic agent for managing both anxiety-related symptoms and molecular deficits associated with neurodegenerative conditions.

## 5.2 Contribution to Knowledge

This research makes several significant contributions to the existing scientific literature:

1. **Establishment of Dual-Action Therapeutic Profile**: This investigation represents among the first to directly demonstrate a dual therapeutic role for the Protease Inhibitor Ritonavir, establishing direct linkage between its behavioral efficacy (anxiolysis) and molecular mechanism (APOE4 gene modulation).

2. **Precise Dosage Optimization**: The study definitively identifies the 400mg/kg concentration as the optimal therapeutic dosage, providing a critical reference point for future preclinical investigations by distinguishing between the suppressive effects of lower concentrations and the normalizing/modulatory effects of the maximum effective dosage.

3. **Novel Behavioral Insights**: The finding that Ritonavir enhances exploratory behavior (increased head-dipping frequency) beyond mere anxiety reduction provides novel insights into its potential utility for restoring general cognitive interest and functional engagement in compromised individuals.

4. **Advancement of Neuroprotective Research**: By demonstrating superior APOE4 modulation compared to the standard reference agent (Donepezil), this research establishes a new avenue for investigating protease inhibitors as potential disease-modifying agents

rather than merely symptomatic treatments, potentially opening new therapeutic paradigms in Alzheimer's disease management.

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## APPENDIX

## SAMPLE CALCULATION OF DOSES

### 1. RITONAVIR

A. Required Dose = 100mg/kg

Stock Solution = 10mg/ml

Weight of Mouse = 25.5g

Therefore, dose of Ritonavir required by a 25.5g mouse will be as follows;

(25.5 x 100)/1000 =2.55mg

Amount of Stock solution required will be;

(2.55 x 1)/10 = 0.255ml

Similar calculations were carried out in all mouse that received Ritonavir 100mg/kg

B. Required Dose = 200mg/kg

Stock solution = 20mg/ml

Weight of Mouse = 21.5g

Therefore, Dose of Ritonavir required by 21.5g mouse will be as follows;

(21.5 x 200)/1000 = 2.15mg

Amount of Stock Solution required will be;

(2.15 x 1)/20 = 0.215ml

Similar Calculations were carried out in all mouse that received Ritonavir 200mg/kg

C. Required Dose = 400mg/kg

Stock Solution = 40mg/ml

Weight of Mouse = 20.7g

Therefore, Dose of Ritonavir required by a 20.7g mouse will be as follows;

(20.7 x 400)/1000 = 8.28mg

Amount of Stock Solution Required will be;

(8.28 x 1)/40 = 0.207ml

Similar Calculations were carried out in all mouse that received Ritonavir 400mg/kg

### 2. DONEPEZIL

Required Dose = 5mg/kg

Stock Solution = 0.5mg/ml

Weight of Mouse = 25.5g

Therefore, Dose of Donepezil required by 25.5g mouse will be as follows;

(25.5 x 5)/1000 = 0.1275mg

Amount of Stock Solution required will be;

(0.1275 x 1)/0.5 = 0.255ml

Similar calculations were carried out in all mouse that received Donepezil 5mg/kg

**3. ASCORBIC ACID**

Required Dose = 100mg/kg

Stock Solution = 10mg/ml

Weight of Mouse = 25.5g

Therefore, Dose of Ascorbic Acid required by a 25.5g mouse will be as follows;

(25.5 x 100)/1000 = 2.55mg

Amount of Stock Solution required will be;

(0.1275 x 1)/10 = 0.255ml

Similar calculations were carried out in all mouse that received Ascorbic Acid 100mg/kg

**4. ALUMINIUM CHLORIDE**

Required Dose = 100mg/kg

Stock Solution = 10mg/ml

Weight of Mouse = 25.5g

Therefore, Dose of Aluminium Chloride required by a 25.5g mouse will be as follows;

(25.5 x 100)/1000 = 2.55mg

Amount of Stock Solution required will be;

(0.1275 x 1)/10 = 0.255ml

Similar calculations were carried out in all mouse that received aluminium Chloride 100mg/kg