



**NEUROTHERAPEUTIC POTENTIAL OF DELTA-9-
TETRAHYDROCANNABINOL ON D-GALACTOSE AND ALUMINIUM
CHLORIDE INDUCED ALZHEIMER-LIKE ANIMAL MODEL**

By

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**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Cognitive impairment and neurodegeneration have been well documented as a disorder of old age diseases including Alzheimer's disease (AD). D-galactose (D-gal) has been identified as senescence agent, whereas aluminium chloride (AlCl_3) is a neurotoxin implicated in the pathogenesis of AD. The cannabinoid agonist delta-9-tetrahydrocannabinol ($\Delta^9\text{THC}$) has evolved as a therapeutic compound for neurodegenerative diseases. The present study has been designed to explore the neurotherapeutic potential of $\Delta^9\text{THC}$ on cognition, brain histology and neurogenesis activity in Wistar rats induced by D-gal and AlCl_3 . The expression of AD pathological hallmarks was also been evaluated. Male albino Wistar rats were injected intraperitoneally with 60 mg/kg D-gal and orally with 200 mg/kg AlCl_3 , once daily for 10 consecutive weeks. After 10 weeks, $\Delta^9\text{THC}$ at 0.75, 1.5 and 3.0 mg/kg and 1 mg/kg donepezil were administered intraperitoneally for 28 days as a part of treatment phase. Behavioural assessments such as novel object recognition (NOR), Morris water maze (MWM), elevated plus maze (EPM) and modified elevated plus maze (mEPM) were evaluated, along with histological examination of the hippocampus. Additionally, neurogenesis markers (GFAP, DCX, NeUN and Calbindin) and AD pathological hallmark markers (BACE 1, APP, p-tau Thr181 and p-tau Thr231) were evaluated in the hippocampus. The results revealed that rats treated with 60 mg/kg D-gal + 200 mg/kg AlCl_3 exhibited cognitive impairment in both spatial and non-spatial learning and memory test, associated with neurodegeneration as evidenced by a decrease in the number of survival pyramidal cells and granule cells ($p < 0.05$), as well as fluctuation in the thickness layer of the hippocampus ($p < 0.05$). It also caused marked decreased in all neurogenesis markers ($p < 0.05$). The expression of BACE1, APP, p-tau Thr181 and p-tau Thr231 were also increased significantly ($p < 0.05$). Treatment with $\Delta^9\text{THC}$, with irrespective of doses, alleviated the D-gal + AlCl_3 induced AD-like pathology in rats. These include improved spatial learning and memory as measured by behavioural tests ($p < 0.05$), ameliorates from pyramidal and granule cell loss ($p < 0.05$) and increased

thickness layer of the hippocampus ($p < 0.05$). Further, Δ^9 THC enhances neurogenesis activity by marked increase of all neurogenesis markers. The expression of AD-related pathological hallmarks was also suppressed by Δ^9 THC. These findings provide scientific evidence and open the opportunity to use cannabinoid agonist (Δ^9 THC) as a potential treatment against AD.

Keywords: Alzheimer's disease, Δ^9 THC, hippocampus, behavioral changes, neurogenesis

SDG: Goal 3 (Good Health and Well-being)



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

POTENSI TERAPEUTIK NEURO DELTA-9-TETRAHYDROCANNABINOL PADA MODEL HAIWAN SEPerti ALZHEIMER YANG DIARUH OLEH D-GALAKTOSA DAN ALUMINIUM KlorIDA

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Kemerosotan kognitif dan kemerosotan saraf telah banyak dilaporkan dalam penyakit yang berkaitan dengan usia tua, seperti penyakit Alzheimer (AD). D-galaktos (D-gal) telah diklasifikasikan sebagai agen penuaan, manakala aluminium klorida (AlCl_3) ialah neurotoksin yang terlibat dalam patogenesis AD. Kanabinoid agonis delta-9-tetrahydrocannabinol ($\Delta^9\text{THC}$) telah berkembang sebagai sebatian terapeutik untuk penyakit neurodegeneratif. Kajian ini telah dibuat untuk meneroka potensi terapeutik neuro $\Delta^9\text{THC}$ terhadap kognisi, histologi otak dan aktiviti neurogenesis kepada tikus yang diaruh oleh D-gal dan AlCl_3 . Ekspresi ciri patologi AD juga telah dinilai. Tikus albino Wistar jantan telah disuntik (i.p) dengan 60 mg/kg D-gal dan 200 mg/kg aluminium klorida secara oral, sehari sekali untuk 10 minggu berturut-turut. Selepas 10 minggu, $\Delta^9\text{THC}$ dos 0.75, 1.5 dan 3.0 mg/kg, dan 1.0 mg/kg donepezil disuntik (i.p) untuk 28 hari, iaitu sebahagian daripada fasa rawatan. Penilaian tingkahlaku seperti pengecaman objek novel (NOR), ujian air berselirat Morris (MWM), berselirat tambah tinggi (EPM) dan berselirat tambah tinggi diubah suai (mEPM) dibuat, bersama-sama dengan pemeriksaan histologi hipokampus. Tambahan pula, penanda aktiviti neurogenesis (GFAP, DCX, NeUN dan calbindin) dan penanda ciri khas patologi AD (BACE1 APP, p-tau Thr81 dan p-tau Thr231) dinilai juga. Keputusan ujian menunjukkan bahawa tikus yang dirawat dengan 60 mg/kg D-gal dan 200 mg/kg AlCl_3 mempamerkan kemerosotan kognitif dalam pembelajaran, dan ujian ingatan spatial dan bukan spatial, yang dikaitkan dengan neurodegeneratif seperti yang dibuktikan oleh penurunan bilangan sel piramidal dan sel granul hidup ($p < 0.05$), serta penurunan lapisan ketebalan hipokampus ($p < 0.05$). Ia juga menyebabkan penurunan yang ketara di dalam semua penanda neurogenesis ($p < 0.05$). Ekspresi BACE1, APP, p-tau Thr181 and p-tau Thr231 juga meningkat dengan ketara. Rawatan dengan $\Delta^9\text{THC}$, tanpa mengira dos, mengurangkan patologi AD ke atas tikus yang disebabkan oleh D-gal dan AlCl_3 . Ia termasuklah peningkatan pembelajaran spatial dan ingatan yang lebih baik seperti yang diukur oleh ujian tingkah laku ($p < 0.05$), membaiki daripada kehilangan sel piramidal dan granul ($p < 0.05$) dan peningkatan lapisan ketebalan hippocampus ($p < 0.05$).

Seterusnya, Δ^9 THC meningkatkan aktiviti neurogenesis dengan peningkatan ketara semua penanda neurogenesis. Ekspresi ciri patologi yang berkaitan dengan AD juga ditindas oleh Δ^9 THC. Penemuan ini memberikan bukti saintifik dan membuka peluang untuk menggunakan agonis kanabinoid (Δ^9 THC) sebagai rawatan yang berpotensi terhadap AD.

Kata Kunci: Penyakit Alzheimer, Δ^9 THC, hipokampus, perubahan tingkahlaku, neurogenesis

SDG: Matlamat 3 (Kesihatan dan Kesejahteraan yang Baik)



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LIST OF ABBREVIATIONS

Δ	Delta
Δ^9 THC	Delta-9-tetrahydrocannabinol
11-OH-THC	11-carboxy-THC
5-HT	Serotonin
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEIs	Acetylcholinesterase inhibitor
AD	Alzheimer's disease
ADI	Alzheimer's disease International
AGEs	Advanced glycation end products
Al	Aluminium
AlCl ₃	Aluminium chloride
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
ApoE2	Apolipoprotein E2
ApoE3	Apolipoprotein E3
ApoE4	Apolipoprotein E4
APP	Amyloid precursor protein
APPs β	Soluble beta amyloid precursor protein
A β	Beta Amyloid
A β 1-40	Beta amyloid 1-40
A β 1-42	Beta amyloid 1-42
A β PP	Amyloid beta precursor protein
BACE-1	Beta-site APP cleaving enzyme-1
BSA	Bovine serum albumin

CA1	Cornu ammonis 1
CA2	Cornu ammonis 2
CA3	Cornu ammonis 3
CAA	Cerebral amyloid angiopathy
CB1	Cannabinoid receptor 1
CBD	Cannabidiol
CBN	Cannabinol
ChE	Cholinesterase
CNS	Central nervous system
CRP	C-reactive protein
CVD	Cardiovascular disease
DAB	Diaminobenzidine
DCX	Doublecortin
DG	Dentate gyrus
D-gal	D-galactose
DI	Discrimination index
DPX	Dibutyl phthalate
EA	Enclosed arm
EPM	Elevated plus maze
FAD	Familial Alzheimer's disease
GABA	γ -aminobutyric acid
GCL	Granular cell layer
GFAP	Glial Fibrillary Acidic Protein
Glu	Glutamate
GSK-3	Glycogen synthase kinase 3
GSK-3 β	Glycogen Synthase Kinase 3 β
H&E	Hematoxylin and Eosin

i.p	Intraperitoneal
IL-6	Interleukin 6
ITL	Initial transfer latency
Kg	Kilogram
MAP	Microtubule – associated protein
mEPM	Modified elevated plus maze
ML	Molecular layer
MWM	Morris water maze
NeUN	Neuronal nuclear antigen
NFTs	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
NOR	Novel object recognition
NSC	Neural stem cells
OA	Open arm
OFT	Open field test
PBS	Phosphate buffer saline
PCs	Progenitor cells
PHF	Paired helical filament
PSEN 1	Presenilin 1
PSEN 2	Presenilin 2
p-tau	Phosphorylated tau
SAD	Sporadic Alzheimer's disease
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SGZ	Sub granular zone
SP	Senile plaques
T2D	Type-2-diabetes

TBI	Traumatic brain injury
TEMED	N,N,N,N-tetramethylenediamine
TL1	First transfer latency
TNF- α	Tumour necrosis factor alpha
WHO	World Health Organization



CHAPTER 1

INTRODUCTION

1.1 Background

Alzheimer's disease (AD) is an irreversible, progressive and age-dependent neurodegenerative disease, which is associated with memory and cognitive impairment (Zakaria et al., 2017). It accounts for more than 60% of cases and affect more than 36 million people worldwide (Benefits, 2018). There are two types of AD; familial and sporadic. The most prevalent type of AD is sporadic, and its aetiology is unknown (Flamier et al., 2018). Although familial and sporadic AD differ in their causes, the progression of the disease appears to be the same. Both types of AD are characterized by presence of senile plaques (SPs), which are primarily composed of various species of β -amyloid ($A\beta$) peptide, and neurofibrillary tangles (NFTs), which are primarily composed of hyperphosphorylated tau proteins (Chiroma et al., 2018). The disease frequently manifests as a decline in short term memory. Symptoms include language difficulties, sleep disturbances, disorientation, mood swing and psychosis as the disease advances. Apart from its numerous behavioural and cognitive deterioration, AD also defined as a disturbance of neurotransmitters levels in the brain, which include acetylcholine (ACh), glutamate (Glu), serotonin (5-HT) and γ -aminobutyric acid (GABA) (Prakash et al., 2014). The disease eventually leads to loss of bodily function, and ultimately, death (Rafii & Aisen, 2015).

Utilization of cannabis for medical purposes has expanded in recent years (Bridgeman and Abazia, 2017). Increased interest in cannabis for medical purposes has been accompanied by renewed scientific interest in substances found in the cannabis plant, specifically cannabinoid (Carliner et al., 2017). Delta-9-tetrahydrocannabinol (Δ^9 THC) which is cannabinoid agonist, is the primary psychoactive compound of *Cannabis sativa* plant (Whiting et al., 2015). Pharmacologically, Δ^9 THC is thought to affect cognition and memory, reward, sensory perception, and behavioural attention (Calabrese & Rubio-Casillas, 2018). It has previously been extensively researched as an anti-emetic, anti-convulsive, anti-inflammatory and analgesic (Eubanks et al., 2006). A number of research have been conducted successfully to investigate the beneficial effect of Δ^9 THC, particularly in the treatment of neurodegenerative diseases (Cao et al., 2014).

According to a study done by Suliman et al. (2018), *in-vivo* findings showed that Δ^9 THC at 0.75 mg/kg significantly increase neurogenesis in the hippocampus of normal aged rats. A similar finding was recently reported in old mice, where a chronic low dose of Δ^9 THC (1.5 mg/kg) increased hippocampal spine density, thus restoring the cognitive function (Bilkei-Gorzo et al., 2017). Furthermore, a four-week treatment with low dose of Δ^9 THC (3 mg/kg per day) showed an improvement of cognitive performance in elder mice (Nidadavolu et al., 2021). Δ^9 THC can also promote the removal of $A\beta$ accumulation in neurons and inhibit the inflammatory response (Currais et al., 2016). Recent research has shown that Δ^9 THC is used to accelerates the removal of toxic clumps of $A\beta$ in the brain, which are thought to trigger the AD progression (Calabrese & Rubio-

Casillas, 2018; Kaplan & Pazarci, 2017). The complete pathway and mechanism of action of the cannabinoid system against AD are currently unknown, however, previous research has demonstrated the involvement of cannabinoid receptor 1 (CB₁) (Campbell & Gowran, 2007). CB₁ receptors are abundant in the brain and involved in learning, memory and cognitive processes that are disrupted early in the course of AD (Riedel & Davies, 2005). It has also been claimed that the cannabinoid system can suppress the neurodegenerative processes that drive the disease progression (Cao et al., 2014). Early research suggests that an oil-based solution of Δ^9 THC improved disturbed behaviour and has neuroprotective properties through interaction with cannabinoid receptors (Jackson et al., 2005).

For several years, mouse models of ageing induced by the combination administration of D-galactose (D-gal) and aluminium chloride (AlCl₃) have been widely utilised to investigate the mechanism of AD (Wei et al., 2017; Luo et al., 2009). D-gal is a senescence agent, whereas AlCl₃ is a neurotoxin linked to the pathogenesis of AD. Mouse exposed with these chemicals have shown a significant decline in cognitive performances, aberrant changes in the structure of hippocampal tissues, accumulation of toxic protein associated with AD, and impairment of the cholinergic system (Awad et al., 2023). Additionally, previous research by Chiroma et al. (2018) also demonstrated that combining these two chemicals can cause cognitive deterioration in rat models, mimicking the changes seen in AD patient. The findings revealed an increased acetylcholinesterase (AChE) activities, high BACE-1 protein expression as well as high phosphorylated tau protein (p-tau). However, there is no evidence that Δ^9 THC can reverse the neurotoxicity and cognitive impairment caused by the combination of D-gal and AlCl₃ in AD-like rat model.

1.2 Problem Statement

AD is the most common type of dementia which accounting for 60-80% of all dementia cases, and imposing a significant economic burden on society (Bridgeman and Abazia, 2017; Duthey & Ph, 2013). According to the World Health Organization (WHO), approximately 55 million people worldwide have dementia, with this figure expected to rise to 78 million in 2030 and 139 million in 2050 (Alzheimer's Association, 2022). Malaysia is expected to become an ageing nation, with the proportion of people 60 and older exceeding 15.3% of the total population in 2030, based on population projections from 2010 to 2040 (Ho, 2018). National Health and Morbidity (2018) stated that the overall prevalence of probable dementia in Malaysia was 8.5% (Sodri et al., 2022). This prevalence is expected to rise over time if there is no intervention taken.

Therapies for AD are remain limited, particularly in terms of slowing or the disease progression. There are currently four approved drugs; three of which are acetylcholinesterase inhibitors and one of which is a N-methyl-D-aspartate (NMDA) receptor antagonist (Ramirez-Bermudez, 2012). Donepezil, memantine, rivastigmine and galantamine are common medications used to treat the symptoms of AD. Existing treatments are symptomatic, aimed at improving cognitive function and allowing the brain to compensate for neuron loss (Reynish et al., 2006). However, none of these treatments are capable of reversing the disease progression; rather they treat disease

symptoms with limited efficacy and produce negative side effects such as stroke, diarrhoea, vomiting and insomnia. This condition has created a demand and urgent need for discovery of novel targets and compounds in order to develop new approaches or strategies for reversing the disease process by targeting the various aspects of AD pathology.

Compounds with pleiotropic activity, which can simultaneously target multiple processes in AD has gained significant attention in the drug development. Compound that can address several of AD processes are believed to provide greater benefits than existing therapies. Thus, Δ^9 THC at non-psychoactive doses has been gained interest to be explored in this present study, which may enhance the therapeutic effect and potentially slow down the AD progression. This study aims to investigate the possible therapeutic benefit of Δ^9 THC by examining cognitive performances, histomorphological changes, neurogenesis activity and Δ^9 THC's ability to reduce A β and tau phosphorylated protein. The findings from this study would provide insightful and exhaustive data on the usage of Δ^9 THC as a potential drug for suppressing the progression of disease. Therefore, it could serve as a foundation for the development of Δ^9 THC as therapeutic approach, thereby enhancing our understanding of clinical settings.

1.3 Hypothesis

- H₀: Treatment with Δ^9 THC did not restore the cognitive performances and reverse morphological changes, inhibit neurogenesis and promote pathological hallmark markers of AD in rats exposed to D-gal and AlCl₃
- H_A: Treatment of Δ^9 THC would restore the cognitive performances, restore the morphological alteration, promote neurogenesis and suppressing pathological hallmark markers of AD in rats exposed to D-gal and AlCl₃

1.4 Objectives of the Study

1.4.1 General Research Objective

To investigate the neurotherapeutic potential of Δ^9 THC on D-gal and AlCl₃ induced AD-like animal model

1.4.2 Specific Research Objectives

1. To determine the neurotherapeutic potential of Δ^9 THC on cognitive performances and anxiolytic properties in rats after D-gal and AlCl₃ exposure.
2. To investigate the neurotherapeutic potential of Δ^9 THC on histomorphological changes in hippocampus of rats after D-gal and AlCl₃ exposure.

3. To evaluate the neurotherapeutic potential of Δ^9 THC on neurogenesis markers in rats after D-gal and AlCl_3 exposure
4. To determine the role of Δ^9 THC in modulating neuropathological hallmark markers of AD after D-gal and AlCl_3 exposure through molecular level



CHAPTER 2

LITERATURE REVIEW

2.1 Background of Alzheimer's disease

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease that degrades memory and cognitive functions over time, impairing the ability to perform daily tasks (Wilson et al., 2012; Fischer et al., 2008). The current global dementia population is estimated to be 55 million people (Alzheimer's Association, 2022). Both genetic and environmental factors influence the risk of developing the disease. This is a massive societal and economic challenge, with ramifications for those living with AD, as well as their family. If current trends persist, the global cost of dementia, including AD is projected to reach US\$2 trillion by 2030, totalling US\$1 trillion in 2018 (Patterson, 2018).

AD is characterized by memory loss, learning difficulty, poor problem-solving skills, language breakdown, behavioural issues such as agitation and sleep changes, and confusion (Waldemar et al., 2007). These alterations occur as the result of neuron destruction and massive synaptic loss in the part of brain which responsible for cognitive functions (Alzheimer Association, 2018). Pathologically, extracellular deposits of beta amyloid ($A\beta$) protein fragments known as senile plaques (SPs) and intracellular twisted strands of tau protein known as neurofibrillary tangles (NFTs) in the brains are the major hallmarks of AD (Kamat, 2015). Patients in the final stages of AD eventually die as a result of other complications associated with the disease. There is presently no treatment for AD, despite its high prevalence and enormous economic burden. Since then, AD has become an important research topic, particularly in terms of how it can be prevented or delayed (Gaugler et al., 2016).

2.2 Types of Alzheimer's disease

2.2.1 Early onset (Familial) Alzheimer's disease

Early onset (Familial) AD is a condition characterized by dementia in people under the age of 65 years with a positive family history, accounting for about 5% of all AD cases and involving at least 230 mutations in three genes coding for proteins (Lleó et al., 2002). The three major causative genes discovered are the amyloid precursor protein (APP) gene on chromosome 21, the presenilin 1 (PSEN1) gene on chromosome 14, and the presenilin 2 (PSEN2) gene on chromosome 1 (Jadhav et al., 2019; Wallonet et al., 2017). These genes were thought to be associated with the $A\beta$ peptide found in senile plaques (SPs) within the brain of people affected with AD (Cheignon et al., 2018). Mutation in these three genes lead to excessive production and accumulation of $A\beta$ (toxic form), which in turn aggregates to form SPs, thus disrupting the neuronal messaging and eventually resulting in neurodegeneration (Wallon et al., 2017; Zhanget al., 2001). Increased toxic $A\beta$ also causes a cascade of pathological processes such as tau hyperphosphorylation,

neurofibrillary tangle formation (NFTs), neuroinflammation and synaptic function loss (J. Hardy & Selkoe, 2002). Certain mutations, on the other hand, are distinguished by distinct neuropathological features, such as severe cerebral amyloid angiopathy (CAA) particularly in APP mutation (Shepherd et al., 2009), and the presence of Lewy bodies in PSEN1 and PSEN2 mutation (Piscopo et al., 2008; Ishikawa et al., 2005). The clinical features of familialAD are heterogeneous, due to different genetic mutations (Larner & Doran, 2006).

2.2.2 Late onset (Sporadic) Alzheimer's disease

Late onset (sporadic) AD is the most common type of AD diagnosed, and there is no known cause (Flamier et al., 2018). It usually affects patients over the age of 65. In contrast, the familial form of AD, which is hereditary, has yet to identify the specific gene involved. Research has shown that apolipoprotein E (apoE) gene on chromosome19 increases the likelihood of acquiring sporadic AD (Poirier, 2003). Several alleles of apoE have been identified, however, only three are the most prevalent; apoE2, apoE3 and apoE4 (Di Battista et al., 2016). Among these three, apoE4 was the most often discovered gene that raised the risk of sporadic AD in around 40% of individuals (Johnson et al., 2014). ApoE has been linked to neuronal growth, tissue repair, nerve regeneration and immunoregulation (Van Cauwenberghe et al., 2016). According to studies, apoE alleles are inherited from one's parent. Inheriting one or two copies of allele E4 from parents greatly increase the chance of developing sporadic AD in 2-3 times (Corder et al., 1993), but it is not guarantee that the person will develop AD. The disruption of A β metabolism in amyloid cascade is the other mechanism responsible for sporadic AD. Beta-site APP cleaving enzyme 1 (BACE 1), an enzyme involved in A β metabolism has been identified to be a significant risk factor for development of sporadic AD (Hébert et al., 2008).

2.3 Risk factors of Alzheimer's disease

2.3.1 Age

The most important risk factor for developing AD is age. The majority of cases occur in people over the age of 65 (Alzheimer Association, 2018). Approximately, 5% old people aged 65 to 74 have AD, whereas the risk of having the disease increases to 50% for those over the age of 85 (Patterson, 2018). As we age, our body's capacity to mend itself becomes less effective. Our brains' ability to self-repair varies between individuals, and these differences contribute to an individual's susceptibility to AD (Alzheimer Association, 2010).

2.3.2 Genetics

In genetics, a mutation is an aberrant alteration that occur in the chemical sequence of the genes (Goldman et al., 2011). Rare occurrences of AD with an early onset (familial) have been linked to mutations in genes on chromosome 1, 14 and 21. If the chromosome is inherited, there is a 50% chance that offspring will get an AD (American Psychiatric

Association, 2018). Late-onset (sporadic) AD does not exhibit a regular pattern of inheritance; nonetheless, a link between the development of AD and ApoE gene has been identified, with the ApoE4 gene demonstrated to increase the risk of developing AD (Bethune et al., 2010). Additionally, people with ApoE2 or ApoE3 variants have low chances of developing AD than those with ApoE4 (Spinney, 2014).

2.3.3 Gender

Due to the changes in hormones that occur at menopause, particularly oestrogen, women are more likely to develop AD than males. It was previously believed that the reduction of these hormones increases likelihood of developing AD. However, hormonal changes are not the primary contributor to the increased prevalence of AD among women (Alzheimer Association, 2010).

2.3.4 Cardiovascular disease (CVD)

It has been proposed that CVD increases the likelihood of developing AD (Love & Miners, 2016). Hypertension, atrial fibrillation, atherosclerosis, and hypercholesterolemia are some of the common risk factors shared by CVD and AD (Vijayan & Reddy, 2016). The mechanisms by which CVD is thought to cause AD are unknown, but high cholesterol levels play a significant role and are consistently linked to the disease. High cholesterol levels have been associated to increased A β levels, causes cognitive impairment. Cholesterol also appears to impair A β degradation (Barbero-Camps et al., 2018).

2.3.5 Type 2 Diabetes (T2D)

T2D is thought to increase the risk of AD by approximately 60% when compared to individuals who do not have diabetes (Chatterjee et al., 2016). T2D and AD have a strong epidemiological link, with some researchers classifying AD as Type 3 diabetes (Li et al., 2015). The scientific literature suggests that there are links between insulin resistance and neurodegenerative processes in the brain, leading to cognitive decline and development of AD (Baglietto-Vargas et al., 2016). Furthermore, insulin is involved in the pathology of AD by activating GSK-3, which causes phosphorylation of tau protein, and aggregates to form NFTs. Insulin also causes in the formation of senile plaques (Pugazhenthil et al., 2017).

2.3.6 Traumatic Brain Injury (TBI)

Recent study suggests that TBI is another risk factor for AD. TBI shares the same molecular pathways identified in AD that contribute to synapse degradation, and ultimately neuronal death in brain regions. Moreover, it may precipitate the onset of cognitive and memory impairment that progress to AD (Blennow et al., 2016).

2.3.7 Other risk factors

A history of clinical depression, stress, and insufficient brain exercise. Smoking, excessive alcohol usage and drug abuse are less well known risk factors (Edwards et al., 2019).

2.4 Pathophysiology of Alzheimer's disease

Two pathological hallmarks have been observed in the brain of individuals with AD; extracellular senile plaques (SPs) and intracellular neurofibrillary tangles (NFTs) (Lane et al, 2018). The plaques are made up of β -amyloid peptides ($A\beta$), whereas the NFTs are made up of the tau protein. The pathophysiology of AD is linked to neuronal injury and death, which begins in the hippocampus brain area that involved in learning and memory processes (Shaffer et al., 2013). As the disease progresses, the brain shrinkage occurred due to neuronal atrophy (Lane et al., 2018).

$A\beta$ is a short peptide that is an aberrant proteolytic by product of the transmembrane protein amyloid precursor (APP) (Vasto et al., 2008). Neurotoxic forms of $A\beta$ peptide ($A\beta_{40}$ or $A\beta_{42}$) are generated by the sequential cleavage of APP initially by β -site APP-cleaving enzyme 1 (BACE1) followed by cleavage via γ -secretase (Cole & Vassar, 2007). Excessive production and accumulation of these $A\beta$ lead to the formation of senile plaques, thereby affecting the inter-connecting neurons and triggering an inflammation cascade that ultimately damages the brain cells, resulting in apoptosis (Sierra-Fonseca & Gosselink, 2018; Heneka et al., 2015). In addition, excitotoxicity also affects $A\beta$ clearance in AD (Mhyre et al., 2012).

On the other hand, aberrant tau protein aggregation is also detected. Tau protein is a microtubule-associated protein that helps to maintain neuronal structure, axonal transport and synaptic plasticity (Fuster-Matanzo et al., 2012; Dixit et al., 2008). Tau is normally controlled by phosphorylation and dephosphorylation. In AD, however, hyperphosphorylated tau (p-tau) accumulates as paired helical filaments (PHFs) that aggregate into NFTs (Roda et al., 2022; Goedert et al., 2006). NFTs formation results in massive synaptic loss and disrupts cognitive functions (Roda et al., 2022; Revett et al., 2013; Mayeux & Stern, 2012). NFTs are first observed in the hippocampus and cerebral cortex of the brain (Kumar & Hassan, 2022).

Most ongoing efforts to discover new therapies are highlighted on disease modification and disease progression; the underlying ideas is that if the onset of the disease can be delayed, or its progression can be hindered. To date, approaches have largely focussed around intervention in the $A\beta$ cascade and tau biology. The other category, regenerating a diseased brain and replacing lost neurons remains very much limited explored (Mufson et al., 2016).

2.5 Causative hypotheses of Alzheimer's disease

Over the years, numerous hypotheses regarding the cause of AD have been developed including amyloid beta, tau, cholinergic dysfunction, glutamate dysfunction, oxidative stress and neuronal damage. On the basis of these theories, many efforts have been made to develop anti-drugs AD. In this section, we highlighted several hypotheses to be discussed.

2.5.1 Cholinergic hypothesis

Cholinergic hypothesis was the first hypothesis proposed to explain the pathogenesis of AD (Contestabile, 2011). According to the cholinergic hypothesis, AD could be caused by a severe decline of cholinergic function in the central nervous system (CNS) (Bartus, 2000). The hypothesis was based on the observation of cholinergic activity loss in experimental animal studies and in the brain of AD patients, which suggested a role of ACh in learning and memory processes (Bartus, 2000). Based on this hypothesis, cholinesterase inhibitors such as donepezil, rivastigmine and tacrine have been approved for the treatment of AD. It has the ability to inhibit acetylcholinesterase, an enzyme that breakdown the ACh neurotransmitter, thus reversing the cognitive dysfunction seen in AD patients (Craig et al., 2011).

2.5.2 Amyloid cascade hypothesis

The amyloid hypothesis of AD was first proposed in the early 1990's (J. A. Hardy & Higgins, 1992). According to this theory, the accumulation of A β peptides into senile plaques in the brain, either due to an increase in production or decrease in clearance, is core feature in the pathogenesis of AD (J. A. Hardy & Higgins, 1992). The production of A β in the brain of AD patients begin with the abnormal cleavage of APP by BACE1 through amyloidogenic pathway, leading to the formation of C-terminal fragment known as C99 transmembrane, which is soluble beta amyloid precursor protein (APPs β). The APPs β are then cleaved into 40-43 amino acid A β peptide which is thought to be toxic and cause neurodegeneration (Rogaeva et al., 2007) (Figure 2.1).

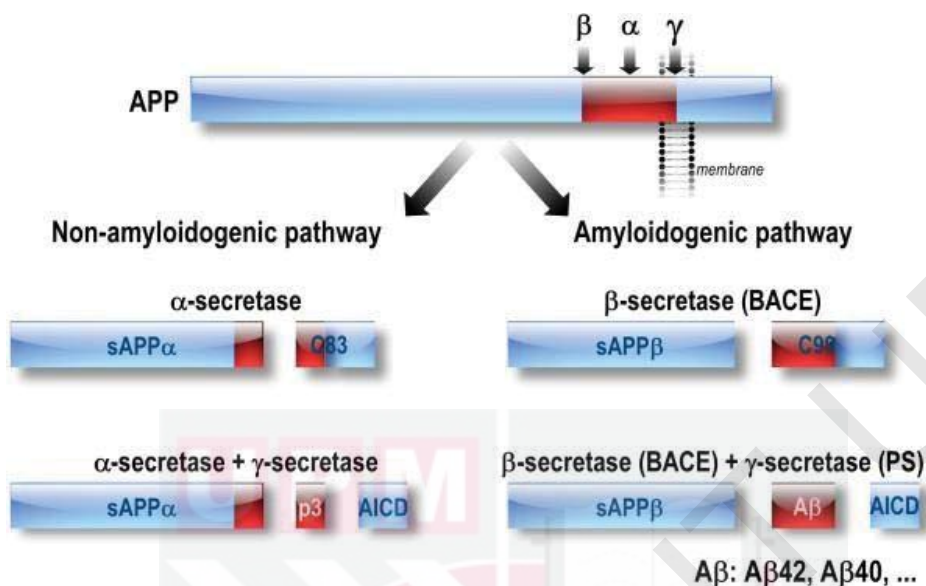


Figure 2.1 : Beta amyloid hypothesis. Through amyloidogenic pathway, abnormal cleavage of APP by BACE1, resulted to the formation of C99 trans-membrane protein and further cleaved by γ -secretase forming A β peptides, which aggregates into plaques. APP-Amyloid precursor protein; BACE1-beta-secretase enzyme; β -beta; γ -gamma; α -alpha
(Source: Del Prete et al., 2014)

2.5.3 Glutamatergic hypothesis

Glutamate serves as the principle excitatory neurotransmitter within the central nervous system (CNS). Glutamate regulates neuronal plasticity, neural transmission, learning and memory processes (Kocahan et al., 2013). Animal studies have shown that glutamate is neurotoxic, leading to the development of lesions that are clinically similar to the human neurodegenerative disorders (Maragos et al., 1987). High densities of glutamatergic neurons are present in the AD-affected tissues, and changes in glutamate signalling play a key role in the development of the disease (Wenk et al., 2006; Francis, 2005).

N-methyl-D-aspartate (NMDA) is essential for synaptic plasticity and is thought to strengthen cognitive abilities (Paoletti et al., 2013). Recently, it was discovered that NMDA activation is linked to AD-like synaptic dysfunction (Kodis et al., 2018). Prolonged Ca^{2+} elevation results in a slow deterioration of synaptic function, which leads to the demise of neuronal cell, that clinically associated with a progressive decline in cognitive abilities. This decline is commonly observed in people diagnosed with AD (Liu et al., 2019). According to glutamate dysfunction hypothesis, memantine is a non-competitive NMDA receptor antagonist used for neuroprotection in the treatment of moderate to severe AD. It provides relief and improves quality of life in AD patients, but it does not prevent agitation or total brain atrophy (Fox et al., 2012).

2.5.4 Tau hypothesis

Tau is a microtubule-associated protein (MAP) that is extensively expressed in the peripheral nervous system and present in the most of tissues. It is an essential component of the cytoskeleton in neuron (Plouffe et al., 2012). Under normal physiological condition, tau undergoes a constant cycle of attachment and detachment from microtubules via phosphorylation by kinase and dephosphorylation by phosphatase, resulting in a continuous dynamic equilibrium that ensures efficient axonal transport (Kosik, 2006). In AD, however, hyperphosphorylation occurred (Lace et al., 2007). This situation would disrupt axonal transport and synaptic metabolism, resulting in dysfunctions that lead to neuronal cell death and loss of cell viability (Wang et al., 2019). Additionally, tau protein hyperphosphorylation causes the proteins to detach from the microtubules and aggregate into PHFs within the neuron, forming NFTs (Noble et al., 2013; Behl, 2012) (Figure 2.2).

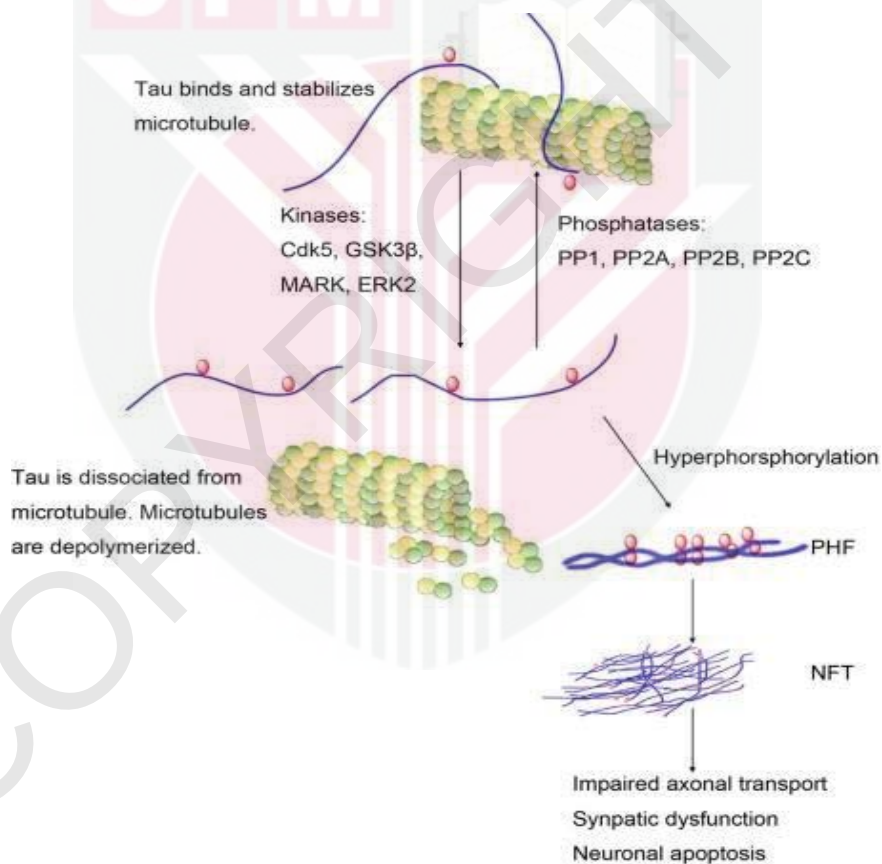


Figure 2.2 : Tau hypothesis. Increasing in the kinase activity and decreasing in the phosphatase activity causes tau hyperphosphorylation. Hyperphosphorylated tau protein is misfolded forming PHFs that are the precursors of NFTs seen in AD. PHFs- paired helical filaments; NFTs- neurofibrillary tangles
(Source: Duan et al., 2012)

2.5.5 Inflammation hypothesis

Although tangles and plaques are well-known hallmarks of AD, the disease brain exhibits additional symptoms which contribute to neurodegeneration. Research study done by Soscia et al. (2010) concluded that A β peptide is engage in immunity and support the role of inflammation in the aetiology of AD. Recent investigations have also shown that neuroinflammation is a characteristic of AD. Microglia-related pathways were found to be a significant AD risk factor (Bolós et al., 2017; Song et al., 2017). It is believed that these pathological changes induce the activation of glial cell, leading to the secretion of proinflammatory cytokines, such as tumour necrosis factor alpha (TNF- α) and interleukin (IL-6), as well as inflammation reactive proteins such as C-reactive protein (CRP) (Chiroma et al., 2018). The elevated concentration of proinflammatory cytokines and CRP might potentially activate glial cells by autocrine or paracrine mechanisms, or both. This activation may lead to an increased production of phosphorylated tau (p-tau) and A β peptide, thus triggering the neurodegeneration (Figure 2.3). Neurogenesis and plasticity are recent approaches to elucidating these pathways, thereby allowing for the exploration of AD treatment interventions (Jevtic et al., 2017).

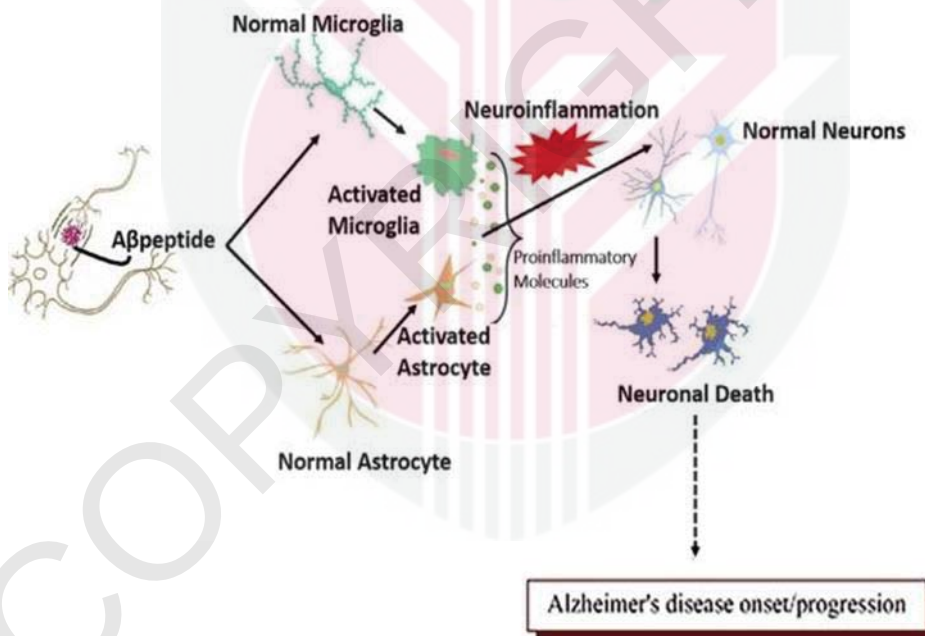


Figure 2.3 : Possible role of microglia or astrocytes in the progression of AD pathology through cytokines-mediated neuroinflammation. Inflammatory stimuli (A β) activate glial cells and astrocytes which in turn produce proinflammatory mediators and inflammatory reaction proteins that have the ability to stimulate more production of A β as well as p-tau. This continuous process promotes neurodegeneration. A β - beta amyloid
(Source: Ahmad et al., 2019)

2.5.6 Oxidative stress hypothesis

Previous research has shown that increasing in oxidative stress is considered to be an early event in AD pathology, as it contributes to membrane damage, cytoskeleton alteration and cell death (Arimon et al., 2020). A large number of oxidatively modified protein in AD animal models and human AD, suggest that oxidative stress plays an important role in AD pathogenesis (Tamagno et al., 2021). Research done by Saeedi et al. (2021) demonstrated that oxidative stress mediates the accumulation of A β , by increasing BACE1 activity. Furthermore, it has been shown that oxidative stress increases the activity of γ -secretase both in-vivo and in-vitro, which mediates hyper production of A β . Another research also demonstrates a correlation between the induction of oxidative stress and the increase of γ -secretase cleavage on APP (Masand et al., 2018).

2.6 Approved therapies

There is no cure for AD. The current pharmacological therapy for AD offers only temporary benefits for a duration of six to eighteen months (Huang et al., 2023). At present, there are four approved drug available to treat AD, which includes donepezil, galantamine, rivastigmine and memantine (Cooray et al., 2020). The approved medicines have limited effects, as it can control the symptoms, however it cannot reduce the pace of its progression or stop the disease (Kim et al., 2018). Moreover, despite an extensive amount of study, the treatments for AD still have limitations and do not deal with the fundamental causes of the disease. These treatments aimed at improving cognitive function through two distinct mechanisms; cholinergic system agonism and N-methyl-D-aspartate receptor (NMDA-receptor) antagonism. Both types of medication are well tolerated by AD patients with mild adverse effects (Korolev, 2014).

2.6.1 Cholinesterase (ChE) Inhibitor

Currently, FDA has approved three cholinesterase (ChE) inhibitors; rivastigmine, donepezil and galantamine (Korolev, 2014). ChE inhibitors are the type of medication that works by inhibiting cholinesterase, an enzyme that degrades the neurotransmitter ACh. Because AD is associated with low levels of ACh, inhibiting or blocking the breakdown of ACh via ChE inhibitor may help to improve brain function (Hempel et al., 2018). The impact of ChE inhibitors on patient cognition and function has been demonstrated to be statistically significant in previous studies (Winblad et al., 2007; Fuentes & Slachevsky, 2005). Additionally, these inhibitors have been found to have positive impacts on the societal and economic burden associated with AD, as indicated by the delay in nursing home placement (Wattmo et al., 2011) and reduction in carer burden (Adler et al., 2014). ChE inhibition's long- term advantages are controversial.

The long-term efficacy studies indicate that there is no observed enhancement in the rate of progression to AD as compared to placebo after 3 years (Wallin et al., 2007).

2.6.2 Glutamatergic agents

Memantine is NMDA-receptor antagonist that target glutamatergic dysfunction (Lipton, 2006), which exerts its mechanism of action by binding to the open state of NMDA receptor channel. Normal NMDA-receptor action permits Ca^{2+} influx for neurotransmission. In AD states, however, elevated NMDA-receptor activity may result in prolonged opening of channels, which subsequently leads to an excessive Ca^{2+} influx, excitotoxicity and ultimately cell death (Lipton & Nicotera, 1998). The use of memantine in clinical trials for the treatment of moderate to severe AD has demonstrated positive effects on patient cognition and function (Wilkinson, 2012). However, the evidence for a positive impact on patient quality of life is inconclusive (Alam et al., 2017).

2.7 Experimental therapy

It is recognised that commencing disease-modifying therapy for AD may not be sufficient, as considerable brain damage has already occurred. Thus, the maximum benefit disease-modifying therapy should theoretically be obtained at an earlier stage (Wu et al., 2011; Aisen et al., 2011) that aims to halt pathologic processes to prevent neurodegeneration and cognitive decline (Grill & Cummings, 2013). Research findings have contributed to a better knowledge of AD pathophysiology and the discovery of specific therapeutic development targets in the brain. Table 2.1 summarizes the experimental therapeutic strategies in AD, adopted from Anand et al.(2014).

Table 2.1 : Experimental therapeutic strategies in AD

Strategies	Modes of action
Tau based therapies	Inhibition of tau phosphorylation Stabilisation of microtubules Blockage of oligomerization of tau Enhancement of tau degradation
Amyloid based strategies	Modulation of secretase enzymes Transport of amyloid Prevention of amyloid accumulation Enhancing clearance of amyloid
Oxidative stress reduction	Exogenous supplementation of antioxidant Supplementing endogenous defence
Others	Caspase inhibitors Growth factor supplementation

(Source: Anand et al. (2014))

2.8 Rats model of Alzheimer's disease

Animal models are valuable tools for studying the disease mechanisms, their pathological processes and evaluating the efficacy of new drugs in the treatment of human diseases (Benedikz et al., 2009). Animal models must closely resemble human disease cognition, particularly with regard to understanding the aetiology of AD

(Benedikz et al., 2009). The two most prevalently used model for AD are rats and mice, both of which are from the rodent family (G, 2008). Among these, rat models have made significant contributions to the understanding and treatment of human disease, thus becoming the animal of choice for most of the behavioural and physiological research (Gibbs et al., 2004). Rats have been favoured by behavioural scientist due to greater intelligence, better ability to learn compared to mice, similarities to human physiological processes and ease measurement of physiological parameters (A. Abbott, 2004). In general, natural rat models of AD do not exist and majority of research are conducted using rat models that mimic the disease phenotypethrough the application of various techniques (Benedikz et al., 2009). The rat models of AD can be categorised into two; i) transgenic model (animals with genetic modifications) ii) non-transgenic model, developed by administering a chemical compound to the rats (Puzzo et al., 2015; Van Dam & De Deyn, 2006).

2.8.1 Transgenic rat models of Alzheimer's disease

The transgenic AD animal models were developed after specific mutations in PSEN1, PSEN2 and APP genes were discovered to be involved in the early onset of AD. These models displayed various characteristics of AD such as behavioural deficits, neuroinflammation, hyperphosphorylated tau, or amyloid accumulation and aggregations (Lewis, 2000). The transgenic animal models only relate to the early onset AD which represent 5% of AD diagnosis in humans while the remaining 95% are late onset AD in which the cause is unknown (Lecanu & Papadopoulos, 2013). Although early onset and late onset of AD have similar clinical presentation, however, late onset AD does not involve any form of gene mutation. The cause of A β accumulation and aggregation in late onset AD is remain elusive. As a result, transgenic animal models are regarded unsuitable for studying the aetiology of late onset of AD (Lecanu & Papadopoulos, 2013). The first transgenic animal model of AD was developed 25 years ago for drug development testing, but there is still no curefor the disease. Therefore, transgenic animals have failed to meet up the expectationsof new drug development. Hence, it could be accomplished by using a pharmacological approach rather than a genetic approach, focusing on the aetiology ofAD (Lecanu & Papadopoulos, 2013).

2.8.2 Non-transgenic rat models of Alzheimer's disease

Scientist began to create a pharmacologically induced rat model of AD (Table 2.2). In general, no genetic manipulation is required for these rat models, which instead rely on chemicals or drugs that can be administered invasively or orally. The benefit of developing this type of animal model is that it mimics the pathogenesis of AD and produces late onset of AD (sporadic) which accounts for 95% of AD diagnosis (Malekzadeh et al., 2017).

D-galactose (D-gal) is common in dairy products and is routinely processed by humans into glucose (Shintani, 2019). Chronic exposure to D-gal, according to Tsai & Yin (2012), causes metabolic alterations that culminate in oxidative damage due to free radicals. D-gal is believed to elicit biochemical changes similar to those observed in

ageing human brain such as reduction in antioxidant enzymes levels (Chiroma et al., 2018; Ullah et al., 2015). D-gal may also react with amines to produce an advanced glycation end products (AGEs) which are linked to natural ageing processes and pathologies to other diseases (Rahman et al., 2022; Xu et al., 2017; Budni et al., 2016). Neurotoxicity induced by chronic D-gal exposure in rodents has frequently been used as a model to investigate the pathophysiology of AD (George et al., 2023; Gao et al., 2015; Zhu et al., 2014). Previous research has demonstrated that persistent treatment of mice to D-gal induces alterations similar to those observed in human such as high A β expression in the hippocampus (Haider et al., 2020; Lin et al., 2014; Qin et al., 2012; Lu et al., 2010). In addition, D-gal induced rat model also exhibits cognitive and learning impairment, oxidative stress, high expression of p-tau protein as well as neuronal death in cerebral cortex and hippocampus (Kenawy et al., 2017; Budni et al., 2016). Other research findings indicate that chronic D-gal exposure can decreased immune response (H. Lei et al., 2003) and cause changes in gene transcription (Tian et al., 2005).

Aluminium (Al) is a well-known neurotoxin widely used in experimental models as it can induce cognitive impairment, which are symptoms linked with AD (Wang et al., 2018). Experiments on both animals and humans have confirmed the existence of cognitive impairment resulting from exposure to Al (Singh et al., 2018; Wang et al., 2018; Bondy, 2010). Chronic administration of Al produced substantial neuronal damage to the hippocampus (Wang et al., 2018). Other mechanisms of Al neurotoxicity include accumulation of A β and NFTs formation (Promyo et al., 2020; Wang et al., 2019). As a result of these mechanism, Al toxicity leads to cognitive decline and neurodegeneration, which act as primary clinical symptoms of AD (Promyo et al., 2020).

In this study, 60 mg/kg of D-gal and 200 mg/kg of AlCl₃ were used as chemical agents to induce AD-like symptoms in a rodent model. According to previous research conducted by Chiroma et al. (2018), similar dosages of these chemicals administered to rodents caused neurodegenerative changes similar to AD, including cholinergic dysfunction and learning impairment. Both of the cerebral cortex and the hippocampus exhibit a high level of amyloidogenic protein and hyperphosphorylated tau expression. A histology investigation also revealed a significant reduction of hippocampal neuron, which resulted in cognitive impairment.

Table 2.2 : Ranges of chemically induced animal models of Alzheimer's disease

Animals	Chemicals	Route of administration/dosage used /induction periods	Findings (behaviour/histology/biochemistry)	References
<i>Sprague Dawley</i> rats (170g – 200g)	Al, Cadmium & Fluoride	Orally 50, 5, 20 mg/kg 90 days	Significant learning deficits Degeneration of pyramidal neurons Swollen neurons & vacuolation apoptotic cells Neuronal degeneration with eosinophilic accumulation Reduced GSH, SOD Increased proinflammatory TNF- α , IL-12 & IL-1 β Increased APP expression & A β 42 levels Increased AChE	(Hussien et al., 2018)
C57BL/6 mice (18-22g)	LPS	Intracerebroventricular (ICV) 40 μ g 19 days	Decreased learning and memory Increased microglial activation, neurodegeneration, lysis, swelling Increased TNF- α , IL-1 β , NLRP3	(Wang et al., 2018)
Albino Wistar rats(180-200g)	AlCl ₃	Orally 300 mg/kg 60 days	Memory impairmentNeuronal loss Pyknosis in CA1 and CA3 regionsEnhanced AChE activity Stimulate production of proinflammatory cytokines TNF- α ,IL-1 & IL-6 Decrease CAT, GSH, GST and MDA levels in hippocampus	(Ravi et al., 2018)
Kunming mice(25-30g)	D-gal and AlCl ₃	Orally, SC 100 mg/kg AlCl ₃ 200 mg/kg D-gal 90 days	Decreased learning and memory Increased pyknotic nuclei Decreased SOD, ChAT, ACh, Increased TNF- α , IL-1 β , MDA, AChE	(Feng et al., 2018)
<i>Sprague Dawley</i> rats (120-180g)	D-gal and AlCl ₃	Intraperitoneal (i.p)60 mg/kg D-gal	Reduced learning and memory Increased AChE, nitric oxide andMDA	(Abulfadl et al., 2018)

Table 2.2 : Continued

		10 mg AlCl ₃ 42 days	Reduced BDNF, SOD, Bcl2, ACh	
Albino Wistar rats(280-300g)	D-gal and AlCl ₃	Orally and i.p 60 mg/kg D-gal 100, 200 & 300 mg/kg AlCl ₃ 10 weeks	Reduced learning and memory Neurodegeneration in hippocampal neurons Increased AChE activity High expression of p-tau	(Chiroma et al., 2018)
Albino Wistar rats(350-400g)	Streptozotocin (STZ)	ICV 2 mg/kg 3-4 months	Cognitive impairment Neuronal loss Ventricular enlargement Accumulation of A β and p-tau Decreased synaptophysin, syntaxin	(Moreira-Silva et al., 2018)
Rats (280-300g)	Streptozotocin (STZ)	ICV 0.5 mg/kg 3-5 weeks 1 mg/kg (9-11 weeks)	Severe impairment in working memory Neurodegeneration Decreased astrocytes in DG, CA1 regions Decreased ChAT mRNA	(Rostami et al., 2017)
<i>Sprague Dawley</i> rats (280-330g)	Streptozotocin (STZ)	ICV 1 and 3 mg/kg 25 days	Learning and memory impairment Stratum Radiatum volumereduction Decreased NeuN in CA1 region Increased GFAP, Iba1	(Zappa Villar et al., 2018)
Albino Wistar Rats	Streptozotocin (STZ)	ICV 15 days	Learning and memory impairment Increased lipid peroxidation and nitric oxide Decreased GSH Increased AChE, TNF- α	(Khadrawy et al., 2022)
Albino Wistar rats (120-150g)	Scopolamine	Intraperitoneal 1 mg/kg 19 days	Deficit in spatial memory Increased MDA, NO Increased cortical Tau Decreased in ATP Increased in ADP and AMP	(Abu Almaaty et al., 2021)
Swiss Albino mice(20-25g)	Scopolamine	Intraperitoneal 1 mg/kg 14 days	Decreased locomotor activity and memory impairment Increased AChE activity and oxidative stress	(Khurana et al., 2021)
Albino Wistar rats (220-250g)	Ethanol	Intragastric route 4 g/kg 64 days	Memory impairment Increased MDA level, GPx activity Decreased	(Vaghef et al., 2019)

Table 2.2 : Continued

			SOD, total antioxidant capacity (TAC) Increased Bax/Bcl-2 ratio and cleaved caspase-9, caspase-3	
C57L/B6 mice(20- 25g)	D-gal	SC injection150 mg/kg 8 weeks	Memory impairment Increased MDA level Decreased SOD, GSH Increased GFAP in CA1, CA3 andDG Increased IL-1 β , TNF- α , IL-6, NF- kB	(Zhong et al., 2020)

Footnotes. APP-Amyloid precursor protein; TNF- α -tumour necrosis factor alpha; IL-1 β - interleukin-1 beta; AChE-acetylcholinesterase; ChAT-acetylcholinesterase; GSH-glutathione; SOD-superoxide dismutase; CAT-catalase; GPx- glutathione peroxidase; CA1-cornu ammonis 1; CA3-cornu ammonis 3; MDA-malondialdehyde; NO-nitric oxide; DG-dentate gyrus; GFAP-glia fibrillary acidic protein; BDNF- brain derived neurotrophic factor

2.9 Behavioural test in AD rat models

Recent research combines molecular investigation with some of behavioural tests to study the efficacy of potential therapies for AD in animal models. Rats and mice have been the primary model and frequently used animal models for behavioural neuroscience research (Ellenbroek & Yoon, 2016). These rodents are suitable models as they exhibit a number of behaviours that closely resemble those of people with AD. Several behavioural assays have been developed to assess the various aspects of cognitive function in animals, providing insight into the effects of experimental treatments, and the potential mechanisms underlying cognitive decline (Puzzo et al., 2015). Impairment of these behavioural task may reflect the cognitive deficits observed in AD.

2.9.1 Learning and memory tests

Learning is the process of acquiring novel information such as new knowledge and abilities (Gross, 2010). The memory processes in the hippocampus are linked to the learning process. Prior to storage in long term memory, new information was acquired through a process known as memory consolidation (McGaugh & Roozendaal, 2009). The hippocampus is required for the development of long-term memory, also known as declarative memory in human or relational memory in animals (Alberini & Ledoux, 2013).

Memory is a behavioural change caused by the retention of information over time (Okano et al., 2000). The hippocampal is bilaterally symmetric in each brain hemisphere and can maintain normal hippocampal function even if one hemisphere is damaged (Di Gennaro et al., 2006). Nonetheless, substantial damage to both hemispheres is deleterious to memory processes, leading to neurodegenerative diseases (Di Gennaro et al., 2006). The following behavioural assays were used to detect AD-related learning and memory defect.

2.9.1.1 Novel Object Recognition test (NOR)

The novel object recognition (NOR) task is based on rodents' natural tendency to investigate novel objects over familiar ones. In the context of NOR test, novelty is operationally defined as the recognition that animals can exhibit a response to a stimuli that is unfamiliar or novel to them. Novel stimuli can alter animal behaviour and rodents' preference for exploring novel objects, reflects their use of recognition and learning memory (Shukitt-Hale et al., 2001). There are no rewards, and the rodents explore the novel object due to their natural curiosity (Baxter, 2010). The rodent is presented with two similar objects in the Phase 1 of the test, then one of two objects is replaced by novel object in the Phase 2 of the test. The total time taken by rodents to explore are recorded (Leger et al., 2013).

Ennaceur and Delacour (1988) investigated the novel object recognition tests for the first time. In the absence of extrinsic reinforcement, they concluded that these tests are simple

behavioural assays of memory that rely solely on the rodent's intrinsic exploratory behaviour. The NOR test has emerged as a widely employed behavioural paradigm for the study of memory alterations. It has also been employed to investigate the effects of various pharmaceutical intervention and brain damage (Goulart et al., 2010).

The way in which the NOR test evaluates the performances of animals may also vary. Depending on the objectives of each study, it can be calculated using several indices, such as discrimination index, index of global habituation or preference index (Gaskin et al., 2010). For discrimination, there are two measures of discrimination behaviour (Ennaceur, 2010). The first measure (D_1) for the habituation phase is the difference between the exploration time for novel (T_N) and familiar objects (T_F), [$D_1 = T_N - T_F$]. The second measure is discrimination index (DI). The formula is [$DI = (T_N - T_F) / (T_N + T_F)$], where T_N and T_F are the exploration times for novel and familiar objects respectively. The outcome of this measurement can vary between a positive score of +1 and negative score of -1. A positive score signifies a greater amount of time spent with a novel object, while negative score indicates a greater amount of time spent with a familiar object. A score of zero indicates a lack of preference (Silver et al., 2011; Aggleton et al., 2010; Baxter, 2010; Oliveira et al., 2010).

The evaluation of animal performance can also be conducted using Index of global habituation. The calculation involves comparing the cumulative duration of exploration for both objects during the familiarization phase with the duration of testing. There should be no statistically significant differences observed in the global index of habituation (Ennaceur, 2010).

The Preference Index is another way to assess cognitive performances in rodents. The aforementioned metric represents the percentage of the time spent exploring one of two objects (A or B) during the training phase, or a novel object (C) during the test phase, divided by the overall time spent exploring both objects. The preference indexes greater than 50% indicate novel object preference, while less than 50% indicate familiar object preference (Hammond et al., 2004).

It is essential to note that the recognition of objects by rodents can be quantified by comparing the exploration time of novel and familiar objects. The measure of recognition is affected by the interval between the time spent with novel object and time spent with familiar object, as well as the time allowed for rodents to investigate the objects during first trial (Ennaceur and Delacour, 1988). In general, when rodents are exposed to both familiar and novel objects, they tend to approach the novel one more frequently and spend more time investigating it (Ennaceur, 2010). The preference for a novel object indicates that the presentation of familiar object is stored in the memory of animals (Ennaceur, 2010). More cognitive abilities are required for the recognition of novelty (Silvers et al., 2011), which then allowing incorporation of new information (Clarke et al., 2010). According to Ennaceur (2010), the definition of exploration is the exploration of an object by directing the nose at a distance of ≤ 2 cm to the object or by touching the object with the nose. In contrast, spinning and running around the object, climbing it, or sitting on it were not considered as exploration.

The NOR task is particularly attractive due to its lack of reliance on external motivation such as reward or punishment. However, it does require a certain level of training or habituation. Moreover, it is feasible to accomplish the task in the short period of time (Silvers et al., 2011). By manipulating retention intervals, the NOR task can be used to study short term memory, intermediate term memory and long-term memory (Wipt & George, 2008). The results of the NOR is influenced by hippocampus (Buckmaster et al., 2004). This brain structure plays a crucial role in the development of recognition memories, and when it is damaged, the performance in recognition memory tasks is impaired (Albasser et al., 2009).

2.9.1.2 Morris water maze (MWM)

Morris water maze (MWM) was invented by Morris (1984). Because the hippocampus is one of the initial brain regions impaired in AD, this test is one of the most popular and well-established behavioural tests for evaluating rodents' spatial learning ability and memory (West, 1993). MWM is performed in a large tank filled with opaque water, with an escape platform hidden beneath the water. The rodents depend on distal cues in order to travel from their initial positions along the edge of an open swimming arena, with the goal of locating the submerged escape platform. The rodents' swimming distance, speed and latency to reach the hidden platform are all recorded using video (Lawlor et al., 2007). Spatial learning is determined by repeated test trials, whereas reference memory is assessed by preference to the platform area when the platform is removed (C. Vorhees, 2010). Normal rodents typically learn to swim to the platform faster over time and reach behavioural asymptote after multiple trials. These learning experiences (training trials) may be separated by days (Daugherty et al., 2015; Goodrich-Hunsaker et al., 2010).

2.9.1.3 T-maze test

The T-maze test is a simple behavioural assay used to assess the rodent cognitive behaviour. The T-maze is an enclosed apparatus in the form of T-shape placed horizontally. Rodents are positioned at the base of the T-shaped apparatus, and allowed to explore one of the goal arms adjacent the other end of the apparatus in this test. After undergoing two consecutive trials, the rodents exhibit a proclivity to explore the arm that was not previously visited on the subsequent trial, thus implying remembrance for the first choice. This phenomenon is referred to as 'spontaneous alternation'. (Deacon & Rawlins, 2006). The technique of reinforcing this behaviour in rats by making them hungry and rewarding them with food is known as 'rewarded alternation'. These two tests (spontaneous and rewarded) are sensitive to the hippocampus, an important brain region for learning and memory. Each trial must be completed in two minutes and the total number of trials might vary based on the needs of the research.

2.9.1.4 Modified elevated plus maze (mEPM)

The mEPM test is a modified version used to determine the long-term spatial learning and memory of rodents. The experiment is predicated on the assumption that rodents possess the ability to acquire and retain knowledge about the specific area that provides them

a sense of safety. The mEPM device and procedure have been previously described by Biala & Kruk (2008).

2.9.2 Motor test

2.9.2.1 Elevated Plus Maze test (EPM)

The elevated plus maze (EPM) is commonly used in assessing anxiety-like behaviour in rodents. It is made of black Plexiglas with two open arms and two enclosed arms, that have equal dimensions and are connected at the central square. The height of the maze was elevated 50cm above the floor (Rico et al., 2019). A total of 5 minutes is given to rodent for exploration in the apparatus. Rodents' time spend in open and enclosed arms are recorded. An increase in the open arm activity indicates anti-anxiety behaviour.

2.9.2.2 Open field test (OFT)

The open field test (OFT) is widely used to evaluate exploratory and anxiety-like behaviours in rats and mice (Pruet & Belzung, 2003). It is especially beneficial for assessing locomotive impairments in mouse models of neuromuscular disease (Rabenet al., 2000), as well as for evaluating the effect of therapeutic agents and it may improve locomotor functions (Malerba et al., 2011). The advantages of using OFT includes, less rat handling throughout the test, no particular training requirements, less rodent stress and it can assess both locomotor and behavioural activities (Nagaraju et al., 2010).

2.10 Cannabis plant

Cannabis is one of the most widely abused drugs in the world. *Cannabis sativa*, also known as marijuana, has long been used for medical purposes in human history. This plant belongs to the *Cannabinaceae* family, and has erected stems that can reach up to 5m in length depending on environmental conditions and genetic variety (Farag & Kayser, 2017). There is a rising public discussion about the legalisation of cannabis for recreational medical use. However, cannabis is rapidly approved globally for medical and recreational uses in recent years (Webster, 2018; Carliner et al., 2017). There has been relatively few research undertaken on medical cannabis, which may be attributed to the fact that cannabis is not legally accessible in some countries. Anti-nociception, anti-inflammation, anticonvulsant, anti-emetic, cancer pain, depression, anxiety disorder, and neurological dysfunction are a few of the cannabis and cannabinoid uses that have been extensively studied and documented over the past two decades (Cousijn et al., 2018). Renewed scientific interest in the medical use of substances found in the cannabis plant, namely cannabinoids, has accompanied increased interest in the medical use of cannabis.

2.10.1 Cannabis species

Cannabis is currently classified into three subspecies; *ssp Sativa*, *ssp Indica* and *ssp Ruderalis* (Figure 2.4). Each subspecies is further subdivided into two main varieties namely wild and cultivated (Stasiłowicz et al., 2021). Cannabis provides a wide range of pharmacological effects in previous herbal traditional methods, making it one of the most ancient plant species to be used. This resulted in the widespread use of cannabis, which has captivated the interest of researchers due to its potential therapeutic effects in the treatment of various diseases, including neurological diseases (Stasiłowicz et al., 2021; Walsh et al., 2021).

Cannabis is comprised of diverse array of bioactive constituents, including cannabinoids, alkaloids, flavonoids, and terpenoids (Andre et al., 2016). These compounds are responsible for the distinctive pharmacological and toxicological characteristics exhibited by cannabis. Presented in Table 2.3 are the primary cannabinoids found in cannabis. Delta-9-tetrahydrocannabinol (Δ^9 THC) is the primarily component responsible for psychoactive effects (Whiting et al., 2015). Today, Δ^9 THC has drawn interest for research and medical applications, and the present study focuses on this phenomenon.

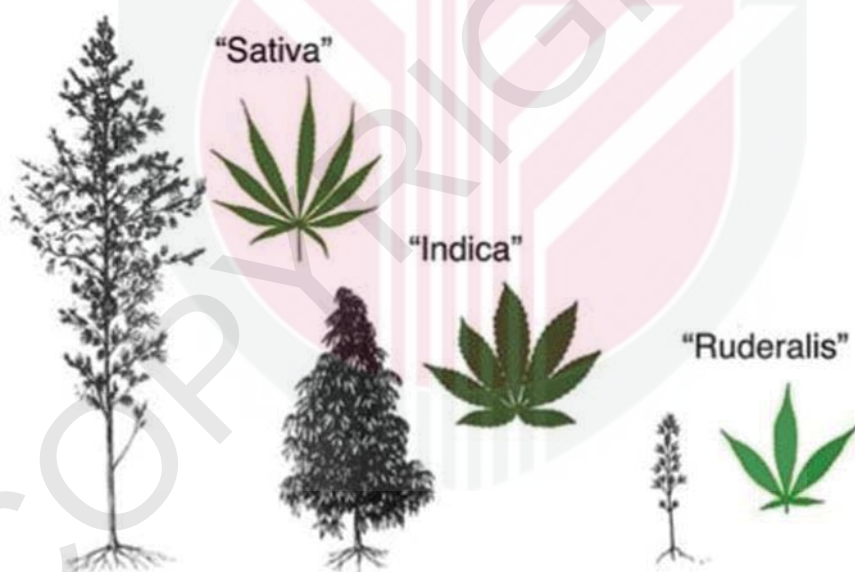


Figure 2.4 : Cannabis species
(Source: McPartland, 2018)

Table 2.3 : Primary cannabinoids in cannabis

Psychoactive components	
Name	Descriptions
Delta-9-tetrahydrocannabinol (Δ^9 THC)	Main psychoactive component, causes psychological and behavioural effect
Delta-8-tetrahydrocannabinol (Δ^8 THC)	Less psychoactive than Δ^9 THC
Cannabinol (CBN)	Less powerful than Δ^9 THC
11-hydroxy- Δ^9 THC (11-OH-THC)	Responsible for psychological effects of cannabis
Non-psychoactive components	
Cannabidiol (CBD)	Has anticonvulsant action
(-) Δ^8 -THC-11-oic acid)	Has analgesic activity

2.11 Delta-9-tetrahydrocannabinol (Δ^9 THC)

2.11.1 Pharmacological action of Δ^9 THC

Δ^9 THC is a tri-cyclic 21-carbon compound with two chiral centres in trans- configuration (Pertwee, 2006) (Figure 2.5). Pharmacologically, Δ^9 THC has high lipid solubility but low aqueous solubility. It is found in cannabis as a mixture of mono- carboxylic acids, that de-carboxylates easily and efficiently when heated (Mechoulam, 2005). Two hypotheses have been proposed to explain the *in-vivo* action mechanism of Δ^9 THC. According to the first theory, Δ^9 THC, which is released as a glucuronide, induces membrane disruption in the brain via non-specific interactions with cellular and organelle membranes (Onaivi, 2007). The second theory proposed that Δ^9 THC interacts with specific cannabinoid receptors (Appendino et al., 2011) in the brain and periphery to produce various physiological effects. Two cannabinoid receptors have been identified; cannabinoid receptor 1 (CB_1) and cannabinoid receptor 2 (CB_2). The CB_1 receptor is predominantly linked to several cognitive processes such as cognition, memory, anxiety, reward, motor coordination, and pain sensory perception. On the other hand, the CB_2 receptor might have a role in the immunosuppressive effects of cannabis (Pertwee, 2006). The CB_1 has garnered significant interest as a potential therapeutics approach in several pathological states, including neuropsychiatric illnesses and neurodegenerative ailments (Zou & Kumar, 2018). Many effects of Δ^9 THC result from perturbation of this complex system, nevertheless, the specific mechanism behind these effects remains unknown.

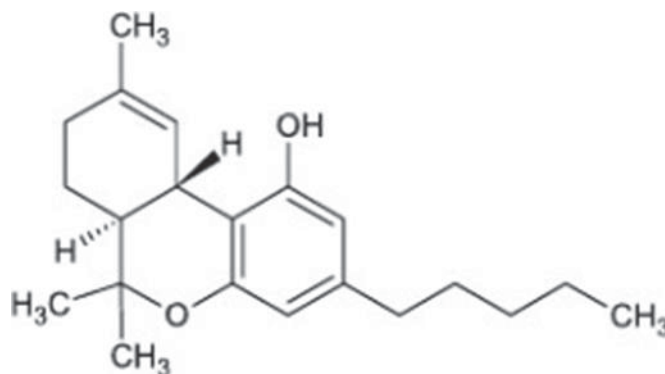


Figure 2.5 : Molecular structure of Δ^9 THC
(Source: Balbino et al., 2014)

2.11.2 Pharmacokinetic of Δ^9 THC

2.11.2.1 Absorption and distribution

The pharmacokinetic of Δ^9 THC administered intravenously or intraperitoneally are comparable to those observed when administered via inhalation (Lucas et al., 2018.) The route of administration influences the bioavailability of Δ^9 THC (Lucas et al., 2018; Vandevenne et al., 2000). Due to its high lipophilicity, Δ^9 THC exhibits a significantly diminished oral bioavailability, which estimated to be as low as 6% (Gaston et al., 2017). In addition, it has been observed that oral Δ^9 THC formulations exhibit substantial hepatic first-pass metabolism, resulting in lower peak plasma levels (Eichler et al., 2012). Δ^9 THC rapidly distributed into well-vascularized organs including lung, heart, brain and liver (Gaston et al., 2018; Dinis-Oliveira, 2016). Body composition and size, as well as disease states influencing the permeability of blood-tissue barriers (Lucas et al., 2018). Approximately 90% of Δ^9 THC present in the blood is distributed throughout plasma, with the remaining 10% being transported in red blood cells.

2.11.2.2 Metabolism and elimination

Drug metabolism is typically characterized by a biphasic mechanism, wherein the compound undergoes a phase I reaction involving functionalization reactions such as oxidation, reduction, or hydrolysis. This is subsequently followed by a phase II reaction involving conjugation reactions, resulting in the formation of two distinct types of metabolites; functionalization products and conjugates (Testa & Krämer, 2008). For Δ^9 THC, microsomal hydroxylation and oxidation catalyzed by cytochrome P450 isoenzymes CYP2C9, CYP2C19, and CYP3A4, producing 11-hydroxy-THC (11-OH-THC) (Watanabe et al., 2007). 11- OH-THC is further oxidized to the inactive 11-carboxy-THC (11-COOH-THC) (Schwope et al., 2011), and eliminated as glucuronic acid conjugate in the urine (Gaston & Friedman, 2017). Metabolism also occurs in extra-hepatic tissues that express CYP450, including the small intestine and brain (Huestis,

2007). The metabolite 11-OH-THC is reported to have psychoactive activity (Lucas et al., 2018).

2.12 Previous research findings on Δ^9 THC

2.12.1 Δ^9 THC and cognition

There is no clear relationship between Δ^9 THC and cognitive enhancement or impairment. Numerous preclinical studies indicate a strong association between Δ^9 THC exposure and cognitive impairment (Levine et al., 2017). An early study found that, high exposure to Δ^9 THC inhibits choline uptake in the hippocampus, thereby limiting acetylcholine synthesis, which causes cognitive impairment (Varvel et al., 2001). Heavy use of Δ^9 THC has also been associated with an increased risk of psychotic disorders in human (Dinis-Oliveira, 2016). Recent research, however, has demonstrated that chronic administration of Δ^9 THC at low concentration can improve cognitive and neurological function (Gruber et al., 2018). Puighermanal and colleagues (2009) investigated mice treated with different dosages of Δ^9 THC (0.3, 1.0, 3.0 and 10 mg/kg, i.p) immediately following NOR training. After a period of 24 hours, doses of 3.0 and 10 mg/kg induced a significant amnesic-like effect, but lower doses did not produce any of memory impairment. Suliman et al. (2017) discovered that rats treated with low doses of Δ^9 THC (0.75, 1.5 and 3.0 mg/kg) spent more time with novel object during NOR test, indicating memory enhancement.

Other researchers demonstrated that Δ^9 THC at extremely low concentration (2.5 nmol/L), can halt the progression of AD by inhibiting the production of its primary pathological marker (beta amyloid) (Cao et al., 2014). Another study demonstrated that a chronically low dose of Δ^9 THC restores cognition in aged mice (12 and 18 months respectively) to levels comparable to those of young, untreated mice. This effect was accompanied by enhanced expression of synaptic marker proteins and increased hippocampal density (Belkei-Gorzo et al., 2017). In addition, the treatment with low dose of Δ^9 THC (3 mg/kg per day) for 4 weeks also enhanced the cognitive performance of aged mice (Nidadavolu et al., 2021).

Compiling these findings, it has been demonstrated that the effect of Δ^9 THC on cognition is a biphasic dose-response effect, which has become an important factor in investigating the therapeutic effects of Δ^9 THC on the brain and cognition associated with AD (Calabrese and Rubio-Casillas., 2018). Thus, it is possible to hypothesize that chronic, low dose treatment with Δ^9 THC could be a potential strategy for reversing cognitive deterioration in AD. Overdosage of Δ^9 THC may induce unfavourable neuropsychological and cognitive adverse effects, hence diminishing its medical value (Chen et al., 2013).

2.12.2 Δ^9 THC and neurogenesis

Cognitive impairment is connected with neuronal degeneration in the region of brain responsible for learning and memory (Götz et al., 2011). Chronic administration of Δ^9 THC has been shown to suppress hippocampal neurogenesis and alter the hippocampus morphology in adult rats. The alteration included a reduction in the mean volume of neurons and number of synapses (D. Kim & Thayer, 2001). Chronic Δ^9 THC treatment also resulted in a significant decrease in the number of newly generated cells (Kochman et al., 2006). In a separate study by Wolf et al. (2010), adult mice treated with Δ^9 THC (6 weeks) displayed poor spatial memory function and decreased proliferation. In contrast, Suliman et al. (2018) found that normal rats exposed to 0.75, 1.5 and 3.0 mg/kg of chronic Δ^9 THC exhibited an increase in neurogenesis. The expression of neurogenesis-related markers was considerably elevated in rats treated with Δ^9 THC. This was demonstrated by increasing of doublecortin (DCX), Nestin, TuJ-1 and glial fibrillary acidic protein (GFAP). Nyffeler et al. (2010) reported that cognitive performance has been observed to improve as a result of enhanced neurogenesis activity. The results of this study indicate that Δ^9 THC can promote neurogenesis at low concentration, while at higher concentration, neurogenesis is inhibited. Δ^9 THC may regulate neurogenesis through activation of CB₁ receptor. Nonetheless, the mechanism by which cannabinoid receptors modulation affects neurogenesis remains unknown and debatable.

2.12.3 Δ^9 THC and neuroinflammation

Nerve cell death and neuroinflammation caused by aggregated A β forming plaques is a common pathologic factor in majority of neurodegenerative diseases, including AD (Leonard & Aricioglu, 2023). These plaques disrupt neuronal communication in the brain, resulting in cognitive impairment (Ransohoff & Brown, 2012). Previous research has demonstrated that accumulation of intracellular A β induces a toxic inflammatory response. Recent findings have shown that Δ^9 THC stimulates the A β clearance and inhibits the inflammatory response by activating CB₁ receptors in brain (Schubert et al., 2020; Currais et al., 2016). According to an *in-vitro* study investigation by Cao et al. (2014), Δ^9 THC is capable to act as anti-A β aggregation agent by reacting directly with A β peptide. Chen et al. (2013) found that Δ^9 THC significantly reduced A β levels and neurodegeneration in 5XFAD transgenic mice by increasing the neprilysin levels, an enzyme responsible for A β degradation.

2.13 Hippocampus

2.13.1 Anatomical structure of hippocampus

The hippocampus is a small, part of limbic system. It is located within the medial temporal lobe of the brain and has a horseshoe-shaped pair structure (Knierim, 2015). The hippocampus is primarily responsible for memory formation; including both short and long-term memory, as well as emotion regulation (Sawangjit et al., 2018). The hippocampus is made up of many cytoarchitecturally different regions, each possibly

responsible for distinct functions (Y. Li et al., 2019; Rebola et al., 2017; McEwen et al., 2016). Studies have shown that cornu ammonis 1 (CA1), cornu ammonis 2 (CA2) and cornu ammonis 3 (CA3) subfields of the hippocampus display a high concentration of cannabinoid receptors (Glass et al., 1997). Batalla et al. (2018) discovered that cannabis exposure has the potential to induce alterations in the neuroanatomy of certain subfields within the hippocampus. Consequently, the hippocampus is recognised as an important biomarker for a variety of psychiatric disorders, including AD (Douaud et al., 2013). Damage to the hippocampus has been seen to impede the formation of new memories (Squire, 2004).

2.13.1.1 Hippocampal proper

The hippocampus is divided into four distinct subdomains; the dentate gyrus (DG), the hippocampus proper, the presubiculum and the subiculum (Weilbacher & Gluth, 2017). Hippocampus proper has subdivisions that include CA1, CA2, CA3 and CA4 that make up the hippocampus structure (Schultz & Engelhardt, 2014). However, as suggested by Insausti & Amaral (2012), the CA4 subfields is excluded. The reason for this is that pyramidal neurons of CA4 are not morphologically similar to the pyramidal neurons of other CA cells and the subfield is frequently regarded as component of dentate gyrus called a hilus. CA1 located adjacent to subiculum, CA2 located at the small boundary between CA1 and CA3, while CA3 located at adjacent with fimbria (Taupin, 2007) (Figure 2.6).

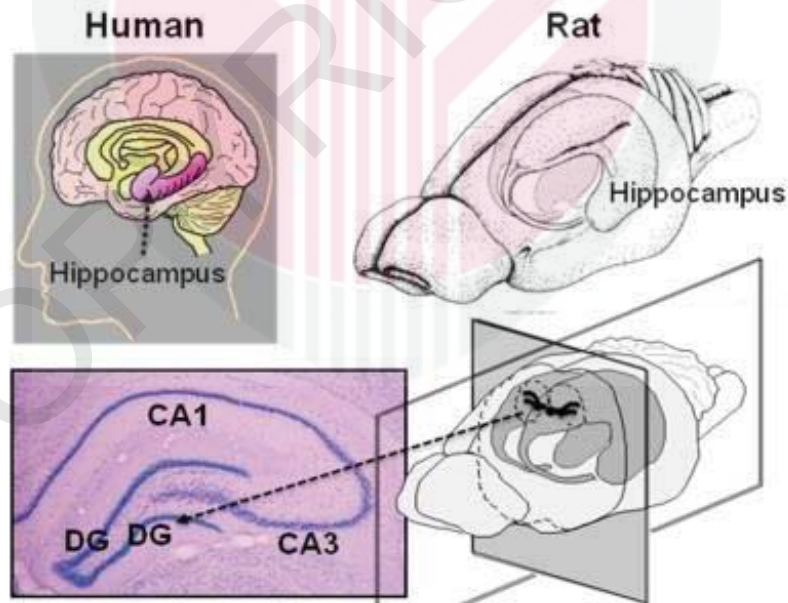


Figure 2.6 : Hippocampus structure. Cornu ammonis (CA1-CA3); dentate gyrus (DG)

(Source: Soya et al., 2011)

2.13.1.2 Dentate gyrus (DG)

Dentate is a trilaminar layer made up of the molecular layer, the granular cell layer and the polymorphic layer (Drew et al., 2013). The molecular layer of DG comprises an outer molecular layer (OML), middle molecular layer (MML) and inner molecular layer (IML) (Nastenko & Veselovsky, 2021). The granular cell layer is a principal cell layer for the DG which is densely packed with dentate granule cells. Subgranular zone (SGZ), a location for neurogenesis, is found in the polymorphic layer which is also known as hilus. It comprises mossy fibres and granule cell precursors (Scharfman & Myers, 2012) (Figure 2.7).

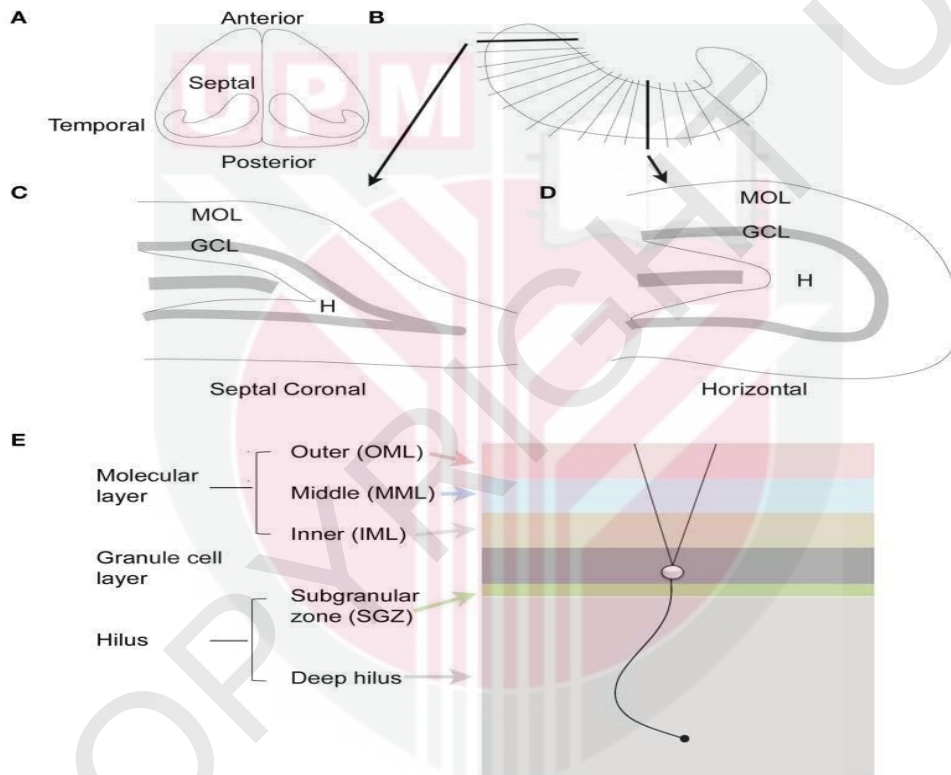


Figure 2.7 : The dentate gyrus layers of the rat. MOL-molecular cell layer; GCL-granular cell layer; H-hilus
(Source: Scharfman & Myers, 2012)

2.13.2 Hippocampal synaptic pathways

Hippocampal synaptic pathway is a circuit that enables the transfer of synapses. The hippocampal connection is known as the tri-synaptic loop. The projection input to the DG is provided by entorhinal cortex (EC) layer II neurons via the perforant pathway. The DG transmit the input to CA3 through mossy fibres. CA3 transmit projections to

CA1 via Schaffer collateral. CA1 neurons return the projections to EC to complete the loop (Knierim, 2015). The second perforant pathway involves the direct projection of an EC layer II neuron to CA3 while the temporoammonic pathway carries the projection from EC layer III neuron to CA1 (Deng et al., 2010) (Figure 2.8).

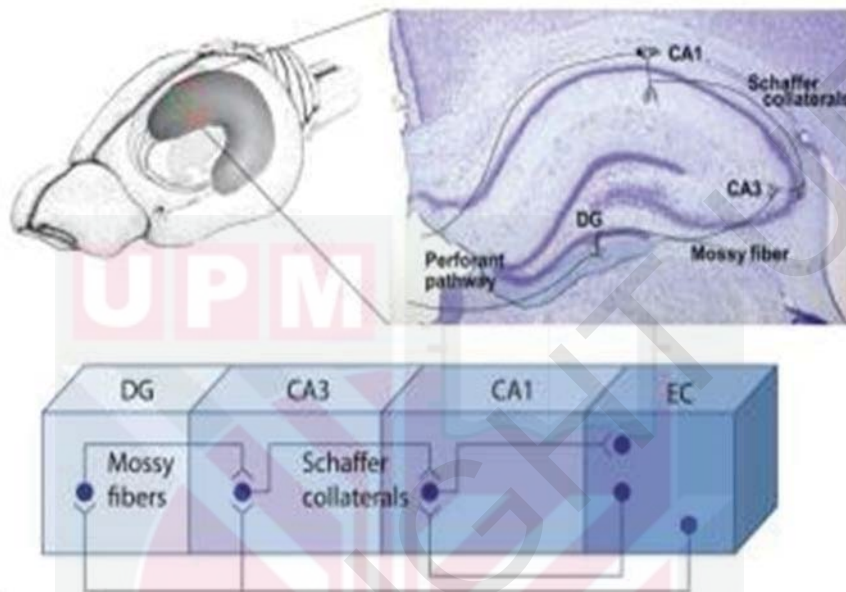


Figure 2.8 : Representative of the rat brain and illustration of hippocampal synaptic pathway. EC-entorhinal cortex; CA-cornu ammonis; DG-dentate gyrus
(Source: H. Xu et al., 2015)

2.14 Neurogenesis

Neurogenesis is the biological process via which progenitor cells give rise to fully functional new neurons (Kempermann et al., 2015). The occurrence of adult neurogenesis in the brain is well established, particularly in dentate gyrus of the hippocampus (Shors et al., 2002). It has been reported that the rate of neurogenesis in the dentate gyrus of the human hippocampus is estimated to be 0.004% each day. In contrast, the daily rate of neurogenesis in the dentate gyrus of mice ranges from 0.3-0.6% (Bergami et al., 2015). The adult mammalian brain's hippocampal dentate gyrus contains neural stem cells or progenitor cells (NS/PCs) that capable of generating new neurons (Van et al., 2005). New neuron production in the dentate gyrus of hippocampus is related to learning and memory processes (Shors et al., 2002). Thus, neurogenesis plays an important strategy which can offers future promise for treating neurodegenerative diseases and others disease-related neuron (Kempermann et al., 2004).

At the subgranular zone, there are two types of neural stem cells (NSC) (Aimone et al., 2014). NSC are self-renewing, multipotent cells known as Type I cell which are divided

into two subtypes; radial astrocytes, a slow dividing cell and horizontal astrocytes, a much rapid dividing cell. These cells divide asymmetrically to produce one daughter cell and one progenitor cell, both of which are referred to as type II cells. Active mitotic division of type II cells produces neuroblasts or also known as type III cells. Neuroblasts undergo slow proliferation to become immature neurons (Patricio et al., 2013).

The immature neurons then migrate to the granular cell layer, where it differentiates, extending its axon into the hilus and projecting more dendritic branches to the molecular layer (Ihunwo et al., 2016). Subsequently, immature neurons will develop into mature neurons that contribute to the hippocampal neuronal network (Quiñones- Hinojosa & Chaichana, 2007). The granular cell layer contains approximately 90% of fully developed granule cells, which are known to have a pivotal function in cognitive processes such as learning and memory (Lopez-Rojas & Kreutz, 2016). The neurogenesis processes were summarised in Figure 2.9.

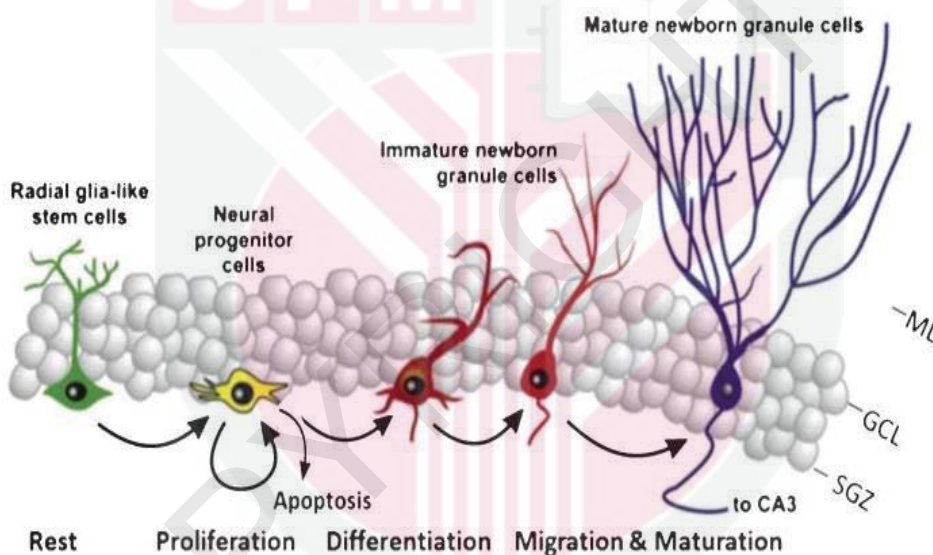


Figure 2.9 : Schematic diagram illustrating the phases of neurogenesis. ML- molecular layer; GCL-granular cell layer; SGZ-subgranular zone
(Source: Bolijn & Lucassen, 2016)

Alternately, the use of distinct markers corresponding to the various stages of neurogenesis. At the proliferation stage, a newly generated cell in dentate gyrus expresses glial fibrillary acidic protein (GFAP) and nestin (Fukuda et al., 2003). The differentiation phase in SGZ shows the cell that has differentiated into an immature neuron. At this point, the cell was nestin-positive but GFAP-negative. Later, the cell will stop expressing nestin and transition towards the expression of doublecortin (DCX) and polysialylated embryonic form of neural cell adhesion molecule (PSA- NCAM) (Fukuda et al., 2003). When a cell expresses both DCX and PSA-NCAM markers, it is in the migration phase and is migrating to the granular cell layer of the DG (Von Bohlen Und Halbach, 2007). The immature neuron moves their dendrites towards the molecular layer

of DG, while simultaneously extending their axonal projections towards hippocampal CA3 pyramidal cell layer. This is referred to as dendritic and axonal targeting phase (Kempermann et al., 2003). Llorens-Martín et al.(2009) discovered that neuron-specific marker protein (NeUN) was expressed during this stage. The final phase is called synaptic integration, in which the new granule cells set up their synaptic contact in order to receive input from the entorhinal cortex (VonBohlen Und Halbach, 2007). Calbindin was expressed in the mature granule cells (Figure 2.10).

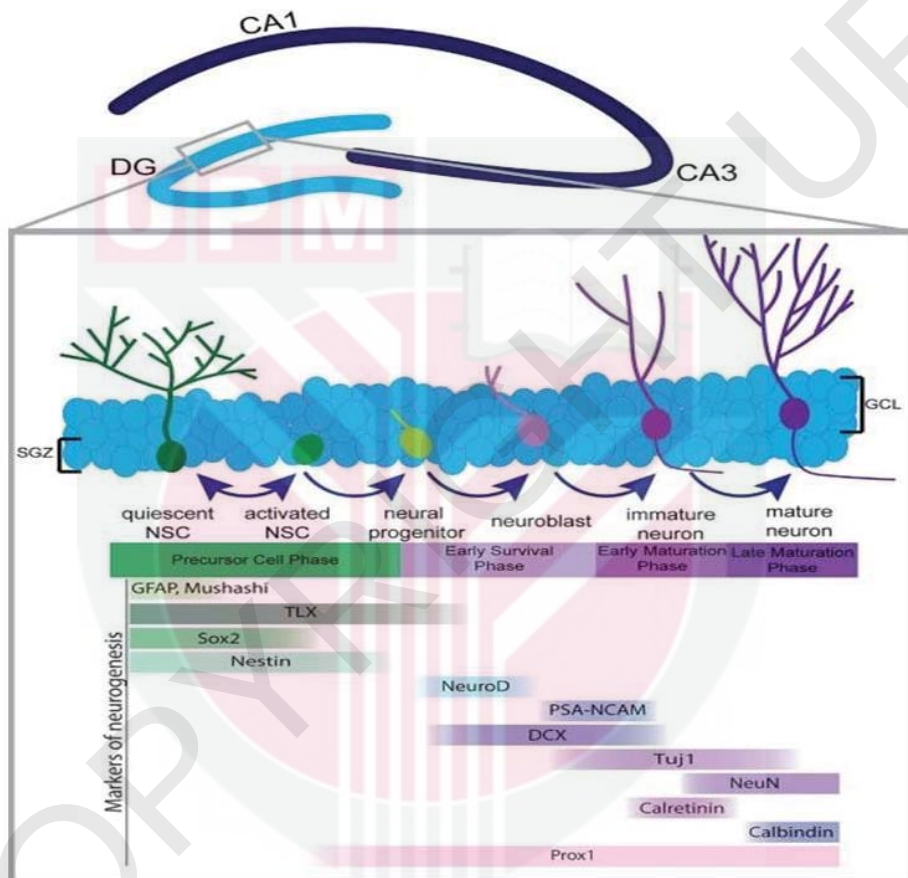


Figure 2.10 : Schematic representation of adult hippocampal neurogenesis in rats. Markers expressed throughout the phases of neurogenesis are shown
(Source: Babcock et al., 2021)

CHAPTER 3

GENERAL METHODOLOGY

3.1 Materials and methods

3.1.1 Drugs and chemical

AlCl₃, D-gal and donepezil were purchased from Sigma-Aldrich, St. Louis, MO, USA, while Δ^9 THC was purchased from Lipomed, Switzerland. D-gal and donepezil were dissolved in distilled water for intraperitoneal injection (i.p). Meanwhile, AlCl₃ was dissolved in normal saline for oral administration (Chiroma et al., 2018). All the drugs and chemicals were freshly prepared prior to the experimental work.

3.1.2 Animals

Seventy male Albino Wistar rats (n=70), 7-8 weeks old weighting 200-250 g were used in this study. The rats were procured from local supplier, Takrif Bestari Enterprise, located in Seri Kembangan, Selangor, Malaysia. The rats were placed in cages containing of 2-3 rats each, and were subjected under the same laboratory settings (temperature 22 ± 25 °C, 12 h light: 12 h dark cycle). The rats were allowed to acclimatize for one week before the experiment began. The rats were provided with standard commercially available rats chow (pellet) and water *ad libitum*. All experimental protocols were carried out during the light cycle at Animal Behavioural Laboratory, Faculty of Medicine and Health Sciences, Universiti of Putra Malaysia with ethical approval from Animal Ethic Committee of Universiti of Putra Malaysia (UPM/IACUC/AUP-R094/2019, Appendix A).

3.1.3 Animal experimental design

Seventy rats were randomly divided into six groups (n=10). Table 3.1 shows the animal groupings and their respective treatments.

Table 3.1 : Animal groups and treatments

Group	Treatment	Dosage
G1: Control	Distilled water (i.p) + 0.9% normal saline (oral)	0.1 ml/100 g b.wt.
G2: AD model	D-gal + AlCl ₃	60 mg/kg D-gal + 200 mg/kg AlCl ₃
G3: Positive control	D-gal + AlCl ₃ + donepezil	60 mg/kg D-gal + 200 mg/kg AlCl ₃ + 1.0 mg/kg donepezil
G4: AD model + Δ^9THC low dose	D-gal + AlCl ₃ + Δ^9 THC	60 mg/kg D-gal + 200 mg/kg AlCl ₃ + 0.75 mg/kg Δ^9 THC
G5: AD model + Δ^9THC medium dose	D-gal + AlCl ₃ + Δ^9 THC	60 mg/kg D-gal + 200 mg/kg AlCl ₃ + 1.5 mg/kg Δ^9 THC
G6: AD model + Δ^9THC high dose	D-gal + AlCl ₃ + Δ^9 THC	60 mg/kg D-gal + 200 mg/kg AlCl ₃ + 3.0 mg/kg Δ^9 THC

This study used a combination of D-gal and AlCl₃ administration to induce Alzheimer's disease in a rat model for 10 weeks (Chiroma et al., 2018). After 10 weeks, the animals were treated with donepezil and three different concentrations of Δ^9 THC respectively for 28 days to examine the potential therapeutic effect. The dosage of donepezil at the concentration of 1.0 mg/kg was adopted from Song et al. (2017). Meanwhile the Δ^9 THC dosages were determined based on previous research (Suliman et al., 2018). It demonstrates that administration of Δ^9 THC at 0.75, 1.5 and 3.0 mg/kg for 28 days improved rats' learning and memory functions, as evidenced in behavioural test and molecular studies.

The animals were evaluated neurobehaviourally after treatment was completed. The following neurobehavioral tests were performed; novel object recognition (NOR), Morris water maze (MWM), elevated plus maze (EPM) and modified elevated plus maze (mEPM). All behavioural tests sessions were conducted between 8 a.m. to 6 p.m. After completion of neurobehavioural test, the rats were decapitated. The brain samples were collected for histological and molecular investigations. The overall experimental design was simplified in Figure 3.1. The overview of thesis chapter was presented in Figure 3.2.

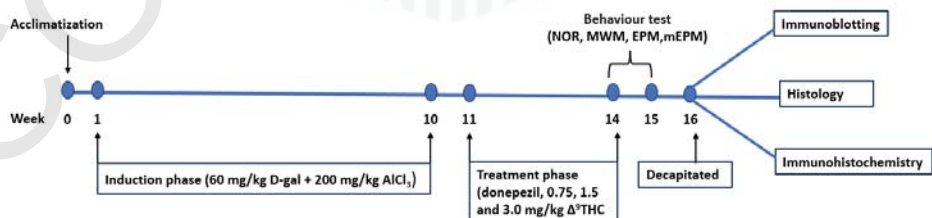


Figure 3.1 : Flowchart of the experimental design for induction of AD-like symptoms in rats and treatment phase. D-gal – D-galactose; AlCl₃ – aluminium chloride; NOR – novel object recognition; MWM – Morris water maze; EPM – elevated plus maze; mEPM – modified elevated plus maze

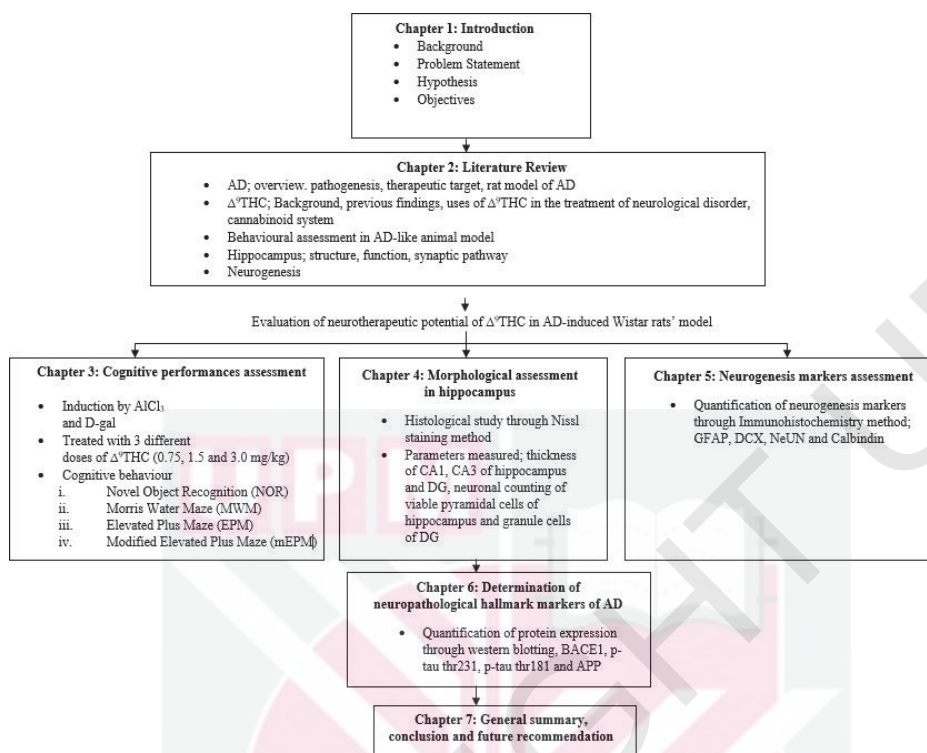


Figure 3.2 : Organization of the thesis chapters. This is the thesis design and a brief schematic presentation of how the experiment was conducted and actualised. AD - Alzheimer's disease; D-gal - D-galactose; AlCl_3 - Aluminium chloride; Δ^9 THC - Delta-9-tetrahydrocannabinol; CA1 – Cornu ammonis 1; CA2 – cornu ammonis 2; CA3 – cornu ammonis 3; DG – dentate gyrus; GFAP – Glial Fibrillary Acidic Protein; DCX - Doublecortin; NeUN – Neuronal Nuclear Antigen; BACE1 – β -site Amyloid Precursor Protein Cleaving Enzyme 1; APP – Amyloid Precursor Protein

CHAPTER 4

NEUROTHERAPEUTIC POTENTIAL OF DELTA-9-TETRAHYDROCANNABINOL ON COGNITIVE PERFORMANCES IN RATS AFTER D-GALACTOSE AND AIC₃ EXPOSURE

4.1 Introduction

Alzheimer's disease (AD) is a neurological ailment that primarily affects senior citizens (Zakaria et al., 2017). AD is marked by cognitive impairment and neurobehavioral disturbances (Kandimalla & Reddy, 2015). Two types of AD have been identified; familial AD, which affects people younger than age 65 (Querfurth & Laferla, 2018) and sporadic AD, which affects those aged 65 and older (Zilka et al., 2006). The common histological hallmarks of AD are characterized by formation of neurofibrillary tangles (NFTs) which composed of hyperphosphorylated tau protein and senile plaques (SPs) constituted of beta-amyloid protein (A β) (Huang & Mucke, 2012). AD is also associated with severe economic effects on the affected individual and their family. More than 36 million people worldwide were impacted by AD, as of 2015 (Rafii & Aisen, 2015). Without an appropriate intervention, the research predicts that this number would expand exponentially to reach 131.5 million by 2050. Much of the predicted increase could occur in developing and under developed nations (Kinney et al., 2018; Prince et al., 2015).

Animal model studies were frequently employed in the field of behavioural neuroscience research. Many studies have used animal models, particularly rats, as they display a range of behaviours that is mimicking to human behaviour in AD. According to a previous study, persistent exposure to D-galactose (D-gal) exacerbates the ageing processes by causing cognitive impairment and neuronal damage. These alterations are particularly prominent in the initial phases of AD (Qu et al., 2015). Neurotoxicity mediated by chronic exposure to D-gal in rats have frequently been employed as an ideal model for investigating the aetiology of AD and for evaluating the new AD therapy (Gao et al., 2015; Zhu et al., 2014).

Aluminium (Al) is a well-known neurotoxic agent utilised extensively in experimental models, it can trigger cognitive impairment and oxidative stress, both of which are symptoms associated with AD (Wang et al., 2018). Research has provided evidence of a correlation between the neurotoxic effects of aluminium in animals and its involvement in the development of neurodegenerative diseases, such as AD in human (Ali et al., 2022). Excessive exposure to Al has been found to induce the over expression of APP, leading to the accumulation of A β plaque on neuronal cells, making Al a potential alzheimerogenic chemical (Mahdi et al., 2019; Mattson, 2004). Additionally, Al also impairs the axonal transports, hinders long-term potentiation, triggers inflammatory responses, and produces synaptic degeneration, leading to significant neurodegeneration (Amjad & Umesalma, 2015). Therefore, rats that have been chronically exposed to Al are regarded as significant animal models for the screening of anti-AD therapies (Ravi & Narasingappa, 2018). As both D-gal and Al have been identified as neurotoxin, combination of these chemicals had been used in this study. In addition, according to the

research conducted by Chiroma et al. (2018), co-administration of 60 mg/kg D-gal and 200 mg/kg AlCl_3 daily for 10 weeks is the most effective method in inducing cognitive impairment in rats. It demonstrates significant neuronal degeneration in the hippocampus and elevated AChE activities (Chiroma et al., 2018).

Throughout history, cannabis has been employed for an extensive duration to address many medical ailments such as cancer, chronic pain, seizures, multiple sclerosis, nausea and anorexia (Chen et al., 2013; Robson, 2001). Delta-9-tetrahydrocannabinol ($\Delta^9\text{THC}$), which mostly found in *Cannabis sativa* plant serve as a principal psychoactive compound that exerts an influence on cognitive processes and behavioural patterns. Previous research found that $\Delta^9\text{THC}$ improved the memory and learning abilities of patients with schizophrenia (Joy et al., 1999). Other investigations cited the negative effects of $\Delta^9\text{THC}$ such as euphoria, anxiety and verbal working memory impairment (Souza et al., 2004). Overdosing on $\Delta^9\text{THC}$ can result in the neuropsychological and cognitive adverse effects that diminish its medical value (Chen et al., 2013). However, recently, there is a growing interest in the medicinal benefits of $\Delta^9\text{THC}$ for human health, particularly in the treatment of AD and other neuropsychiatric disorder (Aso & Ferrer, 2014).

Although numerous pharmacological effects of $\Delta^9\text{THC}$ have been documented, it remains unclear whether $\Delta^9\text{THC}$ has a possible therapeutic effect against D-gal and AlCl_3 induced cognitive impairment in rats. In this study, the approved reference medication donepezil was used to compare the effect of $\Delta^9\text{THC}$. Donepezil is commonly used to treat cognitive impairments such as those caused by AD.

The evaluation of behavioural effects is critical in the *in-vivo* testing of drugs in rats. Rats' attention span, and their short and long-term memory are two aspects of learning that could be measured. For the assessment of cognitive performances in animal models, behavioural tests such as novel object recognition (NOR), Morris water maze (MWM), elevated plus maze (EPM) and modified elevated plus maze (mEPM) have been widely employed. These tests have been recognised to be sensitive to hippocampal dependent learning and memory (Kim et al., 2014). Therefore, the current chapter explored the therapeutic potential of $\Delta^9\text{THC}$ on D-gal and AlCl_3 induced cognitive performances in rat model through neurobehavioral study.

4.2 Material and methods

4.2.1 Equipment

Novel object recognition box (located at the Behaviour Assessment Room, Animal Experimental Unit, Department of Human Anatomy, UPM), Morris water maze tank (located at the Behaviour Assessment Room, Animal Experimental Unit, Department of Human Anatomy, UPM), Elevated Plus maze (located at the Behaviour Assessment Room, Animal Experimental Unit, Department of Human Anatomy, UPM), Indirect white 100 W lamp, Logitech C350 video camera (Logitech, Malaysia), ANY-Maze

tracking software (ANY-Maze™, Stoelting Co., Chicago, U.S.A.) for automated behavioural analysis, stop watch, 70% ethanol for cleaning purposes and distilled water.

4.2.2 Animal and experimental design

Animal husbandry and experimental design refer to Chapter 3, section 3.1.2 and 3.1.3.

4.3 Behavioural test

4.3.1 Novel Object Recognition (NOR)

With minor modifications, this test follows the protocol of Watson et al. (2012) and Bull et al. (2010). The NOR test device was comprised of plexiglas box with dimension of 75 cm x 75 cm base x 40 cm height (Lueptow, 2017). This test consisted of three phases; habituation, acquisition and test phase. During habituation phase (one day before testing), the rat was habituated inside an empty box arena with no stimuli present for 5 minutes. The day following the habituation phase, the rat underwent the acquisition phase with two identical objects (A1 and A2) placed near the wall's corner. The rat was allowed to familiarise with the objects for 5 minutes and the time taken for the rat to explore each object was recorded. Soon after, it was returned back into its home cage for 30 minutes. During test phase, a previously encountered object (A2) was substituted with a novel object (B). The rat was given 5 minutes to explore both objects (Figure 4.1). In order to mitigate errors and minimise biasness, the novel object was consistently positioned in the same location as the previously familiar object, with the sides being alternated between experiments to prevent any potential side preferences. Object exploratory behaviour can be defined as sniffing, licking and touching with its head facing the object at a distance ≤ 2 cm. In contrast, climbing, sitting on the object and turning around from the object were not considered as exploratory behaviour. All the behavioural equipment was wiped clean with 70% alcohol to remove any olfactory cues and debris from the surface before beginning a new test session. In order to assess the rat's preference towards novel object, discrimination index (DI) was calculated by using the following equations: $DI = (B - A1) / (B + A1)$, which B= novel object; A1= familiar object.

A



B



Figure 4.1 : Experimental setup during novel object recognition (NOR) test. (A) During acquisition phase. Two identical objects were placed near the wall's corner of Perspex box; (B) During test phase. One familiar object was substituted with a novel object, and placed at the same positioned as the previous familiar object

4.3.2 Morris Water Maze (MWM)

This was a well-established assay for hippocampal dependent spatial memory in which the rats learned and navigated the spatial location of a platform submerged 2 cm in a circular pool of water using distal cues. The MWM tank was filled with opaque water to a depth of 40 cm and kept at the consistent temperature of $24 \pm 2^{\circ}\text{C}$ (Zhang et al., 2016; Li et al., 2016). MWM tank was equipped with a video tracking camera that was positioned above it. This camera was then connected to a laptop with software ANYmaze™, Stoelting Co., Chicago, USA. With the aid of the software, the tank was divided into four equal quadrants. A platform with a diameter of 10 cm was positioned at the centre of a designated quadrant, referred to as the target quadrant.

The experiment was conducted according to the procedure described by C Vorhees (2010). On the first day of experiment, rats were trained to locate the visible escape platform (2 cm above the water level). The rats were then trained for four consecutive days to locate the hidden escape platform (submerged 2 cm below the water level) while the escape platform quadrant was maintained for the five days of the test. During the place navigation test, each rat participated in three training sessions daily. For each experimental session, the rats were randomly placed in one of three quadrants (starting point), excluding the quadrant containing the escape platform. The rats were then given a maximum of 120 seconds to locate the hidden escape platform in each trial. Rats that successfully located the escape platform were allowed to remain on it for 20 seconds at the end of each trial to facilitate their recognition and familiarisation of the platform's position. In contrast, those rats that were unable to locate the escape platform within the designated time period were guided gently toward the platform and allowed to remain there for 20 seconds as well (Chiroma et al., 2018).

The spatial probe test was conducted 24 hours after the completion of the place navigation test. The rats were placed in the opposite quadrant of the water tank and permitted to explore for the removed escape platform for 30 seconds (Chiroma et al., 2018). The video camera, connected to a computer with ANYmaze software, was used to record and capture several parameters such as swimming trajectories, escape latency, time spent in target quadrant, number of entries to platform quadrant and number of entries to platform zone (Figure 4.2).

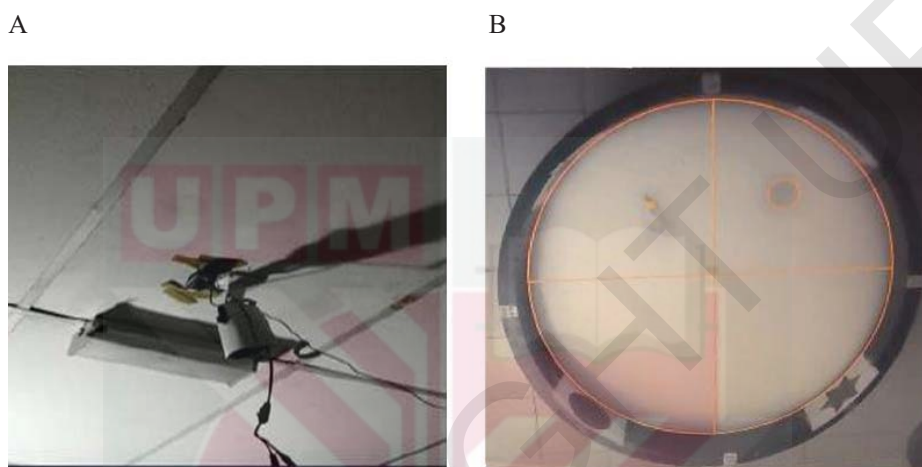


Figure 4.2 : Experimental setup during Morris Water Maze (MWM) test. (A) Logitech camera fixed on the ceiling directly facing the MWM tank to capture the movement, B) ANYmaze video tracking software connected with Logitech camera for recording of rat movement such as swimming trajectories, escape latency, time spent in the target quadrant, number of entries to platform quadrant and platform zone

4.3.3 Modified Elevated Plus Maze (mEPM)

The experimental apparatus employed in this study consisted of a plus-shaped device, featuring two enclosed arms measuring 50 cm in length, 10 cm in width and 50 cm in height, positioned in opposition to each other. Additionally, two open arms, with the same dimensions, were also positioned in opposition to each other. A central square (10 cm x 10 cm) connected all of the arms (Biala & Kruk, 2008) (Figure 4.3). The experiment was carried out in a dark room illuminated by a halogen lamp.

On the first day of experiment, during the acquisition phase, each rat was positioned at one end of an open arm, with its back turned towards the central square. The duration of time that each rat required to reach either of the enclosed arms was measured and referred to as its initial transfer latency (ITL). After recording the ITL, the rats were given 20 seconds to explore the device. The rats that failed to enter the enclosed arm after 90 seconds were gently guided into either of enclosed arms and were allowed to remain there for 20 seconds as well. The rats were considered to be inside either of enclosed arm

when all four of their paws had crossed the boundary line separating the enclosed arm from the central square.

The retention session was conducted 24 hours after acquisition phase. The rats were similarly placed at one end of an open arm, and the first transfer latency (TL1) was recorded. If the rat failed to enter either of the enclosed arm within 90 seconds, the experiment was stopped and its TL1 was recorded as 90 seconds. In order to prevent olfactory queue, the device was cleaned with 70% alcohol between each trial.



Figure 4.3 : Experimental setup during modified elevated plus maze test (mEPM). The device plus (+) shaped with two enclosed arms (EA) and two open arms (OA). All the arms linked by central square

4.3.4 Elevated Plus Maze (EPM)

This paradigm was used to evaluate rats' anxiety-like behaviour (Burne & Brain, 2011). The test was performed on a plus-shaped apparatus with two open arms and two enclosed arms. The rats were placed at the junction of the four arms of the maze, facing an open arm, and the duration in each arm was recorded by a video-tracking system and observer simultaneously for 5 minutes (Leo & Pamplona, 2014). Increased open arm activity indicates anti-anxiety behaviour.

4.4 Statistical analysis

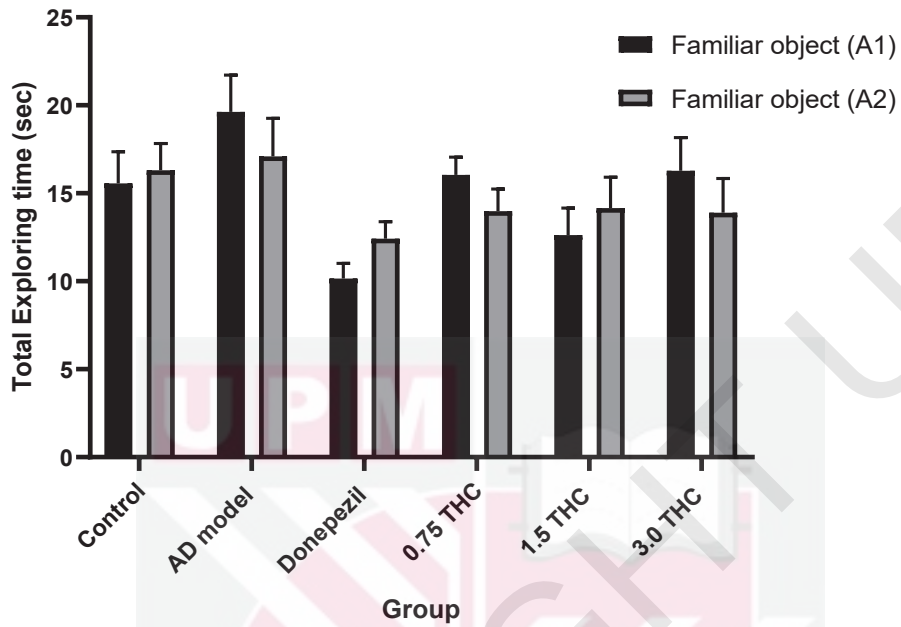
The results from the acquisition and test phase of NOR were analysed using two-way ANOVA, followed by Tukey's multiple comparison test. Meanwhile, discrimination index (DI) of NOR test was analysed using one-way ANOVA followed by Tukey's multiple comparison test. The data of MWM, EPM and mEPM were analysed by one-way (ANOVA) followed by Tukey's multiple comparison test using SPSS version 25.0. The data was presented as mean $\pm$ standard error of mean (SEM). Significance difference was determined by $p < 0.05$.

4.5 Result

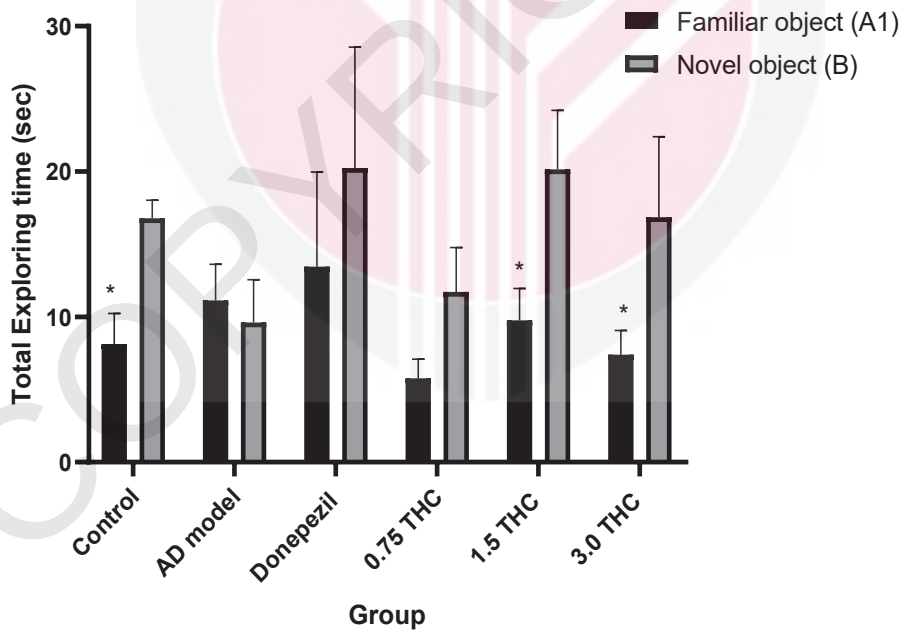
4.5.1 Δ^9 THC ameliorate non-spatial memory in rats induced by D-gal and AlCl_3 in novel object recognition (NOR)

Figure 4.4 (A-C) depicts the ability of the rats to distinguish between familiar and novel objects. During acquisition phase, the rats spent almost equal amount of time exploring two familiar objects (Figure 4.4 A). The two-way ANOVA revealed no statistically significant differences between duration of exploration of two familiar objects (A1 and A2) ($p > 0.05$). In the test phase, treatment groups demonstrated an increase in novel object exploration. A statistically significant differences with $p < 0.05$ in the preferences towards the novel object by control ($16.78\text{s} \pm 1.25$), Δ^9 THC 1.5 mg/kg ($20.15\text{s} \pm 3.05$) and Δ^9 THC 3.0 mg/kg ($16.85\text{s} \pm 3.55$) respectively when compared to AD model ($9.61\text{s} \pm 2.94$) (Figure 4.4 B). In addition, a one-way ANOVA conducted across treatment groups on the discrimination index (DI) that revealed a statistical significance difference [$F(6,37) = 5.419$, $p = 0.015$]. Further, the Tukey's post hoc also revealed a statistically significant different ($p < 0.05$) in DI reduction in the AD model group of rats (-0.15 ± 0.04) when compared to the control (0.39 ± 0.1) and treated groups [donepezil (0.25 ± 0.09); Δ^9 THC 0.75 mg/kg (0.29 ± 0.11); Δ^9 THC 1.5 mg/kg (0.34 ± 0.09); Δ^9 THC 3.0 mg/kg (0.29 ± 0.18)] (Figure 4.4 C). DI is an indicator of their learning ability.

A



B



C

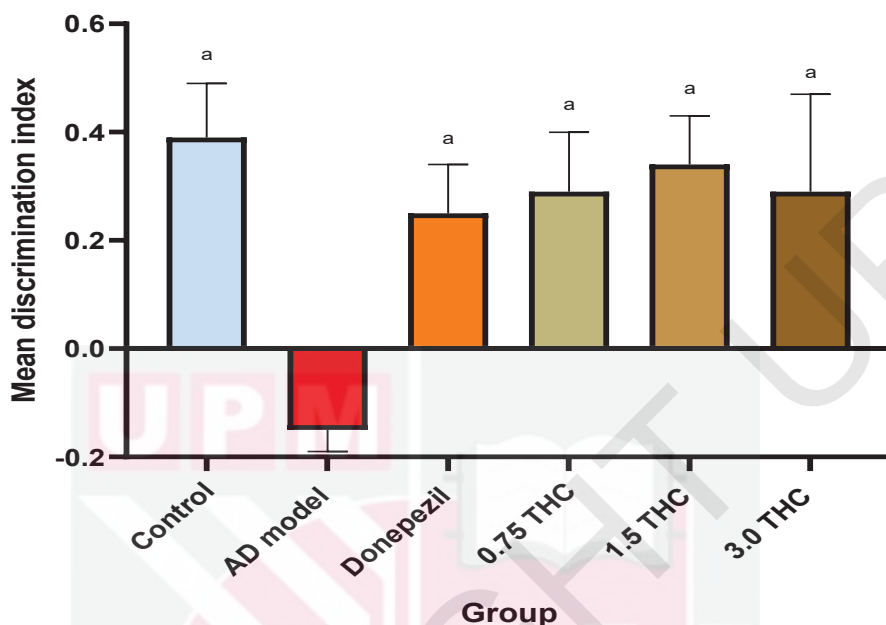


Figure 4.4 : Novel object recognition (NOR) test. Therapeutic effect of Δ^9 THC against D-gal and AlCl_3 induced cognitive performances on rats. A: Acquisition phase; identical objects exploration. B: Test phase; familiar object and novel object exploration. C: Discrimination index (DI). Data was expressed as mean $\pm$ SEM (n=10). ^ap<0.05 vs AD model, *p<0.05 between familiar object and novel object among the same group

4.5.2 Δ^9 THC exhibit spatial learning and memory in rats induced by D-gal and AlCl_3 in Morris water maze (MWM)

Using MWM, the effects of Δ^9 THC treatment in rats induced by D-gal and AlCl_3 on hippocampal-dependent learning and memory was also determined. In the present study, the swimming trajectories, escape latency, time in the target quadrant, number of entries to platform quadrant and number of entries to platform zone were measured as criteria for evaluating rats' spatial learning and memory abilities using this test (Morris, 1984). Figure 4.5 depicts the representative swimming path on the final day (day 5) of the place navigation test. A one-way ANOVA was performed to determine the effects of Δ^9 THC on latency to find escape platform over the five consecutive training days. There was statistically significant interaction between the effect of treatment and days of treatment, [F (6,36) = 5.871, p=0.001]. Tukey's post hoc test revealed that AD model group differed significantly from the control group and all treated groups (p<0.05). However, post hoc test did not reveal any statistically significant differences between control and all treated groups (Figure 4.6A).

One way ANOVA [$F(6,33) = 3.123, p=0.016$] showed a statistically significant difference in the time spent by the rats in the targeted quadrant during probe trial. The AD model ($7.25s \pm 0.64$) spent statistically significant less time in the targeted quadrant when compared to the control ($15.4s \pm 1.92$), $\Delta^9\text{THC}$ 0.75 mg/kg ($14.47s \pm 3.28$), $\Delta^9\text{THC}$ 1.5 mg/kg ($14.61s \pm 1.95$) and $\Delta^9\text{THC}$ 3.0 mg/kg ($13.18s \pm 1.43$), as revealed by Tukey's post hoc (Figure 4.6 B).

The number of entries to platform quadrant was also determined during probe test. A one-way ANOVA revealed statistically significant differences between the AD model group and control group [$F(6,26) = 4.089, p=0.005$]. Tukey's post hoc test revealed that the number of platform quadrant entries by AD model (6.8 ± 0.49) were significantly lower than those of the control group (12.6 ± 0.51) (Figure 4.6 C).

On the other hand, the number of platform zone entries was also measured. As determined by one-way ANOVA [$F(6,25) = 7.754, p=0.001$], there were statistically significant differences between the AD model group and control group. Tukey's post hoc test revealed that the number of platform zone entries by AD model (1.75 ± 0.25) decreased significantly as compared to the control group (4.2 ± 0.374) (Figure 4.6 D).

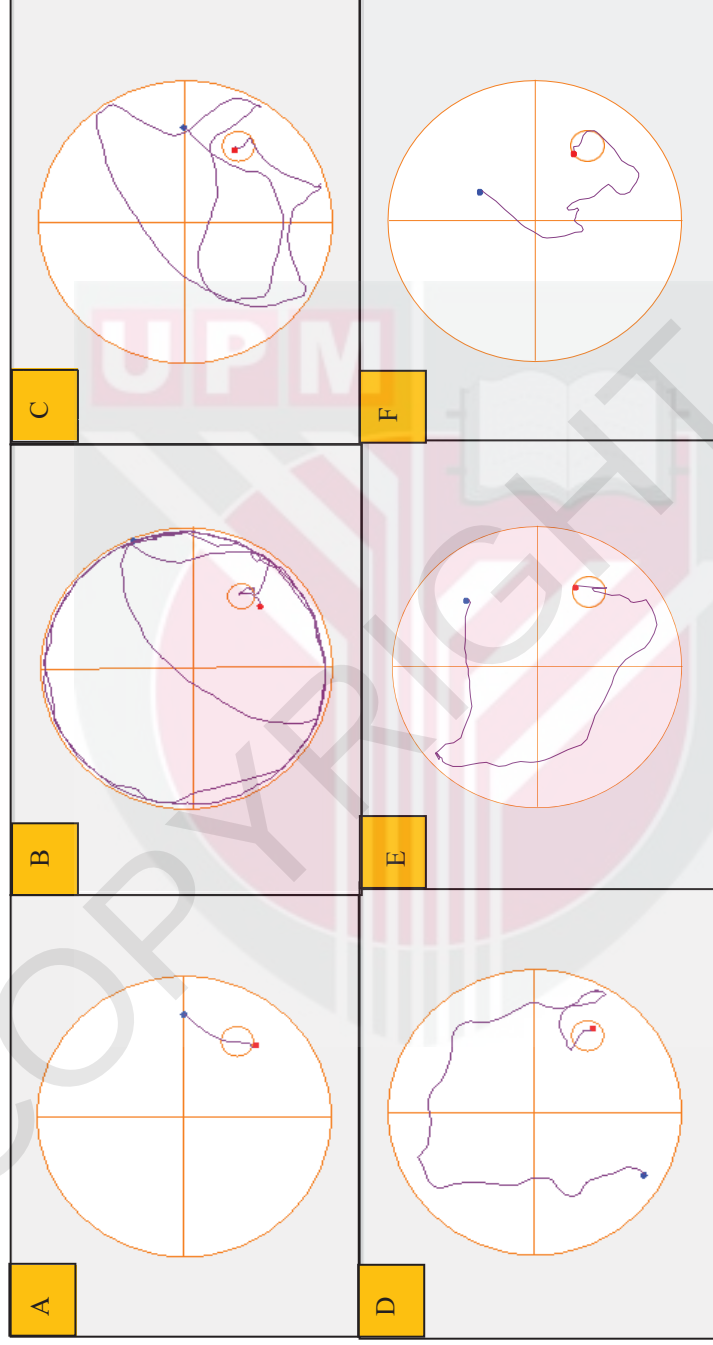
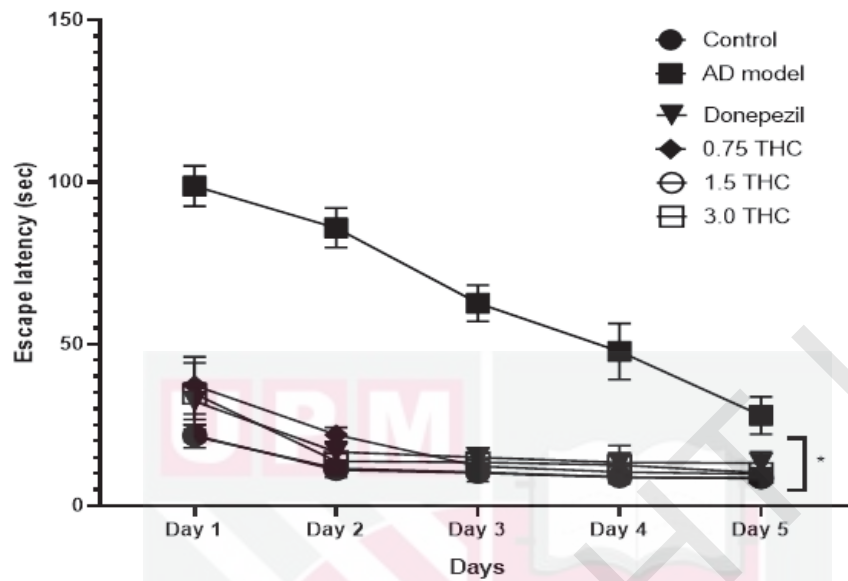
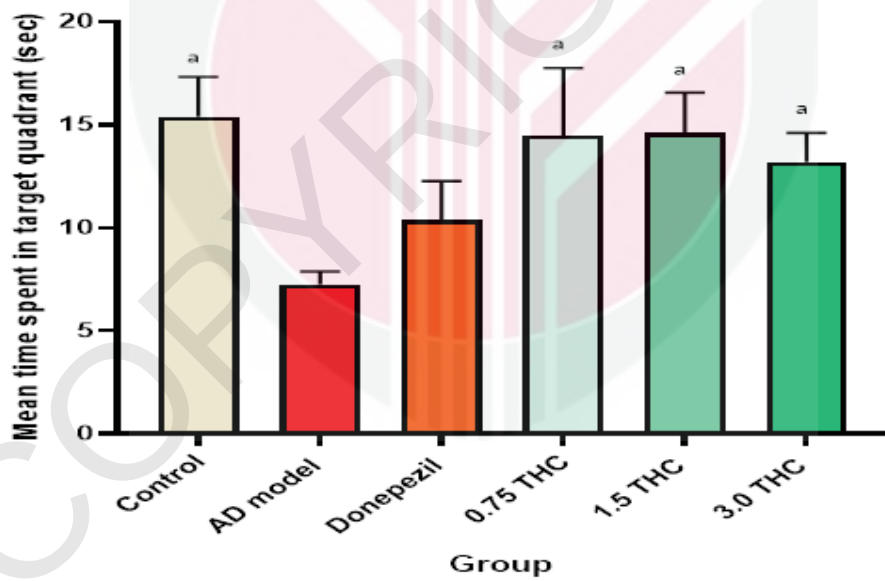


Figure 4.5 : Spatial learning and memory through MWM in rats. Representative of swimming trajectories of each group on trial day 5. (A) Control rats, (B) AD model group, (C, D, E, F) treated group with 1 mg/kg donepezil, 0.75 mg/kg Δ^9 THC, 1.5 mg/kg Δ^9 THC and 3.0 mg/kg Δ^9 THC respectively. Blue dots – starting point during place navigation test; red dots – hidden escape platform point during place navigation test

A



B



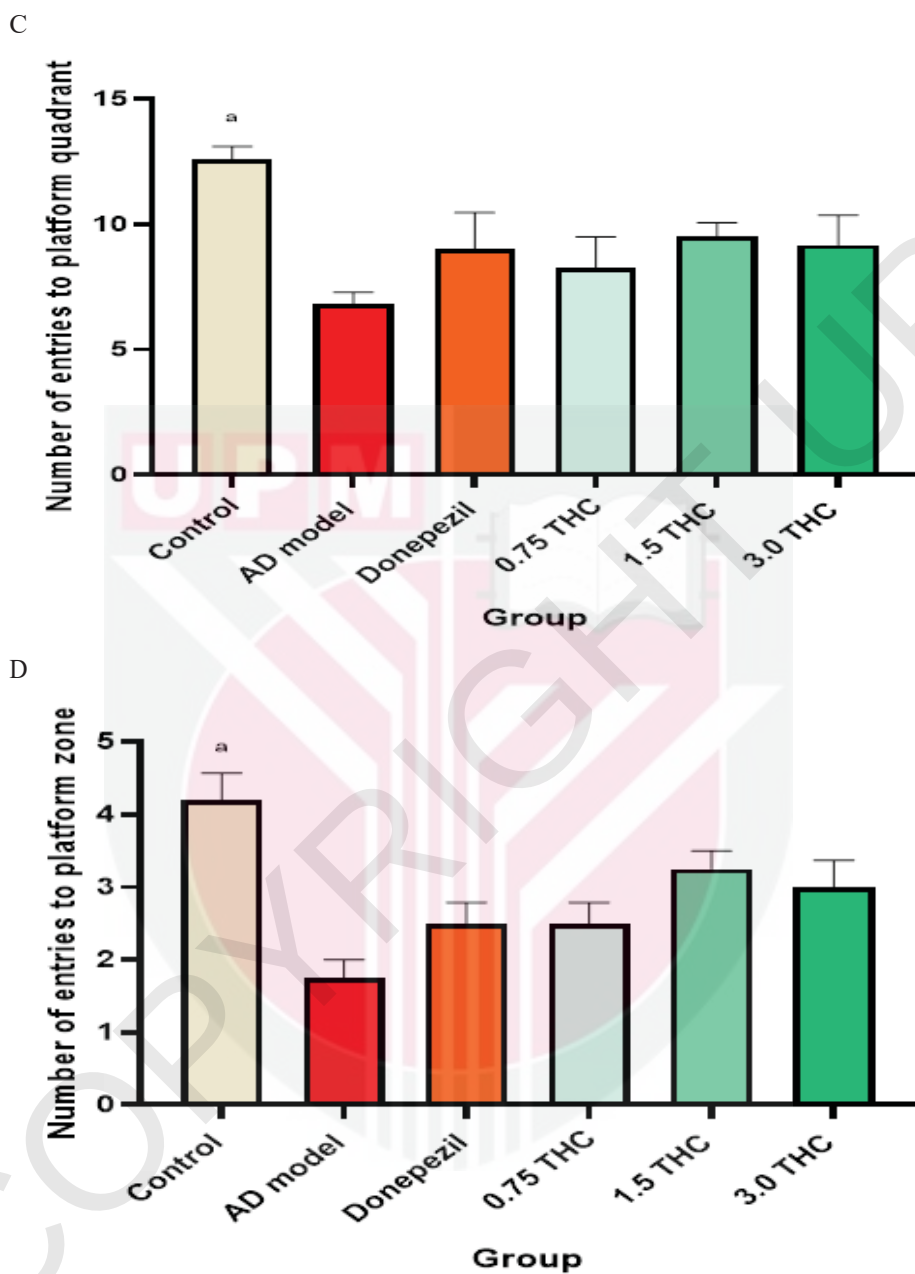


Figure 4.6 : Spatial learning and memory through MWM in rats. (A) the mean escape latency in 5 days to find the escape platform in place navigation test. (B) time spent in target quadrant in probe test. (C) number of entries to the platform quadrant during probe test. (D) number of entries to platform zone during probe test. Data were expressed as mean $\pm$ SEM. ^a $p < 0.05$ vs AD model (n=10)

4.5.3 Δ^9 THC exhibit spatial learning and memory in rats induced by D-gal and AlCl_3 in modified elevated plus maze test (mEPM)

The Δ^9 THC effect against the impairment of spatial learning and memory loss in rats induced by D-gal and AlCl_3 was examined using mEPM. Figures 4.7 and 4.8 depicted the performance of the rats during ITL, and there were no statistically significant differences between groups of rats [$F(6,29) = 0.322$, $p=0.92$]. Figures 4.9 and 4.10 illustrated the 24-hours performance of rats. 1st transfer latency (TL1) differed significantly ($p<0.05$) between the AD model group and all Δ^9 THC-treated groups [$F(6,29) = 3.168$, $p=0.025$]. The treated groups [Δ^9 THC 0.75 mg/kg ($17.4s \pm 3.77$); Δ^9 THC 1.5mg/kg ($16.05s \pm 3.1$); Δ^9 THC 3.0 mg/kg ($17.7s \pm 4.72$)] required less time than the AD model ($34.03s \pm 2.3$) to reach an enclosed arm. The spatial learning and memory of the rats in the AD model declined.

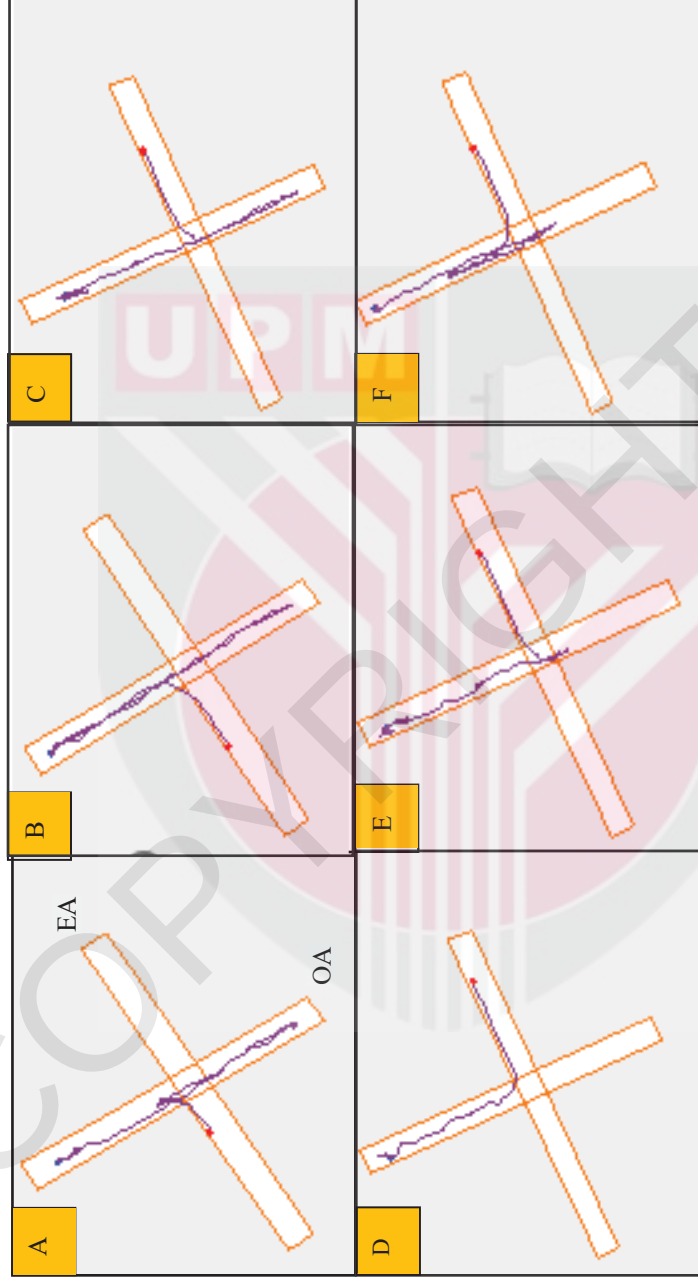


Figure 4.7 : Spatial learning and memory through mEPM in rats. Representative of trajectories of each group during acquisition session. (A) Control rats, (B) AD model group, (C, D, E, F) treated group with 1 mg/kg donepezil, 0.75 mg/kg Δ^9 THC, 1.5 mg/kg Δ^9 THC and 3.0 mg/kg Δ^9 THC respectively. OA-open arm; EA-enclosed arm. Blue dots – starting point during acquisition session; red dots – end point during acquisition session

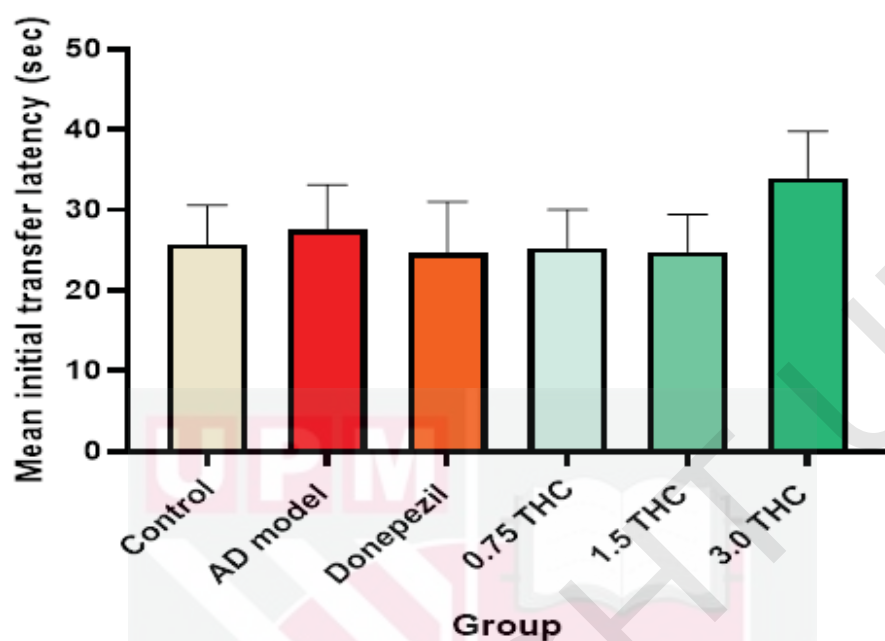


Figure 4.8 : Spatial learning and memory through mEPM in rats. The mean initial transfer latency (ITL). Data were expressed as mean $\pm$ SEM (n=10)

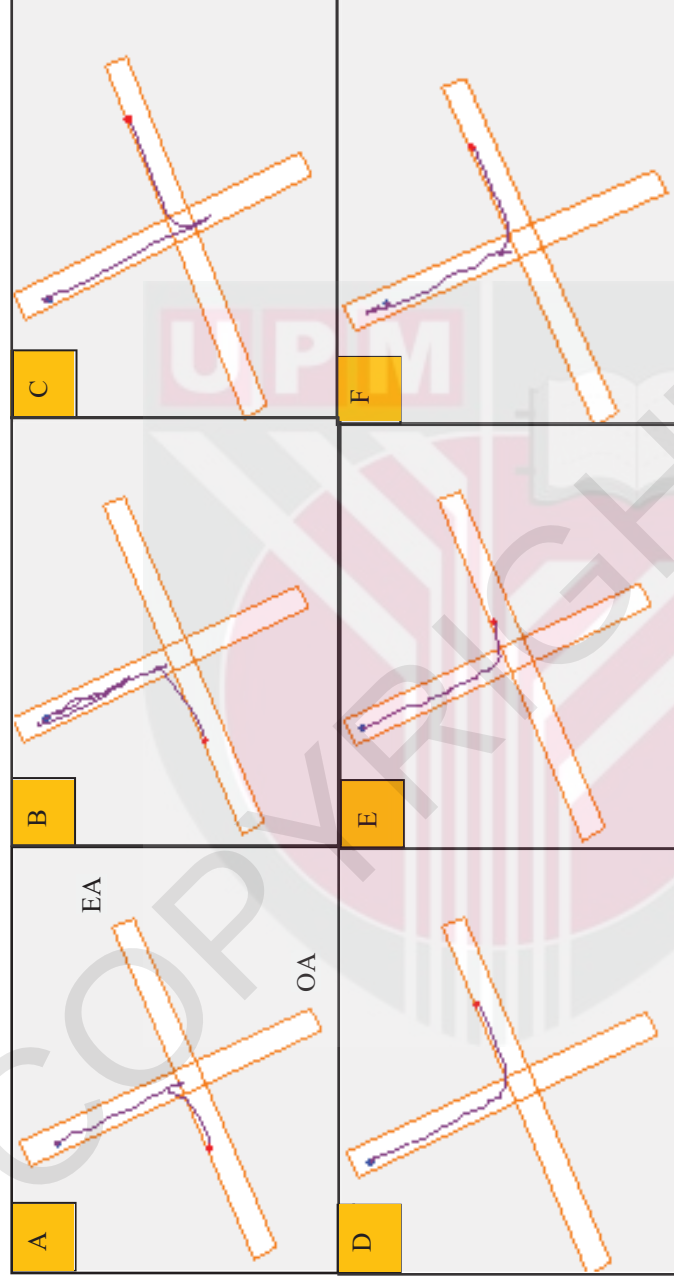


Figure 4.9 : Spatial learning and memory through mEPM in rats. Representative of trajectories of each group during retention session. (A) Control rats, (B) AD model group, (C, D, E, F) treated group with 1 mg/kg donepezil, 0.75 mg/kg Δ^9 THC, 1.5 mg/kg Δ^9 THC and 3.0 mg/kg Δ^9 THC respectively. OA-open arm; EA-enclosed arm. Blue dots – starting point during retention session; red dots – end point during retention session

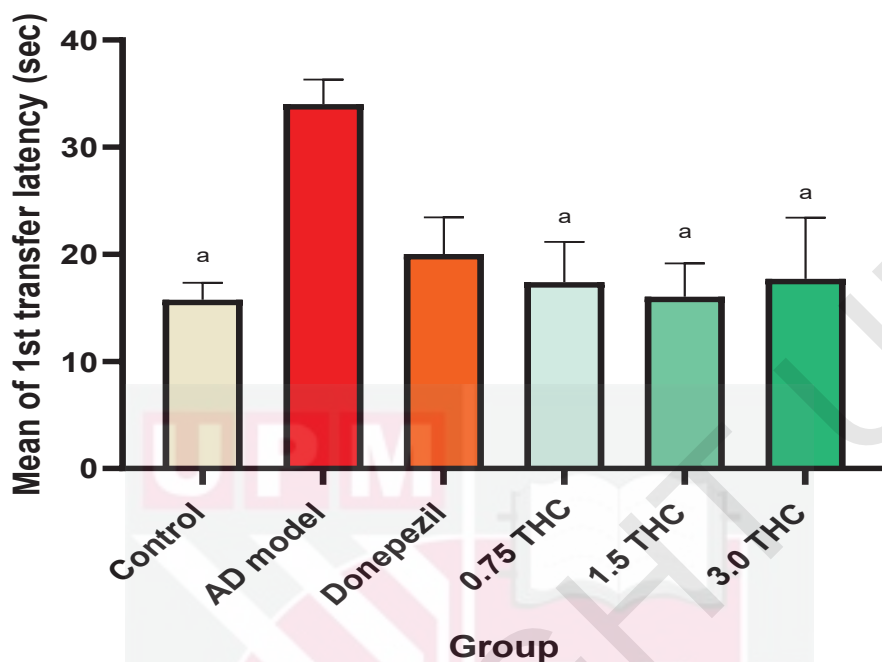


Figure 4.10 : Spatial learning and memory through mEPM in rats. The mean 1st transfer latency (TL1). Data were expressed as mean $\pm$ SEM. ^a $p < 0.05$ vs AD model (n=10)

4.5.4 Δ^9 THC attenuates anxiety-like behaviour in D-gal and AlCl_3 -induced rats in EPM

The percentage of time spent in the open arm increased in all treated groups when compared to the AD group [(F (6,29) = 3.05, $p = 0.019$]. Figure 4.11 demonstrated the trajectories of rats in each group during EPM. Tukey's post hoc test revealed that control ($30.36s \pm 5.92$), donepezil ($22.85s \pm 1.75$) and Δ^9 THC groups of rats [Δ^9 THC 0.75mg/kg ($22.3s \pm 7.35$); Δ^9 THC 1.5 mg/kg ($35.09s \pm 4.07$); Δ^9 THC 3.0 mg/kg ($25.25s \pm 3.59$)] spent significantly ($p < 0.05$) more time on the open arm than the AD group ($11.59s \pm 1.95$) (Figure 4.12). Spending more time in open arms is associated with anti- anxiety behaviours in rats.

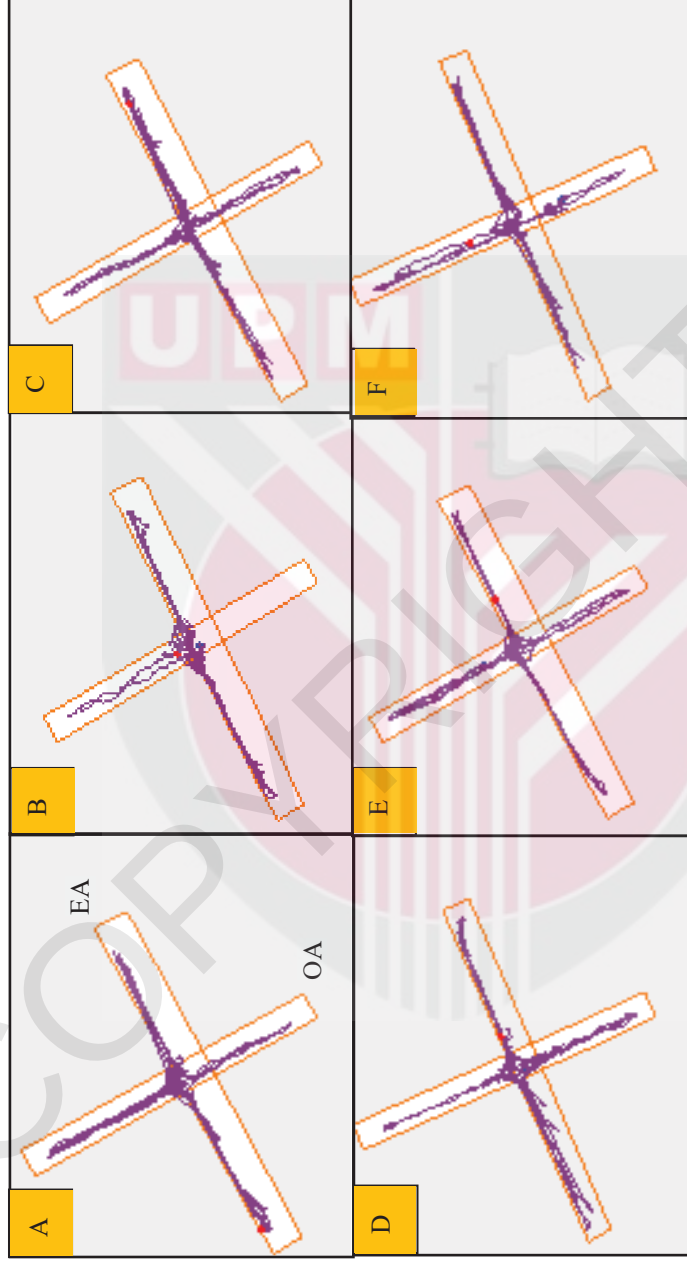


Figure 4.11 : Representative of trajectories of each group during EPM. (A) Control rats, (B) AD model group, (C, D, E, F) treated group with 1 mg/kg donepezil, 0.75 mg/kg Δ^9 THC, 1.5 mg/kg Δ^9 THC and 3.0 mg/kg Δ^9 THC respectively. OA-open arm; EA-enclosed arm. Blue dots – rats' head point; red dots – rats' exploration during EPM

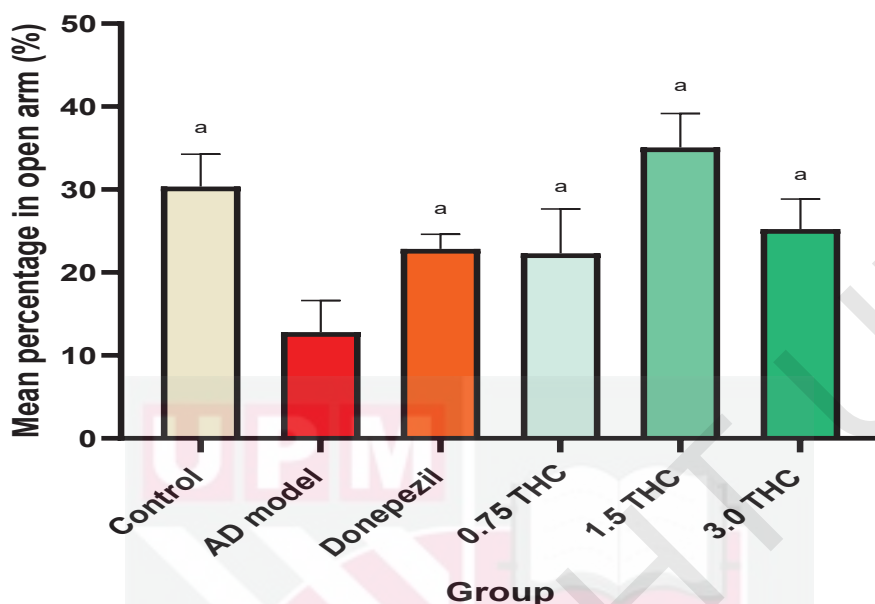


Figure 4.12 : The mean percentage in the open arm. Data were expressed as mean $\pm$ SEM. ^a $p < 0.05$ vs AD model (n=10)

4.6 Discussion

Cognitive impairments have been extensively documented in old age disorders including Alzheimer's disease (AD) (Chiroma et al., 2018). D-gal has been linked to accelerated ageing in rats (Lei et al., 2008), whereas aluminium is a neurotoxic metal that causes cholinergic neuron degeneration and learning impairment (Dumont, 2011). Previous research on rodents have shown that co-administration of D-gal and AlCl_3 could induced neurodegenerative changes similar to AD, such as cognitive and memory dysfunction (Zhang et al., 2016), deficits in the cholinergic system (Prakash & Kumar, 2011), a high level of amyloidogenic protein expressions, and depletion of cells in the cerebral cortex and hippocampus (Haider et al., 2020; Wei et al., 2017; Xiao et al., 2017). The present chapter was aimed at exploring neurotherapeutic potential of $\Delta^9\text{THC}$ on anxiogenic effect and cognitive deficits caused by co-administration of D-gal and AlCl_3 in Wistar rats. In this study, administration of $\Delta^9\text{THC}$ has potentially improve cognitive performances conducted following cognitive impairment by the combine administration of D-gal and AlCl_3 . It was suggested that $\Delta^9\text{THC}$ played a significant role in attenuating the behavioural changes associated with D-gal and AlCl_3 -induced learning and memory in rats.

Evaluation of behavioural effects is a crucial aspect of the *in-vivo* screening of drugs in rats prior to other evaluations such as histology and molecular studies. In the present investigation, cognitive and behavioural changes were evaluated using the NOR, MWM, mEPM and EPM tests. The NOR test is a relatively fast and effective method for

measuring learning and memory in animal model. This task is appealing because it does not rely on external motivation, reward or punishment, but rather on natural exploration, hence it is much less stressful (Silvers et al., 2011).

The findings of this study revealed that rats administered with D-gal and AlCl_3 exhibited decline in non-spatial memory, which supports previous research documenting comparable cognitive impairments in rats exposed to the same neurotoxin (Abulfadl et al., 2018; Xu et al., 2017; Gao et al., 2015). However, $\Delta^9\text{THC}$ treatment led to significant improvements in non-spatial memory in rats, as evidenced by better performances in the NOR test (Figure 4.4 B). Treatment groups of rats in the current study exhibited the capacity to differentiate between novel object and familiar object, demonstrating their cognitive ability (Figure 4.4 B). There were no statistically significant changes were seen in novel object discriminatory abilities between $\Delta^9\text{THC}$ and donepezil treated rats, implying that $\Delta^9\text{THC}$ had similar cognitive enhancing abilities as donepezil in improving non-spatial memory in rats. Discrimination index (DI) is an indicator for preferences to the novel object. The higher the DI value, the better the memory recognition (Figure 4.4 C). The result of NOR is influenced by hippocampus, a brain area which plays an important role in object recognition memory (Aggleton et al., 2010). When there is some damage occurred, the performance in recognition memory tasks is impaired (Albasser et al., 2009), as demonstrated in rats exposed to D-gal and AlCl_3 . The results of the present study confirm to those of Suliman et al. (2018) and Aso et al. (2015) on the cognitive enhancing abilities of object recognition through NOR.

The MWM test is another model that widely used for studying learning and memory behaviour in rats. It is relied on distal cues to find the submerged platform (Wang et al., 2018). The initial trial in MWM was typically characterized by the animals' proclivity to spend most of its time near the walls (thigmotaxis behaviour) or to perform random searches in the pool. However, in the subsequent training trial, the animal demonstrated gradual change in behaviour characterized by progressive active search for the platform. The swimming trajectories of the rats administered with D-gal and AlCl_3 in the present study demonstrated purposely random swimming along the edge of the pool and spending longer time before reaching the hidden platform or in the majority of cases not locating the platform, as compared to the control and treated groups, which showed progressively active search near the platform area and reached the platform in less time (Figure 4.5). These results are suggestive of memory and learning impairment in rats.

The finding from this study was in line with previous research, which also reported cognitive deficits in rodents exposed to D-gal and AlCl_3 (Wei et al., 2017; Zhang et al., 2016). Other parameters that confirm memory impairment in the current study include an increase in escape latency to reach the submerged platform, a decrease in time spent at the target quadrant after the submerged platform was removed, a reduction in the number of platform quadrant entries and platform zone entries during the probe trial. Treatment with $\Delta^9\text{THC}$ attenuated the cognitive deficits induced by D-gal and AlCl_3 in rats, as demonstrated by decreased escape latency to locate the submerged escape platform. In addition, rats treated with $\Delta^9\text{THC}$ spent more time in the target quadrant searching for the removed escape platform during probe test of MWM. The number of entries to platform quadrant and platform zone were also increased in $\Delta^9\text{THC}$ treated

groups. Our findings were consistent with those of Nitzan et al. (2021), who reported that a single injection of ultra-low dose Δ^9 THC (0.002 mg/kg) improves cognitive performance via MWM in a 5xFAD tg-mice AD model. Another study found that Δ^9 THC at 1 mg/kg enhances spatial learning in aged mice in the MWM test (Nidadavolu et al., 2021). There were no statistically significant differences were observed between the Δ^9 THC and donepezil treatment group of rats, showing that Δ^9 THC possesses cognitive enhancing abilities comparable to donepezil, an FDA approved drug for palliative care of AD (Figure 4.6 A-D).

mEPM is a modified version to assess the cognitive functions of rats, specifically their learning and memory processes. This approach is grounded on the fundamental concept that rats are inclined to acquire and retain knowledge of the place that offers them a sense of safety, which refers to enclosed arm of the maze (Yildiz et al., 2007). In the experimental conditions of the current investigation, it was shown that rats administered with D-gal and AlCl_3 demonstrated a decline in their spatial learning abilities, which in line with investigation conducted by Chiroma et al (2018) that utilized the same neurotoxin. Treatment with Δ^9 THC in the present study produced significant enhancement in the spatial learning and memory as seen in this test (Figure 4.9 and Figure 4.10).

In EPM test, the results of present study demonstrated that co-administration of D-gal and AlCl_3 exhibited anxiogenic behaviour, as evidenced by increased in time spent in enclosed arm (Figure 4.12). Anxiety is the most common neuropsychiatric conditions observed in AD patients (Muddasir et al., 2017). Consistent with the findings of Zhang et al (2022) and Muddasir et al (2017), these observations revealed that the use of these inducers decreased the amount of time spent in the open arm during EPM. However, there was an increased time spent in open arm in all treated groups when compared to AD model. The results suggest that rats exhibit anti-anxiety behaviour (Figure 4.12), which was in line with the findings of Kasten et al. (2019), who found that Δ^9 THC at 1 mg/kg significantly enhanced open arm entries in adolescent male C57BL/6J mice, indicating an anxiolytic effect. Contradictory, in the same study, however, Δ^9 THC at 10 mg/kg reduced the effect. Another study also found that 10 mg/kg Δ^9 THC reduced the time spent in open arm of EPM (Long et al., 2010). It might be postulated that the dosage of Δ^9 THC contributes to the changes in anxiety-like activity.

4.7 Conclusion

The present study demonstrates that persistent exposure to D-gal and AlCl_3 impairs the cognitive and memory performances, and induce anxiogenic effect in rats. Treatment with Δ^9 THC restore the cognitive performances and promote anxiolytic effect induced by D-gal and AlCl_3 . The findings also showing that Δ^9 THC possesses cognitive enhancing abilities comparable to donepezil, a reference approved drug for AD. It was proposed that structural changes in the hippocampus of rats caused by Δ^9 THC, a brain region that plays an essential role in the learning and memory processes, influenced the results of behavioural studies. This work represents the first documentation of the therapeutic effect of Δ^9 THC on cognitive abilities of rats induced by D-gal and AlCl_3 .

CHAPTER 5

NEUROTHERAPEUTIC POTENTIAL OF DELTA-9-TETRAHYDROCANNABINOL ON MORPHOLOGICAL CHANGES IN THE HIPPOCAMPUS OF RATS EXPOSED TO D-GALACTOSE AND $AlCl_3$

5.1 Introduction

Brain morphology is the study of the brain's structural characteristic. It is useful for comparing the brains of patients with those of healthy controls (Madan, 2017). By examining the morphology of the brain, it is possible to identify anatomical variation and structural differences in the brain associated with medical conditions such as AD, and to comprehend the brain growth and ageing (Lerch et al., 2017; Somerville, 2016). Additionally, brain morphology might be useful when studying the cognitive ability. With the knowledge of brain morphology, the connection between behavioural measures of memory performance and structural measures such as hippocampal volume can be examined (Olsen et al., 2017; Ritter et al., 2017).

The hippocampus is a part of limbic system of the brain, which is located beneath the medial lobe on both sides of the brain (Jiang et al., 2020). It is a unique structure that regulates emotions and is primarily involved in memory formation (both short term memory and long-term memory) (Knierim, 2015; Gu et al., 2012). The hippocampal circuitry has been classified as having unidirectional and trisynaptic pathways (Li et al., 2009). In summary, the entorhinal cortex is the primary source of input to the hippocampus via its connection to the dentate gyrus (DG). The signal was subsequently transmitted from DG to cornu ammonis 3 (CA3), and then cornu ammonis 1 (CA1). CA1 sequentially projects signal to the subiculum, which relays the hippocampal output to the deep layers of entorhinal cortex (Li et al., 2009). Thus, any damage to the hippocampus may cause difficulties in establishing new memories (Squire, 2004), resulting in cognitive impairment and development of neurodegenerative disorder such as AD.

The hippocampus is made up of several cytoarchitecturally distinct regions, including CA1, CA2, and CA3 regions (Rebola et al., 2017; Oliva et al., 2016; McEwen et al., 2016). According to studies, there is a high degree of variability within the hippocampus, with the CA1, CA2 and CA3 showing high concentrations of cannabinoid receptors, rendering it susceptible to cannabinoid exposure (Glass et al., 1997). Nonetheless, ageing appears to reduce the number of cannabinoid receptor binding sites in the brain region. Study conducted by Batalla et al. (2018), discovered that cannabinoid exposure may affect the neuroanatomy of certain subfields of the hippocampus. The results revealed that cannabinoid exposure increased the volume of CA3 region of hippocampus, consistent with the findings of another study conducted by Orr et al. (2019).

Numerous research supports the notion that cannabis causes cognitive deterioration, particularly with long-term use. According to a report by Bartholomew et al. (2010), cannabis has significant acute impacts on human cognition and visuomotor skills, which

impair young adults' memory. However, evidences from both animals and human studies suggests that cognitive decline severity is dose-dependent (Dragt et al., 2010). Several studies have found that high doses of Δ^9 THC (5-10 mg/kg, daily consume) have harmful effects on mental health (Shrivastava et al., 2011). Positive effects of Δ^9 THC have been demonstrated when used in lower doses (3 mg/kg and below, daily), and nowadays, there is a growing interest in the therapeutic use of Δ^9 THC for neuropsychiatric disorders (Ueberall et al., 2022; Minerbi et al., 2019).

The cannabinoid system is crucial for comprehending the relationship between Δ^9 THC exposure and cognitive performance. This system is susceptible to exogenous cannabinoid such as Δ^9 THC, which are known to have an impact on cognitive and memory functioning (Jager et al., 2010). The cannabinoid receptors especially in the brain also contributed in regulation of learning and memory as well (Ramírez et al., 2005). Cannabinoid receptor expression is altered in animal models and human post mortem samples of AD brain, particularly in the hippocampus and cerebral cortex (Ramírez et al., 2005). Experiments with plant-isolated molecules revealed that Δ^9 THC, via its interaction with CB₁ receptors, is responsible for neuropsychological effects including AD (Matsuda et al., 1990). In the recent research of transgenic Tg_{4. 42} mice expressing human A β ₄₀₋₄₂ that were treated with Δ^9 THC, neuronal death was reduced relative to the control group (Wagner et al., 2019). The results of this investigation may suggest that Δ^9 THC is capable of halting neurodegeneration and promoting neural repair, thereby improving cognition. There is evidence *in vitro* that Δ^9 THC can also modulate glutamate release, oxidant free radicals and calcium influxes (Howlett, 2002; Kreitzer & Regehr, 2001), which in excess, can induce neuronal death in neuroinflammatory diseases (Smith, 2002). Through these previous findings, Δ^9 THC may be a promising candidate for investigating whether it can prevent neurodegeneration in animal model-induced neurotoxicity. Thus, this chapter examines the potential neurotherapeutic effect of Δ^9 THC against D-gal and AlCl₃ induced histological changes in terms of survival pyramidal cells of their hippocampus and survival granule cells in dentate gyrus, as well as structural changes such as hippocampal cell layer thickness specifically in CA1 and CA3 subfields, and dentate gyrus.

5.2 Materials and methods

5.2.1 Drugs and chemicals

All the drugs and chemicals refer to Chapter 3, section 3.1.1

5.2.2 Equipment

The equipment used for histological analysis were Leica TP1020 automated tissue processor, Leica EG1160 tissue embedding station, Leica RM2255 tissue sectioning microtome, Leica HI1210 water bath, Memmert oven and Olympus BX51 TRF microscope.

5.2.3 Animals and experimental design

Animal husbandry and experimental design refer to Chapter 3, section 3.1.2 and 3.1.3. The overall experimental design was simplified in figure 5.1.

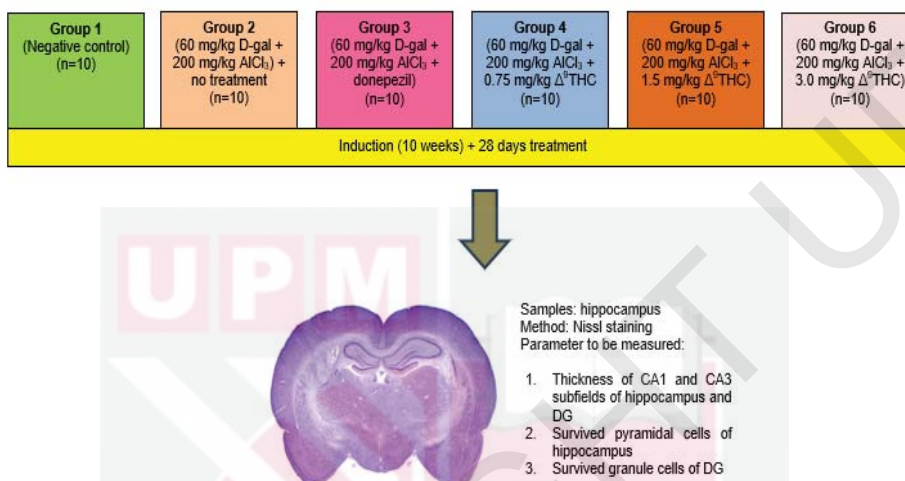


Figure 5.1 : Experimental design for histological studies. 60 mg/kg D-gal + 200 mg/kg AlCl₃ – AD model; D-gal – D-galactose; AlCl₃ – aluminium chloride; Δ⁹THC – delta-9-tetrahydrocannabinol; CA1- cornu ammonis 1; CA3 – cornu ammonis 3; DG – dentate gyrus

5.2.4 Brain samples preparations

Brain retrieval was carried out inside an ice-containing tray and kept in 10% neutral buffered formalin for 7 days prior to further investigation. The histological investigation evaluates the thickness of CA1 and CA3 subfields of the hippocampus, as well as the dentate gyrus (upper blade and lower blade). In addition, the number of viable pyramidal cells in CA1, CA2, CA3 subfields and the number of viable granule cells in dentate gyrus were determined. Using Nissl staining, histological observations and analyses were undertaken.

5.2.4.1 Grossing

The cassettes that were used to hold the entire brain were appropriately labelled. The fixed brain was placed upright on a flat grossing board and cut coronal section of approximately 5 mm thick. Before proceeding to the next step of tissue preparation, the grossed tissues were securely placed into tissue cassettes and soaked in 10% neutral buffered formalin.

5.2.4.2 Tissue processing

The tissues were processed using an automated tissues processor (Leica TP1020) which includes three distinct processes: dehydration, clearing and infiltration. The tissue processing procedures followed, with some changes, the methods published by (Woods & Ellis, 1994). The cassettes were arranged in the provided tissue basket before being stacked into the loading station. This procedure was carried out automatically for 22 hours. Table 5.1 summarizes the automation process.

The first procedure consisted of dehydration to eliminate the tissue fluid, lipids and water-soluble proteins. The dehydration process began with the brain tissues being immersed in a series of increasing concentrations of alcohol solutions: 70%, 90% and 100% until water-free alcohol was reached. A series of increasing concentrations of alcohol solutions allow for the gradual replacement of water content in tissues with alcohol, preventing excessive tissue structure distortion.

The following procedure involved the clearing of brain tissue. Xylene was used as the clearing agent due to its miscible properties towards alcohol and paraffin wax. The clearing processes involved immersing brain tissues in three different xylene immersions, which allow for the gradual replacement of alcohol with xylene, forming clearer and more transparent tissues, and improving cell visibility due to high refractive index of xylene.

The clearing agent was replaced with molten paraffin wax during the infiltration process. The brain tissues were immersed in three different molten paraffin wax immersions to ensure that the clearing agent was gradually removed from the tissue, while also preventing tissue structure shrinkage.

Table 5.1 : Overview of automated tissue processing processes

Process	Reagent	Time
Dehydration	70% alcohol	2 hours
Dehydration	90% alcohol	2 hours
Dehydration	Absolute alcohol	2 hours
Dehydration	Absolute alcohol	2 hours
Dehydration	Absolute alcohol	2 hours
Clearing	Xylene	3 hours
Clearing	Xylene	3 hours
Clearing	Xylene	3 hours
Infiltration	Paraffin wax	1 hour
Infiltration	Paraffin wax	1 hour
Infiltration	Paraffin wax	1 hour

(Source: Woods and Ellis, 1994)

5.2.4.3 Tissue embedding

Following the final infiltration, the tissue cassettes were transferred to the tissue embedding station for embedding process. The integrated tissue embedding centre (Leica EG1160) was used for the tissue embedding process, which includes a pre-warming station, dispensing station and cooling station. The infiltrated brain tissues were removed from the tissue baskets and temporarily stored within the pre-warming station. Prior to beginning the embedding process, paraffin chunks were melted inside the wax reservoir and metal moulds were placed in the pre-warming station.

The infiltrated brain tissues were removed from the cassettes and positioned appropriately in the metal mould. The metal moulds were filled with hot molten paraffin wax approximately 3 mm thick and transferred onto the cooling spot of tissue embedding station until a skin-thick layer of paraffin formed at the base of mould. The processed tissue was removed from the cassettes with lid removed and gently placed at the base of mould in desired orientation. The metal moulds were completely filled with hot molten paraffin wax and allowed to set on the cooling plate of the tissue embedding station. The blocks then were labelled and stored in chiller at 4°C for a final hardening process.

5.2.4.4 Tissue sectioning

The sectioning of brain tissues blocks involved the slicing of brain tissues into extremely thin sections using rotary microtome (Leica RM2255). Brain tissue block was placed and adjusted on the block holder. The square face of the tissue block was readjusted to ensure the upper and lower edges of the block were parallel to the base of the microtome.

The tissue block was carefully trimmed at 10 µm thickness until the tissue area was exposed. The microtome thickness scale was then readjusted to 5 µm, and the tissue blocks were sectioned carefully to produce paraffin ribbon. The ribbon was carefully detached from the edge of microtome blade and placed in a 37°C water bath (Leica HI1210) before mounted on super-frosted glass slides. Slides with mounted sections were drained vertically to remove excess water and dried in the oven at 37°C (Mettmert).

5.3 Nissl staining procedures

The tissues were deparaffinized in an oven for 2 hours 30 mins at 70 °C before began the Nissl staining procedures. The staining of brain tissues followed the modified Nissl staining method described by Mat Taib (2014). The 0.1% cresyl violet stain was prepared by mixing 0.1 g cresyl violet acetate with 100 mL of distilled water. The pH of mixture was adjusted to pH 3.8 by added 10 drops of glacial acetic acid. The cresyl violet stain was filtered using 0.45 µm syringe type filter to remove any undissolved crystals before being stored in the dark condition until further use. Table 5.2 summarizes the steps and processes for Nissl staining.

Table 5.2 : Nissl Staining Processes

Process	Reagent	Time
Deparaffinisation	Xylene	10 minutes
Deparaffinisation	Xylene	10 minutes
Deparaffinisation	Xylene	10 minutes
Rehydration	100% alcohol	5 minutes
Rehydration	100% alcohol	5 minutes
Rehydration	95% alcohol	3 minutes
Rehydration	70% alcohol	3 minutes
Rinsing	Tap water	Tap for 2-3 times
Rinsing	Distilled water	Tap for 2-3 times
Staining	0.1% cresyl violet	3 minutes at 37°C
Rinsing	Distilled water	Tap 2-3 times
Dehydration	95% alcohol	15 minutes
Dehydration	Absolute alcohol	5 minutes
Dehydration	Absolute alcohol	5 minutes
Clearing	Xylene	5 minutes
Clearing	Xylene	5 minutes

The cresyl violet staining procedure has six steps; deparaffinisation, rehydration, staining, rinsing, dehydration and clearing. The deparaffinisation of brain tissue sections involved the removal of paraffin wax through xylene treatment three times for 10 minutes respectively. The rehydration process of tissue sections involved the removal of xylene through absolute alcohol treatment. The tissue sections were immersed in decreasing concentrations of alcohol; beginning with 100% for 10 minutes, 95% for 3 minutes and 70% for 3 minutes. The tissue sections were then rinsed with distilled water before being stained for 3 minutes at 37 °C with cresyl violet. Excess stain was removed by rinsing with distilled water. The sectioned brain tissues were then immersed in alcohol solutions of increasing concentrations; beginning with 95% for 15 minutes, followed by absolute alcohol treatment twice for 5 minutes respectively. The clearing process of stained brain tissues involved the immersion of xylene twice for 5 minutes each before being mounted with dibutyl phthalate in xylene (DPX) and covered with a cover slip.

5.4 Microscopic Observation

A compound research microscope equipped with a digital image acquisition system was used to examine stained brain tissues (Olympus BX51 TRF, Japan). There were four parameters measured; thickness of CA1 and CA3 subfields of the hippocampus as well as the dentate gyrus, number of viable pyramidal cells in CA1, CA2 and CA3 subfields of the hippocampus, and number of viable granule cells in dentate gyrus.

For the thickness purposes, each region; CA1, CA3, upper blade and lower blade of dentate gyrus were measured. Photomicrograph of CA1, CA3 and dentate gyrus were taken at 200x magnification. The thickness of each region was measured using Olympus BX51 TRF software in five random fields per section for evaluation in the minimum of five rats per group (n=5) (Owen et al., 2019).

For counting purposes, with a guide of a pathologist, a total of five sections from both hippocampus and dentate gyrus were collected for assessment in each experimental group, with a sample size of five rats per group (n=5). The quantification of viable pyramidal cells was conducted by summing the counts obtained from at least five randomly selected areas within the mid portions of CA1, CA2 and CA3 subfields of the hippocampus. This analysis was performed at a 200x magnification using a high grade microscope (Olympus BX51 TRF) (Mahdi et al., 2021). The viable granule cells of the dentate gyrus were counted using the same procedures.

5.5 Statistical analysis

Data were analysed using SPSS version 25.0. One-way ANOVA, followed by Tukey's multiple comparison test was employed. The data was presented as mean $\pm$ standard error of mean (SEM). The significant difference was determined by $p < 0.05$.

5.6 Results

5.6.1 Histological result showing Δ^9 THC increases the thickness on the upper blade of dentate gyrus in rats induced by D-gal and AlCl_3

The effects of Δ^9 THC on the neurodegeneration of the dentate gyrus induced by D-gal and AlCl_3 in rats were examined. The dentate gyrus is composed of two parts; the upper and lower blades. Figure 5.2 depicted the photomicrographs of brain sections (upper blade of dentate gyrus). In comparison to the control group of rats, chronic exposure to D-gal and AlCl_3 (AD model) reduced the thickness (5.69 ± 0.24). However, Tukey's post hoc test indicated no statistically significant differences ($p > 0.05$) (Figure 5.3). In addition, there was a statistically significant differences in thickness between Δ^9 THC 1.5 mg/kg and control group ($p < 0.05$).

The rats treated with donepezil 1 mg/kg and Δ^9 THC at doses of 0.75, 1.5 and 3.0 mg/kg showed some changes in thickness. This was demonstrated by increased the thickness in the upper blade of dentate gyrus. Tukey's post hoc analysis revealed that only Δ^9 THC at 0.75 mg/kg (9.42 ± 1.49) and 1.5 mg/kg (13.33 ± 0.48) showed statistically significant differences when compared to the AD model ($p < 0.05$) (Figure 5.3).

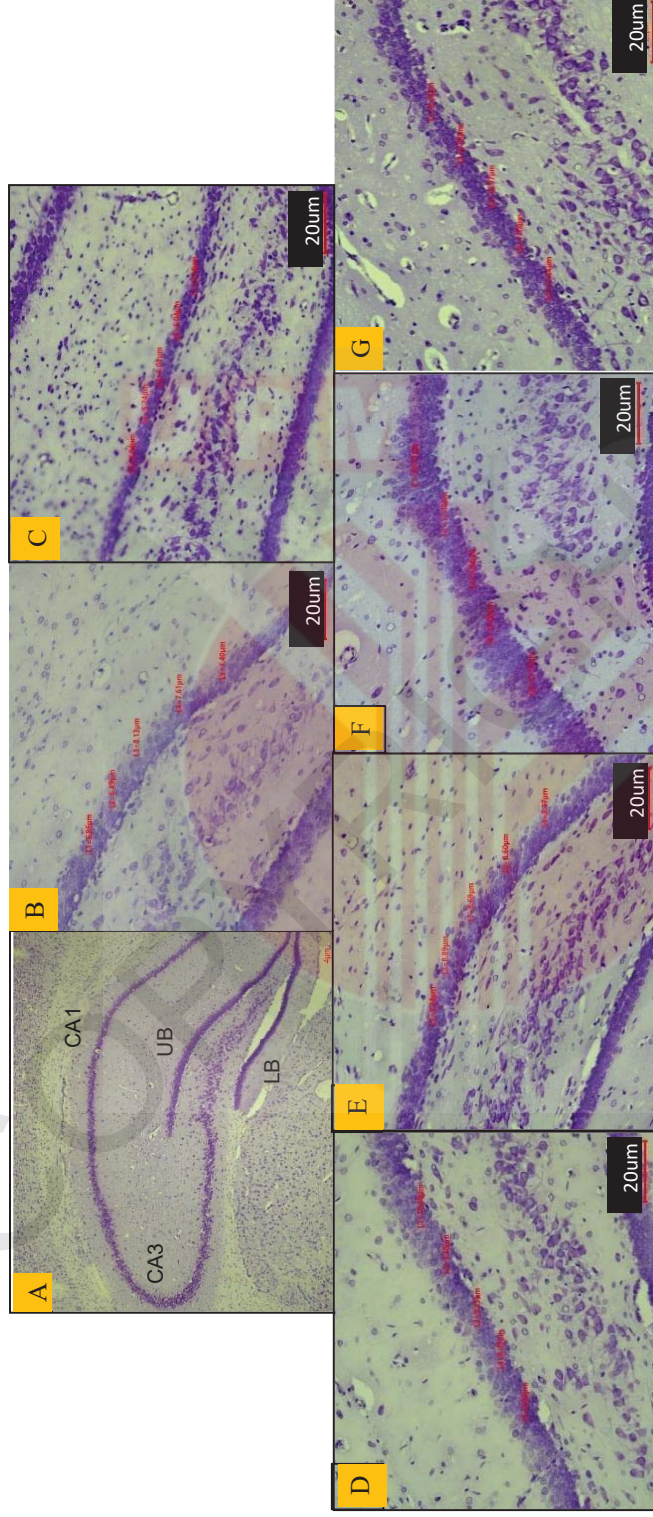


Figure 5.2 : Cresyl violet-stained photomicrographs of brain sections (upper blade of dentate gyrus). (A) The whole hippocampus structure showing all parts, magnification 40x, (B) Control rats, (C) AD model group demonstrates a decrease in thickness, (D, E, F, G) treated with 1 mg/kg donepezil, 0.75 Δ^9 THC, 1.5 Δ^9 THC and 3.0 Δ^9 THC respectively, exhibited an increase in the thickness of upper blade of DG. Magnification of 200x for B-G, scale bar 20 μ m. CA1- cornu ammonis 1; CA3 – cornu ammonis 3; UB – upper blade; LB – lower blade (n=5)

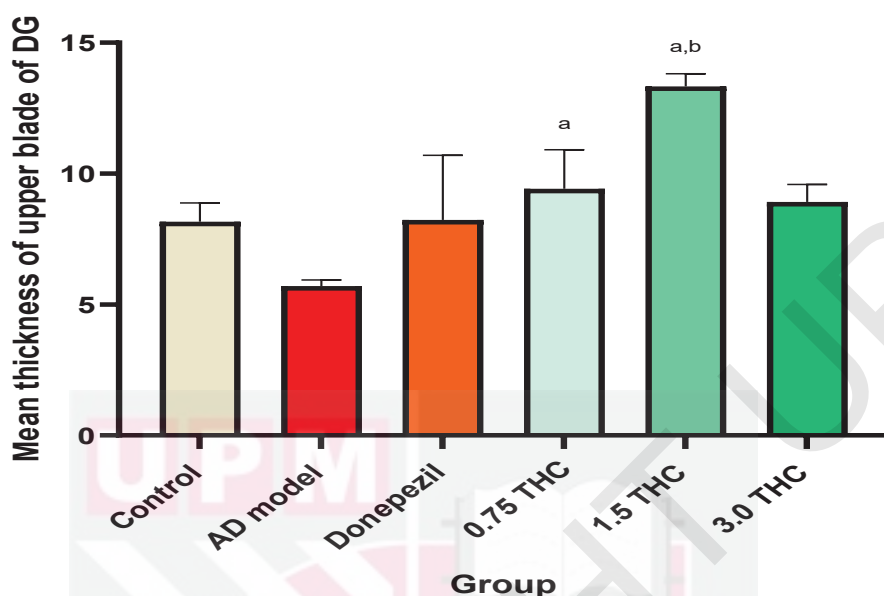


Figure 5.3 : Mean thickness of upper blade of DG for all groups. Data was analyzed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. Data was expressed as mean $\pm$ SEM (n=5). ^ap<0.05 vs AD model; ^bp<0.05 vs control group. DG – dentate gyrus

5.6.2 Histological results showing Δ^9 THC increases thickness on lower blade of dentate gyrus in D-gal and AlCl₃ induced rats

Figure 5.4 depicted the photomicrographs of brain section (lower blade of dentate gyrus). The results showed that chronic exposure to D-gal and AlCl₃ (AD model) (6.83 ± 0.19) resulted in decrease in thickness compared to the control group of rats (8.42 ± 0.12). However, Tukey's post hoc test indicated no significant differences ($p > 0.05$). Meanwhile, treatment with Δ^9 THC at 1.5 mg/kg (11.86 ± 0.34) showed significant differences in thickness as compared to the control group ($p < 0.05$) (Figure 5.5).

Treatment with donepezil 1 mg/kg and Δ^9 THC at the doses of 0.75, 1.5 and 3.0 mg/kg increased the thickness induced by D-gal and AlCl₃. However, only Δ^9 THC doses at 1.5 mg/kg (11.86 ± 0.34) and 3.0 mg/kg (9.02 ± 0.41) showed statistically significant when compared to AD model group ($p < 0.05$). In addition, there was also statistically significant difference between donepezil, Δ^9 THC 0.75, and 3.0 mg/kg when compared to 1.5 mg/kg of Δ^9 THC (Figure 5.5).

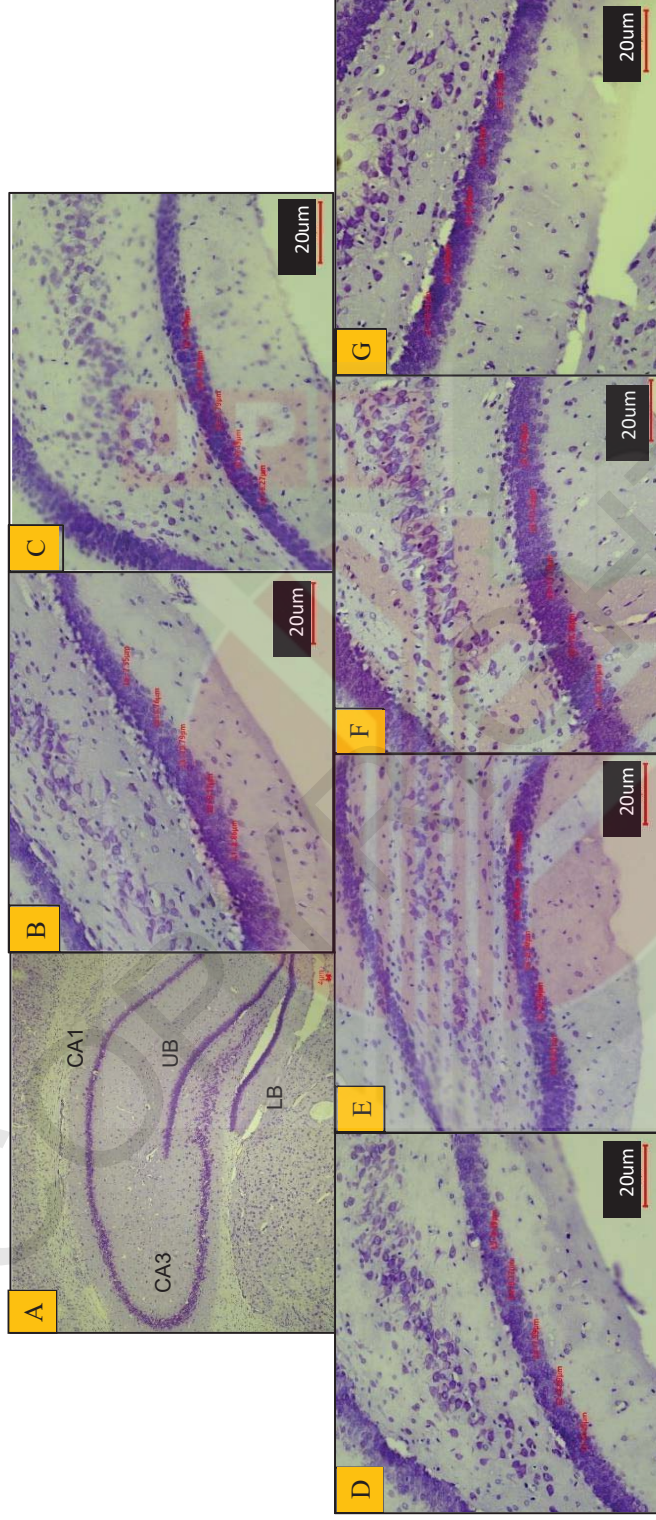


Figure 5.4 : Cresyl violet-stained photomicrographs of brain sections (lower blade of dentate gyrus) (A) The whole hippocampus structure showing all parts, magnification 40x, (B) Control rats, (C) AD model group demonstrates a decrease in thickness, (D, E, F, G) treated with 1 mg/kg donepezil, 0.75 Δ^9 THC, 1.5 Δ^9 THC and 3.0 Δ^9 THC respectively showing increased thickness of lower blade of DG. Magnification of 200x for B-G, scale bar 20um. CA1- cornu ammonis 1; CA3 – cornu ammonis 3; UB – upper blade; LB – lower blade (n=5)

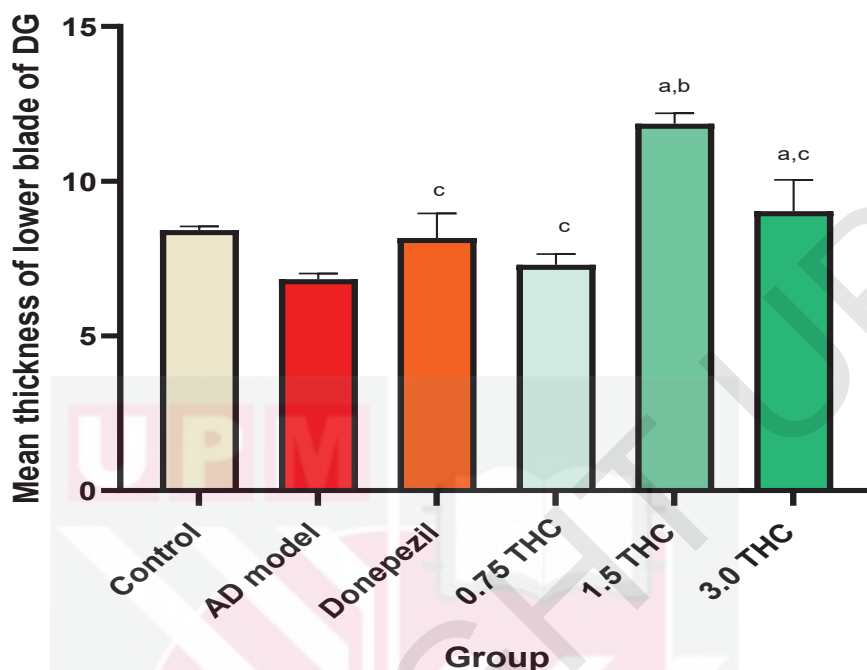


Figure 5.5 : Mean thickness of lower blade of DG for all groups. Data was analyzed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. Data was expressed as mean $\pm$ SEM (n=5). ^ap<0.05 vs AD model, ^bp<0.05 vs control group, ^cp<0.05 vs 1.5 mg/kg Δ^9 THC. DG – dentate gyrus

5.6.3 Histological results showing Δ^9 THC increases cell layer thickness of CA1 subfield in D-gal and AlCl_3 induced rats

Histological sections of the CA1 region of the hippocampus were illustrated in Figure 5.6. The cell layer thickness of CA1 was substantially reduced in AD model group (5.1 ± 0.18) as compared to the control group (7.13 ± 0.33) ($p<0.05$). There was also a significant difference between Δ^9 THC 0.75 mg/kg (9.16 ± 0.6) and Δ^9 THC 1.5 mg/kg (9.20 ± 0.22) when compared to control group ($p<0.05$). Intriguingly, donepezil and all Δ^9 THC treated groups (0.75, 1.5 and 3.0 mg/kg) showed statistically significant increases in thickness with donepezil (7.04 ± 0.51), Δ^9 THC 0.75 mg/kg (9.16 ± 0.6), Δ^9 THC 1.5 mg/kg (9.2 ± 0.22) and Δ^9 THC 3.0 mg/kg (7.98 ± 0.51) respectively as compared to AD model (Figure 5.7).

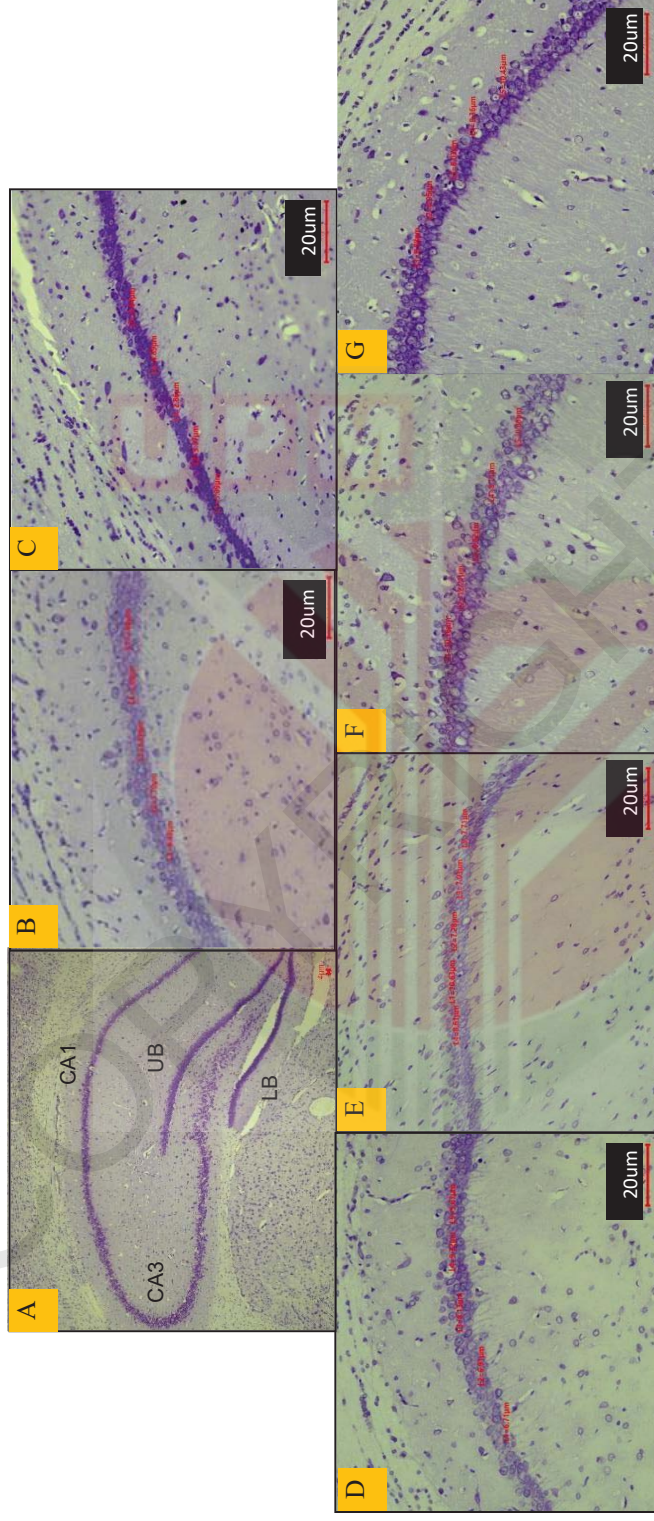


Figure 5.6 : Representative photomicrograph of CA1 subfield cell layer thickness stained with cresyl violet. (A) The whole hippocampus structure showing all parts, magnification 40x, (B) Control rats, (C) AD model group showing decreased in thickness, (D, E, F, G) treated with 1 mg/kg donepezil, 0.75 Δ⁹THC, 1.5 Δ⁹THC and 3.0 Δ⁹THC respectively showing increases in cell layer thickness of CA1 subfield. Magnification of 200x for B-G, scale bar 20um. CA1- cornu ammonis 1; CA3 – cornu ammonis 3; UB – upper blade; LB – lower blade (n=5)

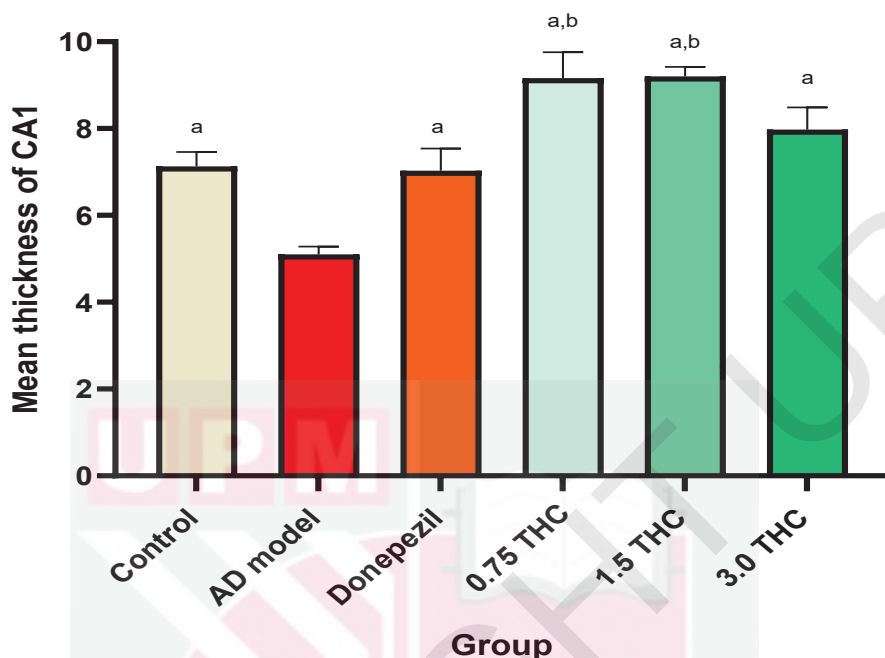


Figure 5.7 : Mean cell layer thickness of CA1 subfield of hippocampus for all groups. Data was analyzed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. Data was expressed as mean $\pm$ SEM (n=5). ^ap<0.05 vs AD model, ^bp<0.05 vs control. CA1 – cornu ammonis 1

5.6.4 Histological results showing Δ^9 THC increases the cell layer thickness of CA3 subfield in D-gal and AlCl_3 induced rats

Histological sections of the CA3 region of the hippocampus were illustrated in Figure 5.8. There was a statistically significant decrease in CA3 cell layer thickness in the AD model group (10.37 ± 0.37) when compared to the control (16.37 ± 1.07) ($p < 0.05$). However, donepezil and all Δ^9 THC treated groups showed statistically significant increases in thickness with donepezil (16.07 ± 1.03), Δ^9 THC 0.75 mg/kg (16.41 ± 1.03), Δ^9 THC 1.5 mg/kg (16.84 ± 0.77) and Δ^9 THC 3.0 mg/kg (16.53 ± 1.01) respectively as compared to AD group. There was no significantly different between all treated group and control group ($p > 0.05$).

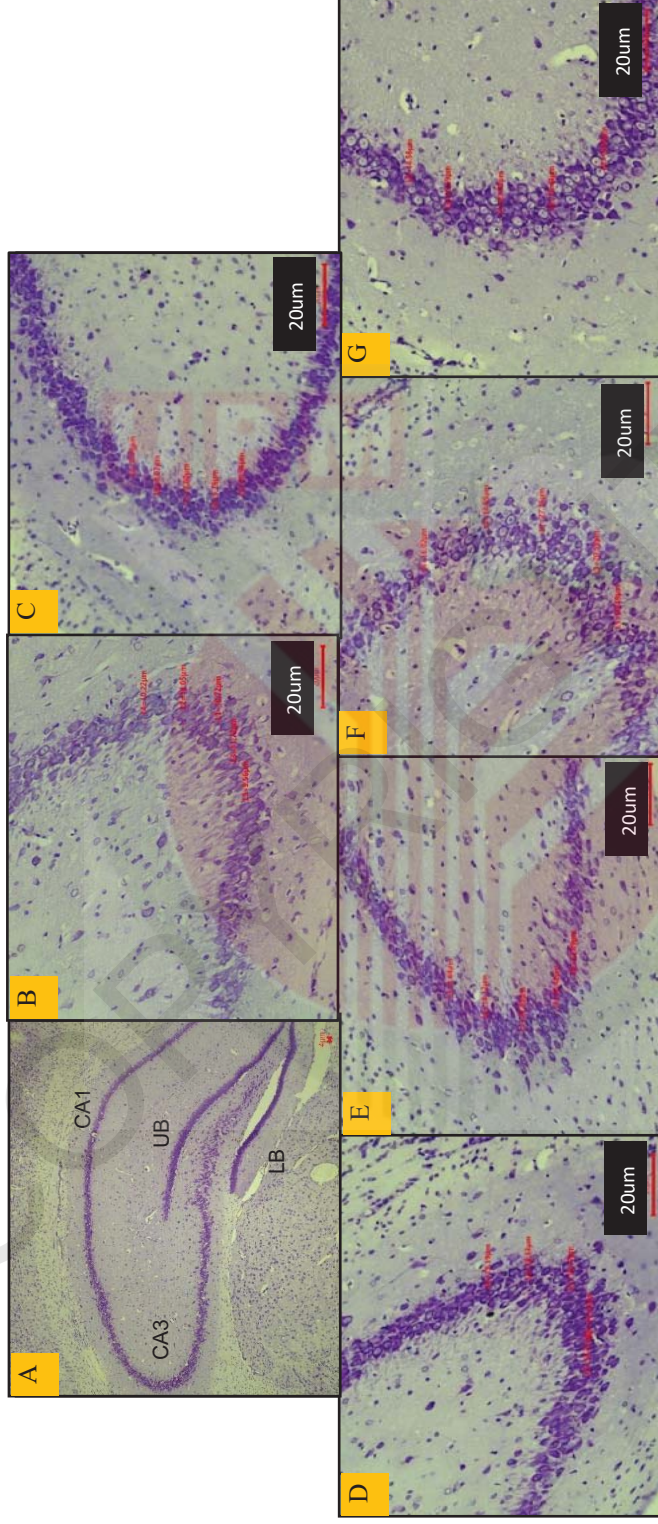


Figure 5.8 : Representative photomicrograph of CA3 subfield cell layer thickness stained with cresyl violet. (A) The whole hippocampus structure showing all parts, magnification 40x, (B) Control rats, (C) AD model group showing decreases in thickness, (D, E, F, G) treated with 1 mg/kg donepezil, 0.75 Δ^9 THC, 1.5 Δ^9 THC and 3.0 Δ^9 THC respectively showing increases in cell layer thickness of CA3 subfield. Magnification of 200x for B-G, scale bar 20um. CA1- cornu ammonis 1; CA3 – cornu ammonis 3; UB – upper blade; LB – lower blade (n=5)

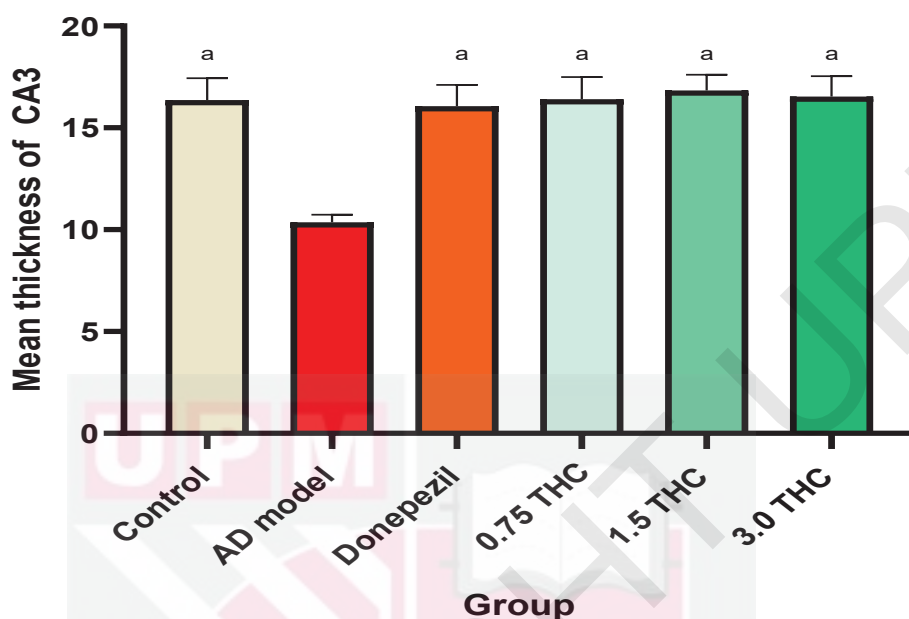


Figure 5.9 : Mean cell layer thickness of CA3 subfield for all groups. Data was analyzed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. Data was expressed as mean $\pm$ SEM (n=5). ^ap<0.05 vs AD model. CA3 – cornu ammonis 3

5.6.5 Histological results showing Δ^9 THC restore against degeneration of the hippocampal subfields, CA1-CA3 pyramidal cells in D-gal and AlCl_3 induced rats

Figure 5.10 and figure 5.11 depicted the CA1-CA3 subfields of the hippocampus for rats in the control and AD model groups. Presence of numerous viable cells (light cytoplasm and dark nucleus) and well-arranged intact layers of pyramidal cells were observed in the control group of rats. However, obvious alterations of the pyramidal cell layers and several hyperchromic cells were observed in the CA1-CA3 hippocampal region of the AD model rats.

One way ANOVA revealed a statistically significant differences in the number of viable pyramidal cells in CA1 [F (6,49) = 33.878, p = 0.001], CA2 [F (6,49) = 24.322, p=0.001] and CA3 [F (6,56) = 48.422, p=0.001] subfields of the hippocampus. Tukey's post hoc test revealed that there was a statistically significant decreases (p<0.05) in the number of viable pyramidal cells within the CA1 (10.98 ± 0.78), CA2(12.00 ± 0.95) and CA3 (7.62 ± 0.48) subfields of AD model group of rats, as compared to the control group; CA1 (18.65 ± 2.08), CA2 (19.98 ± 2.60), CA3 (16.07 ± 0.59) respectively (Figure 5.16).

Figure 5.12 to figure 5.15 depicted the CA1-CA3 subfields of the hippocampus for treated groups. The administration of donepezil and Δ^9 THC at different concentrations alleviates some of histological aberrations earlier observed. Tukey's post hoc analysis confirmed that, Δ^9 THC treatment resulted in significantly increases the number of viable pyramidal cells in CA1 subfield; 0.75 mg/kg (20.10 ± 1.19), 1.5 mg/kg (30.35 ± 0.72) and 3.0 mg/kg (29.00 ± 1.76) groups, when compared to the AD model group (10.98 ± 0.78). There was a statistically significant difference had been observed between Δ^9 THC (1.5 and 3.0 mg/kg) when compared to the control group. In addition, a statistically significant difference in viable pyramidal cells of CA1 has been observed between Δ^9 THC (1.5 and 3.0 mg/kg) and Δ^9 THC 0.75 mg/kg (Figure 5.16). Similar results were also observed ($p < 0.05$) in the CA2 subfield; 0.75mg/kg (24.2 ± 1.41), 1.5 mg/kg (32.28 ± 0.77), 3.0 mg/kg (22.20 ± 1.73), and CA3 subfield; 0.75 mg/kg (15.89 ± 0.56), 1.5 mg/kg (18.13 ± 0.77), 3.0 mg/kg (16.87 ± 0.41), when compared to the AD model group; CA2 (12.00 ± 0.95), CA3 (7.62 ± 0.48) respectively. There was also a statistically significant difference in viable pyramidal cell in CA2 between Δ^9 THC 1.5 mg/kg and control group. (Figure 5.16).

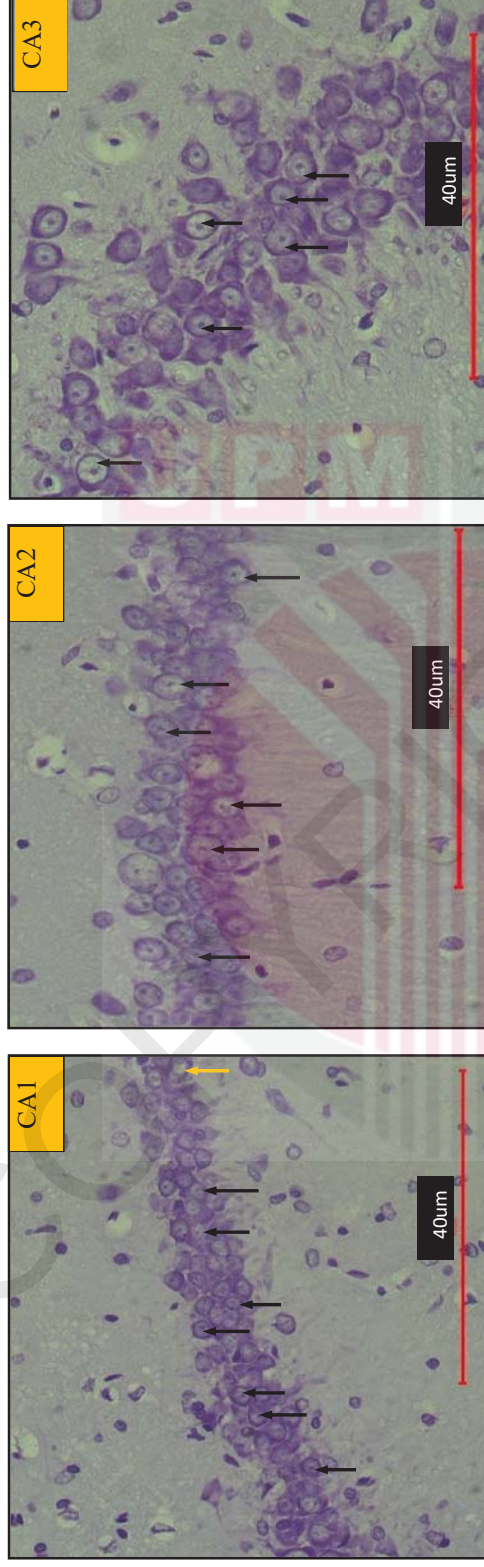


Figure 5.10 : Representative photomicrographs of Nissl stained for control group in the CA1, CA2 and CA3 subfields of the hippocampus. The black arrows indicate viable pyramidal cells and yellow arrows indicate dead pyramidal cells. Images are shown at 400x magnification, scale bar 40um. CA1 – cornu ammonis 1; CA2- cornu ammonis 2; CA3 – cornu ammonis 3. (n=5)

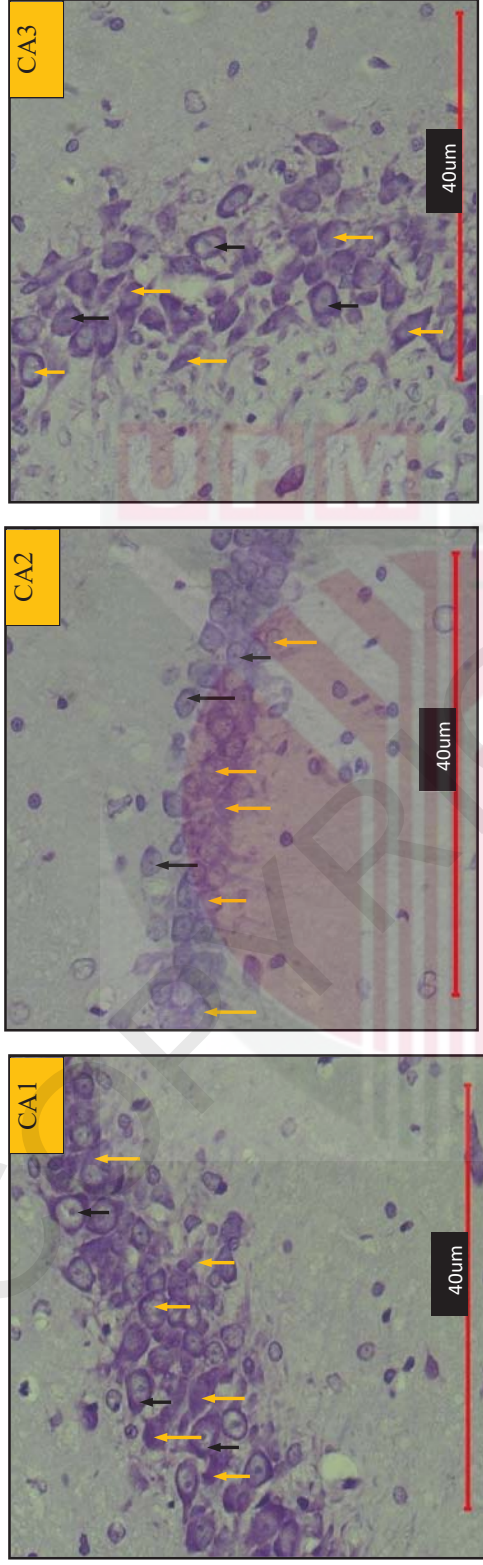


Figure 5.11 : Representative photomicrographs of Nissl stained for AD model group in the CA1, CA2 and CA3 subfields of the hippocampus. The black arrows indicate viable pyramidal cells and yellow arrows indicate dead pyramidal cells. Images are shown at 400x magnification, scale bar 40µm. CA1 – cornu ammonis 1; CA2- cornu ammonis 2; CA3 – cornu ammonis 3. (n=5)

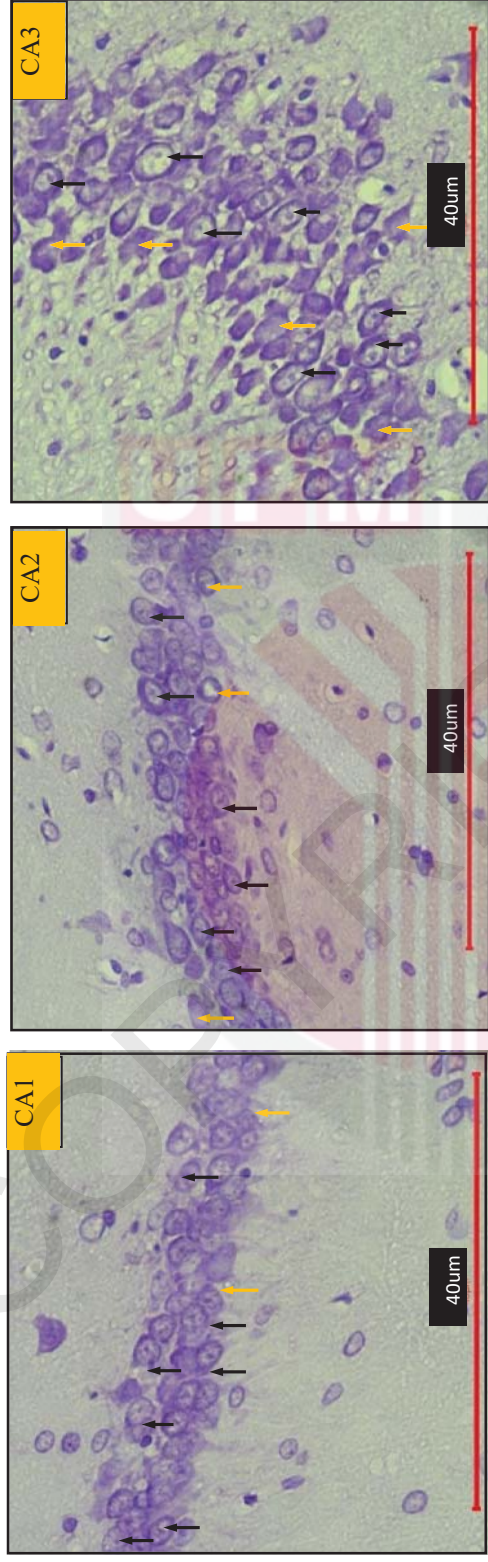


Figure 5.12 : Representative photomicrographs of Nissl stained for donepezil treated group in the CA1, CA2 and CA3 subfields of the hippocampus. The black arrows indicate viable pyramidal cells and yellow arrows indicate dead pyramidal cells. Images are shown at 400x magnification, scale bar 40um. Donepezil = 1 mg/kg. CA1 – cornu ammonis 1; CA2- cornu ammonis 2; CA3 – cornu ammonis 3. (n=5)

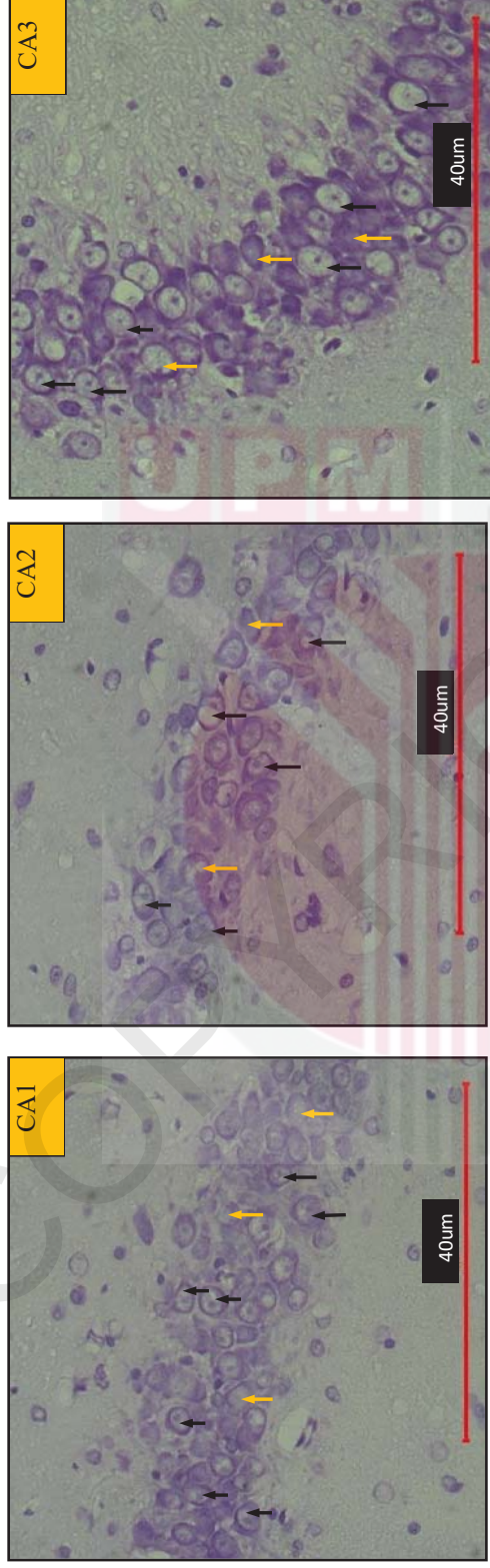


Figure 5.13 : Representative photomicrographs of Nissl stained for 0.75 mg/kg Δ^9 THC treated group in the CA1, CA2 and CA3 subfields of the hippocampus. The black arrows indicate viable pyramidal cells and yellow arrows indicate dead pyramidal cells. Images are shown at 400x magnification, scale bar 40 μ m. CA1 – cornu ammonis 1; CA2- cornu ammonis 2; CA3 – cornu ammonis 3. (n=5)

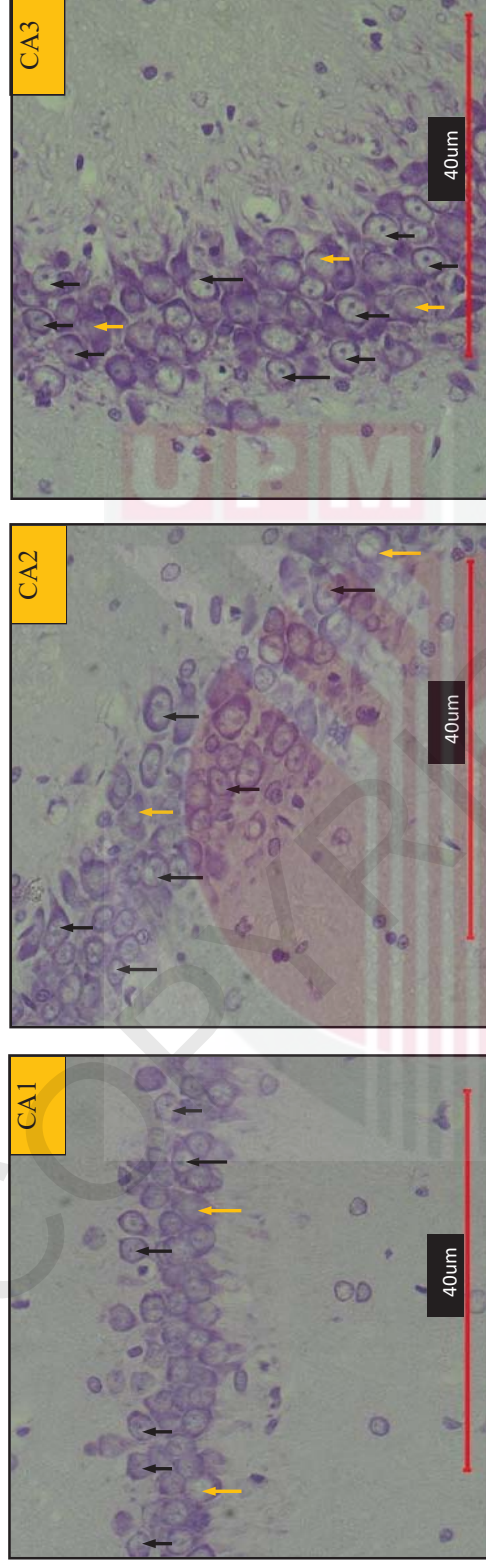


Figure 5.14 : Representative photomicrographs of Nissl stained for 1.5 mg/kg Δ^9 THC treated group in the CA1, CA2 and CA3 subfields of the hippocampus. The black arrows indicate viable pyramidal cells and yellow arrows indicate dead pyramidal cells. Images are shown at 400x magnification, scale bar 40µm. CA1 – cornu ammonis 1; CA2-cornu ammonis 2; CA3 – cornu ammonis 3. (n=5)

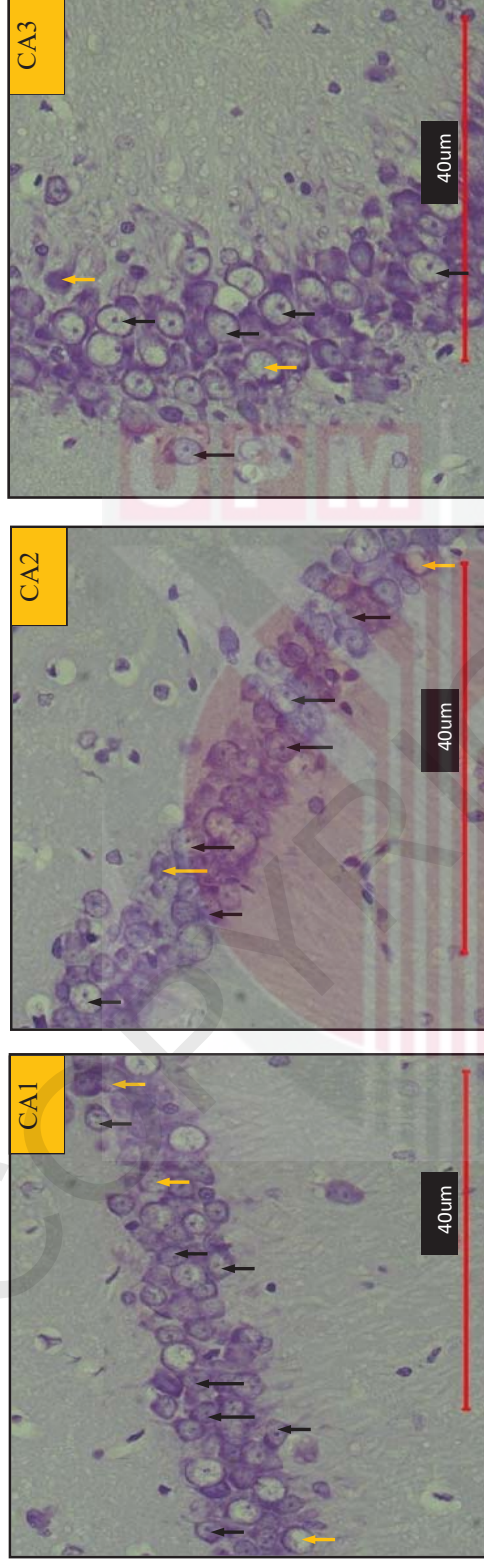
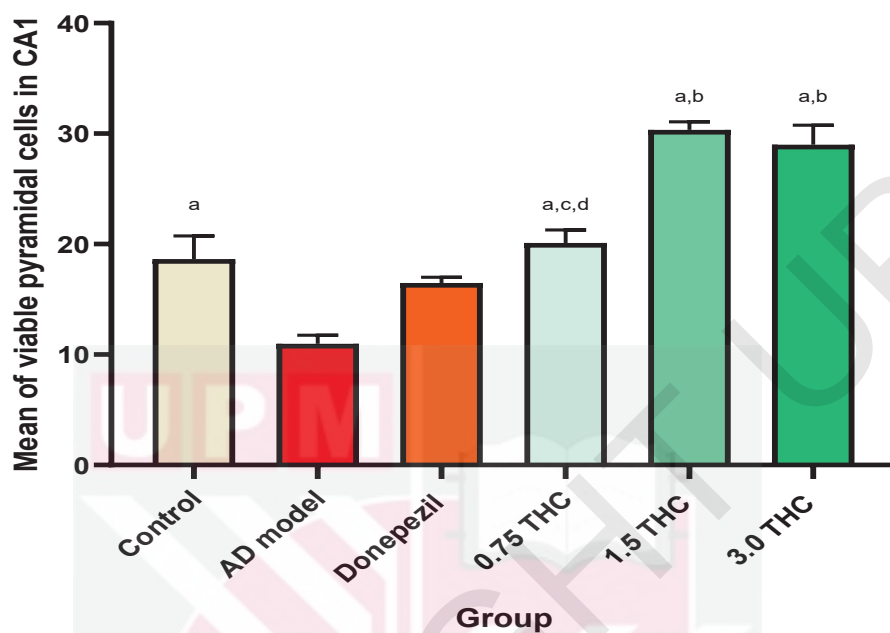
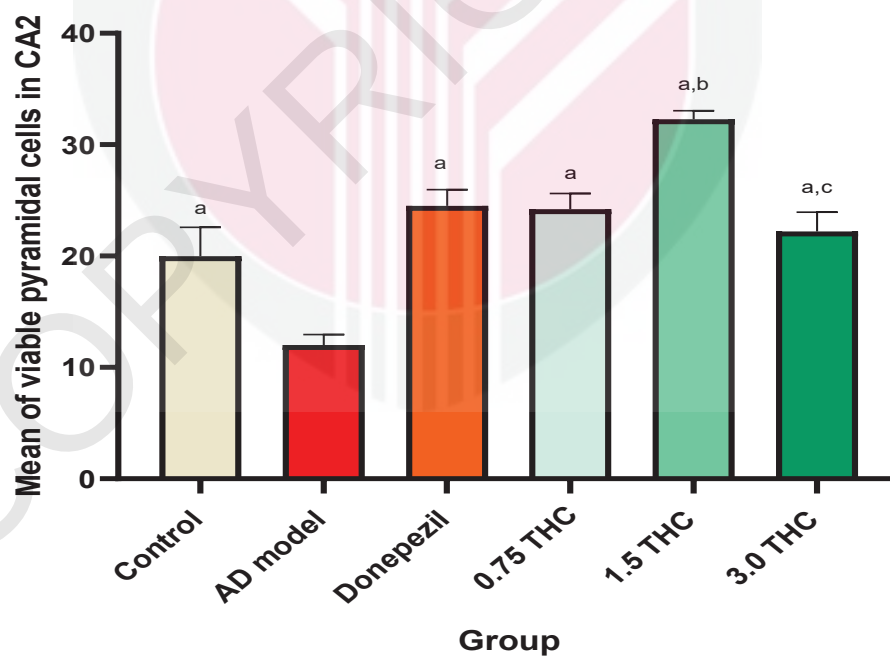


Figure 5.15 : Representative photomicrographs of Nissl stained for 3.0 mg/kg Δ^9 THC treated group in the CA1, CA2 and CA3 subfields of the hippocampus. The black arrows indicate viable pyramidal cells and yellow arrows indicate dead pyramidal cells. Images are shown at 400x magnification, scale bar 40μm. CA1 – cornu ammonis 1; CA2– cornu ammonis 2; CA3 – cornu ammonis 3. (n=5)

A



B



C

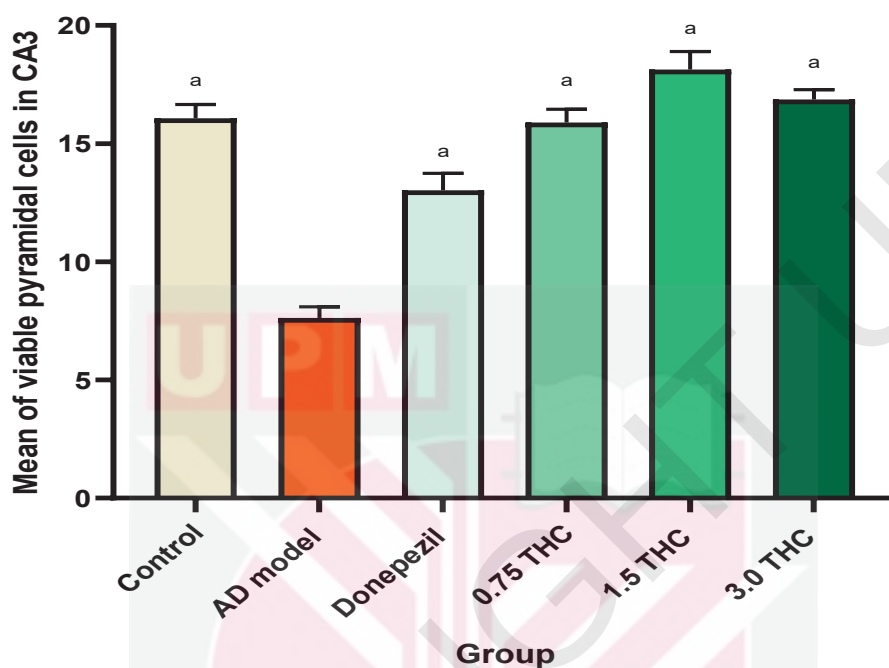


Figure 5.16 : Mean number of viable pyramidal cells in CA1, CA2 and CA3 subfields of hippocampus. Data was analyzed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. Data was expressed as mean $\pm$ SEM (n=5). ^ap<0.05 vs AD model; ^bp<0.05 vs control; ^cp<0.05 vs 1.5 mg/kg Δ^9 THC; ^dp<0.05 vs 3.0 mg/kg Δ^9 THC. CA1 – cornu ammonis 1; CA2- cornu ammonis 2; CA3 – cornu ammonis 3

5.6.6 Histological results showing Δ^9 THC restore against degeneration of the dentate gyrus granule cells in D-gal and AlCl_3 induced rats

The neurotherapeutic potential of Δ^9 THC on the impacts of D-gal + AlCl_3 induced degeneration were also been studied in the dentate gyrus area of rats (upper blade and lower blade). Figure 5.17 and figure 5.18 depicts the histological section of the dentate gyrus for all groups. Histological examinations of dentate gyrus of the control group revealed a normal cytoarchitecture with a dense layer of granule cells containing nuclei at the center of the cells, confirming viable neurons. Chronic exposure of rats to D-gal and AlCl_3 results in the degeneration of granule cells in the upper and lower blade of dentate gyrus respectively.

One-way ANOVA showed statistically significant differences in the number of viable granule cells in the upper blade [$F(6,35) = 34.493$, $p=0.001$] and lower blade [$F(6,35) = 16.257$, $p=0.001$] of the dentate gyrus. Tukey's post hoc test revealed that there was a

statistically significant decreases ($p<0.05$) in the number of viable granule cells in the lower blade of the AD model (24.30 ± 0.46), as compared with control group (33.50 ± 0.67) (Figure 5.18). A statistically significant decreases ($p<0.05$) were also observed in the number of viable granule cells in the upper blade of AD model (13.17 ± 2.03), as compared to the control group 22.27 ± 0.96 (Figure 5.19).

An increasing in the number of viable granule cells have been observed when treated with donepezil and Δ^9 THC respectively. Our findings revealed statistically significant differences ($p<0.05$) in the lower blade of the dentate gyrus between all doses of Δ^9 THC; 0.75 mg/kg (34.8 ± 3.05), 1.5 mg/kg (43.8 ± 1.43), 3.0 mg/kg (43.37 ± 1.97), when compared to the AD model of rats. There was a statistically significant difference has been observed between Δ^9 THC (1.5 and 3.0 mg/kg) as compared to the control group. Similar result was also observed ($p<0.05$) when treated with donepezil (33.6 ± 3.21). For the upper blade, donepezil (33.63 ± 2.29), 0.75 mg/kg Δ^9 THC (34.10 ± 1.68), 1.5 mg/kg Δ^9 THC (41.67 ± 2.15) and 3.0 mg/kg Δ^9 THC (35.77 ± 1.53) showed significant differences when compared to the AD model group and control group ($p<0.05$) (Figure 5.19).

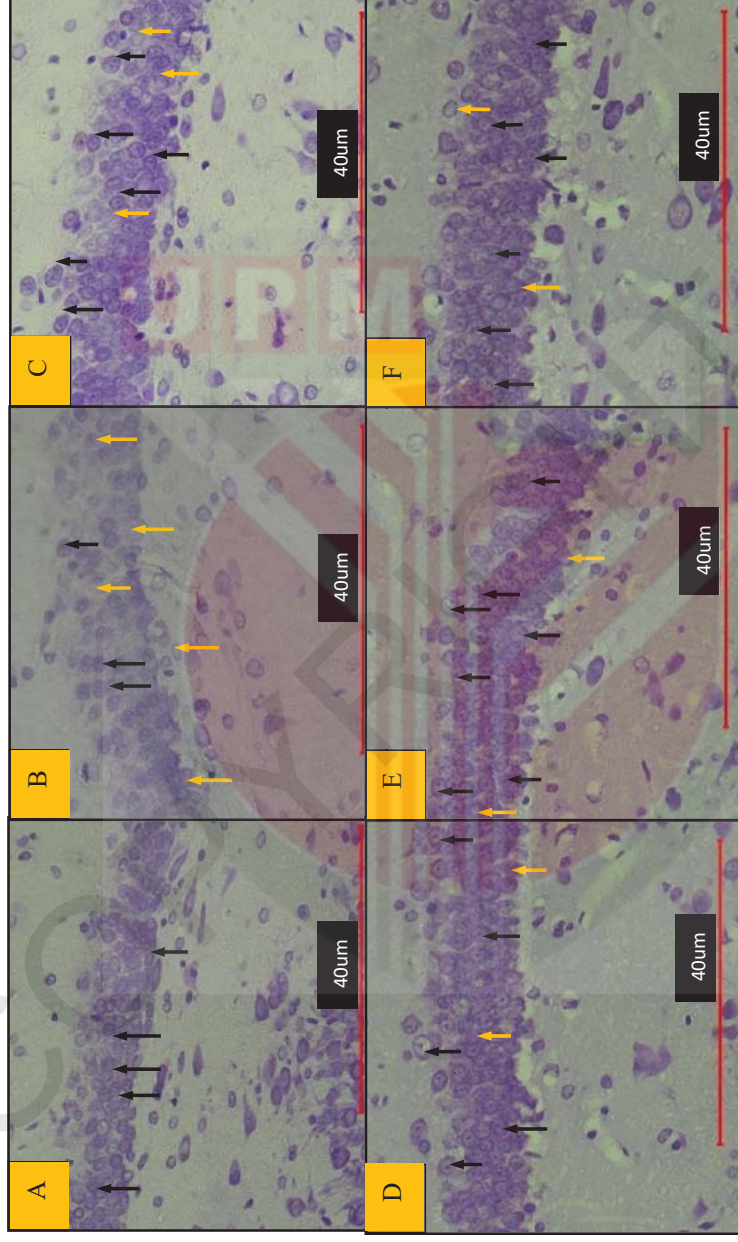


Figure 5.17 : Representative photomicrographs of Nissl stained in the upper blade of dentate gyrus showing the therapeutic effects of Δ^9 THC after being exposed by D-gal and A β 13. (A) Control rats, (B) AD model group, (C, D, E, F) treated with 1 mg/kg donepezil, 0.75 Δ^9 THC, 1.5 Δ^9 THC and 3.0 Δ^9 THC respectively. The black arrows indicate viable granule cells and yellow arrows indicate dead granule cells. Images are shown at 400x magnification, scale bar 40um (n=5)

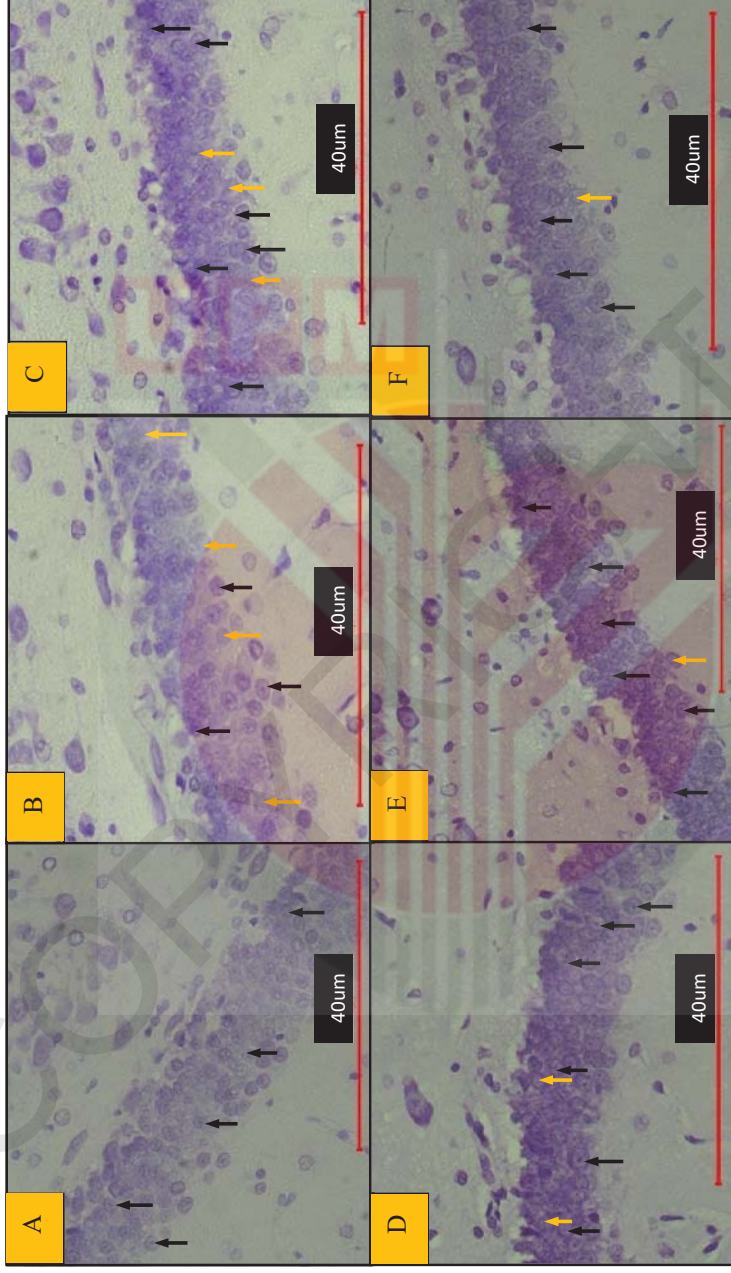


Figure 5.18 : Representative photomicrographs of Nissl stained in the lower blade of dentate gyrus showing the therapeutic effects of Δ^9 THC after being exposed by D-gal and AICl₃. (A) Control rats, (B) AD model group, (C, D, E, F) treated with 1 mg/kg donepezil, 0.75 Δ^9 THC, 1.5 Δ^9 THC and 3.0 Δ^9 THC respectively. The black arrows indicate viable granule cells and yellow arrows indicate dead granule cells. Images are shown at 400x magnification, scale bar 40µm (n=5)

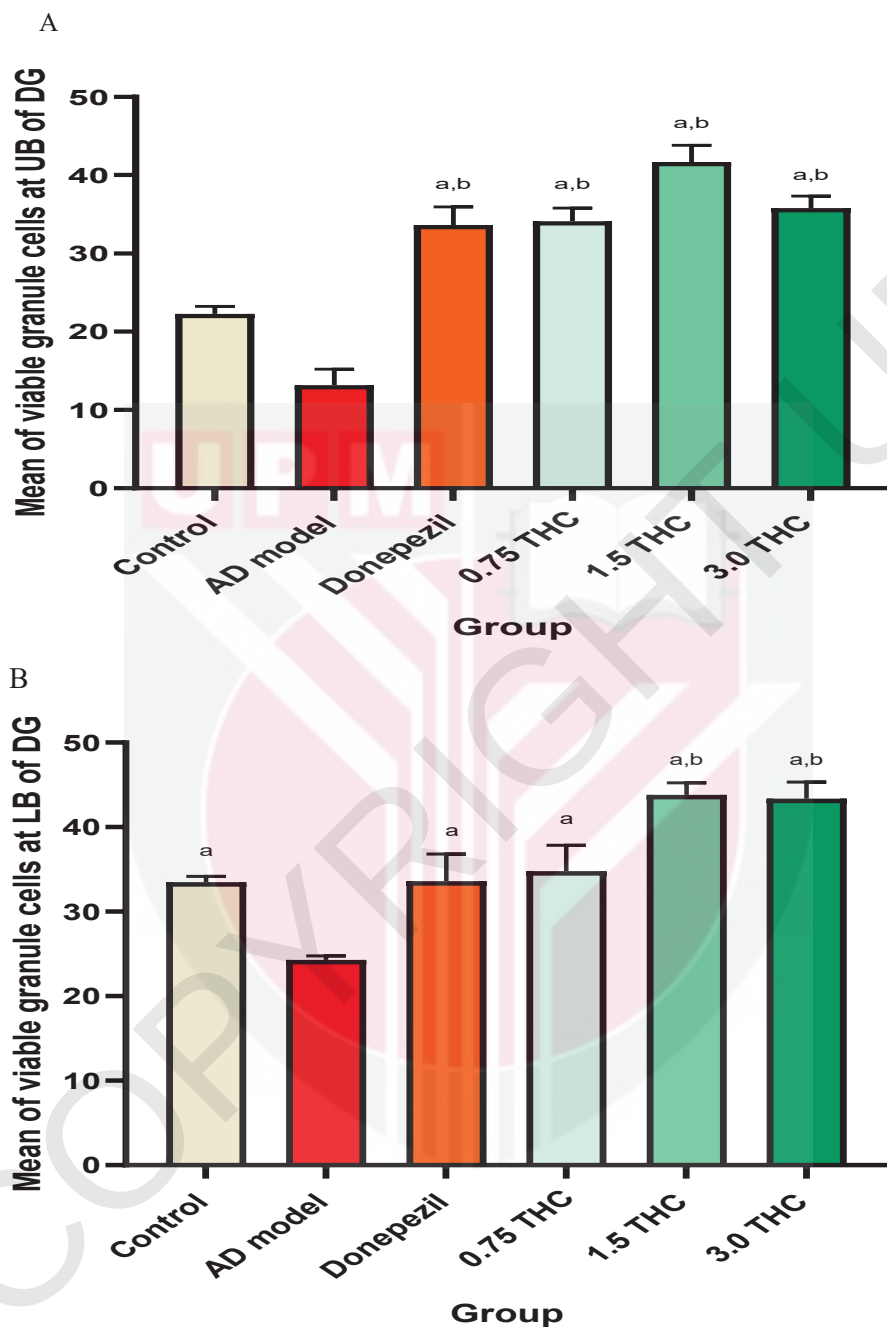


Figure 5.19 : Mean number of viable granule cells of dentate gyrus. Data was analyzed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. Data was expressed as mean $\pm$ SEM (n=5). ^ap<0.05 vs AD model; ^bp<0.05 vs control. UB – upper blade; LB – lower blade; DG – dentate gyrus

5.7 Discussion

Previous research found that prolong administration of D-gal or AlCl_3 in rats resulted in alterations resembling age-related phenomena, including cognitive decline (Zhang et al., 2016), oxidative stress (Kumar et al., 2011; Rodrigues & Ballesteros, 2007) and pathological changes in astrocytes (Lei et al., 2008). It was discovered that combining of D-gal and AlCl_3 administration in rats resulted in cognitive dysfunction and pyramidal cell degeneration in the hippocampus (Chiroma et al., 2018; Zhang et al., 2016). In this study, rats exposed to D-gal and AlCl_3 causes morphological alterations of the hippocampal area, thus disrupt the hippocampal function.

Hippocampus subdivisions include cornu ammonis (CA1, CA2 and CA3) and dentate gyrus (upper blade and lower blade). The dentate gyrus is composed of densely packed tiny granule cells, while the CA regions contains densely packed pyramidal cells (Dekosky et al., 2016). Histologically, the present study observed that chronic exposure to D-gal and AlCl_3 led to noticeable alterations, such as decreases in the thickness cell layer of hippocampus and dentate gyrus area, degeneration of the cell subfields and disorientation of the pyramidal cell layer and granule cell layer respectively. $\Delta^9\text{THC}$ or donepezil treatment in the present study played a significant effect in mitigating symptoms like those in AD, by amended the morphological alterations induced by D-gal and AlCl_3 . It can be observed by high density of viable pyramidal and granule cells, normal pyramidal and granule layers arrangement, and increased the cell layer thickness of hippocampus and dentate gyrus areas. As a result, it is insightful to report that findings from the current study demonstrated that $\Delta^9\text{THC}$ has neurotherapeutic potential in restoring cytoarchitecture of the hippocampus.

According to the study by Lei et al. (2008), increasing thickness in the hippocampus and dentate gyrus area represent elevation of neuronal formation activities. This process is called neurogenesis. Fluctuation on its' thickness reflect the rate of neurogenesis (Yan Li et al., 2009), as seen in AD model group respectively. Since the hippocampus is associated with learning and memory processes of rats, the degenerative changes of cells observed in the hippocampal area could have caused the memory deficits as shown in D-gal and AlCl_3 (AD model) induced rats. Meanwhile, production of new neurons in the dentate gyrus and CA region of hippocampus, specifically on CA1 and CA3 subfields contributed in enhancing learning and memory functions (Shor et al., 2002). These areas involved in encoding and reconstructing a memory (Chauhan et al., 2021). It can be observed by improvement in term of cell layer thickness of hippocampal and dentate gyrus layers of the rats treated with $\Delta^9\text{THC}$ or donepezil. It is noteworthy that $\Delta^9\text{THC}$ treatment, irrespective of its doses, alleviated some of morphological alteration observed in the rats, specifically on the thickness. Our results were consistent with those of a previous study by Abdallah et al. (2010), which demonstrated an increase in neuroproliferation and thickness after $\Delta^9\text{THC}$ exposure. On the other hand, however, Miller et al. (2019) reported that prolonged $\Delta^9\text{THC}$ exposure on Long Evans rat model at 1.5 mg/kg disrupted the morphology, dendritic development and cytoskeleton organization of the prefrontal cortex of the brain, which is also implicated in cognitive function. Further toxicological evaluation of $\Delta^9\text{THC}$ in different animal model should be assessed, as our findings was contradict with previous study done by Miller et al

(2019).

Our observation using Nissl staining method, hippocampal CA1, CA2, CA3 neurons as well as upper blade and lower blade of dentate gyrus of rats exposed with D-gal and AlCl_3 were morphologically perturbed. It was presented by significantly reduced the number of viable cells when compared to the control and treated groups ($p < 0.05$). High density of pyknotic cells observed, as indicated by darkly stained cells with cytoplasmic shrinkage. Meanwhile, the number of viable cells significantly increased in the rats treated with $\Delta^9\text{THC}$ or donepezil. The pyknotic cells and the extent of aberration was lower than AD model group. An increasing of viable cells by treated groups showing a strength signal connection between dentate gyrus and CA region, thus enhance memory.

Our result, however, was contradictory with the recent findings done by Landucci et al. (2022) revealed that prolonged exposure to $\Delta^9\text{THC}$ caused morphological alteration in neurons and modifications in the expression of synaptic proteins *in-vitro*. $\Delta^9\text{THC}$ exposure for 72 hours caused significantly decreased PSD95 protein levels in the hippocampus, as indication that $\Delta^9\text{THC}$ modify post-synaptic protein. Further, it also induced disorganization of CA1 stratum pyramidalis and showing a sign of morphological alterations with large number of pyknotic neurons and less densely packed of pyramidal neurons (Landucci et al., 2022). Another study done by Rubino et al. (2009), also demonstrated that rats treated with $\Delta^9\text{THC}$ for 10 days significantly decreases post-synaptic marker PSD95, leading to cognitive impairment. Chan et al. (1998) also demonstrated that *in-vitro* treatment of hippocampal neuron with $\Delta^9\text{THC}$ induces cell death. There was another study, however, reported that $\Delta^9\text{THC}$ administration ameliorate the synaptic plasticity by inducing the transcription of BDNF and Klotho genes, showing the beneficial impact of $\Delta^9\text{THC}$ specifically in enhancing the cognitive functions (Bilkei-Gorzo et al., 2017). Compiling data on previous findings, it has been suggested that the effect of $\Delta^9\text{THC}$ could be in biphasic fashion (Calabrese & Rubio-Casillas, 2018). It was demonstrated that low level of $\Delta^9\text{THC}$ may have the capacity to slow down or halt the AD progression by ameliorating the survival of neuronal cells as shown in our findings. Conversely, higher overdosage of $\Delta^9\text{THC}$ may cause negatively impact.

In the early stages of AD, the hippocampus shows rapid loss of neuronal cells (Rao et al., 2022). Neuronal cell loss or neurodegeneration happens typically because of the accumulation of two abnormal proteins; β -amyloid and tau, which act as major pathological hallmarks of AD. This condition contributes to the loss of synapse and loss of communication between neurons (Pham et al., 2010). The synaptic deterioration manifests with memory and learning impairment as it has been well established observed in the human AD brain (Shankar et al., 2008). Less synapses are found in the post-mortem brains of patient with AD especially in the hippocampus and cortex brain regions (Gutierrez & Limon, 2022; Schulz-Schaeffer, 2010). According to our findings, it may postulate that $\Delta^9\text{THC}$ may exerted the neurotherapeutic properties through maintenance the survival of cells and cell layers for the normal function of the brain. This process is known as neurogenesis. Neurogenesis could become one of strategy approach and provide new opportunities for cellular therapy in the central nervous system, particularly for neurodegenerative diseases like AD (Taupin, 2006).

5.8 Conclusion

The histomorphological results from the present study suggests that Δ^9 THC may have potential therapeutic benefits of remedying AD in rats, by restoring the cell layers and survival of cells for proper brain function. In the next chapter of the present study, neurogenesis markers related to our histology findings were discussed.



CHAPTER 6

NEUROTHERAPEUTIC POTENTIAL OF DELTA-9-TETRAHYDROCANNABINOL ON NEUROGENESIS MARKERS IN RATS EXPOSED TO D-GALACTOSE AND AlCl_3

6.1 Introduction

Alzheimer's disease (AD) is a severe multifactorial disorder marked by memory loss, cognitive decline, and debilitating neurodegeneration (Lambert et al., 1998). It is documented that disruption in synaptic activity triggering memory loss and cognitive impairments (Forner et al., 2017). Cognitive impairment is connected with neuron degeneration in regions of the brain involved in learning and memory (Götz et al., 2011). At advanced level, AD impairs in language and behaviour.

Neurogenesis is the process of producing new neurons from neural progenitor cells (NPCs) or neural stem cells during brain development (Dard et al, 2019; Kempermann et al., 2015). Several studies hypothesised that neurogenesis contributed to the hippocampal learning and memory (Suliman et al., 2018). Altman and colleagues (1965) presented the first evidence that new neurons were generated specifically at the subgranular zone (SGZ) of dentate gyrus in adult rats. Neurogenesis was believed to happen, offering hope for the future treatment of neurodegenerative diseases or others neuron-related illness (Kempermann et al., 2003).

It has been demonstrated that increasing neurogenesis may enhance the production of new neurons, promoting and strengthening the neuronal network, thus restoring the cognitive function in rats (Griffiths et al., 2020). For adult neurogenesis, there are multiple stages involved until the functional neurons are formed (Lieberwirth et al., 2016). In this present study, the hippocampus was used to examine the neurotherapeutic potential of $\Delta^9\text{THC}$ on neurogenesis activity in rats exposed to D-gal and AlCl_3 by measuring the neurogenesis markers. In cognitively impaired caused by D-gal and AlCl_3 , the potential of $\Delta^9\text{THC}$ to restore the neuronal loss through the production of neurogenesis markers (GFAP, DCX, NeUN and calbindin) has not yet been documented. This is the first study to investigate the influence of $\Delta^9\text{THC}$ on these neurogenesis markers in rats induced by D-gal and AlCl_3 .

Glial fibrillary protein (GFAP) is classified as an intermediate filament-III protein that is mostly observed in astrocytes, non-myelinating Schwann cells in the PNS and enteric glial cells in the CNS (McKeon & Benarroch, 2018). GFAP is essential for the cytoarchitecture and mechanical strength of astrocytes, as well as their supportive activities on the physiology of adjacent neurons and maintenance of the blood-brain barrier (McKeon & Benarroch, 2018; Eng et al., 2000). Immunohistochemistry and western blotting analysis revealed that GFAP-expressing astrocytes and microglia are located in the CA1 layers of the hippocampus (Perez-Nievas & Serrano-Pozo, 2018), corroborating of high amounts of GFAP in the brain. GFAP is implicated in the aetiology

of several numbers of neurologic disorder (Yang & Wang, 2015; Hol & Pekny, 2015), hence GFAP expression regulation serves as the primary biomarker.

Doublecortin (DCX) is a microtubule-associated phosphoprotein that has been proposed as a signal for the development of new neurons (Nacher et al., 2004). Throughout brain development, DCX is necessary for neuronal migration and differentiation (Friocourt et al., 2007). In AD, DCX expression diminishes, which may directly lead to memory loss, that can be restored by increasing neurogenesis (Moreno-Jimenez et al., 2019). Another study by Nakagawa et al. (2022) indicated that the number of doublecortin-positive immature neurons in the dentate gyrus decreases overtime, especially in the early stages of AD, and reduces further in later stages of AD. Neuronal nuclear protein, commonly known as NeUN is an additional neurogenesis marker that researchers employ actively for neuronal differentiation and identification of mature neurons (Gusel'nikova & Korzhevskiy, 2015).

Early stages of AD, neurodegeneration are marked by calcium dysregulation, neuronal loss and synaptic dysfunction (J. Hardy & Selkoe, 2002). Calbindin is one of the most important calcium-binding proteins, serving as a Ca^{2+} buffer (Connections et al., 1992). Ca^{2+} levels have been found to be altered in AD patients, resulting in a disturbed calcium-binding proteins homeostasis that disrupts calcium signalling. Hence, it results in neuronal degeneration and neural transmission dysfunctions (Palotás et al., 2002). Calbindin also correlates with learning and memory, since significantly decreased calbindin levels have been discovered in the dentate gyrus of AD (Palop et al., 2003).

Chronic treatment of cannabinoid agonist such as $\Delta^9\text{THC}$ has been observed to induce changes in the structure of the hippocampus. The alteration entails a reduction in the mean volume of neuron and the number of synapses (Kim & Thayer, 2001). Prolonged therapy with $\Delta^9\text{THC}$ significantly reduced the number of newly generated cells (Kochman et al., 2006). However, to date, cannabinoids are increasingly recognized for their functions in neuronal development processes, likely via activation of cannabinoid receptors (Prenderville et al., 2015). Based on earlier findings, $\Delta^9\text{THC}$ did enhance the neurogenesis process found in the normal rats, as observed using immunohistochemistry (IHC) method (Suliman et al., 2018). In this study, IHC was used to quantify protein markers implicated in the mechanism of neurogenesis in the hippocampus rats exposed with D-gal and AlCl_3 . The selected markers were GFAP, DCX, NeUN and calbindin.

6.2 Materials and methods

6.2.1 Drugs and reagents

In addition to the drugs and chemicals mentioned before (refer to section 3.1.1), xylene, alcohol, Rabbit monoclonal [EP3478] to Calbindin (Abcam), Goat Anti-Rabbit IgG H&L (HRP) (Abcam), Silanized Glass Slide (MUTO), EnVision™ FLEX Wash Buffer (20x) (DAKO), EnVision™ FLEX Target Retrieval Solution, High pH (50x) (DAKO), EnVision™ FLEX Target Retrieval Solution, Low pH (50x) (DAKO), Mouse and

Rabbit Specific HRP/DAB IHC Detection Kit - Micro-polymer (Abcam), blocking solution (Abcam), Rabbit monoclonal [EPR12763] to NeUN - Neuronal Marker (Abcam), Rabbit polyclonal to GFAP (Abcam), Rabbit polyclonal to Doublecortin (Abcam), Dako Antibody Diluent(DAKO) were also used in this study.

6.2.2 Equipment

Disposable microtome blades high profile stainless steel (Feather), analytical weighing balance (Sartorius, Germany), 0.45 μ m filter membrane (Whatman Nylon Membrane Filters, Whatman International Ltd Mainstone England), automated tissue processor (Leica TP1020), paraffin embedding station (Leica EG1160), tissue microtome (Leica RM2255), and water bath.

6.2.3 Animals

Animal husbandry and experimental design was referred to Chapter 3, section 3.1.1 and 3.1.2 respectively. The overall experimental design was simplified in Figure 6.1.

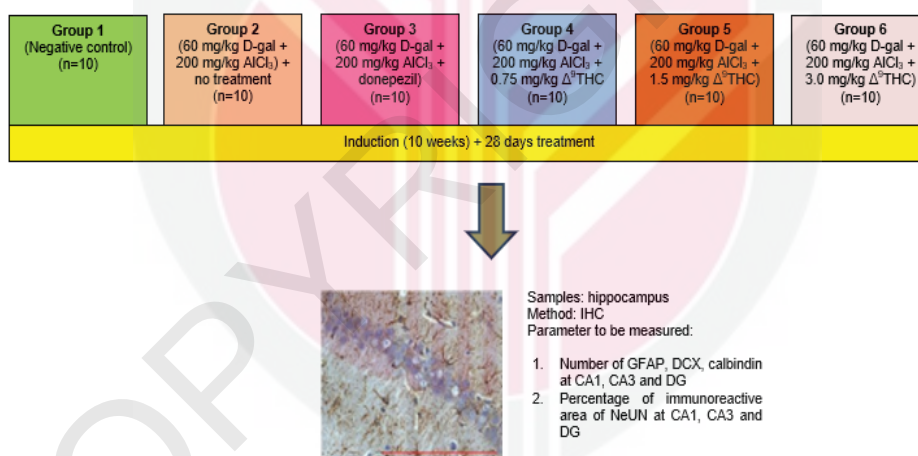


Figure 6.1 : Experimental design for IHC study. 60 mg/kg D-gal + 200 mg/kg AlCl₃ – AD model. D-gal – D-galactose; AlCl₃ – aluminium chloride; Δ^9 THC – delta-9-tetrahydrocannabinol; CA1 – cornu ammonis 1; CA3 – cornu ammonis 3; DG – dentate gyrus

6.2.4 Brain samples preparation

Brain retrieval was carried out inside an ice-containing tray and kept in 10% neutral buffered formalin for 7 days prior to further investigation. The samples were then placed in the tissue cassette in preparation for tissue processing with an automated tissue

processor (Leica TP1020) (refer to the Chapter 5, section 5.2.5.2). Using microtome, the trimming and sectioning process was carried out (Leica RM2255). The sample was cut 3-4 μm thickness using a microtome blade (Feather, Japan). A minimum of 5 sections were collected from each animal. The cut samples were placed in the 40 °C water bath (Leica HI1210) before being fished using silanized glass slides and was allowed to dry at room temperature (refer to Chapter 5, section 5.2.5.3 and 5.2.5.4).

6.2.5 Immunohistochemistry (IHC) procedures

The slides were baked at 60 °C for 2 hours 30 minutes. The slides were then dewaxed by soaking them in the xylene (3 times, 10 minutes each), followed by 100% alcohol (2 times, 5 minutes each), 95% alcohol (3 minutes) and 70% alcohol (3 minutes). The slides were then ready for IHC staining.

The slides were immersed in the distilled water for 1 minute. The slides were then soaked in EnVision™ FLEX Target Retrieval Solution, High pH (NeUN and calbindin) and EnVision™ FLEX Target Retrieval Solution, low pH (GFAP and DCX) for 30 minutes at 95 °C. The slides were allowed to cool down for 20 minutes in the retrieval buffer at room temperature. Following that, it was rinsed twice with washing buffer for 3 minutes each. The slides were then incubated in blocking solution for 30 minutes at room temperature before being incubated with primary antibodies for GFAP (1:2000; 20 minutes at room temperature), DCX (0.5 $\mu\text{g}/\text{ml}$; overnight at 4 °C), NeUN (1:3000; 1 hour at room temperature) and calbindin (1:1000; 1 hour at room temperature).

The slides were then incubated for 15 minutes at room temperature with Goat Anti-Rabbit IgG H&L (HRP) secondary antibody following a second rinsing with washing buffer. The slide was then rinsed and incubated with 0.05% w/v 3, 3'-diaminobenzidine (DAB) for 15 minutes. The slide was finally counterstained with hematoxylin and eosin (H&E). GFAP, DCX and calbindin immunopositive cells were quantified using Image J software, and the percentage of NeUN immunoreactive areas was calculated using the same software [(immunoreactive area divided by total area) x 100]. The areas that we focussed included cornu ammonis 1 (CA1), cornu ammonis 3 (CA3) and dentate gyrus (DG).

6.2.6 Statistical analysis

The data was analysed by one-way ANOVA followed by Tukey's post hoc test using SPSS version 25.0. The results were presented as mean $\pm$ SEM. $p < 0.05$ was used to determine the significance difference.

6.3 Results

6.3.1 Δ^9 THC ameliorate the neurogenesis marker by increasing the number of immunopositive GFAP cells in the CA1, CA3 and DG of the hippocampus against D-gal and AlCl_3 induced rats

Figures 6.2, 6.4 and 6.6 are photomicrographs of GFAP+ immunoreactive neurons in the CA1, CA3 and DG regions, respectively. All rat groups displayed a statistically significant differences in the expression of GFAP+ in the CA1 subfield of the hippocampus [$F(6,35) = 72.95, p=0.001$]. The number of GFAP+ cells was significantly increased in rats' groups treated with donepezil (73.67 ± 2.19) and Δ^9 THC at all doses; 0.75 (86.00 ± 4.17), 1.5 (136.33 ± 4.22), 3.0 (113.5 ± 4.11) when compared to AD model (35.33 ± 1.74) ($p<0.05$). Among treated groups, a significant difference has been observed between Δ^9 THC (1.5 and 3.0 mg/kg) when compared to Δ^9 THC 0.75 mg/kg and donepezil respectively. There was also a statistically significant difference between Δ^9 THC 1.5 mg/kg and control group ($p<0.05$) (Figure 6.3)

A significantly marked decreased in the number of GFAP+ cells was observed in the CA3 subfield of AD model (60.33 ± 3.92), as compared to control (111.00 ± 5.51) ($p<0.05$). However, Δ^9 THC-treated rats exhibit a significant increase in the number of GFAP+ cells; Δ^9 THC 0.75 mg/kg (103.17 ± 6.15), Δ^9 THC 1.5 mg/kg (140.67 ± 9.69), Δ^9 THC 3.0 mg/kg (143.33 ± 10.89) when compared to the AD model. Among these treated groups, there was a significant difference between Δ^9 THC (1.5 and 3.0 mg/kg) when compared to Δ^9 THC 0.75 mg/kg and donepezil respectively ($p<0.05$). In addition, the number of GFAP+ cells were also significantly increased between Δ^9 THC (1.5 and 3.0 mg/kg) as compared to the control group (Figure 6.5).

Similar finding was observed on the number of GFAP+ cells in the dentate gyrus of AD model (31.83 ± 1.83), when compared to control (70.00 ± 2.48) ($p<0.05$). Treatment with Δ^9 THC at all doses showed GFAP+ cells upregulation; 0.75 mg/kg (74.0 ± 2.31), 1.5 mg/kg (122.17 ± 5.42), 3.0 mg/kg (158.33 ± 4.33) when compared to AD model group. There was a statistically significant difference between Δ^9 THC (1.5 and 3.0 mg/kg) when compared to Δ^9 THC 0.75 mg/kg and donepezil respectively. A significant difference also has been observed in the number of GFAP+ cells in dentate gyrus between Δ^9 THC (1.5 and 3.0 mg/kg), when compared to the control group (Figure 6.7).

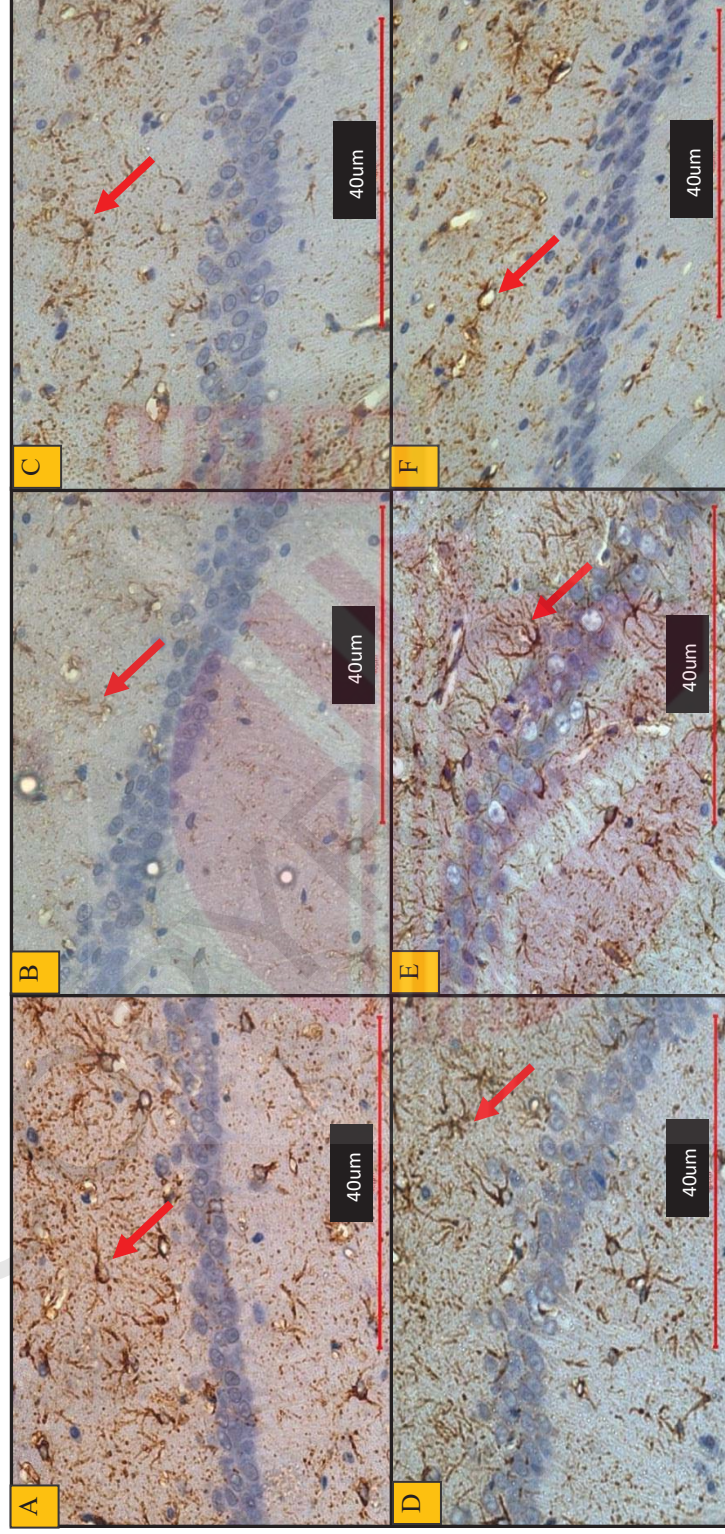


Figure 6.2 : Representative photomicrograph of CA1 subfield of the hippocampus by immunohistochemistry (IHC). (A) Control rats, (B) AD model group showing decreased in the number of GFAP+ cells, (C, D, E, F) treated group with 1 mg/kg donepezil, 0.75 Δ^9 THC, 1.5 Δ^9 THC and 3.0 Δ^9 THC respectively showing increased in the number of GFAP+ cells. Magnification of 400x for A-F, scale bar 40um. Red arrow represents the GFAP+ cells (includes at the top and bottom region of CA1 region)

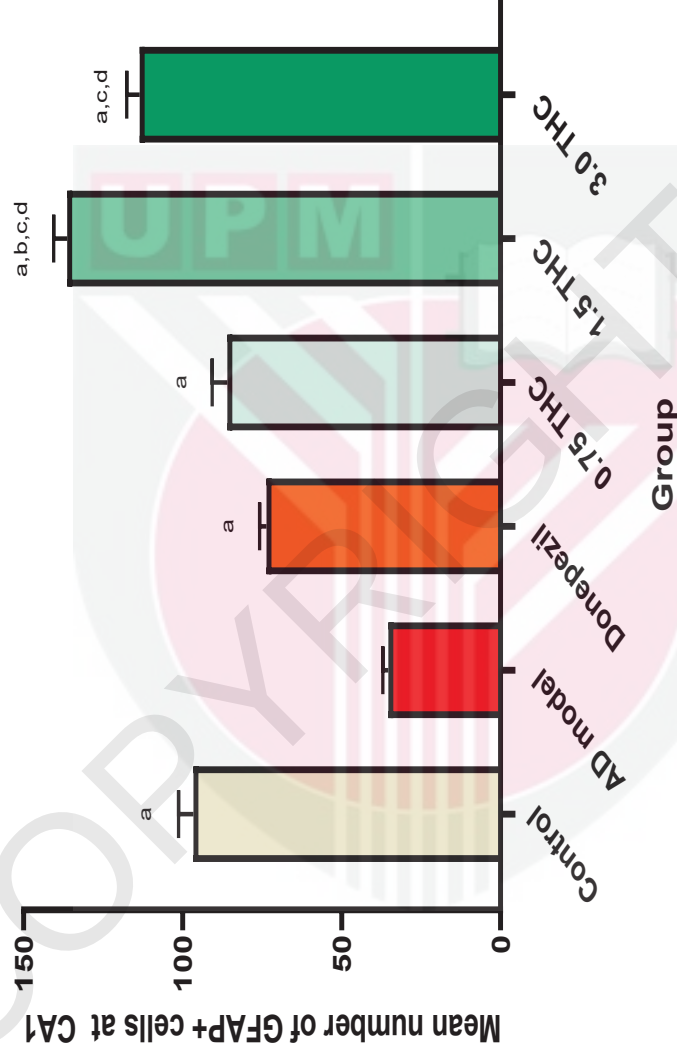


Figure 6.3 : Data showed the mean number of GFAP+ cells $\pm$ S.E.M at CA1 subfield of the hippocampus ($n=5$). The GFAP expression was obtained through paraffin-IHC technique. The experimental includes control, AD model (60 mg/kg D-gal + 200 mg/kg AICl₃, donepezil (AD model + 1 mg/kg donepezil), 0.75 THC (AD model + 0.75 mg/kg Δ^9 THC), 1.5 THC (AD model + 1.5 mg/kg Δ^9 THC) and 3.0 THC (AD model + 3.0 mg/kg Δ^9 THC). Data was analysed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. ^a $p<0.05$ vs AD model; ^b $p<0.05$ vs control; ^c $p<0.05$ vs 0.75 mg/kg Δ^9 THC; ^d $p<0.05$ vs donepezil. CA1 – cornu ammonis 1

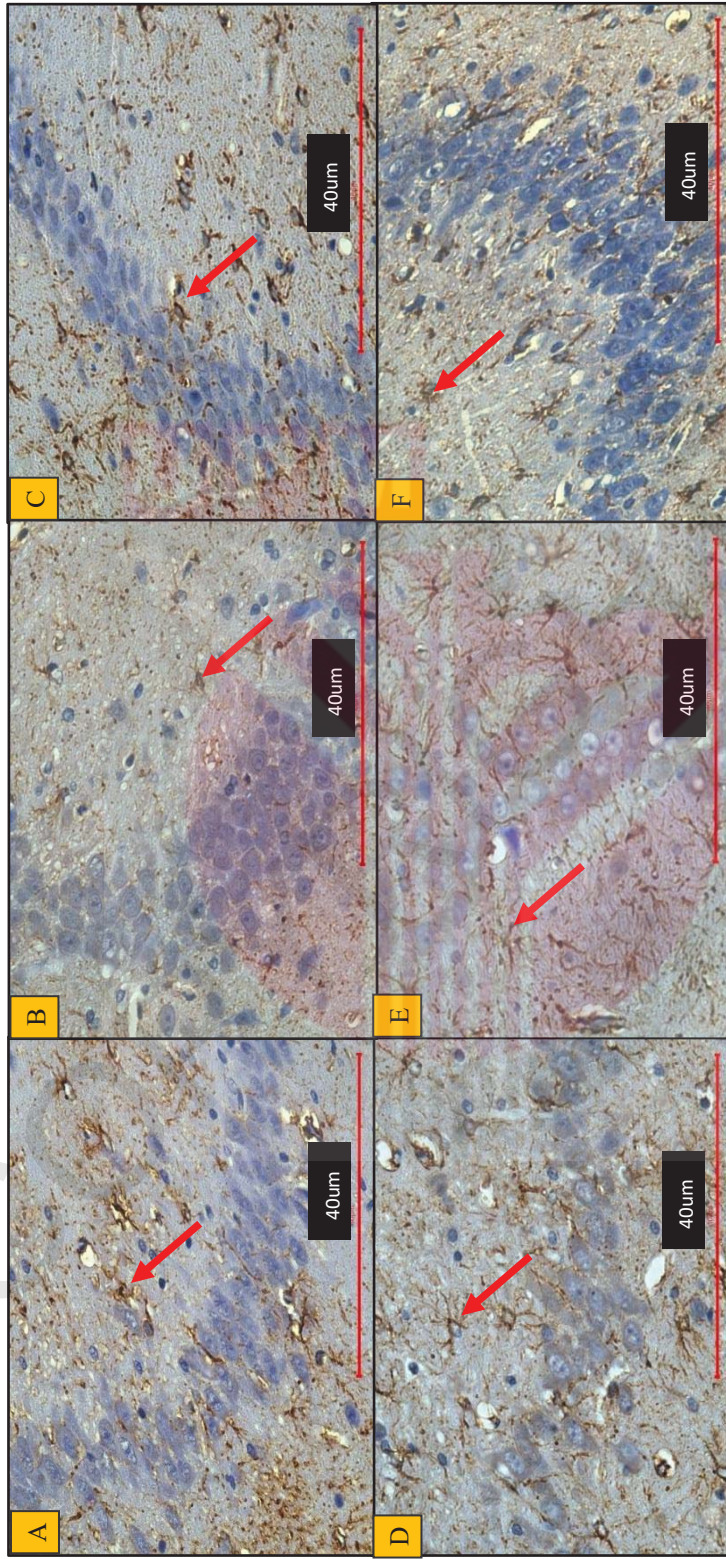


Figure 6.4 : Representative photomicrograph of CA3 subfield of the hippocampus by immunohistochemistry (IHC). (A) Control rats, (B) AD model group showing decreased in the number of GFAP+ cells. (C, D, E, F) treated group with 1 mg/kg donepezil, 0.75 Δ^9 THC, 1.5 Δ^9 THC and 3.0 Δ^9 THC respectively showing increased in the number of GFAP+ cells. Magnification of 400x for A-F, scale bar 40um. Red arrow represents the GFAP+ cells

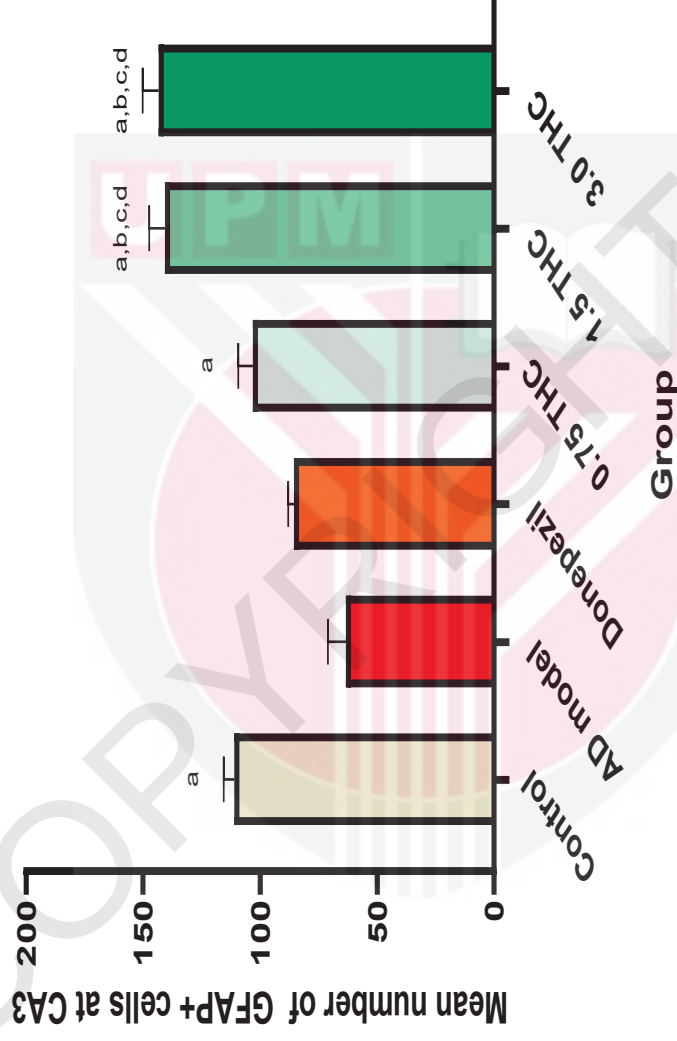


Figure 6.5 : Data showed the mean number of GFAP+ cells $\pm$ S.E.M at CA3 subfield of the hippocampus (n=5). The GFAP expression was obtained through paraffin-IHC technique. The experimental includes control, AD model (60 mg/kg D-gal + 200 mg/kg AlCl_3), donepezil (AD model + 1 mg/kg donepezil), 0.75 THC (AD model + 0.75 mg/kg $\Delta^9\text{THC}$), 1.5 THC (AD model + 1.5 mg/kg $\Delta^9\text{THC}$) and 3.0 THC (AD model + 3.0 mg/kg $\Delta^9\text{THC}$). Data was analysed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. ^a $p < 0.05$ vs AD model; ^b $p < 0.05$ vs control; ^c $p < 0.05$ vs 0.75 mg/kg $\Delta^9\text{THC}$; ^d $p < 0.05$ vs donepezil. CA3 – cornu ammonis 3

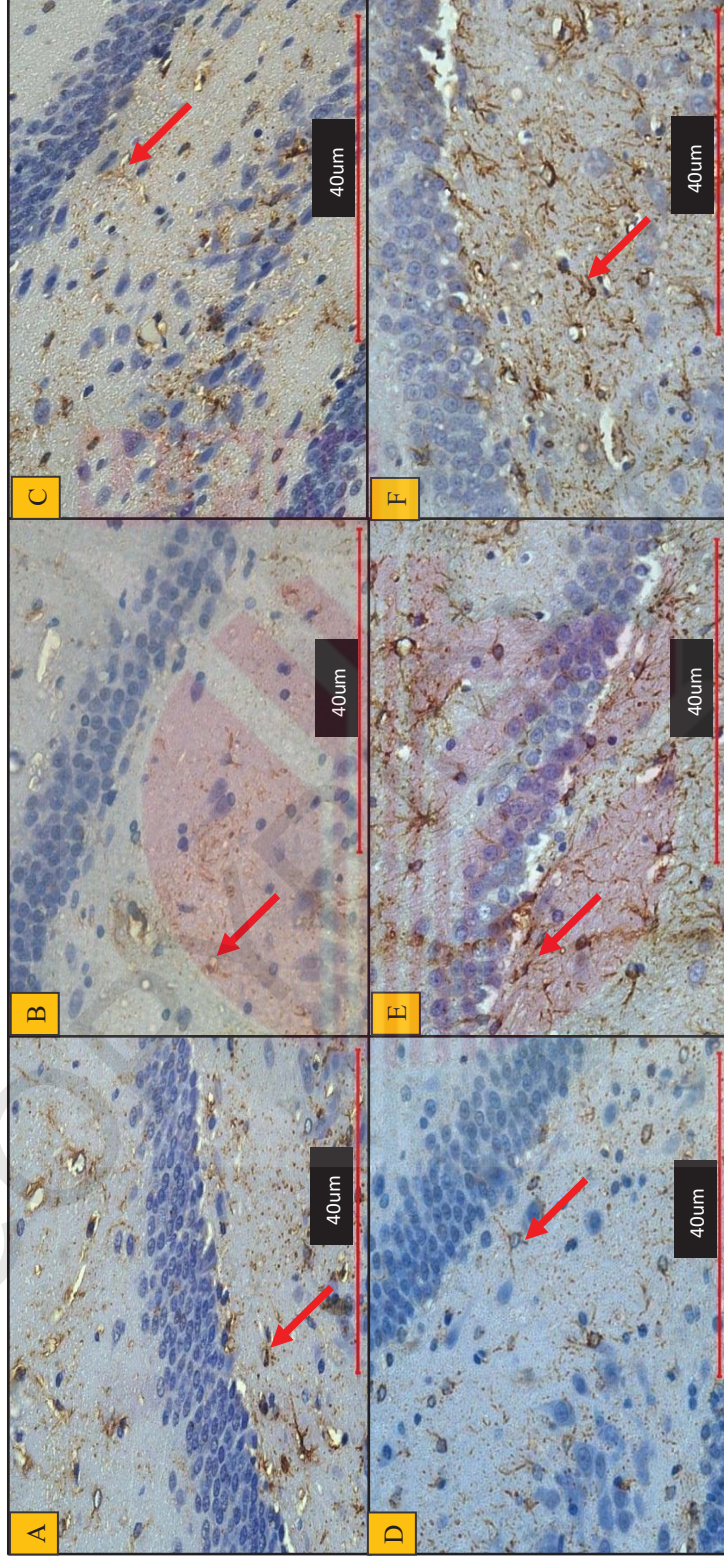


Figure 6.6 : Representative photomicrograph of dentate gyrus (DG) by immunohistochemistry (IHC). (A) Control rats, (B) AD model group showing decreased in the number of GFAP+ cells, (C, D, E, F) treated group with 1 mg/kg donepezil, 0.75 Δ^9 THC, 1.5 Δ^9 THC and 3.0 Δ^9 THC respectively showing increased in the number of GFAP+ cells. Magnification of 400x for A-F, scale bar 40µm. Red arrow represents the GFAP+ cells (includes upper and lower blade of DG)

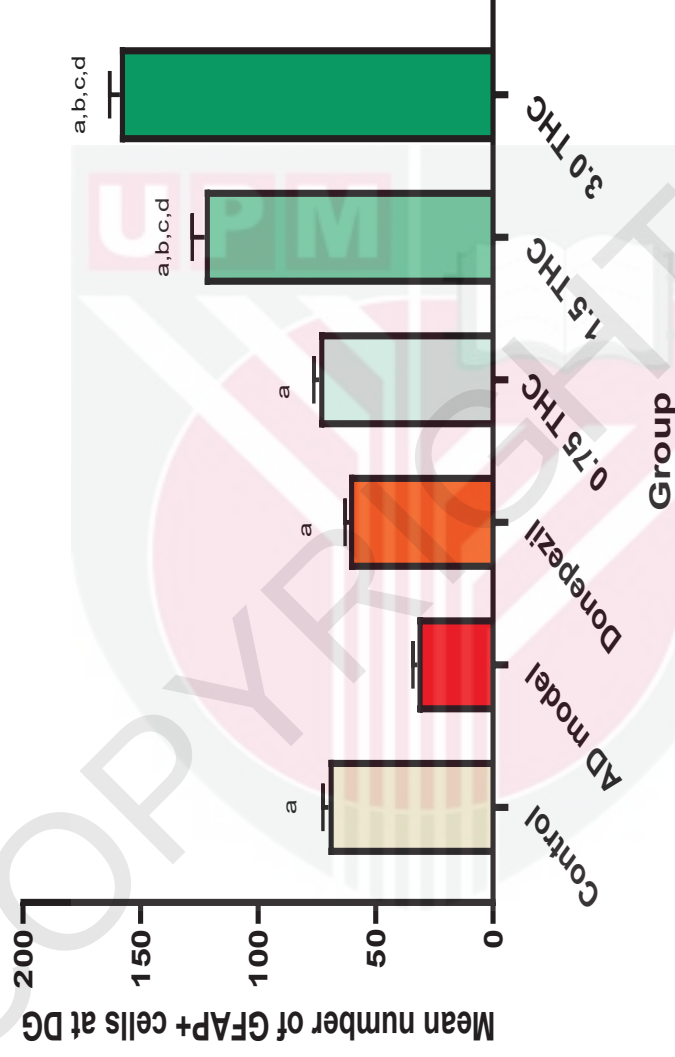


Figure 6.7 : Data showed the mean number of GFAP+ cells $\pm$ S.E.M at dentate gyrus (n=5). The GFAP expression was obtained through paraffin-IHC technique. The experimental includes control, AD model (60 mg/kg D-gal + 200 mg/kg AICl₃), donepezil (AD model + 1 mg/kg donepezil), 0.75 THC (AD model + 0.75 mg/kg Δ^9 THC), 1.5 THC (AD model + 1.5 mg/kg Δ^9 THC) and 3.0 THC (AD model + 3.0 mg/kg Δ^9 THC). Data was analysed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. ^ap<0.05 vs AD model; ^bp<0.05 vs control; ^cp<0.05 vs 0.75 mg/kg Δ^9 THC; ^dp<0.05 vs donepezil . DG – dentate gyrus

6.3.2 Δ^9 THC ameliorates the neurogenesis marker by increasing the number of doublecortin (DCX) immuno-positive cells in the CA1, CA3 and dentate gyrus of hippocampus against D-gal and AlCl_3 induced rats

Figures 6.8, 6.10 and 6.12 depicted the photomicrograph of the CA1, CA3 and dentate gyrus that showing DCX+ cells. One-way ANOVA showed a statistically significant [$F(6,35) = 75.65, p = 0.001$] differences in the expression of DCX+ in the CA1 subfield of the hippocampus among all the rats' groups. The number of DCX+ cells was significantly reduced in AD model (45.67 ± 2.86), when compared to the control group (74.17 ± 0.79) ($p < 0.05$). Tukey's post hoc revealed a significantly increase in the number of DCX+ cells ($p < 0.05$) in the treated group of rats; donepezil (71.17 ± 2.33), $0.75 \Delta^9\text{THC}$ (69.33 ± 1.69), $1.5 \Delta^9\text{THC}$ (84.50 ± 2.59) and $3.0 \Delta^9\text{THC}$ (77.17 ± 2.04) when compared to AD model (45.67 ± 2.86). There was a significant difference between $\Delta^9\text{THC}$ 1.5 mg/kg and control group (Figure 6.9).

In the CA3 subfield of the hippocampus, the result demonstrated that there was a significantly reduced in the number of DCX+ cells of AD model group as compare to the control group ($p < 0.05$). Tukey's post hoc analysis showed a statistically marked increase on the number of DCX+ cells in donepezil (62.5 ± 1.57) and $\Delta^9\text{THC}$ treated of rats; 0.75 mg/kg (64.17 ± 2.69), 1.5 mg/kg (72.5 ± 2.89), and 3.0 mg/kg (70.33 ± 1.58), when compared to AD model (29.33 ± 1.94). There was also a significant difference between $\Delta^9\text{THC}$ 1.5 mg/kg and control group (Figure 6.11).

Our findings also revealed a statistically significant reduction in the number of DCX+ cells of AD model group on the dentate gyrus area ($p < 0.05$). The number of DCX+ cells, however, was significantly increased ($p < 0.05$) in the treated group of rats; donepezil (68.17 ± 1.85), $0.75 \Delta^9\text{THC}$ (80.33 ± 2.40), $1.5 \Delta^9\text{THC}$ (81.67 ± 2.63), $3.0 \Delta^9\text{THC}$ (93.00 ± 3.47) when compared to AD model (27.83 ± 3.26). Among these treated groups, $\Delta^9\text{THC}$ 1.5 mg/kg showed a significant difference in the number of DCX+ cells when compared to the control group (Figure 6.13).

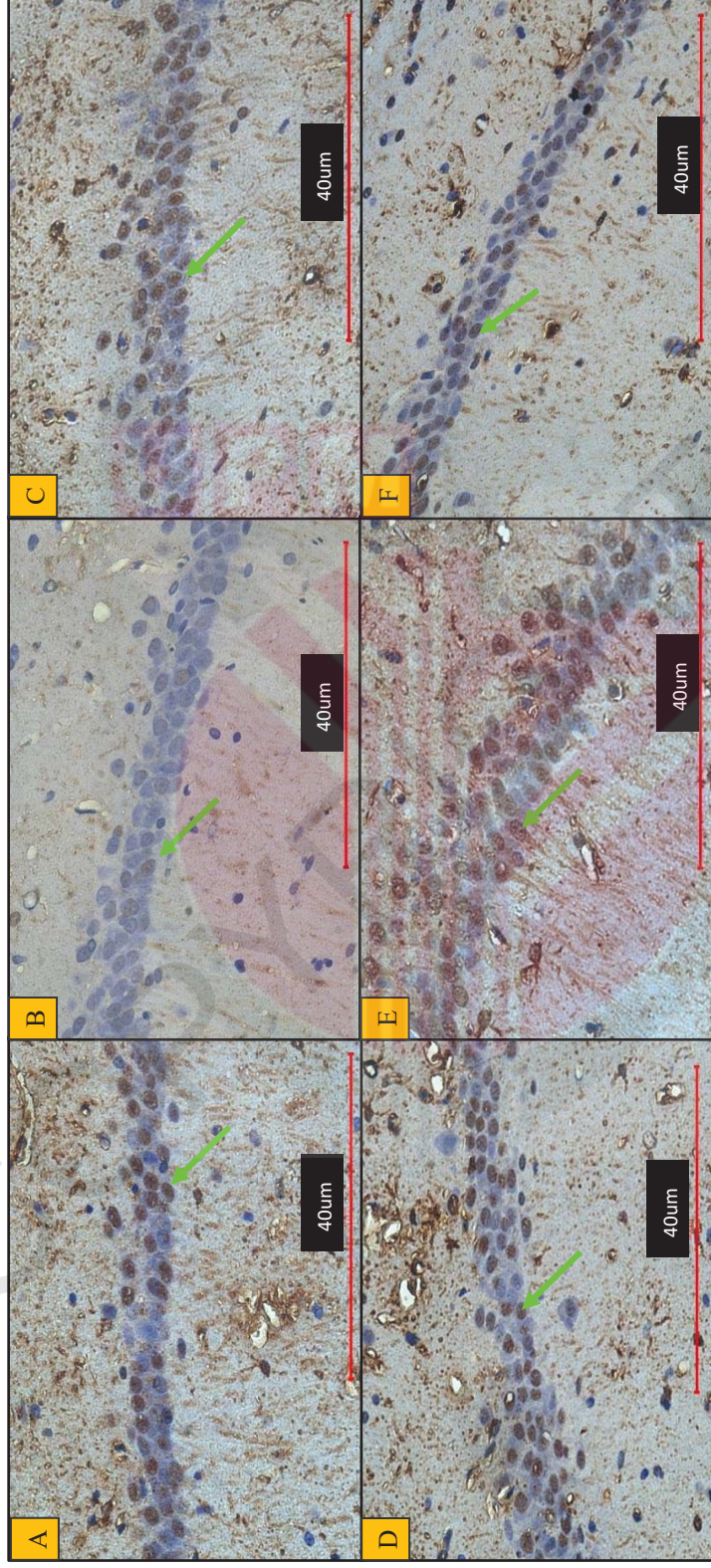


Figure 6.8 : Representative photomicrograph of CA1 subfield of the hippocampus by immunohistochemistry (IHC). (A) Control rats, (B) AD model group showing decreased in the number of DCX+ cells, (C, D, E, F) treated group with 1 mg/kg donepezil, 0.75 Δ^9 THC, 1.5 Δ^9 THC and 3.0 Δ^9 THC respectively showing increased in the number of DCX+ cells. Magnification of 400x for A-F, scale bar 40 μ m. Green arrow represents the DCX+ cells

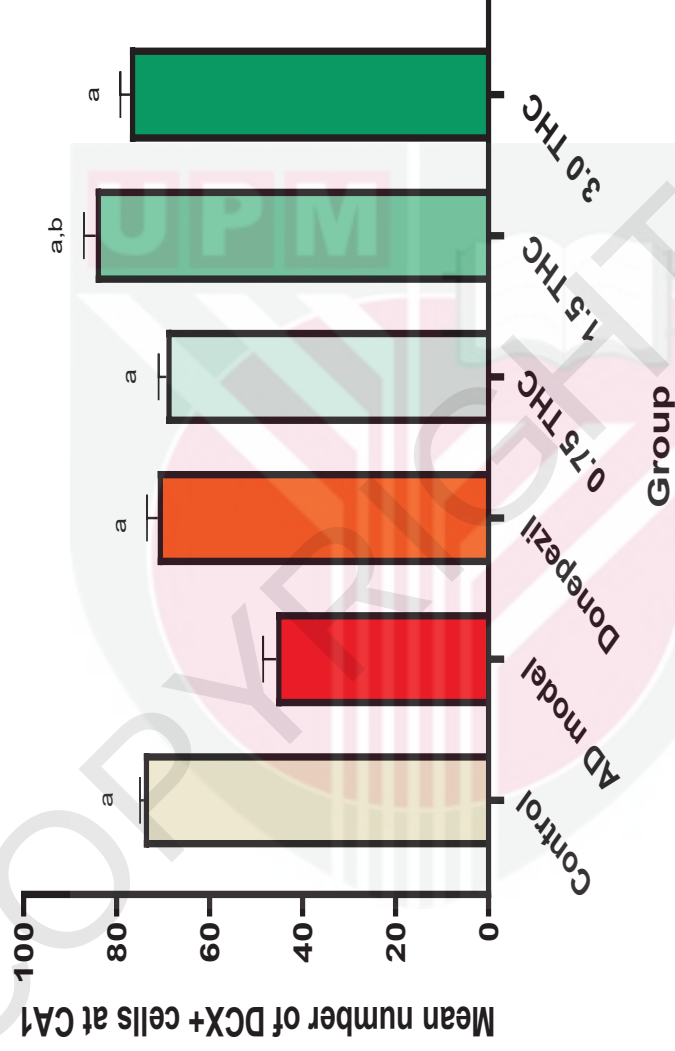


Figure 6.9 : Data showed the mean number of DCX+ cells $\pm$ S.E.M at CA1 subfield of the hippocampus (n=5). The DCX expression was obtained through paraffin-IHC technique. The experimental includes control, AD model (60 mg/kg D-gal + 200 mg/kg AlCl_3), donepezil (AD model + 1 mg/kg donepezil), 0.75 THC (AD model + 0.75 mg/kg $\Delta^9\text{THC}$), 1.5 THC (AD model + 1.5 mg/kg $\Delta^9\text{THC}$) and 3.0 THC (AD model + 3.0 mg/kg $\Delta^9\text{THC}$). Data was analysed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. ^a $p < 0.05$ vs AD model; ^b $p < 0.05$ vs control. CA1 – cornu ammonis 1

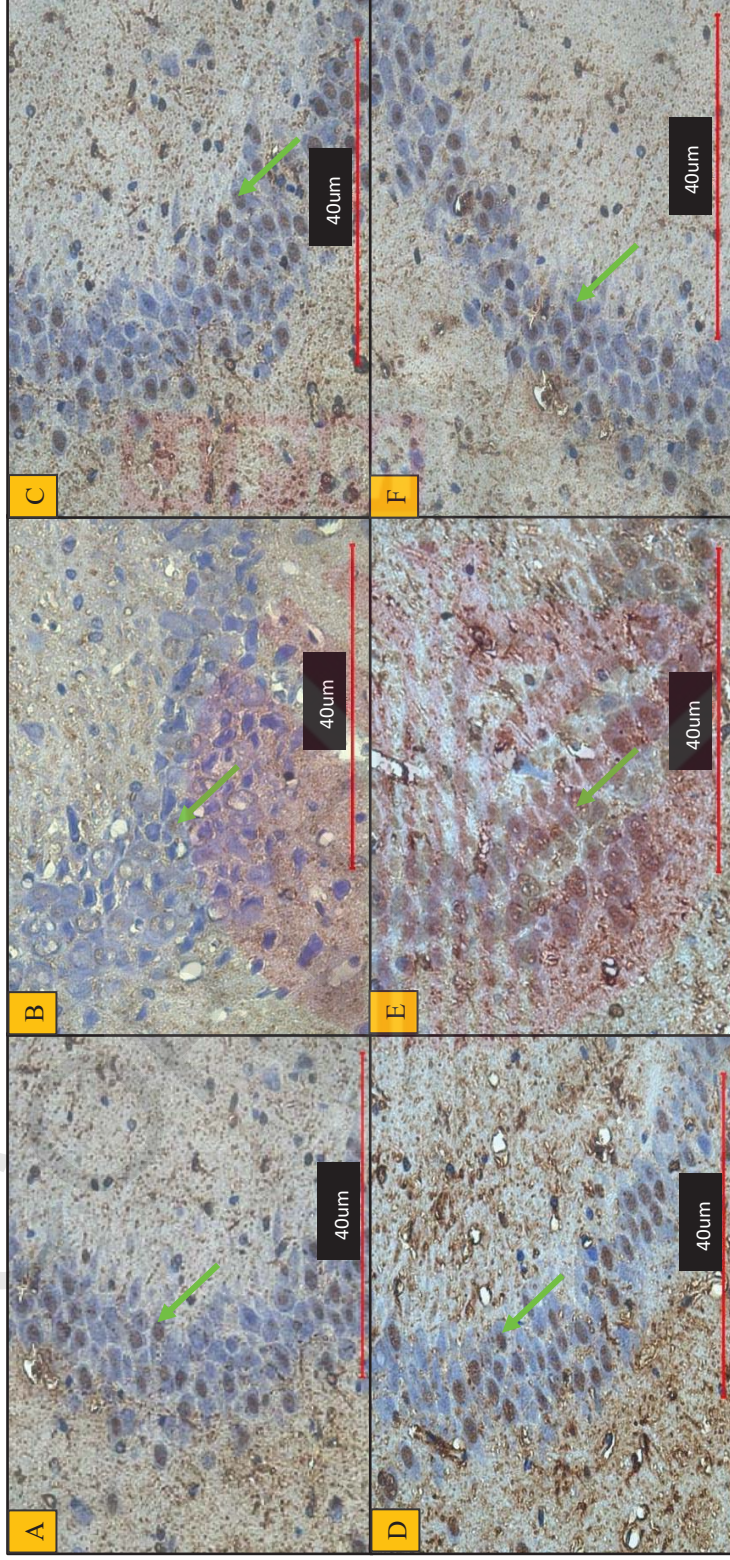


Figure 6.10 : Representative photomicrograph of CA3 subfield of the hippocampus by immunohistochemistry (IHC). (A) Control rats, (B) AD model group showing decreased in the number of DCX+ cells, (C, D, E, F) treated group with 1 mg/kg donepezil, 0.75 Δ^9 THC, 1.5 Δ^9 THC and 3.0 Δ^9 THC respectively showing increased in the number of DCX+ cells. Magnification of 400x for A-F, scale bar 40um. Green arrow represents the DCX+ cells

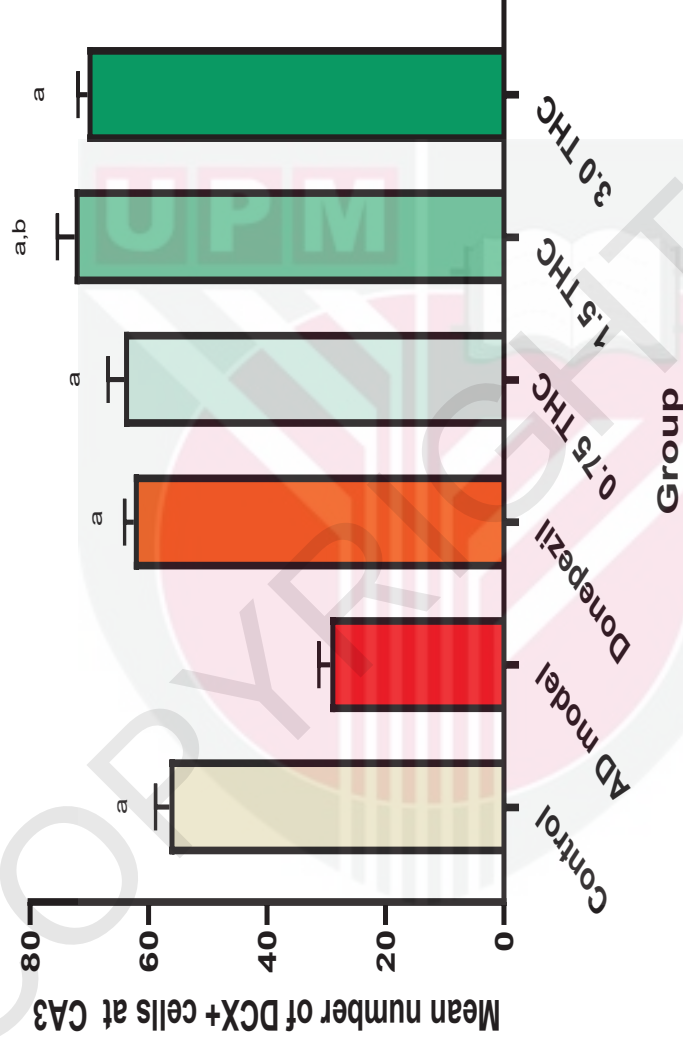


Figure 6.11 : Data showed the mean number of DCX+ cells $\pm$ S.E.M at CA3 subfield of the hippocampus (n=5). The DCX expression was obtained through paraffin-IHC technique. The experimental includes control, AD model (60 mg/kg D-gal + 200 mg/kg AlCl_3), donepezil (AD model + 1 mg/kg donepezil), 0.75 THC (AD model + 0.75 mg/kg $\Delta^9\text{THC}$), 1.5 THC (AD model + 1.5 mg/kg $\Delta^9\text{THC}$) and 3.0 THC (AD model + 3.0 mg/kg $\Delta^9\text{THC}$). Data was analysed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. ^a $p < 0.05$ vs AD model; ^b $p < 0.05$ vs control. CA3 – cornu ammonis 3

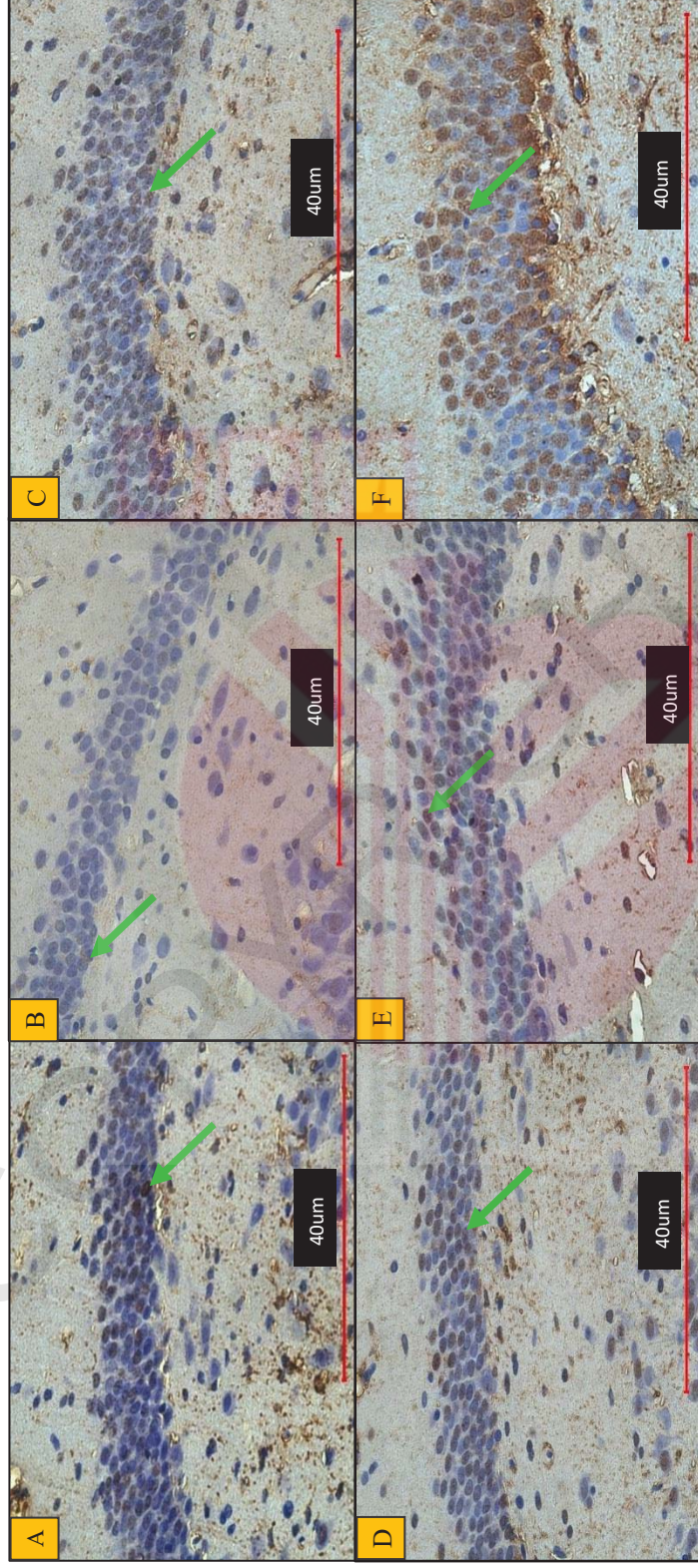


Figure 6.12 : Representative photomicrograph of dentate gyrus by immunohistochemistry (IHC). (A) Control rats, (B) AD model group showing decreased in the number of DCX+ cells, (C, D, E, F) treated group with 1 mg/kg donepezil, 0.75 Δ^9 THC, 1.5 Δ^9 THC and 3.0 Δ^9 THC respectively showing increased in the number of DCX+ cells. Magnification of 400x for A-F, scale bar 40um. Green arrow represents the DCX+ cells

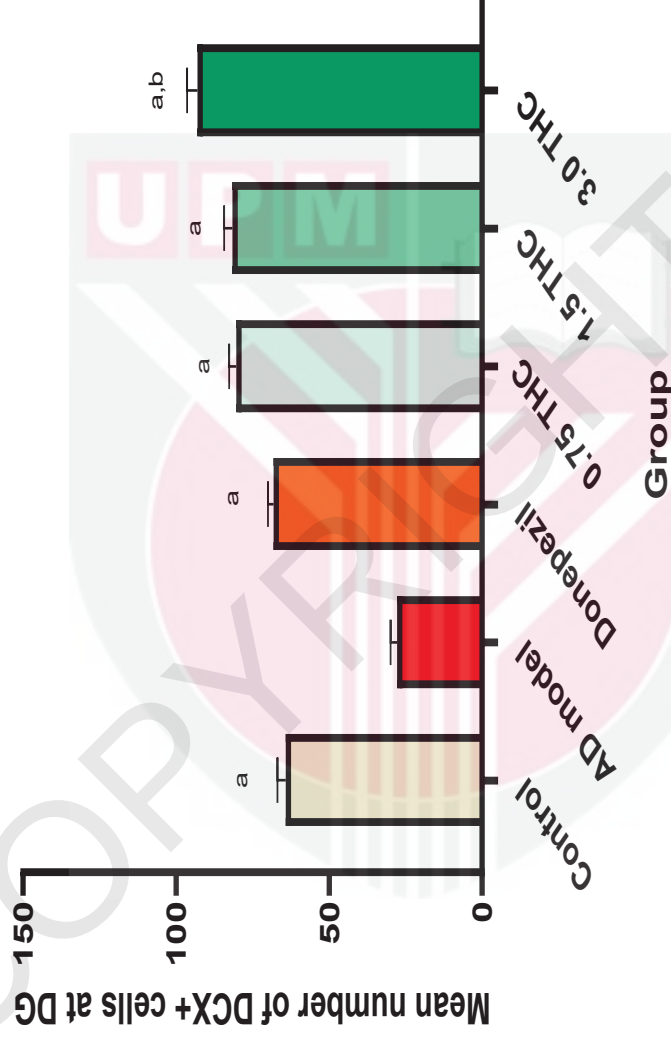


Figure 6.13 : Data showed the mean number of DCX+ cells $\pm$ S.E.M at dentate gyrus (n=5). The DCX expression was obtained through paraffin-IHC technique. The experimental includes control, AD model (60 mg/kg D-gal + 200 mg/kg AICl₃), donepezil (AD model + 1 mg/kg donepezil), 0.75 THC (AD model + 0.75 mg/kg Δ^9 THC), 1.5 THC (AD model + 1.5 mg/kg Δ^9 THC) and 3.0 THC (AD model + 3.0 mg/kg Δ^9 THC). Data was analysed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. ^ap<0.05 vs ADmodel; ^bp<0.05 vs control. DG – dentate gyrus

6.3.3 Δ^9 THC ameliorates the neurogenesis marker by increasing the percentage of immunoreactive NeUN at CA1, CA3 and dentate gyrus in rats induced by D- gal and AlCl_3

The expression of NeUN immunoreactive cells in the CA1 subfield of the hippocampus of all the rats' groups is shown in Figure 6.14. One-way ANOVA revealed statistically significant [$F(6,35) = 54.6, p=0.001$] differences in the expression of NeUN+ in the CA1 areas of the hippocampus among all the rats' groups. Further, Tukey's post hoc confirmed a marked increase in the percentage of NeUN immunoreactive area in treated group of rats; donepezil (8.61 ± 0.31), $0.75 \Delta^9\text{THC}$ (12.66 ± 1.10), $1.5 \Delta^9\text{THC}$ (13.63 ± 1.03) and $3.0 \Delta^9\text{THC}$ (13.04 ± 0.87) when compared to AD model (2.07 ± 0.37). There was a significant difference has been observed between all $\Delta^9\text{THC}$ treated group and control group ($p<0.05$) (Figure 6.15).

Our findings also revealed that, the percentage of NeUN immunoreactive area was significantly reduced in the CA3 subfield of rats' hippocampus administered with D- gal and AlCl_3 as compared to the control group ($p<0.05$) (Figure 6.16). As shown in figure 6.17, the rats treated with donepezil (22.04 ± 0.87) or $\Delta^9\text{THC}$ at all doses; $0.75 \Delta^9\text{THC}$ (31.16 ± 1.34), $1.5 \Delta^9\text{THC}$ (34.81 ± 0.96) and $3.0 \Delta^9\text{THC}$ (34.28 ± 1.03) exhibited a statistically significant increase in the percentage of NeUN immunoreactive area when compared to group (12.13 ± 1.24) ($p<0.05$). A significant difference also have been observed between all $\Delta^9\text{THC}$ treated groups when compared to the donepezil and control group respectively ($p<0.05$).

Rats exposed with D-gal and AlCl_3 (AD model) demonstrated significantly reduced the percentage of NeUN immunoreactive area in dentate gyrus as compared to control group ($p<0.05$) (Figure 6.18). Post hoc Tukey's test revealed that donepezil or $\Delta^9\text{THC}$ treated group were statistical significantly increased in the percentage of NeUN immunoreactive area; donepezil (11.38 ± 0.66), 0.75 mg/kg (20.61 ± 1.15), 1.5 mg/kg (25.04 ± 1.04) and 3.0 mg/kg (25.13 ± 1.16) when compared to AD model (2.82 ± 0.31) ($p<0.05$). The results also demonstrated that there was a significant difference between all $\Delta^9\text{THC}$ treated group as compared to control and donepezil respectively ($p<0.05$). Among $\Delta^9\text{THC}$ treated group, a significant difference was observed between $\Delta^9\text{THC}$ (1.5 and 3.0 mg/kg) and $\Delta^9\text{THC}$ 0.75 mg/kg (Figure 6.19).

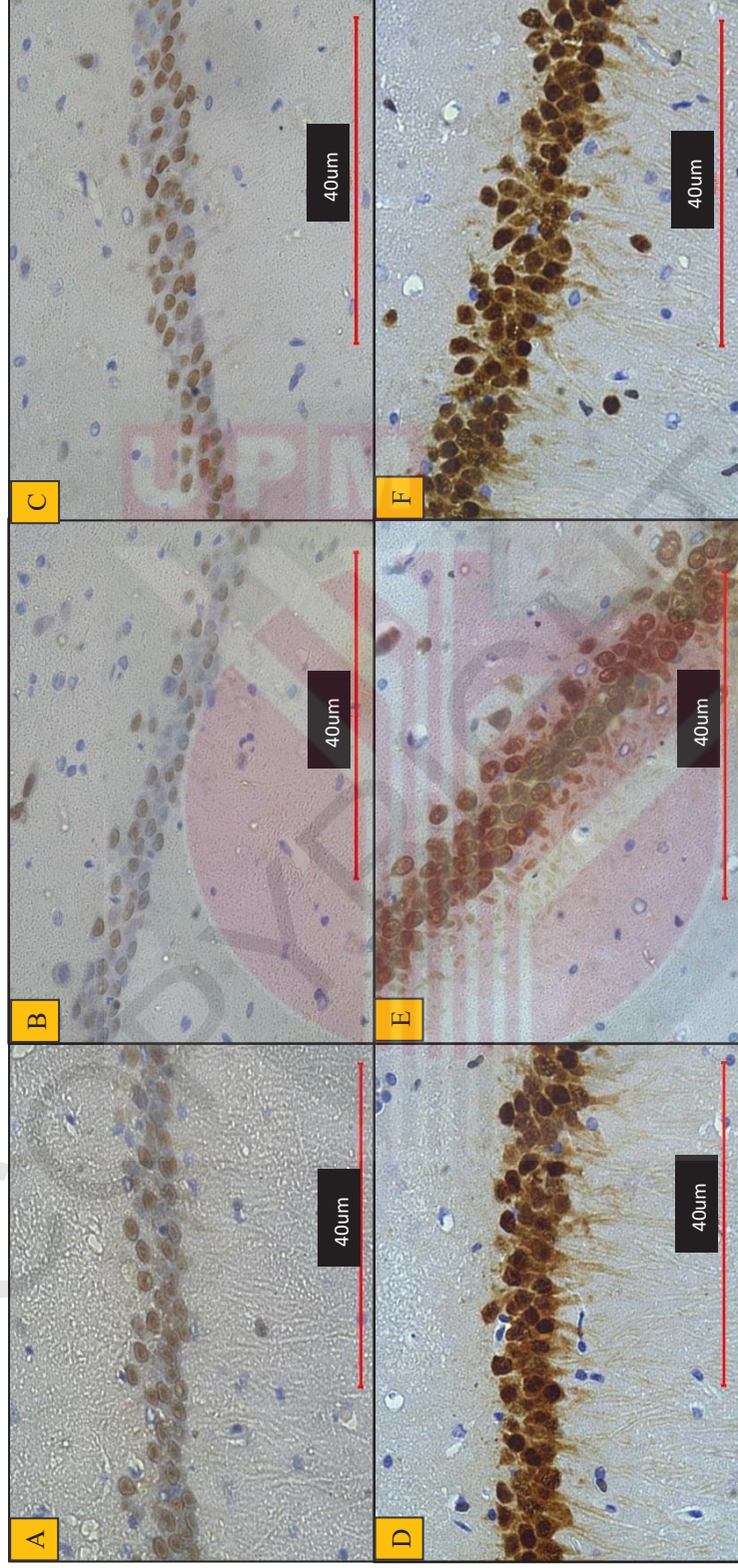


Figure 6.14 : Representative photomicrograph of CA1 subfield of the hippocampus by immunohistochemistry (IHC) for NeuN expression. (A) Control rats, (B) AD model group, (C, D, E, F) treated group with 1 mg/kg donepezil, 0.75 Δ^9 THC, 1.5 Δ^9 THC, 1.5 Δ^9 THC and 3.0 Δ^9 THC respectively. Magnification of 400x for A-F, scale bar 40um

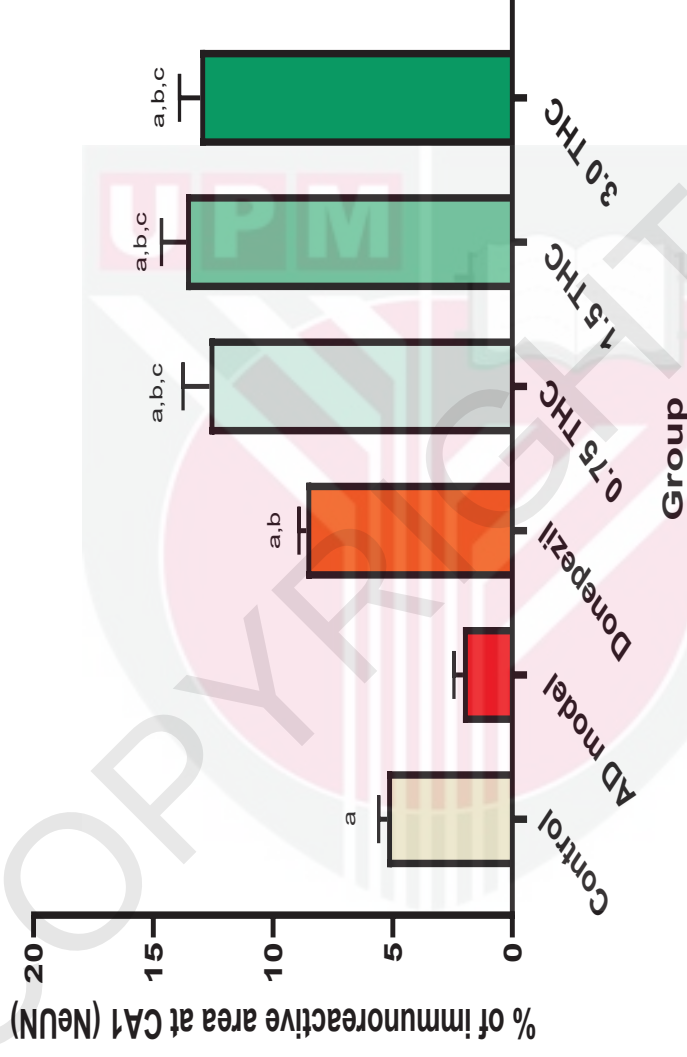


Figure 6.15 : Data showed the percentage of NeUN immunoreactive area $\pm$ S.E.M at CA1 subfield of the hippocampus (n=5). The NeUN expression was obtained through paraffin-IHC technique. The experimental includes control, AD model (60 mg/kg D-gal + 200 mg/kg AlCl_3), donepezil (AD model + 1 mg/kg donepezil), 0.75 THC (AD model + 0.75 mg/kg $\Delta^9\text{THC}$), 1.5 THC (AD model + 1.5 mg/kg $\Delta^9\text{THC}$) and 3.0 THC (AD model + 3.0 mg/kg $\Delta^9\text{THC}$). Data was analysed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. ^a $p < 0.05$ vs control; ^b $p < 0.05$ vs AD model; ^c $p < 0.05$ vs donepezil. CA1 – cornu ammonis 1

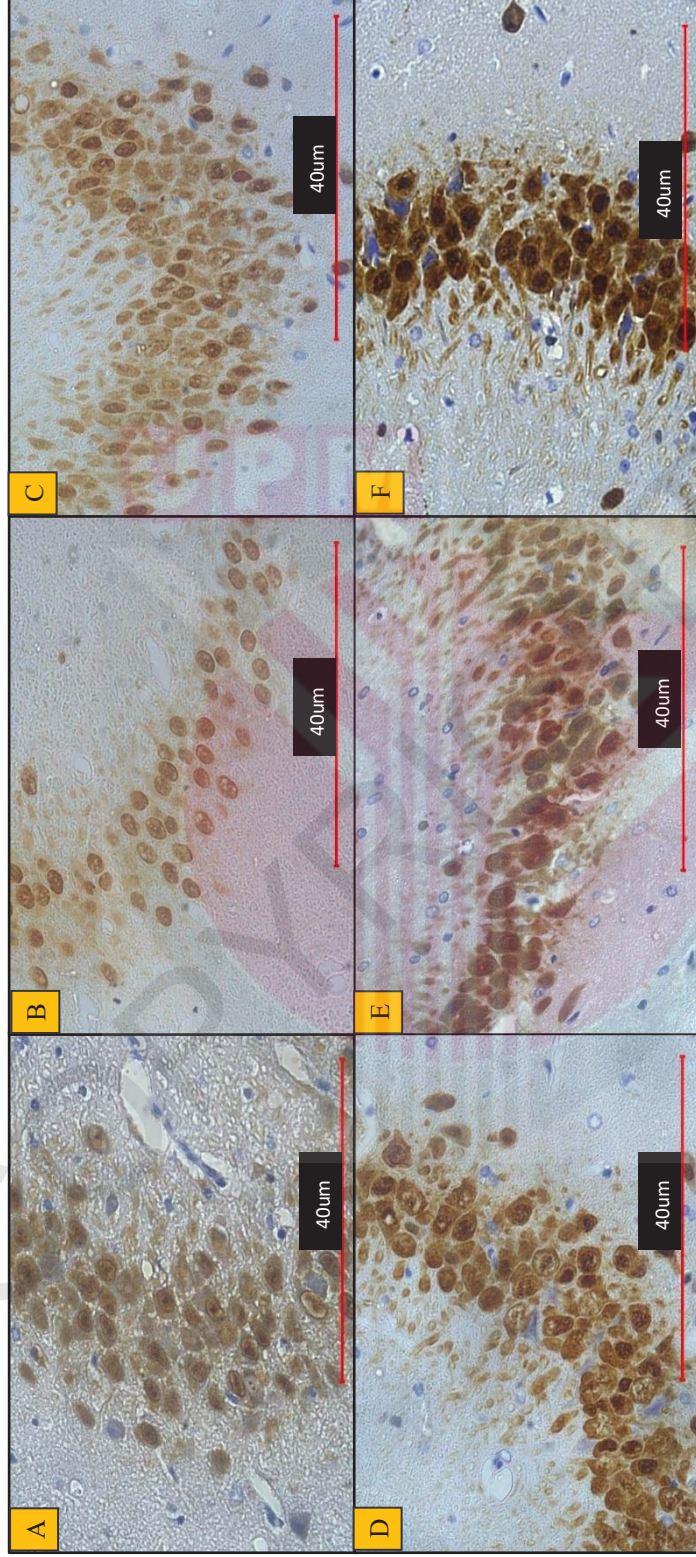


Figure 6.16 : Representative photomicrograph of CA3 subfield of the hippocampus by immunohistochemistry (IHC) for NeUN expression. (A) Control rats, (B) AD model group, (C, D, E, F) treated group with 1 mg/kg donepezil, 1.5 Δ^9 THC, 0.75 Δ^9 THC and 3.0 Δ^9 THC respectively. Magnification of 400x for A-F, scale bar 40um

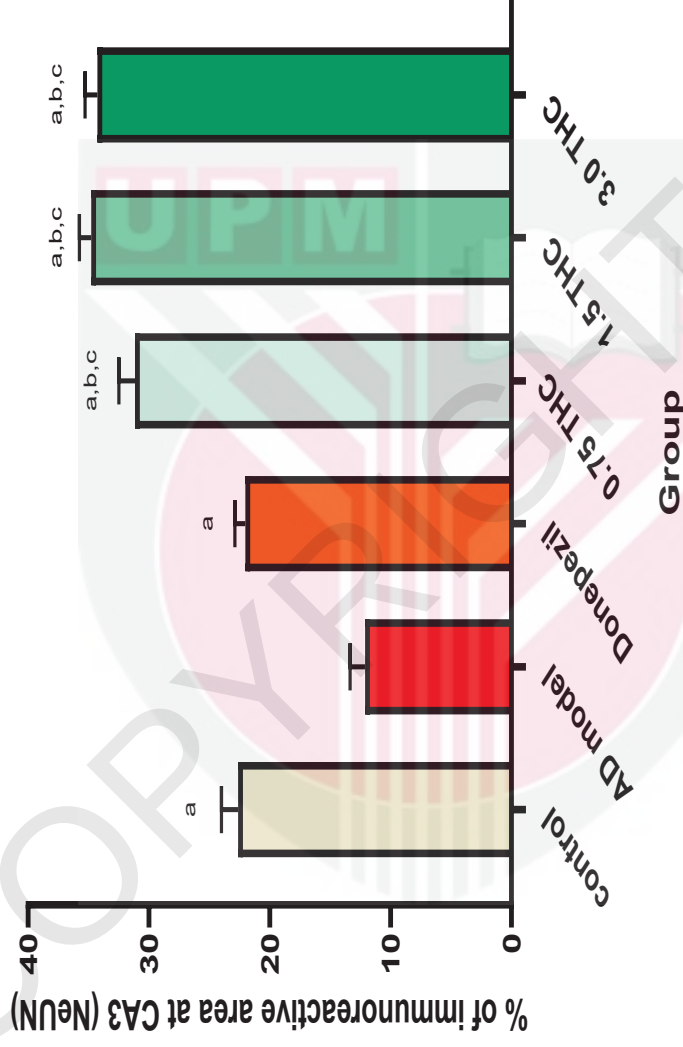


Figure 6.17 : Data showed the percentage of NeUN immunoreactive area $\pm$ S.E.M at CA3 subfield of the hippocampus (n=5). The NeUN expression was obtained through paraffin-IHC technique. The experimental includes control, AD model (60 mg/kg D-gal + 200 mg/kg AlCl_3), donepezil (AD model + 1 mg/kg donepezil), 0.75 THC (AD model + 0.75 mg/kg $\Delta^9\text{THC}$), 1.5 THC (AD model + 1.5 mg/kg $\Delta^9\text{THC}$) and 3.0 THC (AD model + 3.0 mg/kg $\Delta^9\text{THC}$). Data was analysed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. ^a $p < 0.05$ vs control; ^b $p < 0.05$ vs AD model; ^c $p < 0.05$ vs donepezil. CA3 – cornu ammonis 3

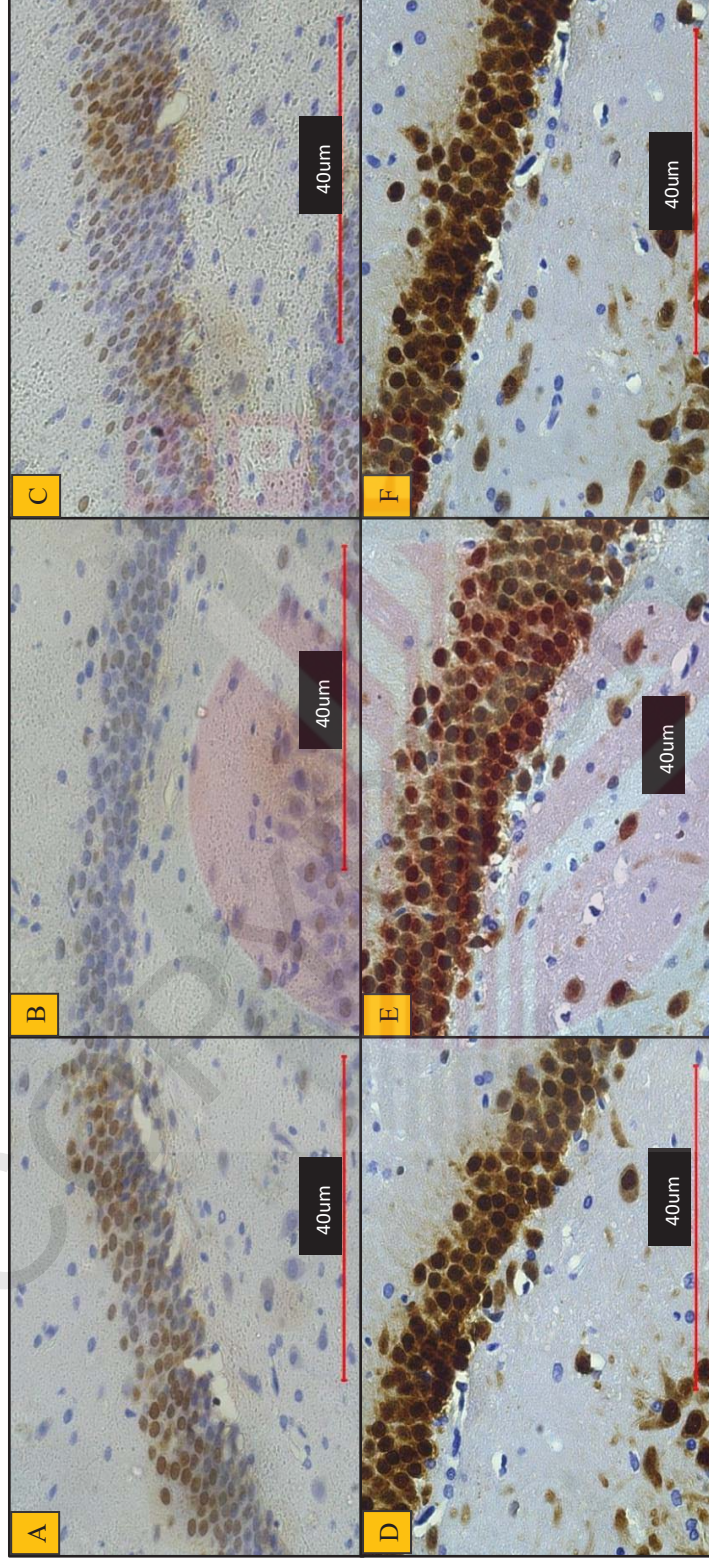


Figure 6.18 : Representative photomicrograph of dentate gyrus by immunohistochemistry (IHC) for NeuN expression. (A) Control rats, (B) AD model group, (C, D, E, F) treated group with 1 mg/kg donepezil, 0.75 Δ^9 THC, 1.5 Δ^9 THC and 3.0 Δ^9 THC respectively. Magnification of 400x for A-F, scale bar 40um

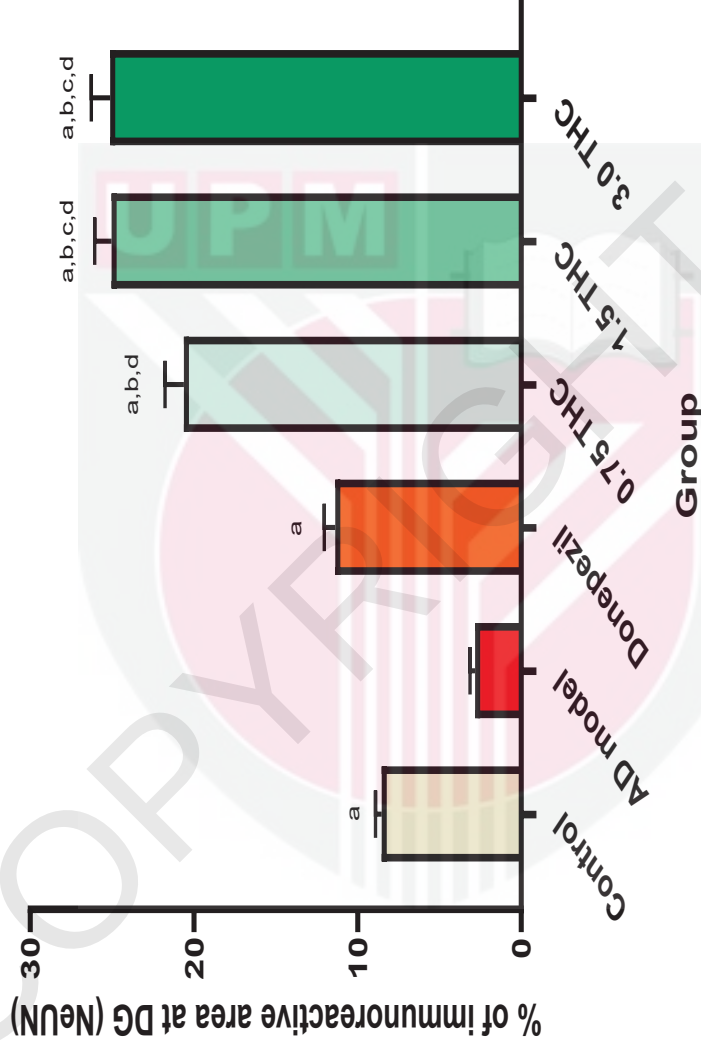


Figure 6.19 : Data showed the percentage of NeUN immunoreactive area $\pm$ S.E.M at dentate gyrus (n=5). The NeUN expression was obtained through paraffin-IHC technique. The experimental includes control, AD model (60 mg/kg D-gal + 200 mg/kg AlCl_3), donepezil (AD model + 1 mg/kg donepezil), 0.75 THC (AD model + 0.75 mg/kg $\Delta^9\text{THC}$), 1.5 THC (AD model + 1.5 mg/kg $\Delta^9\text{THC}$) and 3.0 THC (AD model + 3.0 mg/kg $\Delta^9\text{THC}$). Data was analysed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. ^a $p < 0.05$ vs AD model; ^b $p < 0.05$ vs control; ^c $p < 0.05$ vs 0.75 $\Delta^9\text{THC}$; ^d $p < 0.05$ vs donepezil. DG – dentate gyrus

6.3.4 Δ^9 THC ameliorates the neurogenesis marker by increasing the number of calbindin+ cells at CA1, DG of the hippocampus and increasing the percentage of calbindin immunoreactive area at CA3 subfield in rat hippocampus induced by D-gal and AlCl_3

Result showed that there was a statistically significant reduced in the number of calbindin+ cell in the CA1 region of hippocampus in AD model group, when compared to the control [$F(6,35) = 58.38, p = 0.001$]. The number of calbindin+ cells increased significantly in all treated groups in comparison to the AD model ($p < 0.05$). There was also a significant difference between all Δ^9 THC treated group and control group ($p < 0.05$) (Figure 6.20 and figure 6.21).

The result also reported that the number of calbindin+ cells in the dentate gyrus was statistically substantially reduced in rats administered with D-gal and AlCl_3 (AD model) as shown in Figure 6.25 ($p < 0.05$). In contrast, post hoc Tukey's test demonstrated that all treated groups significantly increased the number of calbindin+ cells; donepezil (100.50 ± 3.23), 0.75 Δ^9 THC (121.17 ± 3.86), 1.5 Δ^9 THC (143.67 ± 4.55) and 3.0 Δ^9 THC (155.00 ± 4.04) in comparison with AD model (43.17 ± 2.55) ($p < 0.05$). A significant difference also has been observed in the number of calbindin+ cells between all Δ^9 THC treated groups as compared to the control group ($p < 0.05$). Among these Δ^9 THC treated group, the results demonstrated that there was a significant between Δ^9 THC (1.5 and 3.0 mg/kg) and Δ^9 THC 0.75 mg/kg ($p < 0.05$) (Figure 6.24 and figure 6.25).

On the other hand, the expression of calbindin immunoreactive cells in the CA3 areas of the hippocampus of all the rats' groups was determined and shown in Figure 6.22. One-way ANOVA showed a statistically significant [$F(6,35) = 53.31, p = 0.001$] differences in the expression of calbindin+ in the CA3 subfield of the hippocampus among all the rats' groups. Further, Tukey's post hoc confirmed a marked increase in the percentage of calbindin+ immunoreactive area in treated group of rats ($p < 0.05$); donepezil (31.28 ± 1.39), 0.75 Δ^9 THC (44.27 ± 1.59), 1.5 Δ^9 THC (45.36 ± 2.38) and 3.0 Δ^9 THC (44.2 ± 2.51) when compared to AD model (14.01 ± 1.29). There was a significant difference between all Δ^9 THC treated groups and control group ($p < 0.05$). In addition, a significant difference was also demonstrated between Δ^9 THC (1.5 and 3.0 mg/kg) and donepezil ($p < 0.05$) (Figure 6.23).

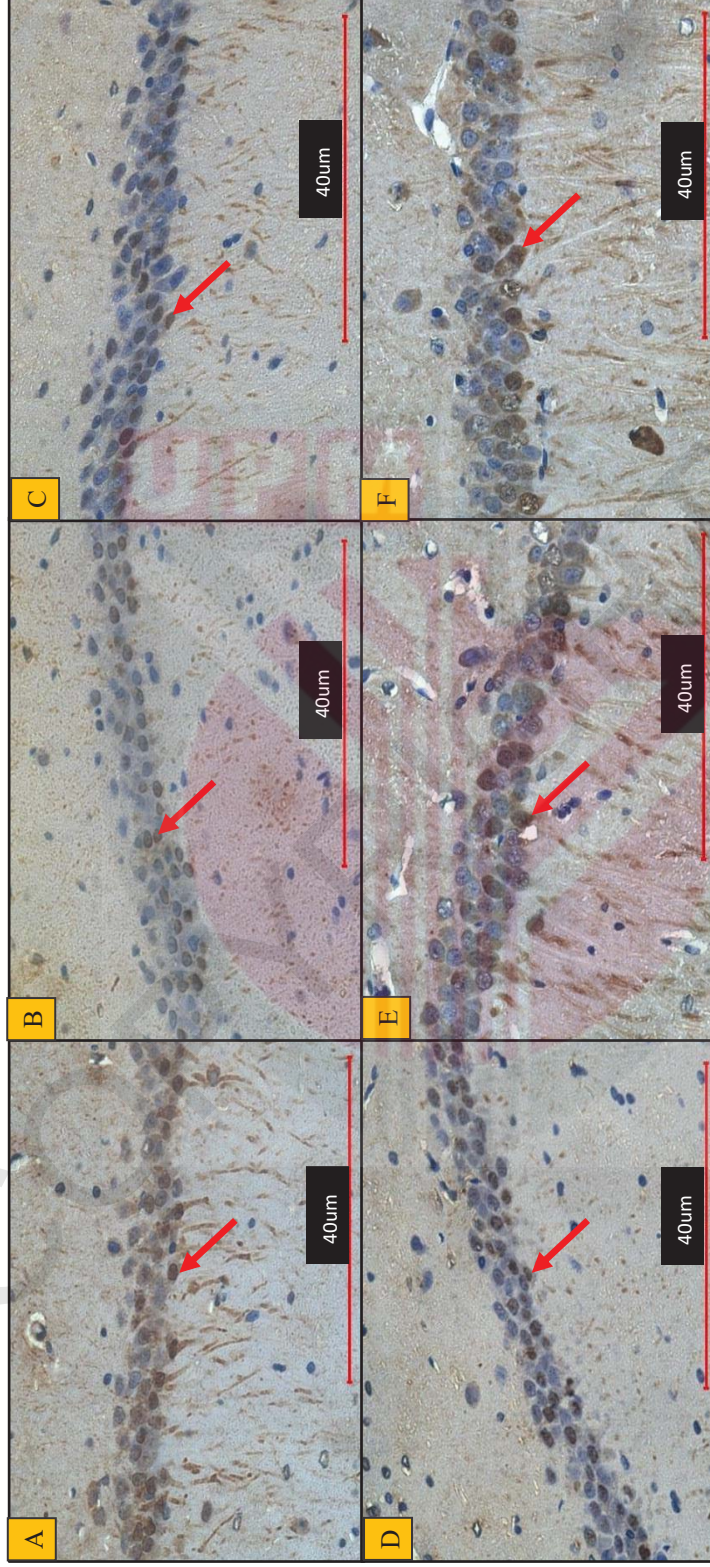


Figure 6.20 : Representative photomicrograph of CA1 region of hippocampus by immunohistochemistry (IHC). (A) Control rats, (B) AD model group, (C, D, E, F) treated group with 1 mg/kg donepezil, 0.75 Δ^9 THC, 1.5 Δ^9 THC and 3.0 Δ^9 THC respectively. Magnification of 400x for A-F, scale bar 40um. Red arrow represents the calbindin+ cells

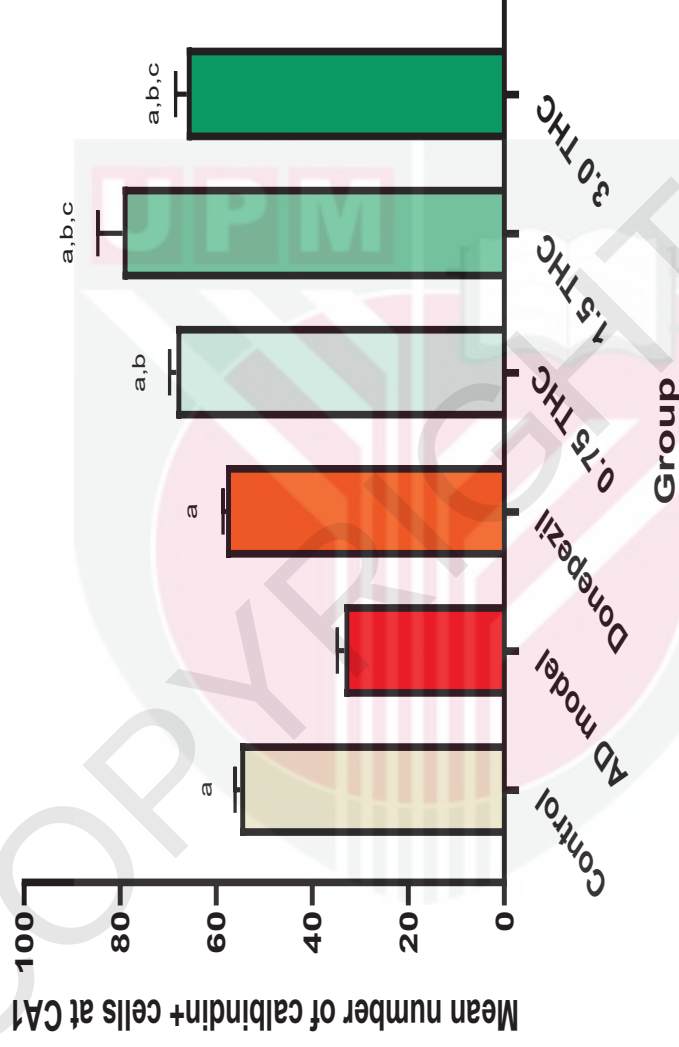


Figure 6.21 : Data showed the mean number of calbindin+ cells $\pm$ S.E.M at CA1 subfield of the hippocampus ($n=5$). The calbindin expression was obtained through paraffin-IHC technique. The experimental includes control, AD model (60 mg/kg D-gal + 200 mg/kg AlCl_3), donepezil (AD model + 1 mg/kg donepezil), 0.75 THC (AD model + 0.75 mg/kg $\Delta^9\text{THC}$), 1.5 THC (AD model + 1.5 mg/kg $\Delta^9\text{THC}$) and 3.0 THC (AD model + 3.0 mg/kg $\Delta^9\text{THC}$). Data was analysed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. ^a $p<0.05$ vs AD model; ^b $p<0.05$ vs control; ^c $p<0.05$ vs donepezil. CA1 – cornu ammonis 1

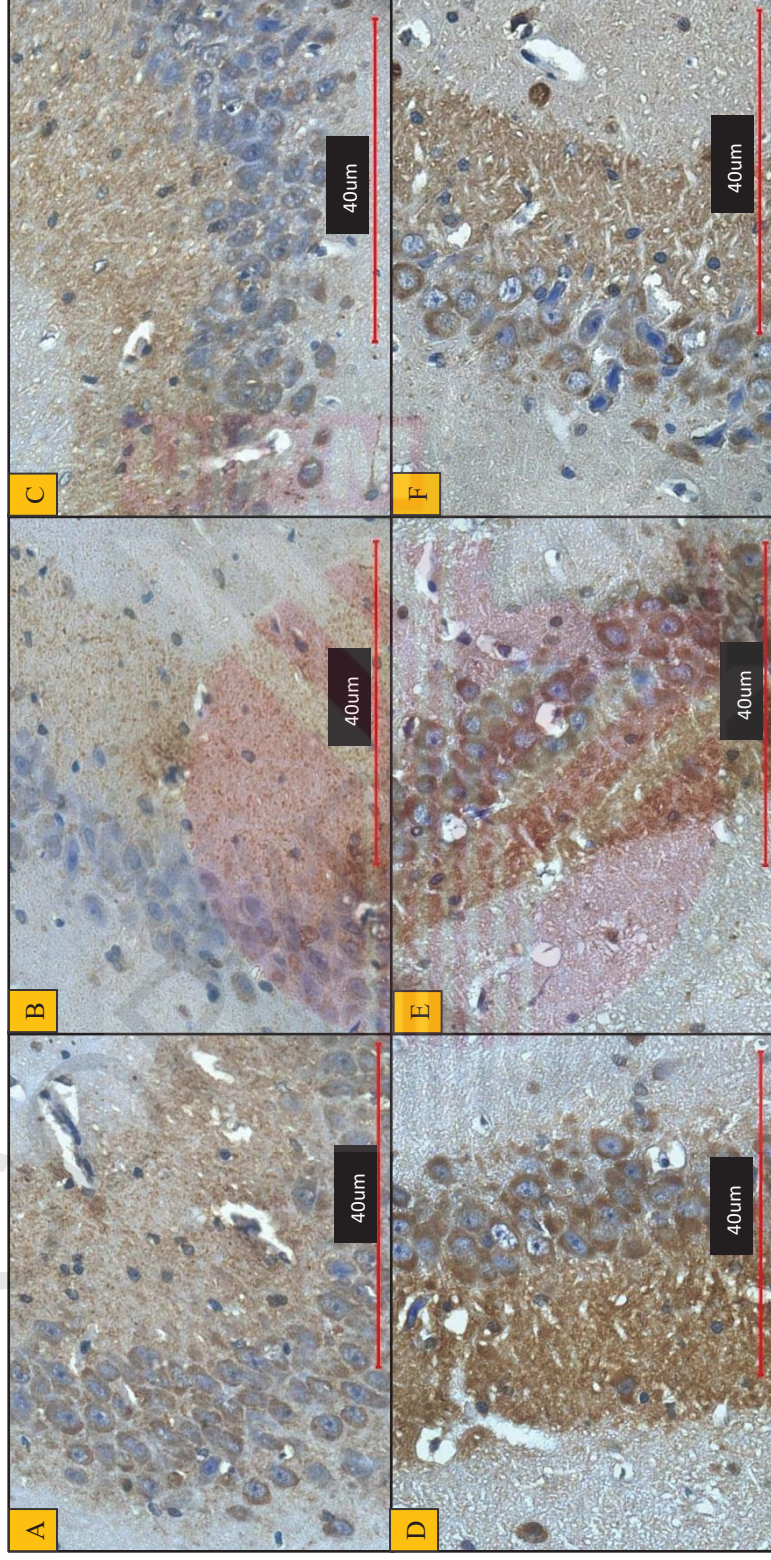


Figure 6.22 : Representative photomicrograph of CA3 subfield of the hippocampus by immunohistochemistry (IHC) for calbindin expression. (A) Control rats, (B) AD model group, (C, D, E, F) treated group with 1 mg/kg donepezil, 0.75 Δ^9 THC, 1.5 Δ^9 THC and 3.0 Δ^9 THC respectively. Magnification of 400x for A-F, scale bar 40um

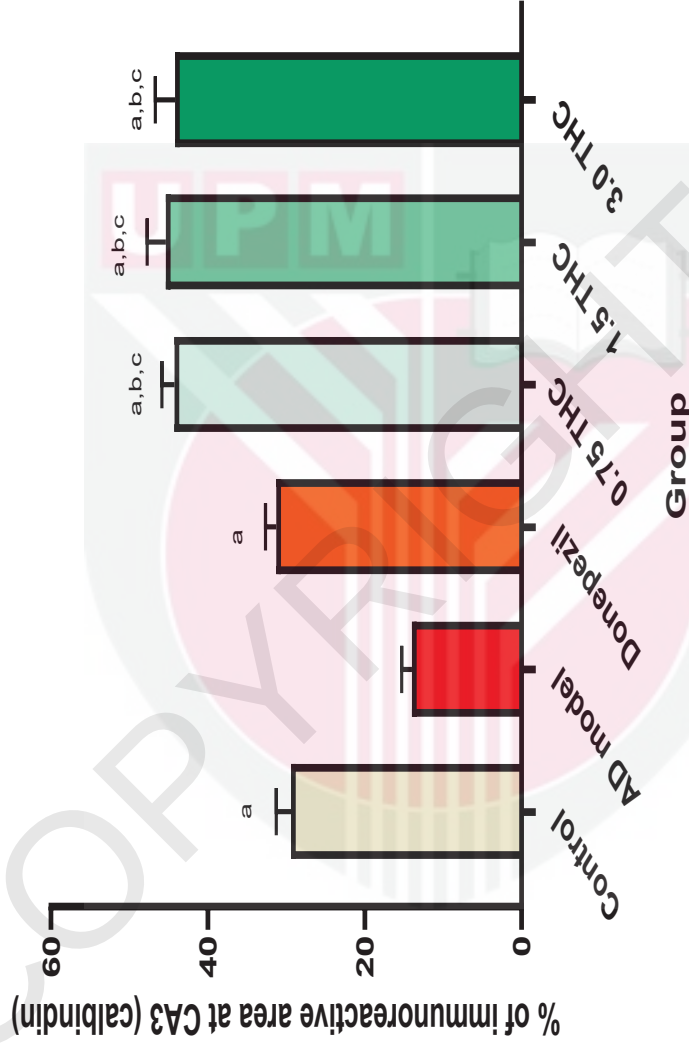


Figure 6.23 : Data showed the percentage of calbindin immunoreactive area $\pm$ S.E.M at CA3 subfield of the hippocampus (n=5). The calbindin expression was obtained through paraffin-IHC technique. The experimental includes control, AD model (60 mg/kg D-gal + 200 mg/kg AICl₃), donepezil (AD model + 1 mg/kg donepezil), 0.75 THC (AD model + 0.75 mg/kg Δ^9 THC), 1.5 THC (AD model + 1.5 mg/kg Δ^9 THC) and 3.0 THC (AD model + 3.0 mg/kg Δ^9 THC). Data was analysed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. ^ap<0.05 vs AD model; ^bp<0.05 vs control; ^cp<0.05 vs donepezil. CA3 – cornu ammonis 3

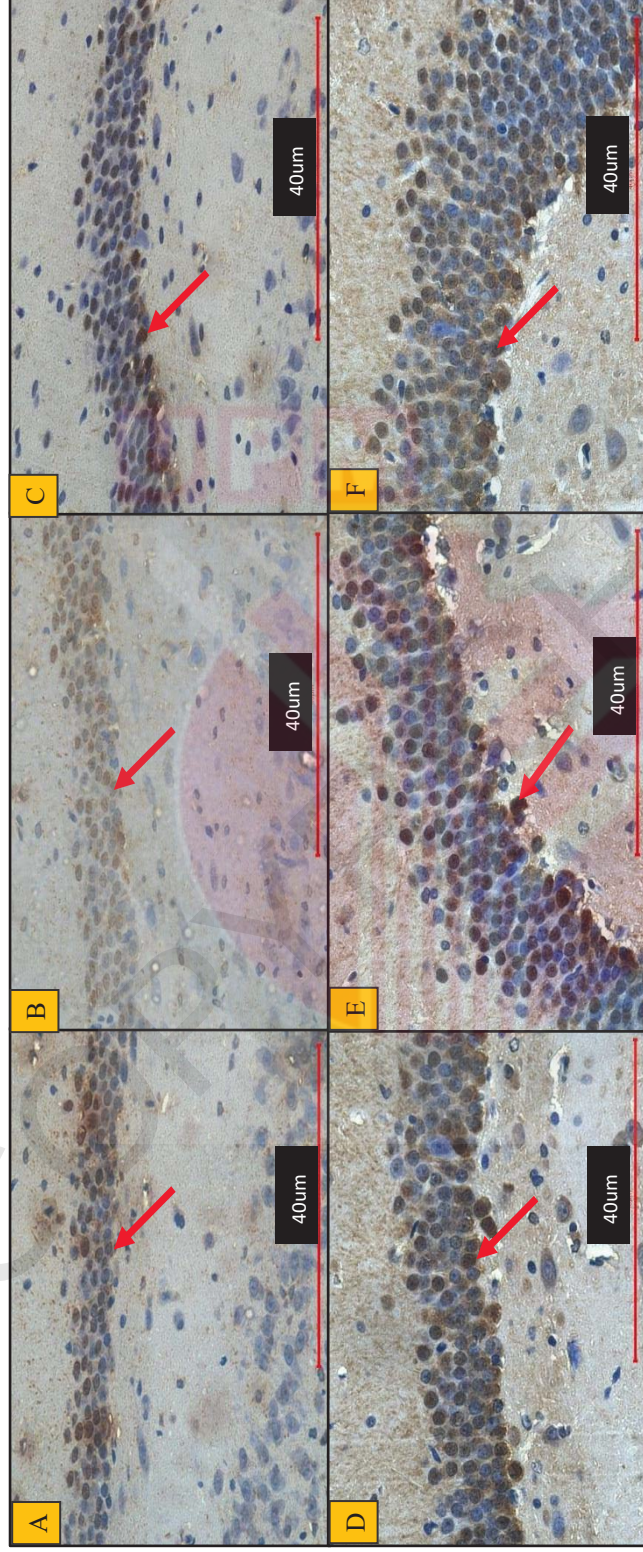


Figure 6.24 : Representative photomicrograph of dentate gyrus by immunohistochemistry (IHC). (A) Control rats, (B) AD model group, (C, D, E, F) treated group with 1 mg/kg donepezil, 0.75 Δ^9 THC, 1.5 Δ^9 THC and 3.0 Δ^9 THC respectively. Magnification of 400x for A-F, scale bar 40μm. Red arrow represents the calbindin+ cells

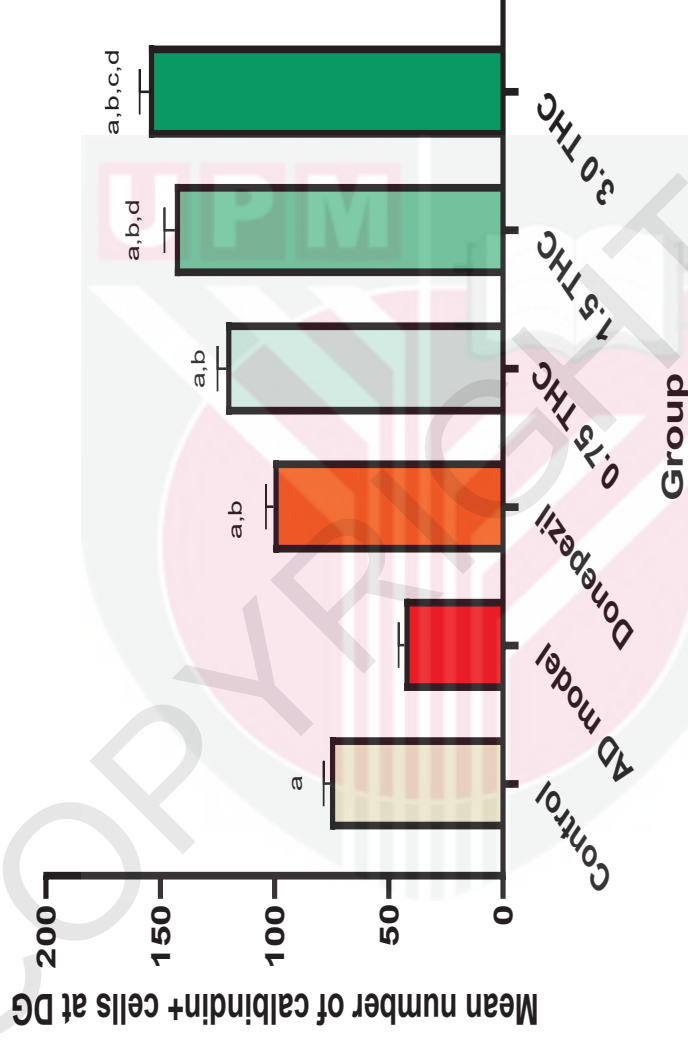


Figure 6.25 : Data showed the mean number of calbindin+ cells $\pm$ S.E.M at dentate gyrus (n=5). The calbindin expression was obtained through paraffin-IHC technique. The experimental includes control, AD model (60 mg/kg D-gal + 200 mg/kg AlCl_3), donepezil (AD model + donepezil), 0.75 THC (AD model + 0.75 mg/kg $\Delta^9\text{THC}$), 1.5 THC (AD model + 1.5 mg/kg $\Delta^9\text{THC}$) and 3.0 THC (AD model + 3.0 mg/kg $\Delta^9\text{THC}$). Data was analysed using one-way ANOVA followed by Tukey's post hoc test among the group of rats. ^a $p < 0.05$ vs AD model; ^b $p < 0.05$ vs control; ^c $p < 0.05$ vs 0.75 mg/kg $\Delta^9\text{THC}$; ^d $p < 0.05$ vs donepezil. DG – dentate gyrus

6.4 Discussion

Alzheimer's disease (AD) is a neurodegenerative disease that develops with age and is characterized by massive synaptic and neuronal loss in brain. In normal condition, the adult hippocampus develops new neurons continually throughout life; this process is known as neurogenesis. The hippocampus, which is essential for learning and memory, is among the first brain regions impaired by AD (Kot et al., 2022). Neurogenesis impairment may contribute to a cognitive deterioration associated with AD. A decline in neurogenesis in the hippocampus indicates age-related memory loss in rats and humans (Kot et al., 2022; Marchalant et al., 2009). Thus, enhancing neurogenesis receives the greatest attention and is another promising strategy that may improve cognitive deficits associated with AD.

Restoration through increasing hippocampal neurogenesis would serve as endogenous repair mechanism and contribute to the structural and functional plasticity of the hippocampus. In this work, cognitive functions were assessed using immunohistochemical procedures (IHC) by examining the expression of neurogenesis-related protein markers in the hippocampus, namely the CA1 and CA3 subfields, and the dentate gyrus. These regions were observed due to their significance in learning and cognitive processes, as well as their susceptibility to neurodegeneration (Evstratova & Tóth, 2014).

The current work established that rats treated chronically with D-gal and AlCl_3 exhibited a decrease in neurogenesis activity in the hippocampus, as indicated by a decrease in the examined markers; GFAP, DCX, NeuN and calbindin. This condition may cause the memory loss. Notwithstanding, $\Delta^9\text{THC}$ treatment promoted neurogenesis activities in the hippocampus following prolonged exposure to D-gal and AlCl_3 , as indicated by an increase in neurogenesis markers. An increase in neurogenesis activity was linked to better learning and memory processes. This result was consistent with the findings of Suliman et al. (2018), who reported that acute and chronic administration of $\Delta^9\text{THC}$ promoted neurogenesis in the hippocampus of the normal rats.

During the process of hippocampal neurogenesis, neural stem cells undergo a well-defined sequence of cellular stages in order to differentiate into fully developed and functional neurons. This progression begins with Type 1 cells (radial glia-like cells), and continues with Type 2a and 2b cells (neural progenitor cells). Subsequently, the cells transition into Type 3 cells (neuroblast), which then undergo migration and final maturation to ultimately become mature functional neurons (Abbott & Nigussie, 2020). This set of cellular states can be distinguished through the successive activation of distinct molecular markers (Abbott & Nigussie, 2020; Kuhn et al., 2018; Zhang & Jiao, 2015). The newly generated neuron originating from neural stem cells will integrate into pre-existing neural networks inside the granular neurons layer of the dentate gyrus to participate in learning and memory processes (Augusto-Oliveira et al., 2019; Zhang & Jiao, 2015).

Δ^9 THC administration was associated with an increase in the number of GFAP- positive cells in the hippocampus, indicating an increase in neuronal cell proliferation activity (McKeon & Benarroch, 2018). Δ^9 THC was hypothesised to enhance new neuron formation in the hippocampus as a result of GFAP expression. This is consistent with previously established findings where the cannabinoids have been known to stimulate neurogenesis in the adult brain (Jin et al., 2004). Despite the facts that the plasma GFAP marker was not examined in the current investigations, the results demonstrated that this marker is essential for neurogenesis. The expression of GFAP-positive cells in the hippocampus served as the primary neurogenesis marker (Garcia et al., 2004).

Neuronal migration is a critical process in the neurogenesis. Doublecortin (DCX) is a crucial component in neurogenesis and the disruption of neuronal migration contributes to multiple neuropathological conditions such as AD. Treatment with Δ^9 THC led to the upregulation of DCX-positive cells in the hippocampus. Increasing of DCX expression indicates a successful migration (Valeri & Mazzon, 2021). The findings suggest that Δ^9 THC accelerates the migration of neuroblasts, which is in line with the previous work done by Von and Halbach (2007). There was another experiment conducted by Valeri & Mazzon (2021), who reported Δ^9 THC (2 μ m) can induce neuronal differentiation and migration in mice embryonic stem cell. Another study found that DCX was engaged from late stage 2 of the neurogenesis process (migration and differentiation) to the early phase of stage 5 (synaptic integration), indicating that it was involved in both mitotic and post-mitotic neuron (Brown et al., 2003).

Mature neurons were found to be highly expressed in the Δ^9 THC-treated group of rats, as evidenced by an increase in NeuN immunoreactive percentage. These findings suggest that increased hippocampal neurogenesis is also related to enhancement of cell maturation. In addition, many intrinsic and extrinsic factors influence the proliferation and maturation of newborn neurons in the hippocampus (Hashizume et al., 2018). Current study showed Δ^9 THC may act as an intrinsic regulatory factor in hippocampal neurogenesis, by interact with CB₁ receptors which abundantly present in the brain, especially the hippocampus (Hashizume et al., 2018). Activation of these receptors by Δ^9 THC may modulate the proliferation, differentiation, and survival of neural progenitor cells, which influencing the overall process of neurogenesis.

Calbindin is an additional marker being evaluated in this study. This marker represents the final stage of neurogenesis, which involves synaptic integration, forming functional mature neurons (Todkar et al., 2012). Higher expression of calbindin is indicative of strong synaptic integration, and vice versa (Todkar et al., 2012). A marked increase in the number of calbindin-positive cells (CA1 subfield and DG) and immunoreactive percentage (CA3 subfield) in Δ^9 THC treated groups of rats, suggests that Δ^9 THC is capable in reversing the cognitive impairment induced by D-gal and AlCl₃.

According to the research conducted by Von Bohlen Und Halbach (2007), there are five steps involved in neurogenesis; proliferation, differentiation, migration, axonal and dendritic targeting, as well as synaptic integration. All the neuronal markers investigated represent the entire mechanism of new neuron development. New functioning neurons are required to restore cognitive impairment caused by D-gal and AlCl₃ exposure-induced

AD and to maintain normal brain function (Griffiths et al., 2020). GFAP expression indicates the rate of proliferation of newly formed neuroblast (Garcia et al., 2004), whereas DCX expression represent the migration process of neuroblast (Rao & Shetty, 2004). On the other hand, NeUN is commonly utilised marker for mature neurons (Saito et al., 2018), and calbindin is important in synaptic integration to create functional mature neuron (Li et al., 2017).

6.5 Conclusion

The findings of this study suggest that Δ^9 THC can restoring the normal function of neuronal cells in the hippocampus induced by D-gal and AlCl_3 , through the process of neurogenesis. The subsequent chapter explored the molecular research on the therapeutic potential of Δ^9 THC in reducing the major neuropathological hallmarks of AD.

CHAPTER 7

NEUROTHERAPEUTIC POTENTIAL OF DELTA-9-TETRAHYDROCANNABINOL ON BACE 1, AMYLOID PRECURSOR PROTEIN, p-TAU Thr181 AND p-TAU Thr231 HALLMARK MARKERS EXPOSED BY D-GALACTOSE AND $AlCl_3$ IN RATS

7.1 Introduction

Alzheimer's disease (AD) is a complex slowly neurodegenerative disorder characterized in its earliest stages by a decline in learning and memory (Li et al., 2016). The pathological features of AD can be recognized by the accumulation of amyloid peptide ($A\beta$) and tau aggregates in several cortical brain regions, leading to the formation of senile plaque (SPs) and neurofibrillary tangles (NFTs) respectively (Neddens et al., 2018). These changes initiate significant synapse loss and neuronal cell death in the hippocampus region of the brain, which is crucial for learning and memory process. Analyses of AD brains have revealed that the massive synapse loss that occurs in the disease strongly correlates substantially with cognitive impairment (Serrano-Pozo et al., 2011).

Synapses are the fundamental units responsible for memory storage and transport the information in the brain. These synapses consist of two main components; presynaptic and postsynaptic (Fu & Ip, 2017). Many AD transgenic animal models also exhibit synaptic degeneration, accompanied by deficits in learning and memory (Sheng et al., 2018; Forner et al., 2017). Cognitive impairment in AD appears to be caused by a sequence of pathological alterations that affect synapses (Tu et al., 2014; Pooler et al., 2014). $A\beta$ peptide, a major component of senile plaques (SPs), plays a pivotal role in the progression of the disease (Li et al., 2017). These peptides are proteolytic fragments resulting from the improper cleavage of amyloid precursor protein (APP) (Bloom, 2014), which produce the two primary isoforms of $A\beta$; $A\beta_{40}$ and $A\beta_{42}$. $A\beta_{42}$ is believed to be the most amyloidogenic of these peptides and may disrupt the synaptic function in the earliest stages of AD (Selkoe, 2008; Yang et al., 2006).

Another feature of AD is intracellular neurofibrillary tangles (NFTs), which are primarily composed of hyperphosphorylated tau (p-tau). Tau is a microtubule-associated protein that is essential for microtubule assembly and stabilization, and is mostly expressed in the axon of neuron (Yipeng et al., 2016). In AD, there is dysregulation in the activity of kinases and phosphatase, leading to an excessive of phosphorylation of tau, thus increase the ability to self-assemble into tangles paired helical filament (PHF) (Götz et al., 2001). These tangles become accumulated and aggregated, resulting in the loss of axonal or dendritic transport and disassembly of the microtubules in AD (Goedert et al., 2010). These changes also result in synaptic detriment (Chabrier et al., 2014). Growing evidence has been demonstrated that the reduction in total synapse number is associated with an increase in p-tau and $A\beta$ levels (Takahashi et al., 2010). In the diseased brain, p-tau sites are commonly associated with tau aggregation mechanism, which involves the destabilization of microtubules, leading the transition from pre-tangles to the formation of NFTs (Moszczynski et al., 2015; Lund

et al., 2013). Luna-Muñoz et al. (2005) have reported that the early occurrence of Thr231 tau phosphorylation is observed in the neuronal pathogenesis of AD.

These findings contribute significantly to the prospective treatment of AD. In light of the importance of addressing the key pathological hallmark of AD, it is crucial to propose a chemical or substances that specifically halts or slows the progression of the disease. Previous chapter results (Chapter 5 and Chapter 6) demonstrated that Δ^9 THC attenuated cognitive impairment in D-gal and AlCl_3 induced rats by decreasing hippocampal neuronal death and enhancing neurogenesis activity. Whether Δ^9 THC also retard the formation of A β peptide and hyperphosphorylated tau protein to prevent the cognitive impairment induced by D-gal and AlCl_3 in rats remains unknown. Hence, this chapter addresses the effect of Δ^9 THC in reducing major pathological hallmark of D-gal and AlCl_3 -induced AD like rat model. This chapter focusing by examining the level of BACE1, APP, p-tau Thr181, and p-tau Thr231 using western blotting method.

7.2 Materials and methods

7.2.1 Drugs and chemicals

In addition to the drugs and chemicals mentioned before (refer to section 3.1.1), rabbit monoclonal [EPR19523] to BACE 1 from Abcam AB183612, rabbit monoclonal [Y188] to amyloid precursor protein from Abcam AB32136, phosphor-tau (Thr181) monoclonal antibody (AT270) from Thermoscient MN1050, phosphor-tau (Thr231) polyclonal antibody from Thermoscient 44-746G, beta actin monoclonal antibody from Elabscience E-AB-20078, Goat anti-mouse IgG peroxidase /HRP conjugated from Elabscience E-AB-1001 were purchased. Pierce™ BCA protein assay kit was purchased from Thermo Fisher Scientific. RIPA lysis buffer and phosphatase inhibitor cocktail were purchased from Nacalai Tesque 08714-04 and 07575-51 respectively together with SIGMAFAST™ protease inhibitor cocktail tablet R&M S8830-2TAB. Stacking gel buffer, resolving gel buffer, 30% Acrylamide/Bis solution 29:1, PrecisionPlus Protein™ Kaleidoscope Prestained protein standard, Laemli buffer, N,N,N',N'-Tetramethylethylenediamine and Ammonium Persulphate were purchased from Biorad. Sodium Dodecyl Sulphate (SDS), Glycine, Methanol, Sodium fluoride, Sodium chloride were purchased from R&M while Tris and DTT were purchased from biobasic. Tween 20 and β -mercaptoethanol were purchased from Nacalai Tesque.

7.2.2 Equipment

Analytical weighing balance (Sartorius, Germany), pH meter (Mettler-Toledo, Greisensee, Switzerland), Eppendorf single channel pipettes P1000 (100-1000 uL), P200 (20-200 uL) and P10 (0.5-10 uL) (Eppendorf, Hamburg, Germany), Prot/Elec Pipet Tips 1-200 ul from biorad were used in this study. Others equipment used included Versamax microplate reader (LLC, Sunnyvale, Ca, USA), refrigerated centrifuge (Thermoscientific, Rockford, IL, USA), 0.45 um nitrocellulose membrane from Biorad. The apparatus for protein electrophoresis, Biorad vertical electrophoresiscell and Biorad PowerPac™ family were also used (Biorad Laboratories, Hercules, CA, USA).

7.2.3 Animals

Animal husbandry and the experimental design was referred to Chapter 3, section 3.1.2 and 3.1.3 respectively. The overall experimental design was simplified in Figure 7.1.

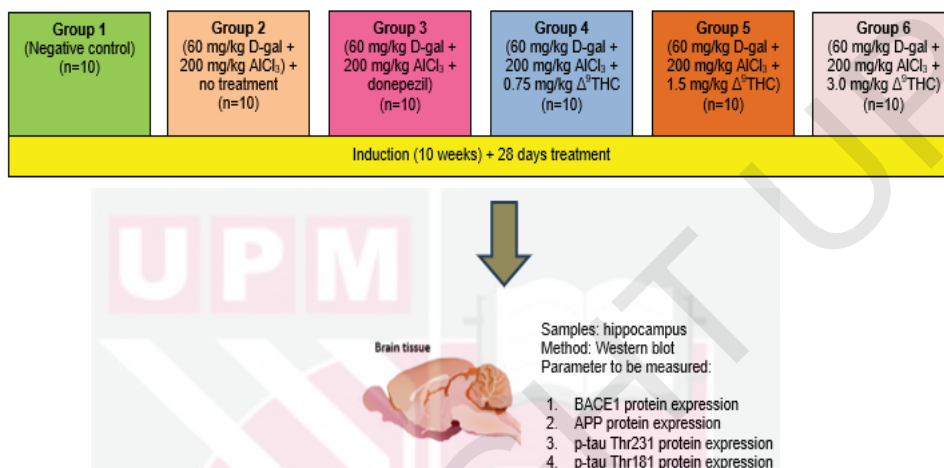


Figure 7.1 : The experimental design for the evaluation of Δ^9 THC therapeutic effect on neuropathological markers exposed by AlCl₃ and D-gal-induced rat model. D-gal – D-galactose; AlCl₃ – aluminium chloride; Δ^9 THC – delta-9-tetrahydrocannabinol; BACE1 – beta secretase 1; APP – amyloid precursor protein; p-tau – phosphorylated tau

7.3 Assessing neuropathological markers of AD

7.3.1 Determination of beta secretase 1 (BACE 1), amyloid precursor protein (APP), p-tau Thr181 protein and p-tau Thr231 protein

7.3.1.1 Brain Tissues Collection and Preparation of Brain Lysate

The brains specifically hippocampus was collected immediately after decapitation and stored at -80 °C till further analysis. Using the western blot technique, the relative expression of BACE 1, APP, p-tau Thr 181 protein and p-tau Thr231 protein in the hippocampus was analysed. With minor modifications, the western blot procedure follows the protocol of He et al. (2007).

The frozen hippocampus tissue was thawed for 30 minutes and washed with distilled water to eliminate any clotted blood. The tissue was weighed at 0.01 g before mixed with 400 ul RIPA lysis buffer together with 15 ul phosphatase inhibitor and 15 ul

protease inhibitor. The mixture was homogenized using homogenizer for 10 minutes on ice (D130, Wiggan Hauser, USA). The lysate was then centrifuged at 13000 rpm for 10 minutes at 4 °C. The resultant supernatant and pellet were aliquoted and stored at -80 °C. Using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, USA), the protein concentration in each sample was determined, and its absorbance at 562 nm was read using a microplate reader. Samples with 20 µg were aliquoted and kept at -80 °C for later use.

7.3.1.2 SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE) and Electrotransfer

For the gel preparation, 10% resolving gel and 4% stacking gel were applied (Appendix F1). 20 µl of brain lysate containing 20 µg of protein sample were added to 20 µl of Laemmli sample buffer supplemented with 1:19 dilution of β-mercaptoethanol and heated at 95 °C for 5 minutes. The samples were vortexed, centrifuged at 1000 rpm for 1 minute and loaded into the gel 20 µl per well. Known molecular weight marker (Precision Plus Standard Kaleidoscope Marker) was loaded into the gel as well.

The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was run at 100 V for 60 minutes followed by 150 V for another 30 minutes. The separated protein was then transferred onto 0.45 µm nitrocellulose membrane using wet transfer method for 60 minutes at 100 V. The membrane was stained with Ponceau S dye to observe any presence of protein bands.

7.3.1.3 Blocking and Antibody Incubation

Blocking of non-specific binding was conducted by incubating the membrane with 5% w/v skimmed milk powder in TBST Tween 20 for 2 hours at 4 °C (BACE1 and APP) or 5% BSA in TBST Tween 20 for overnight at 4 °C (p-tau Thr231 and p-tau Thr181). The membrane was then incubated with primary anti-BACE 1 (1:1000), anti-APP (1:1000), anti-p-Tau Thr181 (2 µg/ml), anti-p-Tau Thr231 (1:1000) overnight in the blocking solution at 4 °C. The next day, the membrane was washed for three times with TBST Tween 20 for 5 minutes each and incubated with secondary horseradish peroxidase (HRP) antibody in blocking solution (1:5000) for 2 hours at 4 °C. The membrane was again washed for three times at 10 minutes each.

7.3.1.4 Detection and Visualization

The membrane was incubated with chemiluminescence HRP substrate (1:1 of WesternBright™ ECL and WesternBright™ Peroxidase) for 5-10 minutes. The membrane was viewed using gel documentation system (Infinity System, Vilber Lourmat, Germany) and images containing protein of interest were obtained.

7.3.1.5 Secondary Probing

The membrane was incubated with stripping buffer for 5 minutes at room temperature. The membrane was then washed with TBST Tween 20 three times for 5 minutes each. Non-specific binding was blocked by incubating the membrane in 5% w/v skimmed milk powder or 5 % BSA for 2 hours at 4 °C. The membrane was washed with TBST

Tween 20 for three times at 5 minutes respectively and incubated with housekeeping antibody, β -actin for 1 hour at room temperature (43 kDa, 1:5000). The membrane was then washed with TBST Tween 20 for three times at 5 minutes, before incubated with horseradish peroxidase (HRP) antibody in blocking solution for 2 hours at 4 °C (1:5000) and washed again with TBST Tween 20 three times at 10 minutes respectively. The membrane was incubated with chemiluminescence HRP substrate for 5 minutes and viewed with gel documentation system.

7.3.1.6 Quantification of protein and data analysis

The image J system was used to quantify the protein levels (Davarinejad, 2015). The data were normalised by dividing the mean intensity of the protein of interest with housekeeping protein (β -actin) for each reading. One-way analysis of variance (ANOVA) was performed with SPSS version 25.0 software followed by post-hoc Tukey's Multiple Comparison Test where applicable for inter-group comparison, with $p < 0.05$ considered a significant difference.

7.4 Results

7.4.1 Effect of Δ^9 THC on BACE-1 expressions in the hippocampus of D-gal and AlCl_3 induced rats.

The expression of BACE-1 in the rat hippocampus was evaluated by immunoblotting, as shown in Figure 7.2. A marked increase in the level of BACE-1 was observed in the hippocampus of the AD model (2.05 ± 0.15) when compared to control. One-way ANOVA revealed statistically significant differences in BACE-1 level [$F(6,35) = 32.66, p = 0.001$]. The Tukey's post hoc test revealed a significant reduction in BACE-1 level in the treated group of rats ($p < 0.05$); donepezil (0.53 ± 0.09), $0.75 \Delta^9$ THC (0.57 ± 0.15), $1.5 \Delta^9$ THC (0.39 ± 0.11) and $3.0 \Delta^9$ THC (0.5 ± 0.17) when compared to AD model group of rats (2.05 ± 0.15). There was also a statistically significant difference between $1.5 \Delta^9$ THC and control group.

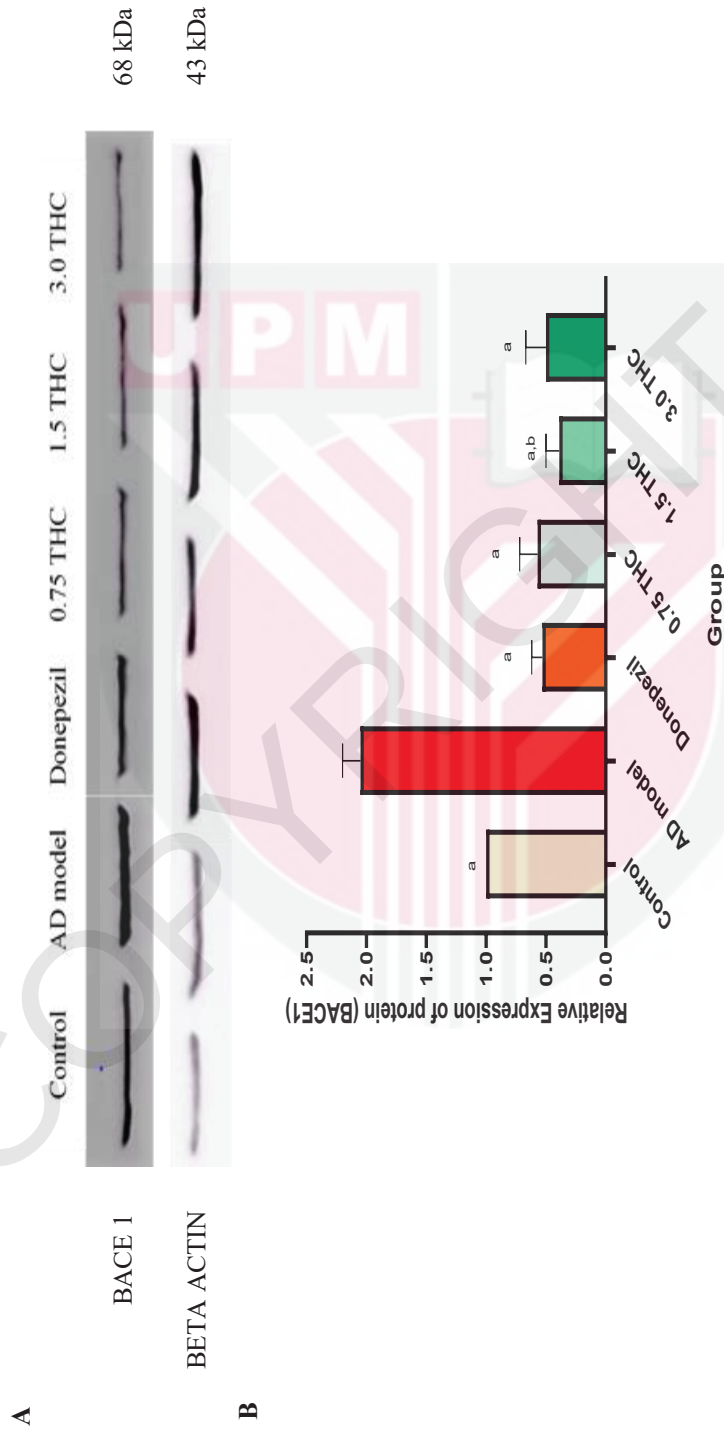


Figure 7.2 : Therapeutic effect at different doses of Δ^9 THC in D-gal and AlCl_3 induced rats on BACE 1 protein expression in hippocampus. (A) Representative immunoblot of BACE 1 and beta actin (loading control) proteins measured by western blot analysis in hippocampus. (B) Relative protein density of BACE 1 in hippocampus analysed by Image J software. The data were normalised by dividing the mean intensity of BACE1 with housekeeping protein (β -actin) for each reading. Data was presented as mean $\pm$ SEM, $n=5$. ^a $p<0.05$ vs AD model; ^b $p<0.05$ vs control

7.4.2 Effect of Δ^9 THC on APP expressions in the hippocampus of D-gal and AlCl_3 induced rats.

Figure 7.3 depicts the immunoblot examination of APP expression. One-way ANOVA revealed a statistically significant increase in the level of APP in the AD model as compared to the control group [$F(6,35) = 14.32, p=0.001$]. A marked decreases in APP level were observed in the donepezil (0.89 ± 0.14), $0.75 \Delta^9$ THC (0.79 ± 0.25), $1.5 \Delta^9$ THC (0.54 ± 0.10) and $3.0 \Delta^9$ THC (0.68 ± 0.24) groups of rats when compared to AD model (2.41 ± 0.74).



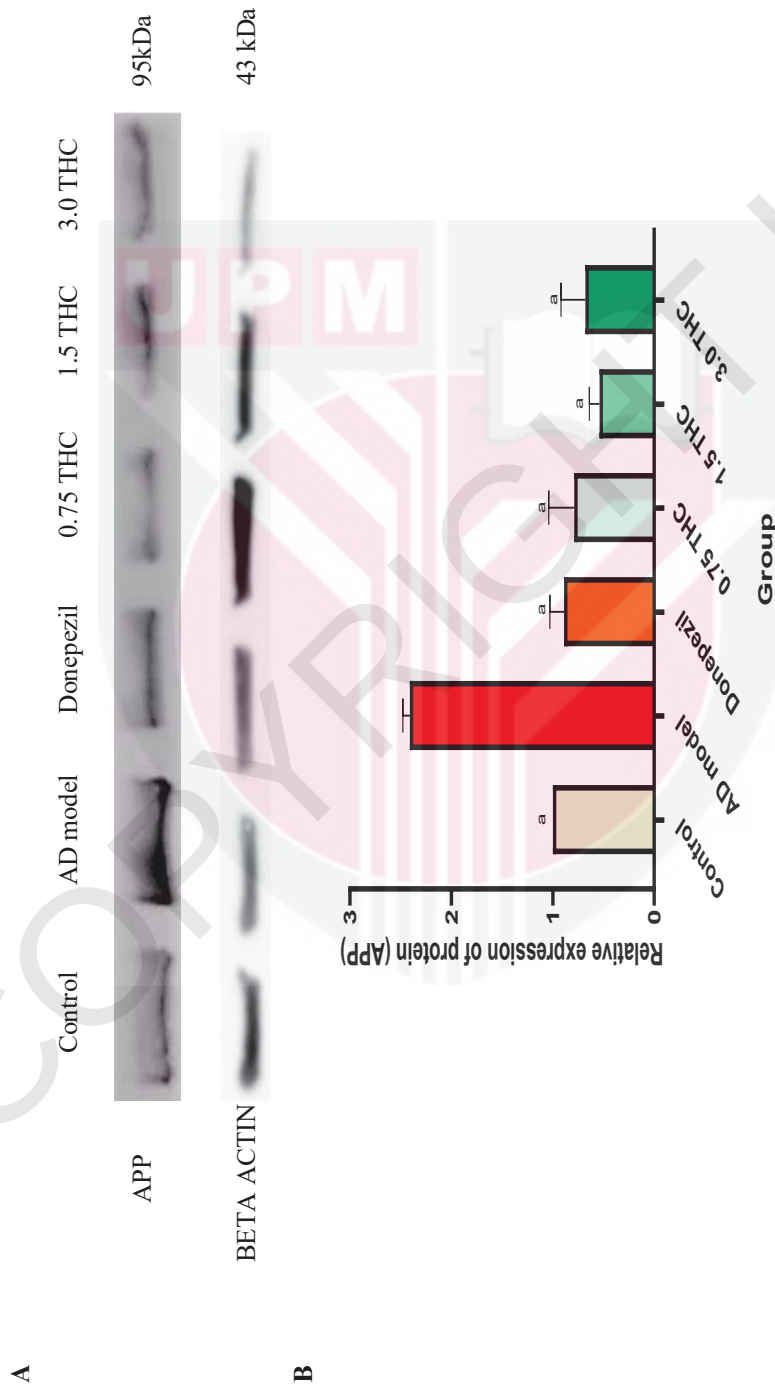


Figure 7.3 : Therapeutic effect at different doses of Δ^9 THC in D-gal and AlCl_3 induced rats on APP protein expression in hippocampus. (A) Representative immunoblot of APP and beta actin (loading control) proteins measured by western blot analysis in hippocampus. (B) Relative protein density of APP in hippocampus analysed by Image J software. The data was normalised by dividing the mean intensity of APP with housekeeping protein (β -actin) for each reading. Data was presented as mean $\pm$ SEM, n=5. ^ap<0.05 vs AD model

7.4.3 Effect of Δ^9 THC on p-tau protein expressions in the hippocampus of D-gal and AlCl_3 induced rats

The presence of hyperphosphorylated tau protein is an early indicator of AD, hence p-tau Thr181 and p-tau Thr231 were being assessed in this study. Our finding showed, there was a statistically significant differences of p-tau Thr181 across all rat's groups [$F(6,35) = 12.19, p=0.001$]. One way ANOVA demonstrated that the rats exposed to D-gal and AlCl_3 had a significantly higher level of p-tau Thr181 ($p<0.05$) as compared to control. Furthermore, Tukey's post hoc analysis revealed a significant reduction in p-tau Thr181 level in all treated groups of rats ($p<0.05$); donepezil (0.91 ± 0.67), $0.75 \Delta^9\text{THC}$ (0.63 ± 0.09), $1.5 \Delta^9\text{THC}$ (0.62 ± 0.07) and $3.0 \Delta^9\text{THC}$ (0.70 ± 0.12) when compared to the AD model (1.64 ± 0.22) (Figure 7.4).

In addition, p-tau Thr231 was also being examined in this study. Our finding showed, there was a statistically significant downregulated in p-tau Thr231 level of all treated groups when compared to AD model (1.42 ± 0.26) ($p<0.05$). The relative protein expression of p-tau Thr231 in the treated groups were as follows; donepezil (0.76 ± 0.13), $0.75 \Delta^9\text{THC}$ (0.66 ± 0.14), $1.5 \Delta^9\text{THC}$ (0.55 ± 0.11) and $3.0 \Delta^9\text{THC}$ (0.59 ± 0.13). However, there were no statistically significant between the model group and control group ($p>0.05$) (Figure 7.5).

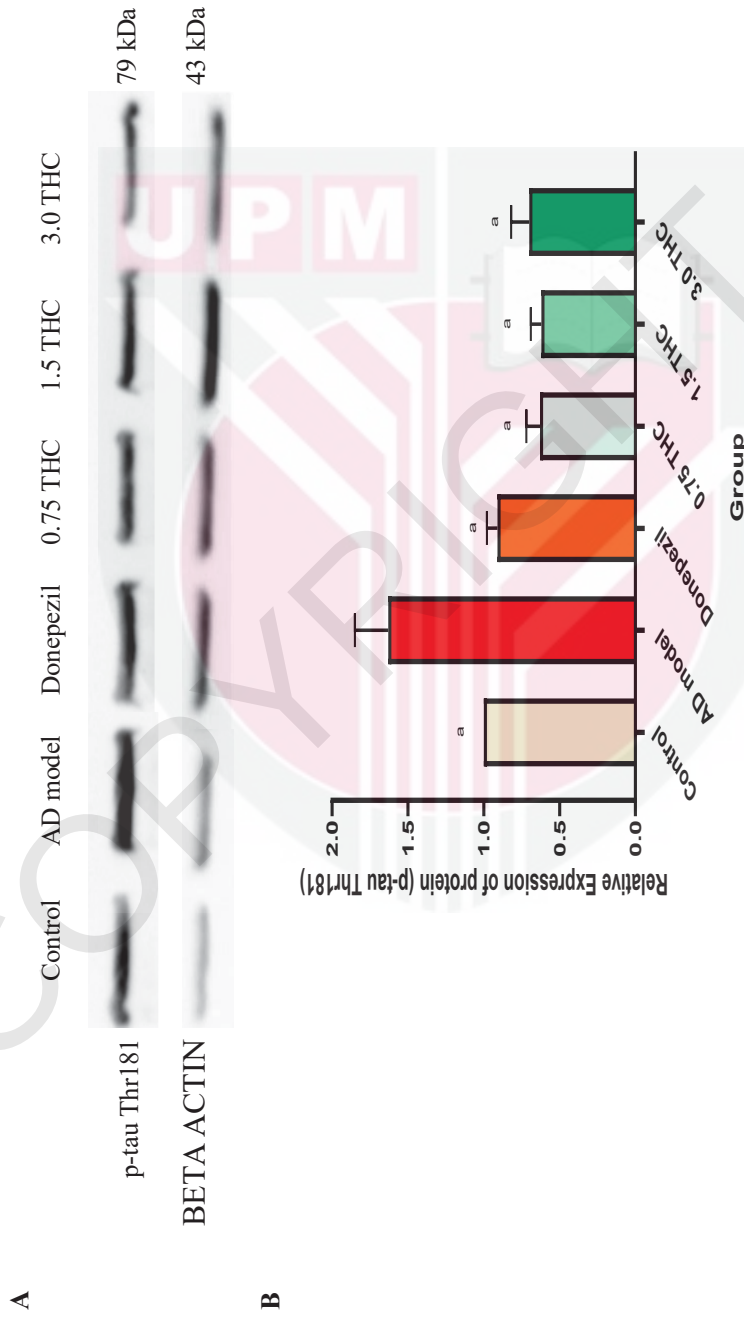
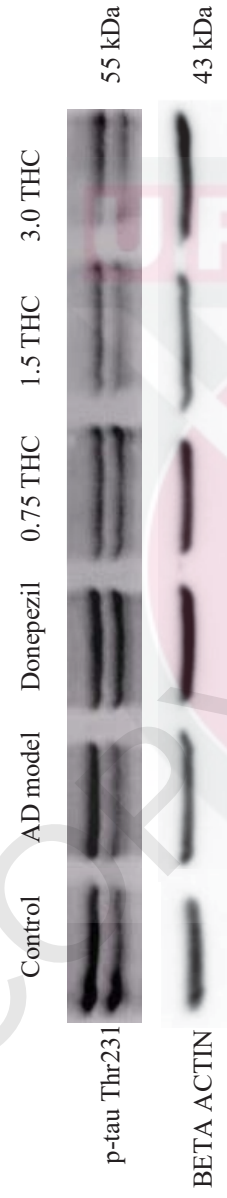


Figure 7.4 : Therapeutic effect at different doses of Δ^9 THC in D-gal and AlCl_3 induced rats on p-tau Thr181 protein expression in hippocampus. (A) Representative immunoblot of p-tau Thr181 and beta actin (loading control) proteins measured by western blot analysis in hippocampus. (B) Relative protein density of p-tau Thr181 in hippocampus analysed by Image J software. The data was normalised by dividing the mean intensity of p-tau Thr181 with housekeeping protein (β -actin) for each reading. Data was presented as mean $\pm$ SEM,

n=5. ^ap<0.05 vs AD model



B

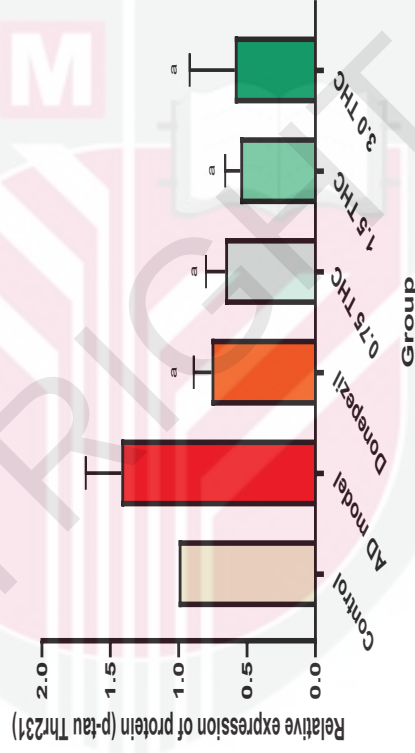


Figure 7.5 : Therapeutic effect at different doses of Δ^9 THC in D-gal and AlCl_3 induced rats on p-tau Thr231 protein expression in hippocampus. (A) Representative immunoblot of p-tau Thr231 and beta actin (loading control) proteins measured by western blot analysis in hippocampus. (B) Relative protein density of p-tau Thr181 in hippocampus analysed by Image J software. The data was normalised by dividing the mean intensity of p-tau Thr231 with housekeeping protein (β -actin) for each reading. Data was presented as mean $\pm$ SEM, n=5. ^ap<0.05 vs AD model

7.5 Discussion

Progressive cognitive impairment in AD is caused by an imbalance of A β regulation and hyperphosphorylated tau protein, which result in the production of plaques and neurofibrillary tangles respectively, over time (Li et al., 2016; Hardy & Selkoe, 2002). These processes contribute to synapse loss, and ultimately causes neuronal cell death. Inhibition of phosphorylated tau protein and regulation of A β synthesis are further strategies for reducing the progression of AD (Li et al., 2016). In order to explore the Δ^9 THC effect, we are evaluating the expression of several pathological markers, including amyloid precursor protein (APP), beta secretase (BACE-1), p-tau Thr181 and p-tau Thr231 protein expressions.

β -site APP-cleaving enzyme I (BACE1) is a major β -secretase enzyme in the brain that cleaves APP during the amyloidogenic pathway, creating neurotoxic forms of A β (Cole & Vassar, 2007). These A β is thought to be detrimental when it is excessively being produced, disrupting the homeostatic balance of amyloid (Gouras et al., 2015). Previous experimental works demonstrated that over expression of A β and disruption in A β clearance owing to excitotoxicity led to protein misfolding, resulting in the formation of plaques, that reflect an aberrant pathological lesion (Jack et al., 2018). Moreover, the accumulation of plaques in synapses affect the interconnecting neurons, resulting in the loss of signal transmission from one neuron to the next (Sierra-Fonseca & Gosselink, 2018; Heneka et al., 2015).

Induction of D-gal and AlCl₃ in rats resulted in the overexpression of BACE1, leading to an increase in A β level. Δ^9 THC treatment, demonstrates the neurotherapeutic potential by reducing BACE1 protein expression significantly. This result suggests that Δ^9 THC may be able to reduce the most prominent pathogenic feature of AD. Thus, reducing the likelihood of senile plaques formation. In another perspective from other experimental work done by Abate et al. (2021), who reported Δ^9 THC was effective in lowering A β level in 5XFAD transgenic mice by increasing neprilysin, an enzyme involved in A β degradation. *In-vitro* study also showed that Δ^9 THC has anti- aggregation properties by reducing the fluorescence intensity in the thioflavin test, as conducted by Cao et al. (2014).

APP is a transmembrane protein with a single membrane-spanning domain (Weyer et al., 2011). Several researchers have hypothesised that APP is involved in brain development by altering neuronal intercellular connection and providing neuroprotection (Sadleir et al., 2016; Savonenko et al., 2015). However, little is known regarding the precise function of APP (Savonenko et al., 2015). Our research indicated that D-gal and AlCl₃ induce a high degree of APP relative protein expression, resulting in increased A β peptide synthesis. Δ^9 THC altered the impact by decreasing the APP level. It has been proposed that Δ^9 THC may involve in a clearance mechanism event. However, the detailed mechanism of action is unclear, hence, further research is required to better comprehend the underlying process.

In AD, during the amyloidogenic pathway, it triggers the enzymatic activity of β -secretase to cleave A β peptide (A β 40 or A β 42) in a greater proportion (Savonenko et al., 2015). Among these two types of peptides, A β 42 is believed to be neurotoxic and causes neurodegeneration (Acx et al., 2014). Accumulation and aggregation of these A β peptide resulting to the formation of plaques later, which is the major hallmark in AD brain (Attems et al., 2011). Hence, it has emerged as a chief neurotoxic peptide (Sajjad et al., 2018). Based on our findings, decreasing APP and BACE1 by Δ^9 THC may provide strategies and opportunities for lowering A β synthesis, thereby halting the progression of AD.

Several investigations have indicated that A β aggregation may also result in tau hyperphosphorylation. In a mouse model, researchers discovered clear evidence of a correlation between A β deposition and tau over expression. When mice were exposed to a high quantity of A β , modest alterations in endogenous tau resulting in hyperphosphorylation were found (Stancu et al., 2014). The chain of reactions beginning with the aggregation of extracellular beta-amyloid and culminating in the hyperphosphorylation of tau will ultimately result in the formation of tau aggregates (Kumar et al., 2023). *In vivo* gene expression studies reveal that A β accumulation causes an increase in microtubule associated protein tau mRNA, resulting in an increase of tau protein concentration in cerebrospinal fluid (Sajjad et al., 2018). Yet, inadequate understanding of the underlying mechanism of action. Additionally, the tau aggregates are deposited within the neurons and NFTs are first observed in the hippocampus, causing cognitive decline that is one of the most prominent symptoms of AD (Kumar et al., 2022).

In rats induced by D-gal and AlCl₃, high levels of p-tau Thr181 and p-tau Thr231 were detected, confirming the development of AD in the rat model. Both of these markers are promising biomarkers for axonal abnormalities and detected in the preclinical stage of AD as well as NFTs in the post mortem AD brains (Hirota et al., 2022). Intriguingly, administration of Δ^9 THC as a treatment result in the downregulation of these expressions. According to the findings of the present study, Δ^9 THC may slow down and halt the AD progression by lowering the p-tau Thr181 and p-tau Thr231, hence preventing cognitive deterioration. However, the exact mechanisms which lead to these changes are remain unclear. Study postulate that, it might be through the cannabinoid mediated pathway, whereby Δ^9 THC interact with cannabinoid receptors producing these actions. Therefore, by understanding the concept of pathogenesis of a disease, it may be possible to identify a Δ^9 THC path for developing therapeutic approaches particularly in slowing the disease progression of AD.

7.6 Conclusion

The findings of the present study suggest that Δ^9 THC may have significant therapeutic benefits for AD, particularly in decreasing the primary pathogenic markers BACE1, APP, and hyperphosphorylated tau protein. These researched hallmarks lead directly to the production of senile plaques and neurofibrillary tangles, two of the most prominent indications of AD. With the decrease of these protein markers, Δ^9 THC's ability to slow

or reverse the progression of AD could be better understood. It also could provide an important research database prior to the necessity for additional investigations.



CHAPTER 8

SUMMARY, CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH

8.1 Summary and conclusion

AD is a multi-faceted neurological condition that normally begins with progressive memory loss, and gradually worsens and incapacitates the patients. AD is currently the major cause of dementia worldwide. With the global rapid increase in the aging population, the number of people with AD increases dramatically, making AD a dire global health burden among the elderly. Although there are therapies available that may help some symptoms, there is no cure for AD. The USFDA approved drugs available in the market are used primarily for symptomatic management and they do have adverse effects.

Cannabinoid agonist delta-9-tetrahydrocannabinol (Δ^9 THC), the primary psychoactive component of cannabis, is mostly found in *Cannabis sativa* plant (Calabrese, 2018). It is widely established that Δ^9 THC binds to cannabinoid receptors in order to induce physiological changes. According to the previous research, continuous administration of Δ^9 THC at low concentration improves the neurological function of aged animals and promotes hippocampal neurogenesis in normal aging rats (Suliman et al., 2018; Jiang et al., 2005). Moreover, Δ^9 THC has been shown to protect against cognitive impairment caused by inflammation (Fishbein-Kaminietsky et al., 2014; Assaf et al., 2011). In light of the favourable effects of Δ^9 THC on cognition and memory, non- psychoactive doses of Δ^9 THC were chosen in the present investigation to evaluate its therapeutic effect on AD-like rat model.

The present study was designed to evaluate the neurotherapeutic effects of Δ^9 THC on cognitive abilities of the AD-like rat model, beside examining the therapeutic effect of Δ^9 THC on the histological alteration in the rat brain. Further, the therapeutic effect of Δ^9 THC on neurogenesis activity and pathogenic protein markers of the rat model were also evaluated. The effects of Δ^9 THC were compared with donepezil, USFDA approved drug for the treatment of mild to moderate AD. Donepezil is a selective inhibitor of the enzyme AChE, the primary function of which is to hydrolyse the neurotransmitter ACh to its inactive metabolites acetate and choline (Zambrano et al., 2019).

Using neurotoxic compound, AD symptoms and behavioural abnormalities can be modelled in animals, specifically rodents. In the current investigation, rats administered with 60 mg/kg D-gal and 200 mg/kg AlCl_3 daily for 10 weeks has been emerged as the ideal model for AD-like neurotoxicity and cognitive impairment (Chiroma et al., 2018). Behavioural studies revealed cognitive deficits in spatial and non-spatial learning and memory function in the AD-like rat model. In addition, a marked neuronal degeneration together with thickness layer reduction were observed in the hippocampus brain area of rats as revealed by Nissl's stain. Furthermore, the AD-like rat model exhibited a downregulation of neurogenesis markers, as evidenced by a decrease in the number of

positive-GFAP cells, positive-DCX cells, and positive-calbindin cells, as well as a reduction in the percentage area of NeuN immunoreactive.

At molecular level, AD-like rats displayed an upregulation of neuropathological markers of AD, including BACE1, APP, p-tau Thr181 and p-tau Thr 231. Previously, similar model of AD with cognitive deficits, biochemical alterations and neurodegeneration was also been optimised in the rat model (Chiroma et al., 2018). The established AD-like rat model in this present study is simple, effective, and economical non-transgenic rat model, that could help in understanding the aetiology of AD. Further, it could be a useful technique for screening the new drugs and modifying agents.

Δ^9 THC could be employed as potential agent in uncovering the aetiology of AD. Δ^9 THC mitigated the cognitive deficits observed in the AD-like rat model, according to the findings of the current study's behavioural evaluations. Rats treated with Δ^9 THC performed better than AD-like rat models in both spatial and non-spatial learning and memory tests, as demonstrated by their increased time in the novel object of the NOR test, decreased escape latency, increased time spent in the target quadrant during the probe test, increased number of platform quadrant and platform zone entries during the MWM test. In addition, less time required to reach the enclosed arm have been observed in rats treated with Δ^9 THC during mEPM test. Δ^9 THC treatment also acts as anti-anxiolytic, demonstrated by increased time spent in open arms during the EPM test. However, these cognition enhancing effects of Δ^9 THC have not yet been reported in human studies as well as in cognitively impaired rats.

Histological examination of survived pyramidal and granule cells of the rats' hippocampal neurons indicated that Δ^9 THC mitigated against neurodegeneration and morphological aberrations seen in the AD-like rat model. Some of aberrations prevented by Δ^9 THC in the AD-like rat model included changes in the shape of pyramidal and granule cells evidenced by darkly stained nucleus and sparse arranged cells in the hippocampus. Improvement in hippocampus thickness and the number of viable cells can also be detected. The result clearly suggests that Δ^9 THC exerts its therapeutic effects via the restoring of the survival of the cells and cell layers, thereby promoting normal brain function. Δ^9 THC also has therapeutic potential in preserving normal cytoarchitecture. According to our knowledge, the current study is the first to report on the neurotherapeutic potential of Δ^9 THC on the brains of D-gal and AlCl_3 induced AD-like rat model stained with Nissl.

Neurogenesis impairment may contribute to cognitive deterioration associated with AD. Our research reveals that Δ^9 THC promoted neurogenesis activities in the hippocampus of AD-like rat model, as indicated by an increase in neurogenesis markers. It is evidenced by an increase in the number of GFAP-positive cells, DCX- positive cells, calbindin-positive cells and NeuN immunoreactive percentage area. These markers illustrate the entire of neurogenesis process. An increase in neurogenesis was associated with enhanced learning and memory processes. The results of this study strongly suggest that Δ^9 THC may have neurotherapeutic potential in AD, notably in restoring the normal function of neuronal cells in the hippocampus of AD-like rat model via neurogenesis.

Some of the most prominent neuropathological hallmarks are also being explored for molecular characteristics. The markers that contributed to the production of senile plaques and neurofibrillary tangles were the focus of our study. From the findings of the current study, it is apparent that Δ^9 THC treatment may slow down or halt the AD progression by reducing the primary pathological indicators. This was accomplished by reducing the protein expressions the BACE1, APP, p-tau Thr181 and p-tau Thr231. Thus, by investigating the pathophysiology of AD, it may possible to determine the exact mechanisms of Δ^9 THC for the development of treatment methods, specifically to retard or slow the advancement of AD.

From the findings of the present study, it is evident that Δ^9 THC treatment modulates the D-gal and AlCl_3 induced AD-like cognitive impairment and its associated pathologies in rats. These was achieved through enhanced cognition, decreased neurodegeneration, increased neurogenesis activity and decreased expression of pathogenic protein markers (Figure 8.1). All these findings provide scientific evidence to support the investigation of Δ^9 THC as a potential and beneficial compound for the treatment of AD.

8.2 Recommendation for future research

An exploration in detail the key pathway through the Δ^9 THC exerts its beneficial therapeutic effects in AD is highly recommended. Although, it is generally accepted that Δ^9 THC may act on cannabinoid receptors, the exact mechanism remains unknown. In addition, before Δ^9 THC can be developed into more clinically useful pharmacological formulation, a comprehensive toxicological evaluation of non- psychoactive dosages of Δ^9 THC in different animal models is necessary. It should also cover the preclinical evaluation of Δ^9 THC's pharmacokinetics properties, such as absorption, distribution, metabolism and excretion. It is also essential to determine if a longer duration of Δ^9 THC was used. Like our research, the results were provided following 28 days of the treatments. More research is needed to determine whether Δ^9 THC at the same concentration produces the same positive benefits when used longterm.

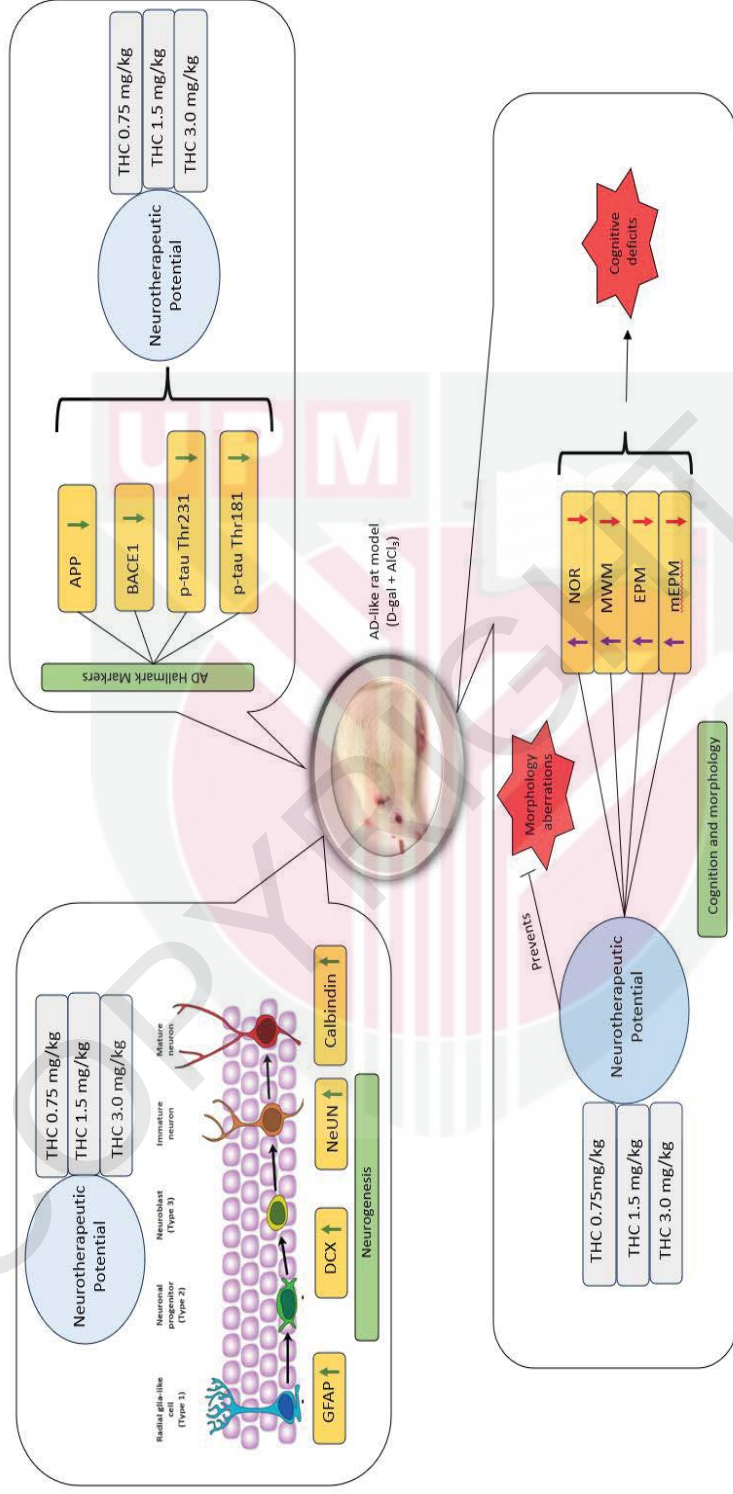


Figure 8.1 : Summary of neurotherapeutic effect of Δ^9 THC on D-gal and AlCl_3 induced AD-like rat model. Administration of D-gal and AlCl_3 in rat resulted to cognitive impairment, neuron degeneration, reduced neurogenesis activity and increased expression of pathogenic protein markers. Intriguingly, treatment with Δ^9 THC ameliorates the cognitive deficits and neuron degeneration, increased neurogenesis activity and decreased the expression of pathogenic protein markers

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Appendix B

Animal Experimental Work

Appendix B1

Sample of rats



Appendix B2

Body weight of rats



Appendix B3

Intraperitoneal injection (i.p)



Appendix B4

Brain sample collection



Appendix C

Preparation of Drugs

Appendix C1

Preparation of Delta-9-tetrahydrocannabinol (Δ^9 THC)

Stock solution of Δ^9 THC: 100 mg/ml in 100% ethanol solution

Total volume of Δ^9 THC in one vial is 1 ml

To prepare 3 mg/ml,

0.9 mL (stock solution) + 29.1 mL 0.9 % NaCl = 30 mL (total volume), ethanol 3%

To prepare 1.5 mg/ml,

10 mL (3 mg/ml) + 9.7 ml 0.9 % NaCl + 0.3 mL ethanol = 20 mL (total volume),
ethanol 3%

To prepare 0.75 mg/ml,

10 mL (3 mg/ml) + 29.1 mL 0.9 % NaCl + 0.9 mL ethanol = 40 mL (total volume),
ethanol 3%

Appendix D
Neurobehavioral Study Setup

Appendix D1
Novel Object Recognition (NOR)



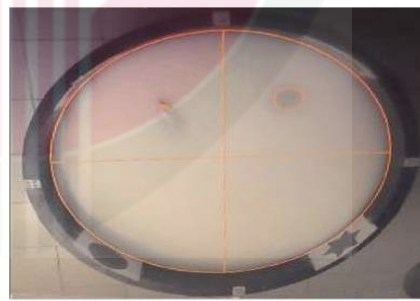
Acquisition phase



Test phase

Experimental setup during novel object recognition (NOR) test.

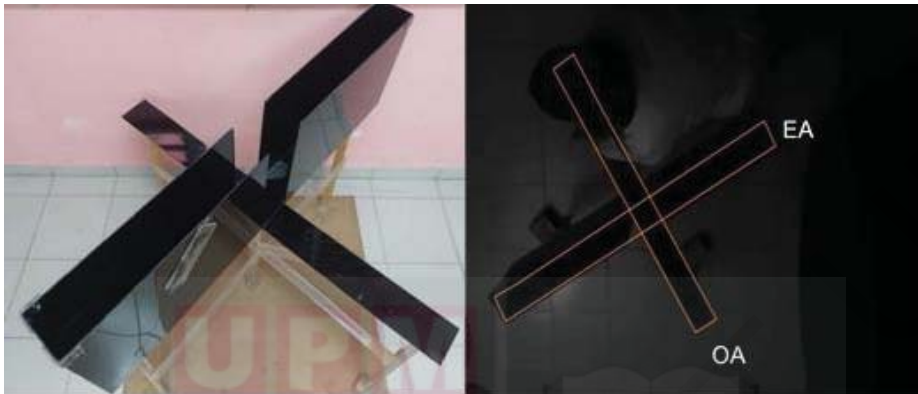
Appendix D2
Morris Water Maze (MWM)



Experimental setup during Morris Water Maze (MWM) test.

Appendix D3

Elevated Plus Maze (EPM) and modified Elevated Plus Maze (mEPM)



Experimental setup during elevated plus maze (EPM) and modified elevated plus maze (mEPM).

Appendix E

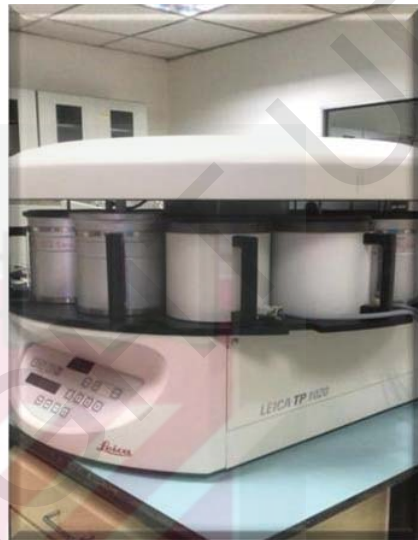
Histology study

Appendix E1

Histological Study (Cresyl Violet Staining)



Paraffin tissue block



Automated tissue processor



Tissue embedder



Microtome

Tissue staining protocol

Slide dewaxation (60°C, ~ 2hours 30 minutes)



Xylene, 3X, 10 minutes each



100% alcohol, 2X, 5 minutes each



95% alcohol, 3 minutes



70% alcohol, 3 minutes



Rinsed under running tap water



Rinsed with distilled water



Soaked in 0.1% cresyl violet (3 minutes, 37°C)



Rinsed with distilled water



95% alcohol, 15 minutes



100% alcohol, 2X, 5 minutes



Xylene, 2X, 5 minutes

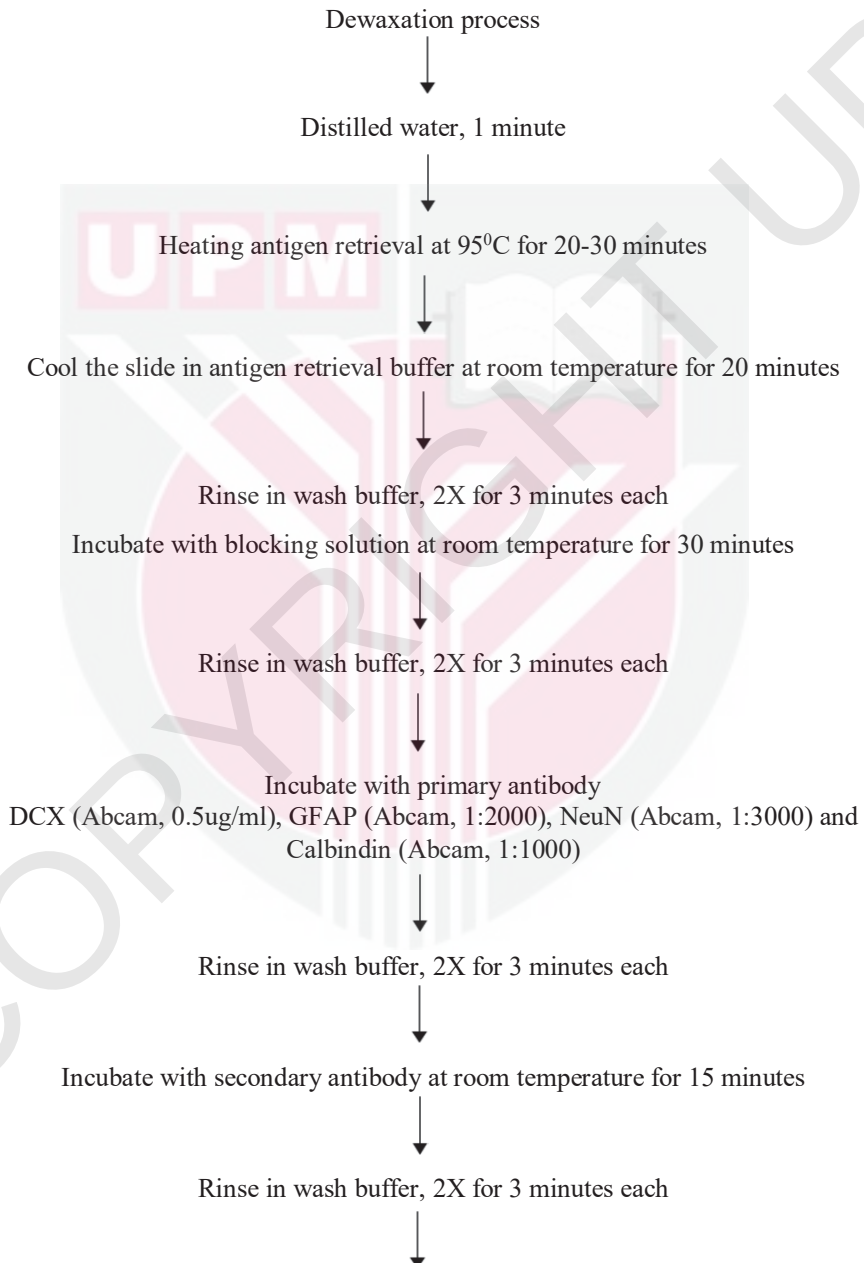


Mounted with DPX

Appendix E2

Quantification of Protein Marker (Immunohistochemistry)

IHC staining protocol



Incubate with 0.05 % DAB in TBS



Rinse in wash buffer, 2X for 3 minutes each



Hematoxylin, 1 minute



Running tap water, 2 minutes



70 % alcohol, 2 minutes



80 % alcohol, 2 minutes



95 % alcohol, 3X for 2 minutes each



100 % alcohol, 2X for 2 minutes each



Xylene, 2X for 2 minutes each



Mounting with DPX

Appendix E3

Antibody for IHC

i. Anti-GFAP antibody

Product name	: Anti-GFAP antibody ab7260
Description	: Rabbit polyclonal to GFAP
Host species	: Rabbit
Species reactivity	: Mouse, rat
Procedure	:

The section was pre-treated using heat mediated antigen retrieval with sodium citrate buffer (pH 6, epitope retrieval solution) for 20 minutes. The section was then incubated with ab7260 at 1/2000 dilution for 15 minutes at room temperature and detected using an HRP conjugated compact polymer system. DAB was used as the chromogen. The section was then counterstained with hematoxylin and mounted with DPX

ii. Anti-Doublecortin antibody

Product name	: Anti-doublecortin antibody ab18723
Description	: Rabbit polyclonal to doublecortin
Host species	: Rabbit
Species reactivity	: Mouse, rat
Procedure	:

The section was pre-treated using heat mediated antigen retrieval with sodium citrate buffer (pH 6, epitope retrieval solution) for 20 minutes. The section was then incubated with ab18723 at 0.1µg/ml dilution for 15 minutes at room temperature and detected using an HRP conjugated compact polymer system. DAB was used as the chromogen. The section was then counterstained with hematoxylin and mounted with DPX

iii. Anti-NeUN antibody

Product name	: Anti-NeUN antibody [EPR12763] – Neuronal marker ab177487
Description	: Rabbit monoclonal [EPR12763] to NeUN – Neuronal marker
Host species	: Rabbit
Species reactivity	: Mouse, rat, goat, sheep
Procedure	:

Sections were de-paraffinized and subjected to heat mediated antigen retrieval using citric acid. The sections were blocked using 1% BSA for 10 minutes at 21°C. ab177487 was diluted 1/800 and incubated with the sections for 2 hours at 21°C.

iv. Anti-calbindin antibody

Product name	: Anti-calbindin antibody [EP3478] ab108404
Description	: Rabbit monoclonal [EP3478] to calbindin Host
species	: Rabbit
Species reactivity	: Mouse, rat, human Procedure :

The section was pre-treated using heat mediated antigen retrieval with sodium citrate buffer (pH 6, epitope retrieval solution) for 20 minutes. The section was then incubated with ab108404 at 1/100 dilution for 15 minutes at room temperature and detected using an HRP conjugated compact polymer system. DAB was used as the chromogen. The section was then counterstained with hematoxylin and mounted with DPX.

v. Secondary antibody

Product name	: Goat anti-rabbit IgG H&L (HRP) ab6721 Host
species	: Goat
Target species	: Rabbit

Appendix F

Western blotting techniques

Appendix F1

Preparation of gel

1 gel	4 % (ml)	5 % (ml)	6 % (ml)	7 % (ml)	8 % (ml)	9 % (ml)	10 % (ml)	11 % (ml)	12 % (ml)	13 % (ml)	14 % (ml)	15 % (ml)
DdH ₂ O	3.05	2.85	2.7	2.55	2.35	2.2	2.05	1.85	1.7	1.55	1.35	1.2
30% acrylamide	0.65	0.85	1.0	1.15	1.35	1.5	1.65	1.85	2.0	2.15	2.35	2.5
Resolvingbuffer (1.5M)		1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Stackingbuffer (0.5M)	1.25											
10 % SDS	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
10 % APS	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
1 gel	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025

2 gel	4 % (ml)	5 % (ml)	6 % (ml)	7 % (ml)	8 % (ml)	9 % (ml)	10 % (ml)	11 % (ml)	12 % (ml)	13 % (ml)	14 % (ml)	15 % (ml)
DdH ₂ O	6.1	5.7	5.4	5.1	4.7	4.4	4.1	3.7	3.4	3.1	2.7	2.4
30% acrylamide	1.3	1.7	2.0	2.3	2.7	3.0	3.3	3.7	4.0	4.3	4.7	5.0
Resolvingbuffer (1.5M)		2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Stackingbuffer (0.5M)	2.5											
10 % SDS	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
10 % APS	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Total	10.06	10.06	10.06	10.06	10.06	10.06	10.06	10.06	10.06	10.06	10.06	10.06

Appendix F2

Buffer for Western Blotting

Polyacrylamide gel

100 mL of Resolving buffer: 18.17 g of Trizma in 100 mL of deionized water and adjust pH 8.8 using HCl

100 mL Stacking buffer: 6.057 g of Trizma in 100 mL of deionized water and adjust pH 6.8 using HCl

Laemmli buffer (loading buffer)

Reagent	Amount (g)
0.5 M Tris	2.5 ml
Glycerol	2.0 ml
10 % SDS	2.0 ml
Deionized water	2.5 ml
Beta mercaptoethanol	1.0 ml
2.5% bromophenol blue	40 µl

10x Running Buffer (Electrophoresis buffer): 1000 ml

Reagent	Amount
Tris	30.3 g
Glycine	44 g
SDS (add last)	10 g
Dissolved in 1000 ml deionized water, do not adjust	

Transfer buffer (Blotting buffer): 5000 ml

Reagent	Amount (g)
Tris Glycine	15.15 g
Deionized water	4 L
Methanol	1 L

Blocking buffer

5 % w/v skimmed milk powder in Tris-buffered saline Tween 20 (TBST 20)

Tris-buffered saline 0.1 % Tween 20 (TBST 20): 10 L

Reagent	Amount (g)
25 mM Tris	30.285
125 mM Sodium Chloride (NaCl)	73.05
Deionized water	1 L (adjust to pH 7.6)
Tween 20	10 ml (to the final concentration of 0.1%)

Appendix F3

Antibody for western blot

i. Anti-BACE1 antibody

Product name : Anti-BACE1 antibody [EPR19523]
Description : Rabbit monoclonal [EPR19523] to BACE1
Host species : Rabbit
Species reactivity : Mouse, rat, human
Function :

Responsible for the proteolytic processing of the amyloid precursor protein (APP). Cleaves at the N-terminus of A-beta peptide sequence, between residues 671 and 672 of APP, leads to the generation and extracellular release of beta-cleaved soluble APP and a corresponding cell-associated C-terminal fragment which is later released by gamma-secretase

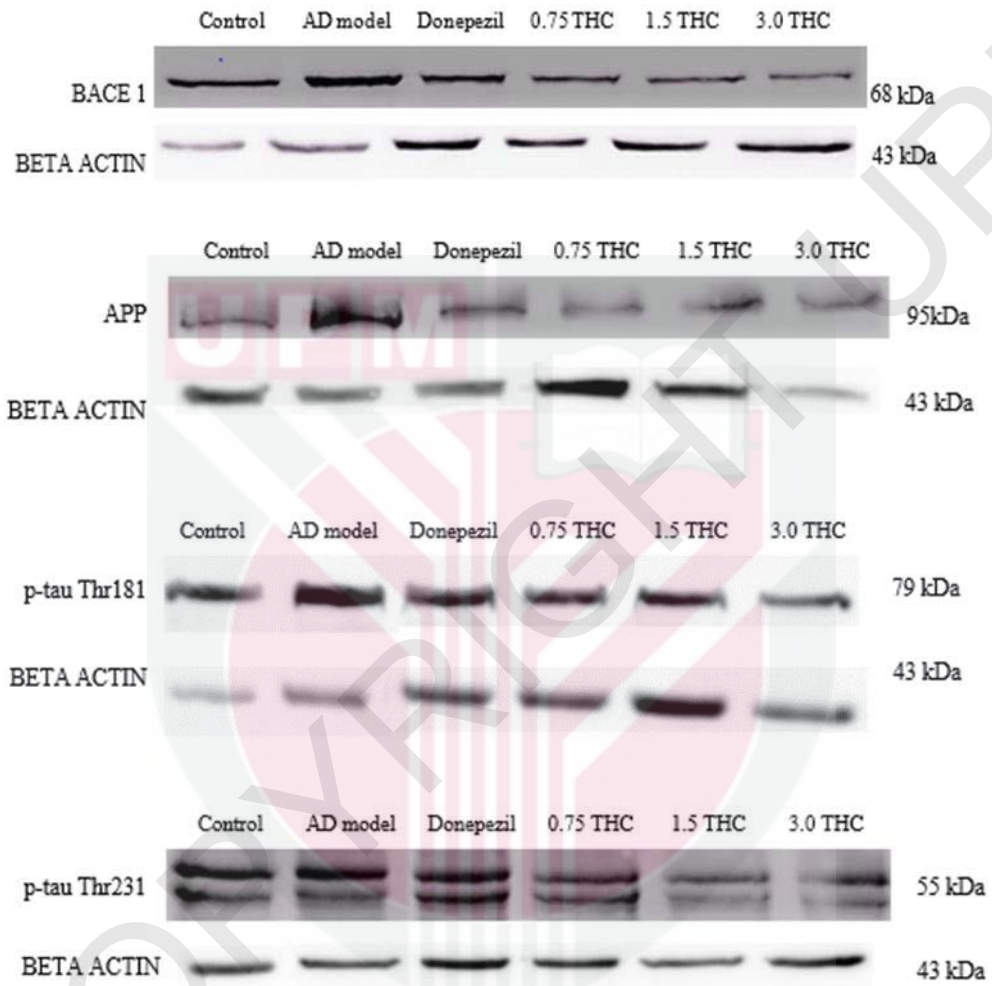
ii. Anti-Amyloid Precursor Protein antibody [Y188] ab32136

Product name : Anti-amyloid Precursor Protein antibody [Y188]
Description : Rabbit monoclonal [Y188] to amyloid precursor protein
Host species : Rabbit
Species reactivity : Mouse, rat, humanFunction :

Responsible for the proteolytic processing of the amyloid precursor protein (APP). Cleaves at the N-terminus of A-beta peptide sequence, between residues 671 and 672 of APP, leads to the generation and extracellular release of beta-cleaved soluble APP and a corresponding cell-associated C-terminal fragment which is later released by gamma-secretase

Appendix F4

Example of immunoblot



LIST OF PUBLICATIONS

Zakaria, F.N., Moklas, M.A.M., Baharuldin, M.T.H., Mat Taib, C.N, Hamid, H.A., Hamid, H.A., & Chiroma, S.M. (2024). Delta-9-tetrahydrocannabinol ameliorates aluminium chloride-induced Alzheimer's-like cognitive deficit in rats via neurogenesis in their hippocampus. *Journal of Young Pharmacist*, 16(1):33-41 (**JCR, Q3**)

Zakaria, F.N., Moklas, M.A.M., Baharuldin, M.T.H., Mat Taib, C.N, Hamid, H.A., Hamid, H.A., & Mahdi, O. Combination of D-galactose and aluminium chloride induced Alzheimer-like pathology symptom in rat hippocampus. *Journal of Health and Translational Medicine – (SCOPUS, Q4)* Accepted

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Awards

Young Investigator Award, 2023 at International Anatomical and Biomedical Scientific Conference (IABS, 2023)