

Delta-9-Tetrahydrocannabinol (Δ^9 THC) Treatment Improves Neuronal Survivability by Ameliorating Neuronal Apoptosis and Increasing Calbindin Expression in the Pyramidal Cells of Alzheimer-Induced Rat Hippocampus

Arisha Abdullah Mas'od ¹, Fatin Nadzirah Zakaria ^{1,*}, Razif Dasiman ^{1,*}, Samaila Musa Chiroma ², Mohamad Aris Mohd Moklas ³, Zulkhairi Amom ¹

¹ Department of Basic Sciences, Faculty of Health Sciences, Universiti Teknologi MARA, Selangor Branch, Puncak Alam Campus, 42300 Bandar Puncak Alam, Selangor, Malaysia; 2022213244@isiswa.uitm.edu.my (A.A.M.); fatinn9604@uitm.edu.my (F.N.Z.); razifdasiman@uitm.edu.my (R.D.); zulkha2992@uitm.edu.my (Z.A.);

² Newcastle University Medicine Malaysia (NUMed), 1, Jalan Sarjana 1, Kota Ilmu, Educity@Iskandar, 79200 Iskandar Puteri, Johor, Malaysia; Musa-Chiroma.Samaila@newcastle.edu.my;

³ Department of Human Anatomy, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia; aris@upm.edu.my;

* Correspondence: fatinn9604@uitm.edu.my (F.N.Z.); razifdasiman@uitm.edu.my (R.D.);

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Abstract: Alzheimer's disease (AD) is the most common form of dementia, an age-dependent neurodegenerative process that involves the gradual deterioration of neurons, leading to a decline in cognitive abilities. Because the endocannabinoid system (ECS) is highly expressed in the hippocampus, Delta-9-tetrahydrocannabinol (Δ^9 -THC) has often been associated with learning and memory. This research is designed to evaluate the effects of Δ^9 THC on cellular and physiological changes in the hippocampus induced by varying doses during the early stage of developmental AD, focusing on neuronal survival and possible alterations aimed at ameliorating neuronal apoptosis and upregulating the expression of neurogenesis protein markers. Male albino Wistar rats were first exposed to a combination of 60 mg/kg D-gal intraperitoneally with 200 mg/kg AlCl_3 orally, once daily for ten consecutive weeks, to induce AD. Doses of Δ^9 THC at 0.75, 1.5, and 3.0 mg/kg were administered to all groups for 28 days, and 1 mg/kg of donepezil was used as a positive control measure. The results revealed that Δ^9 THC significantly alleviated cognitive impairments by reducing neuronal apoptosis, increasing neuronal survivability, and preventing morphological aberrations. The evaluation of the immunoreactivity area of calbindin-positive-stained cells indicated that Δ^9 THC successfully promotes calbindin expression as one of the neurogenesis protein markers at the corpus ammonis (CA) region. This suggests that Δ^9 THC might be a promising strategy that may remediate AD-associated cognitive deficits.

Keywords: Alzheimer's disease; cannabis; Δ^9 THC; hippocampus; cognitive impairment.

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1. Introduction

Alzheimer's Disease (AD) is the most common form of dementia, especially among the elderly. It is a progressive neurological disorder characterized by the presence of plaques and neurofibrillary tangles in the brain, leading to the gradual degeneration and apoptosis of neuronal cells [1,2]. Currently, the World Health Organization (WHO) has stated that around 55 million people have dementia globally, and this number is expected to rise to 78 million in 2030 and 139 million in 2050 [3]. The overall prevalence of probable dementia in Malaysia was 8.5%, as reported by the National Institutes of Health (NIH) [4]. Prevalence is expected to grow worldwide in the coming years as the proportion of older people in the overall population increases in nearly every country. However, current treatments available barely reduce or slow down the progression of AD, particularly symptomatic drugs aiming at alleviating progressive memory-declining aspects, such as targeting the natural cholinergic degradation in the brain and compensating glutamate neurotransmission [5]. The strongest available FDA-approved pharmacological approaches developed for AD are acetylcholinesterase inhibitors (donepezil, rivastigmine, galantamine, and a combination of memantine and donepezil), N-methyl-D-aspartate (NMDA) receptor partial antagonist (memantine), and recently discovered amyloid-targeting antibody drug (lecanemab) [6]. These drugs aim to increase communication between nerve cells to counteract cognitive decline in people living with dementia (PLWD). Altogether, these points underscore the urgency of further global efforts to investigate the more in-depth effects of Δ^9 THC-based treatments and identify their intrinsic roles in remediating disease progression.

Considering that the progressive memory decline in AD is characterized by $A\beta$ and tau aberrancies across different biological substrates that stimulate neuronal apoptosis, targeting multiple substrates simultaneously will help stop or slow the progression of the disease. Over the years, Δ^9 -tetrahydrocannabinol (Δ^9 THC), the psychoactive component of *Cannabis sativa*, an annual herbaceous plant in the *Cannabis* genus, a species of the *Cannabaceae* family, has attracted researchers' interest for its role in modulating brain memory and cognitive functions. The main target of Δ^9 THC is the endocannabinoid system (ECS), which is vital in regulating numerous brain functions, including learning, memory, mood, anxiety, drug addiction, nutritional behavior, pain perception and modulation, and cardiovascular function. The components in the system include cannabinoid receptors, endogenous ligands, secondary messengers, and endocannabinoid degradation pathways [7]. There are three primary ECS receptors that correlate with the psychotropic and cognitive effects of Δ^9 THC, namely the Cannabinoid Type 1 Receptor (CB1), the Cannabinoid Type 2 Receptor (CB2), and G-protein-coupled receptor 55 (GPR55) [8]. ECS modulates excitatory and inhibitory neural activity by blocking presynaptic voltage-gated calcium channels, reducing neurotransmitter release, and regulating signalling pathways involved in synaptic maturation and plasticity [9,10]. Researchers often associate Δ^9 THC with learning and memory functions because the endocannabinoid system (ECS) is highly expressed in the hippocampus and cortex, and cannabinoid agonists have been shown to interfere with the underlying pathophysiology of neurodegenerative processes. Δ^9 THC may be therapeutically useful for targeting several underlying pathophysiological processes linked to neuropsychiatric symptoms by interrupting downstream neurodegenerative processes. This may be due to Δ^9 THC, which has been shown to competitively inhibit acetylcholinesterase and prevent $A\beta$ aggregation, thereby interfering with amyloidogenesis [11]. In addition, activation of both CB1 and CB2 has been shown to

have beneficial effects, inducing hippocampal neurogenesis and reducing A β toxicity (plaque deposition) in rodents, as well as in other pre-clinical and clinical studies of Alzheimer's disease [12].

In the endocannabinoid system, cannabinoids exert their effects through agonism of CB1 and CB2 receptors, which respond to both external and endogenous agonists. CB1 receptors are found mainly in the central nervous system (CNS) and peripheral nervous system (PNS) [13]. The CB1 receptor is one of the most abundant G protein-coupled receptors (GPCRs) in the CNS and inhibits adenylate cyclase when activated, preventing the conversion of AMP to cyclic AMP [14]. Expression of CB1 receptors is high in the neocortex, hippocampus, basal ganglia, cerebellum, and brainstem [15]. Due to the localization of CB1 receptor in the CNS, together with both of its endogenous ligands (endocannabinoids (eCB)) and enzymes involved in the synthesis and degradation, it is responsible for multiple pathophysiological events such as memory deficits and neurodegenerative disorders. CB2 receptors are predominantly expressed in the immune system (tonsils, spleen, and leukocytes) and haemopoietic cells [16]. This receptor exhibits a more defined pattern of expression in the brain than CB1 receptors. In the CNS, CB2 receptor expression is primarily localized to microglia, resident macrophages, and is associated with inflammation [17,18]. Microglial cells play a significant role in AD and other diseases associated with the basal ganglia. Hence, there is a relevant relationship between the selective localization of CB2 receptors and their neuromodulatory effect on microglia function, such as inflammation. Recent discoveries also indicate that CB2 receptors, expressed in neurons, can regulate synaptic function while also being involved in drug abuse and synaptic plasticity [19].

AD is subdivided into two types: early-onset familial AD (< 65 years of age), in which brain changes occur 15-20 years before symptom onset, and late-onset sporadic AD (> 65 years of age). After the clinical onset, AD presents a progressive, declining course made up of different stages (early, middle, or late). The stages differ and depend on the patient's cognitive status, especially their degree of brain disability [20]. Sporadic AD is the most common and consists of a widely studied genetic risk factor, gene-encoding apolipoprotein E. Only approximately 5% of AD cases are categorized as familial AD caused by the inheritance of an autosomal dominant mutation in the genes encoding amyloid precursor protein (APP), presenilin 1 (PS1), or presenilin 2 (PS2). Both PS1 and PS2 are enzymes involved in APP production [21]. Furthermore, the aberrant cleavage of APP into A β peptides of 40 residues (A β 40) or of 42 residues (A β 42) is proven to be the cause of mutations in APP, PS1, and PS2; these mutations would eventually lead to the formation of A β plaques, contributing to neuronal apoptosis in AD [21]. Due to the complex multi-factorial and limited understanding of the mechanism of action of AD by Δ^9 THC, this research was designed with behavioral, histological and neuroimaging approaches to evaluate the effects of Δ^9 THC on neuronal survivability; both viability and apoptosis, and its potential to enhance neurogenesis by modulating the expression of the neurogenesis protein marker in order to regain back the capabilities in learning and memory. Neuronal survival was determined by counting CA1 pyramidal cells using H&E staining to assess the extent of neurodegeneration and any other potential changes in survival. Meanwhile, immunofluorescence was performed to evaluate the expression of calbindin-positive cell markers. Calbindin was targeted as it plays a major role in neurogenesis, specifically neuronal differentiation and development by acting as a calcium buffer and regulating neuronal excitability. Thus, the findings from this study will provide a broader spectrum of pharmacological effects and demonstrate how they can help open future

opportunities to include Δ^9 THC as one of the neurotherapies among the other AD treatments available.

2. Materials and Methods

2.1. Reagents and equipment.

AlCl_3 , D-gal, and donepezil were purchased from Sigma-Aldrich (St. Louis, MO, USA), whereas Δ^9 THC was obtained from Lipomed (Switzerland). D-gal and donepezil were dissolved in distilled water for intraperitoneal injection (i.p). AlCl_3 was dissolved in normal saline for oral administration [22]. Prior to the experimental work, all drugs and chemicals were freshly prepared. Meanwhile, for immunofluorescence; primary conjugated antibody (Abcam Anti-Calbindin antibody [EP3478] ab108404), secondary conjugated antibody (Abcam Goat Anti-Rabbit IgG H&L [Alexa Fluor 647] preadsorbed ab150083), DAPI staining solution (Abcam, ab228549, US), fluorescence mounting medium (Agilent Dako, US), Target Retrieval Solution (TRS) high pH buffer (Agilent Dako EnVision FLEX, DM828, US), isolator hydrophobic marker pen (Abcam PAP pen, ab2601, UK), hydrogen peroxide blocking reagent (Abcam, ab64218, UK), protein block (Abcam, ab64226, UK), and phosphate buffered saline (PBS) (concentrated 20X) were also used in this research for neurogenesis study analysis respectively.

2.2. Animals.

A total of 70 healthy male Albino Wistar rats weighing 200-250 g were obtained from Selangor, Malaysia. Before beginning the experiment, the rats were acclimatized for one week in the standard laboratory settings (temperature $22 \pm 30^\circ\text{C}$, 12h light: 12h dark cycle) with two to three rats per cage. These rats had unrestricted access to food and water. All experimental protocols were performed in the Animal Behavioural Laboratory, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, with approval from the Animal Ethical Committee of Universiti Putra Malaysia (UPM/IACUC/AUP-R094/2019).

2.3. Experimental design.

The rats will be randomly divided into seven groups (n=10). Table 1 shows the animal groups and treatments they received. Group 1 served as a normal group, which will be treated with distilled water + 0.9% normal saline; Group 2, AD model group, will receive 60 mg/kg D-gal + 200 mg/kg AlCl_3 ; Group 3, AD group, will not receive any treatment; Group 4 act as positive control group will receive 60 mg/kg D-gal + 200 mg/kg AlCl_3 together with donepezil treatment at 1.0 mg/kg. Groups 5, 6, and 7 will serve as treatment groups and receive 60 mg/kg D-gal + 200 mg/kg AlCl_3 , together with Δ^9 THC at 0.75 mg/kg, 1.5 mg/kg, and 3.0 mg/kg, respectively. Groups 2 and 3 were similar because one group generated an AD model, and the other determined whether there was potential for self-recovery in apoptotic cells and for the expression of protein markers indicating the potential for neurogenesis.

A combination administration of AlCl_3 and D-gal was used in this study to induce Alzheimer's disease in a rat model. The total induction was ten weeks. The drug treatments were administered for 28 days at three different concentrations of Δ^9 THC to investigate the potential therapeutic effect. Donepezil 1.0 mg/kg was used for symptomatic treatment of AD, and its main physiological function is the hydrolysis of the Ach neurotransmitter [23]. All

solutions were prepared immediately before the experiment and administered daily via intraperitoneal injection (i.p.), with a volume of 0.1 ml/100 g body weight. This was exceptional for AlCl₃ as it was orally administered instead. Table 1 indicates the animal groups and their respective treatments conducted in this research.

Table 1. Animal groups and treatment.

Group	Treatment	Dosage
Negative control (normal)	Distilled water + 0.9% normal saline	0.1 ml/100 g b.wt.
AD model	D-gal + AlCl ₃	60 mg/kg D-gal + 200 mg/kg AlCl ₃
AD model with no treatment (self-recovery)	D-gal + AlCl ₃ + no treatment	60 mg/kg D-gal + 200 mg/kg AlCl ₃ + no treatment
Positive control	D-gal + AlCl ₃ + donepezil	60 mg/kg D-gal + 200 mg/kg AlCl ₃ + 1.0 mg/kg donepezil
AD model + Δ ⁹ THC 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
AD model + Δ ⁹ THC 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
AD model + Δ ⁹ THC 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

2.4. Morphological study (hematoxylin and eosin staining).

The rats were decapitated after the induction weeks were over. The brain was immediately removed and placed in an ice-filled tray, then preserved in 10% neutral buffered formalin for 7 days until further analysis. The brain tissues were preserved in 10% neutral buffered formalin for 7 days until further analysis. The fixed brain underwent grossing and tissue processing using an automated tissue processor (Leica TP1020). The tissues then proceeded to the embedding process to form tissue blocks before being sectioned. Using a rotary microtome (Leica HistoCore MULTICUT - Semi-Automated Rotary Microtome, US), the tissue blocks were carefully trimmed to a thickness of 10 μm until the area of tissue exposed. The thickness scale of the microtome was readjusted to 5 μm to produce a paraffin ribbon. The extent of neuronal survival in the CA1 region was assessed by staining sections with H&E using an autostainer (Leica Autostainer XL Staining System ST5010). The H&E staining method follows a basic protocol consisting of 9 steps: dewaxing, dehydration, hematoxylin, differentiation, bluing, eosin, dehydration, clearing, and cover-slipping. Hematoxylin is used for illustrating the nuclear detail in cells. Meanwhile, eosin is used as a counterstain to distinguish cytoplasmic components from nuclei.

A compound research microscope equipped with a digital image acquisition system was used to examine stained brain tissues (Leica IC550 HD, Germany). Two parameters were measured: the number of apoptotic cells and the number of viable pyramidal cells in the CA1 subfield of the hippocampus. A photomicrograph of CA1 was taken at 200x magnification. The cell count for each parameter was measured using ImageJ software in five random fields per section, with a minimum of five rats per group (n=5). For quantification, a total of 5 hippocampal sections were collected for assessment in each experimental group, with a sample size of 5 rats per group (n=5). The quantification of apoptotic pyramidal cells was conducted by summing the counts obtained from at least five randomly selected areas within the mid portions of the CA1 subfield of the hippocampus. This analysis was performed at 200x magnification using a high-grade microscope (Leica IC550 HD) and Leica Application Suite (LAS V3.3, Leica Microsystems, Germany) viewing software. The viable pyramidal cells in CA1 were counted using the same procedures described.

2.5. Neurogenesis study (Immunofluorescence).

The paraffin-embedded cortex tissues were sectioned with a 3 μm thickness using a rotary microtome (SLEE MAINZ, CUT5062, Germany). The tissues were fixed using silanized coated slides (Muto Pure Chemicals Co. Ltd., Japan) at 40°C in a water bath. This was followed by air-drying the slides at room temperature for a few days before proceeding to dry the slides on a hotplate (Pro Scientific, HPS-7C, USA) at 60-62°C for 1.5 hours. Then, the slides were dewaxed by soaking three times in xylene for 5 mins, twice in 100% alcohol for 3 mins, and once in 95% alcohol and 70% alcohol for 3 mins each. Next, the slides were baked in a Target Retrieval Solution (TRS) high pH buffer (Agilent Dako EnVision FLEX, DM828, US) for 30 mins at 95°C using a laboratory oven (Lab Tech, LCO-3050H, UK) before being cooled down for 10 mins at room temperature to avoid thermal shock to the tissues. After that, the cortex tissues on the slide will be marked in a circle with an isolator hydrophobic marker pen (Abcam PAP pen, ab2601, UK) to prevent mixing of adjacent antiserum. The slide will be double-blocked with hydrogen peroxide blocking reagent (Abcam, ab64218, UK) and protein block (Abcam, ab64226, UK) for 30 mins each, respectively, before washing in phosphate-buffered saline (PBS) for 5 mins in between. Then, the slides will be removed from the wash tank, and residual PBS will be discarded by tapping the edge of the slide against a pad of tissue paper. The slide should be wiped around the isolator ring if required. Next, the tissues will be incubated with the primary conjugated antibody (Abcam Anti-Calbindin antibody [EP3478] ab108404) overnight at 1:200, then washed in PBS for 10 minutes the next day.

Next, the secondary conjugated antibody (Abcam Goat Anti-Rabbit IgG H&L [Alexa Fluor 647] preadsorbed, ab150083) was incubated overnight at 1:500, then washed again in PBS for 10 minutes. The slides were rinsed thoroughly 7-8 times before incubation with DAPI staining solution (Abcam, ab228549, US) at 1:500 overnight to counterstain, using a fluorescence mounting medium (Agilent Dako, US). Compared with fresh-frozen tissues or cell cultures, overnight incubation with DAPI ensured that the thick hippocampal tissue, previously embedded in paraffin, was thoroughly stained for the targeted proteins of interest. During the optimization process, a short incubation time with DAPI resulted in lower fluorescence than overnight incubation. This may be due to the fact that the hippocampal tissues are thicker than the cells, and because the tissues were embedded in paraffin wax and fixed beforehand. The optimized method suggests incubating for longer hours to give it ample time to penetrate the tissues thoroughly and react with the protein markers of interest. The next day, the slides were finally mounted using a coverslip. The photomicrographs were examined with an inverted fluorescence microscope (Nikon Eclipse TI-S 612519). The specific Calbindin positive immunostaining areas were calculated, and 6 representative pictures were taken from the cortex of each animal at the CA1 region using the Nikon Imaging Software (NIS-Elements Viewer, Japan Optical Industries Co., Ltd, Japan). A minimum of 5 animals per group were used for quantification. Calbindin+ cells were quantified, and the percentage of immunoreactive area and fluorescence intensity were calculated using ImageJ software (Rasband, W.S., ImageJ, US).

2.6. Statistical analysis.

Statistical analyses were performed using SPSS, version 29.0. One-way ANOVA was used to analyze neuronal viability, neuronal apoptosis, the percentage of calbindin-immunoreactive area, and fluorescence intensity. The significant results were further analyzed

using Tukey's post hoc test. The data obtained were summarised as mean \pm standard error of the mean (SEM), and the significance of the difference will be determined by $p < 0.05$.

3. Results

3.1. Delta-9-tetrahydrocannabinol (Δ^9 THC) increases the neuronal viability of pyramidal cells in the CA1 Region.

The neurotherapeutic potential of Δ^9 THC on the survivability of pyramidal cells was studied in the CA1 region of the rats' hippocampus. Neuronal viability in the control group showed normal cytoarchitecture, with an array of densely packed cells with distinct nuclei to distinguish viable from apoptotic cells. As shown below, the photomicrographs depict the condition of the histological sections of viable pyramidal cells at CA1 subfields for all groups (b-h) (blue arrow) (figure 1).

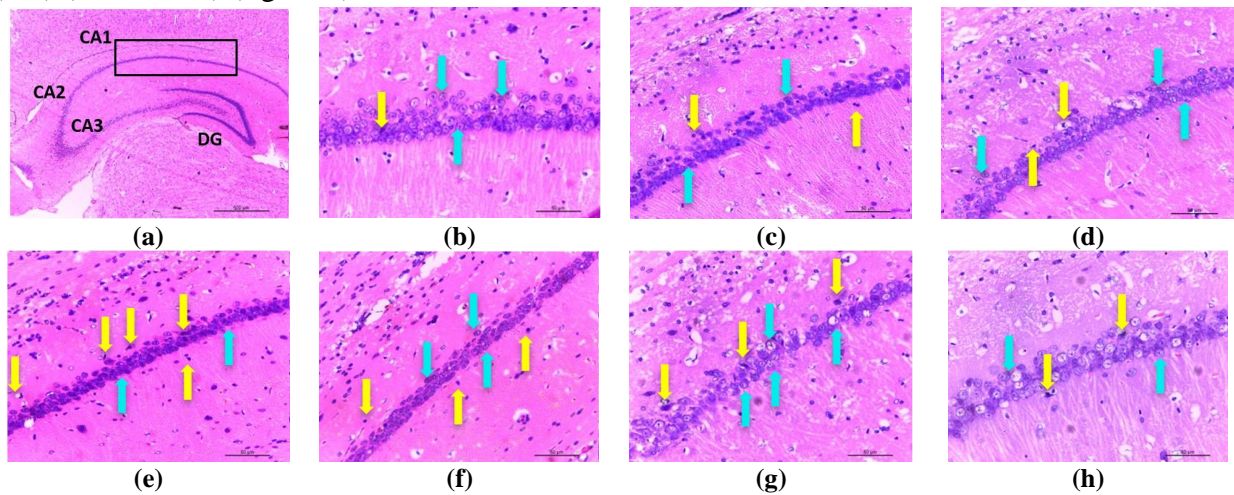


Figure 1. Representative photomicrograph of H&E-stained pyramidal cells at CA1 region of rat hippocampus after being administered by Δ^9 THC: (a) Hippocampus at 50x magnification with the black box indicates CA1 region of the hippocampus; (b) Control rat (negative control); (c) AD model; (d-h) AD model with no treatment (self-recovery), drug treatment with 1 mg/kg donepezil, 0.75 mg/kg Δ^9 THC, 1.50 mg/kg Δ^9 THC, and 3.0 mg/kg Δ^9 THC respectively. The blue arrows indicate viable pyramidal cells, and the yellow arrows indicate dead pyramidal cells. Images are shown at 400x magnification, scale bar 50 μ m.

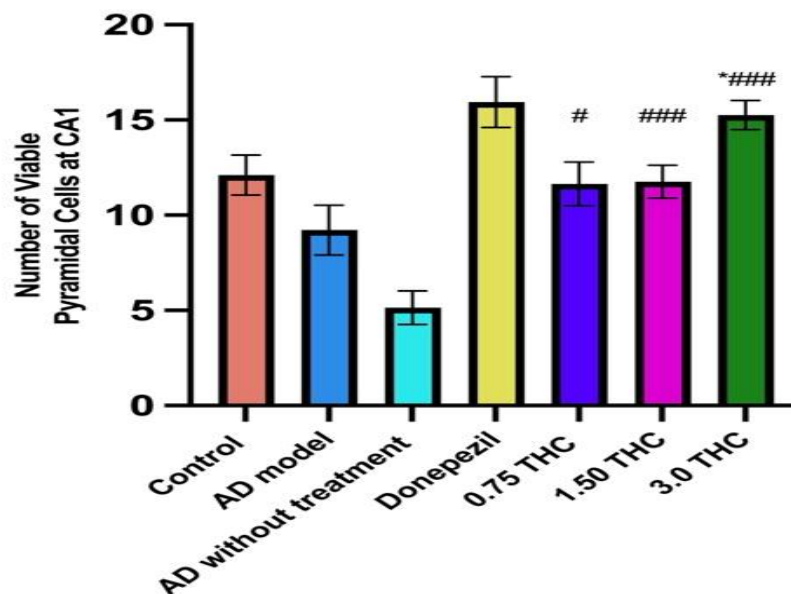


Figure 2. Mean number of viable pyramidal cells in the CA1 region. Data were expressed as mean \pm SEM (n=10). * $p < 0.05$ vs AD model, # $p < 0.05$ vs without treatment group.

After comparing the effectiveness and significances of the treatment, one-way ANOVA analysis shows a significant difference ($p < 0.05$) in the number of viable pyramidal cells at CA1 subfields [$F(6,63) = 11.60, p < 0.001$] between different groups of rats (figure. 2). Next, Tukey's post hoc test revealed that only high dose 3.0 mg/kg Δ^9 THC shows a statistically significant increase ($p < 0.01$) in the number of viable pyramidal cells at CA1 subfield (15.26 ± 0.77) compared to the AD model (9.22 ± 1.31). However, as compared to the AD model without treatment (5.14 ± 0.88), a significantly marked upregulation in the number of viable pyramidal cells was observed in all three Δ^9 THC treatment groups ($p < 0.001$); 0.75 mg/kg Δ^9 THC (11.64 ± 1.15), 1.5 mg/kg Δ^9 THC (11.76 ± 0.87) and 3.0 mg/kg Δ^9 THC (15.26 ± 0.77) respectively.

3.2. Delta-9-tetrahydrocannabinol (Δ^9 THC) reduces neuronal apoptosis of pyramidal cells in the CA1 region.

Analysing the morphological structures of neuronal cells (Figure 1), the yellow arrows indicate histological sections of apoptotic pyramidal cells in the CA1 subfield for all groups. Compared to the control group (normal healthy rats), histological examinations of the AD model group and the AD model without treatment group showed a slightly lower number of clear, darkened nuclei, indicating neuronal apoptosis has occurred in Groups 2 and 3. A distinct difference can be seen between the positive control group, donepezil, and the Δ^9 THC treatment groups because the treatment groups morphologically showed more of a condensed layer of circular-shaped pyramidal cells with visible nuclei, indicating a lesser number of apoptotic cells, whereas a higher number of viable cells were found in the Δ^9 THC treatment groups. However, only the administration of medium and high doses of Δ^9 THC; 1.50 mg/kg Δ^9 THC (2.70 ± 0.30), 3.0 mg/kg Δ^9 THC (2.10 ± 0.14) has resulted in a significant reduction of number of apoptotic cells but not low dose 0.75 mg/kg Δ^9 THC (3.48 ± 0.13) compared to AD model group (4.42 ± 0.77). One-way ANOVA demonstrated statistically significant differences in the number of apoptotic pyramidal cells in CA1 subfields [$F(6.63) = 6.51, p < 0.001$] between different groups of rats. Tukey's post hoc test has revealed a statistically significant decrease ($p < 0.05$) in the number of apoptotic pyramidal cells in the CA1 subfield of the AD model group of rats (4.42 ± 0.77) compared to 1.50 mg/kg Δ^9 THC (2.70 ± 0.30) and 3.0 mg/kg Δ^9 THC (2.10 ± 0.14), respectively.

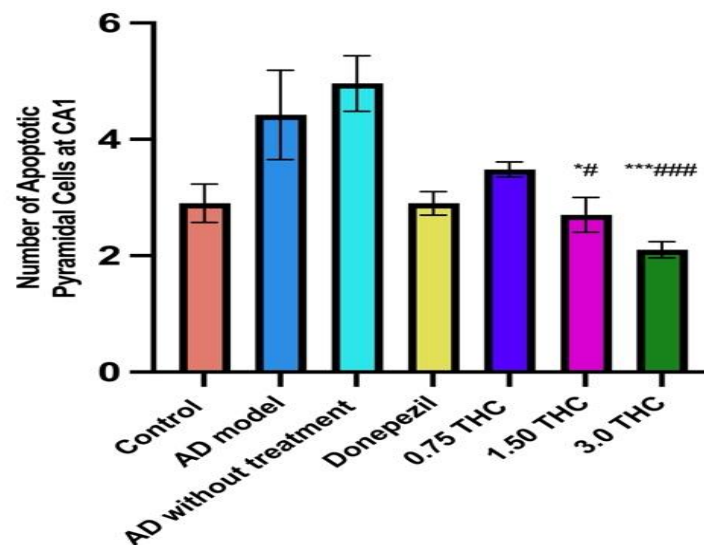


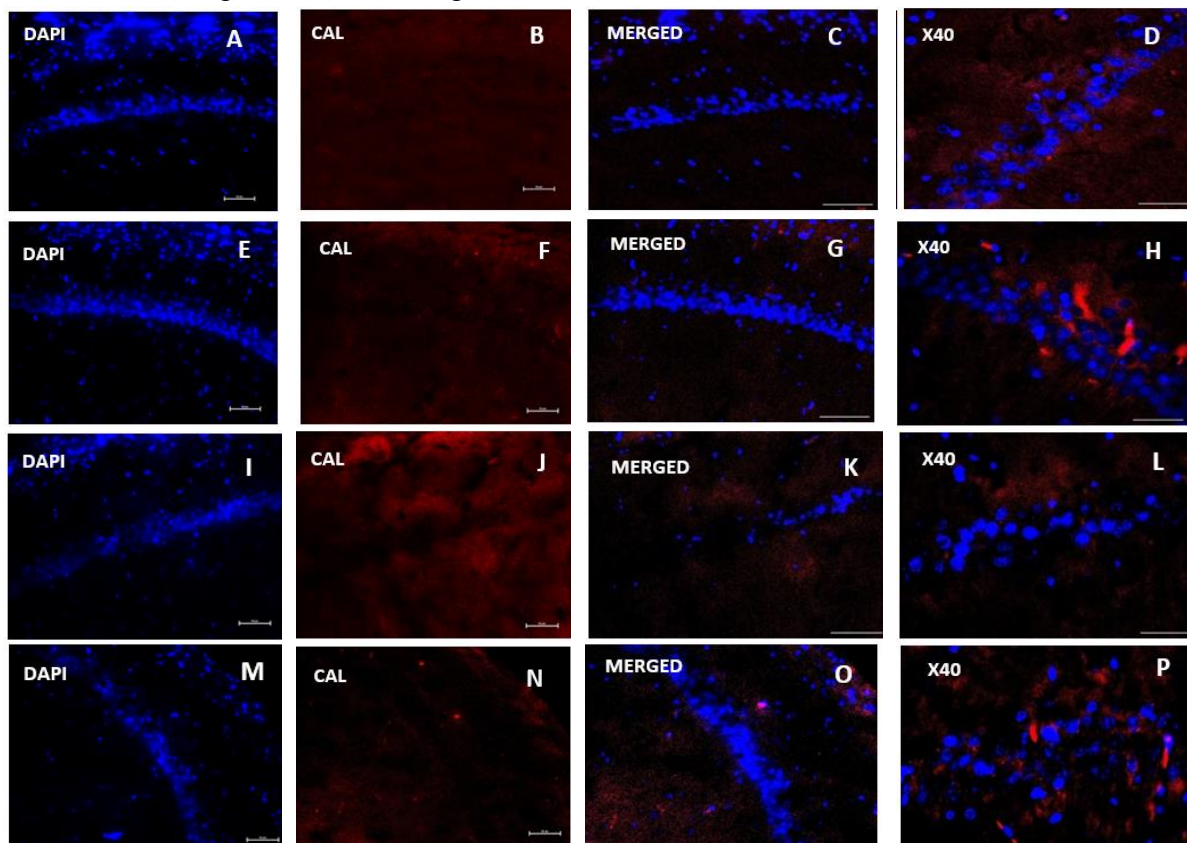
Figure 3. Mean number of apoptotic pyramidal cells in the CA1 region. Data were expressed as mean \pm SEM (n=10). * $p < 0.05$ vs AD model, # $p < 0.05$ vs without treatment group.

In the group without treatment, the medium dose 1.50 mg/kg Δ^9 THC (2.70 ± 0.30) showed a significant decrease ($p < 0.05$); meanwhile, the high dose 3.0 mg/kg Δ^9 THC (2.10 ± 0.14) showed a very significant decrease ($p < 0.001$) relative to the control group. It was postulated that there were no changes in Group 3; hence, there was no self-recovery potential of AD-induced pyramidal cells. As shown below, the graph compares groups regarding their mean differences in the number of apoptotic cells in the CA1 region, expressed as mean (SEM), against the AD model and AD without treatment (figure). 3).

3.3. *Delta-9-tetrahydrocannabinol (Δ^9 THC) promotes calbindin expression protein marker at CA1 region of the hippocampus.*

3.3.1. Δ^9 THC increases the percentage area of calbindin-positive stained cells (calbindin+).

To determine Δ^9 THC's capacity to restore neuronal loss through improved neuronal survival and neurogenesis, a neuroimaging technique for detecting protein marker expression is necessary. The one used in this study is immunofluorescence labeling. The principle is based on the ability to individually excite fluorophores and distinguish their emissions in their spectra [24]. Immunofluorescence is widely used in both *in vivo* and *in vitro* animal models of AD that use cannabis-based treatments and has shown promising results in studying neurogenesis [25, 26, 27, 28]. However, there is no isotype control in this study, as it is designed only to determine or localize the targeted protein and to clearly differentiate the positive and negative populations, which is supported by a previous method, H&E staining. Even so, double blocking was used to help in refraining a non-specific background signal from the specific antibody signal. The photomicrographs below are representative of immunofluorescently stained pyramidal cells in the CA1 region of the hippocampus. Meanwhile, the intensified increase in immunofluorescent signal in the treated groups showed Δ^9 THC's ability to upregulate calbindin, a neurogenesis marker (figure 4).



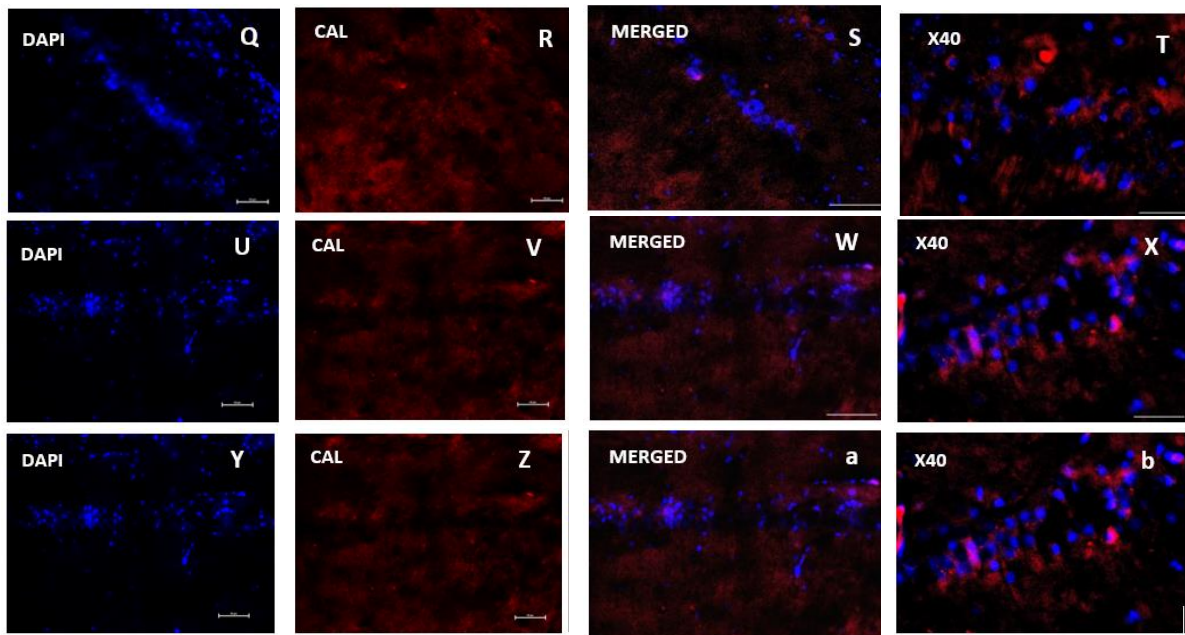


Figure 4. Representative photomicrographs of immunofluorescence for calbindin in (A-D) control; (E-H) AD model; (I-L) AD without treatment; (M-P) donepezil; (Q-T) 0.75 mg/kg Δ^9 THC; (U-X) 1.50 mg/kg Δ^9 THC; (Y-b) 3.0 mg/kg Δ^9 THC at 200x and 400x magnification, respectively. Merged photomicrographs consist of DAPI + calbindin at 200x magnification. The percentage area of calbindin-positive stained pyramidal cells at the CA1 region of a rat hippocampus has been quantified.

The results of the one-way ANOVA demonstrated a statistically significant difference in calbindin-positive cells [$F(6, 35) = 11.25, p < 0.001$]. A significant increase in the percentage area of calbindin+ cells was observed in the CA1 region of the hippocampus across all three Δ^9 THC treatment groups ($p > 0.001$) compared to the AD model and AD without treatment, reflecting how Δ^9 THC increases calbindin expression with increasing dosage. Δ^9 THC at all doses showed calbindin+ cells upregulation; 0.75 mg/kg Δ^9 THC (14.93 ± 1.17), 1.50 mg/kg Δ^9 THC (17.40 ± 1.84) and 3.0 mg/kg Δ^9 THC (19.05 ± 1.41) when compared to AD model (9.27 ± 0.35), AD without treatment (9.03 ± 0.52) and donepezil (11.49 ± 1.15) respectively. Rats exposed to D-gal and $AlCl_3$ (induced AD model) and the AD without treatment group showed a significant reduction in the percentage of calbindin+ immunoreactive area in pyramidal cells in CA1 compared to the control group ($p < 0.05$).

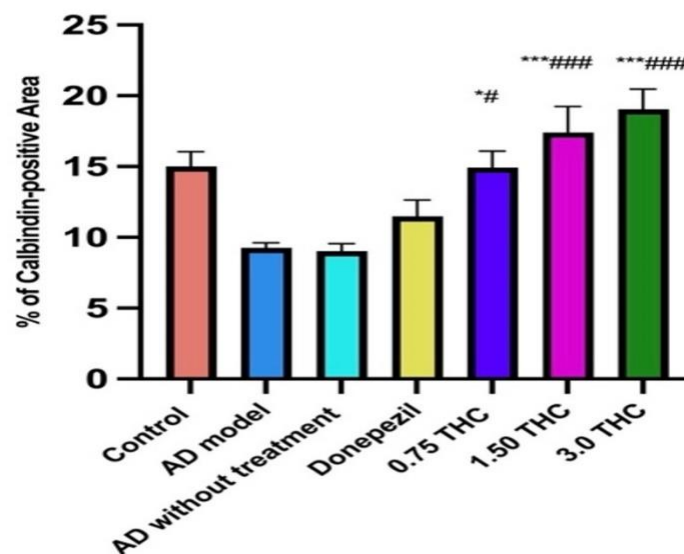


Figure 5. Mean percentage area of calbindin-positive cells in the pyramidal cells at the CA1 region. Data were expressed as mean \pm SEM (n=10). * $p < 0.05$ vs AD model, # $p < 0.05$ vs without treatment group, * $p < 0.05$ vs control.

Post hoc Tukey's test revealed that the donepezil or Δ^9 THC-treated group demonstrated a significant increase in calbindin+ immunoreactive area ($p < 0.001$) when compared to the AD model and AD without the treatment group, at which the mean percentage area of calbindin-positive area among pyramidal cells at the CA1 region in all the treated groups was quantified (Figure 5).

3.3.2. Δ^9 THC increases the fluorescence intensity of calbindin-positive stained cells (calbindin+).

The percentage area of positively stained cells differs from fluorescence intensity in that the mean fluorescence (or mean fluorescence intensity per pixel) is the total fluorescence divided by the number of pixels measured. Thus, it is proportional to the concentration [29]. This study aligns with this study and provides insights into the abundance of the neurogenesis protein marker in the pyramidal cells of the CA1 region. Meanwhile, calculating the percentage area of positive-stained cells, calbindin+, is useful for drawing a fine-line comparison between neuronal apoptosis and neuronal survival and for assessing how the rate of neuronal survival varies across the treatment groups. One-way ANOVA demonstrated that the fluorescence intensity of the calbindin+ immunoreactive area was significantly affected [$F(6,35) = 11.26, p < 0.001$]. Fluorescence intensity was significantly reduced in the AD model and the AD without treatment group ($p < 0.05$). However, across all three Δ^9 THC treatment groups, fluorescence intensity increased significantly. Across the treated drug groups, the fluorescence intensity of the calbindin+ immunoreactive area was significantly increased as follows: 0.75 mg/kg Δ^9 THC ($p < 0.05$), 1.50 mg/kg Δ^9 THC ($p < 0.001$), and 3.0 mg/kg Δ^9 THC ($p < 0.001$). The mean fluorescence intensity of calbindin+ area proven to be increased accordingly; donepezil (29.41 ± 2.90), 0.75 mg/kg Δ^9 THC (38.08 ± 2.98), 1.50 mg/kg Δ^9 THC (44.37 ± 4.70), 3.0 mg/kg Δ^9 THC (48.57 ± 3.59) respectively in comparison to AD model (23.64 ± 0.88) and AD without treatment (23.02 ± 1.32) respectively. Post hoc Tukey's test revealed that rats that were AD-induced in both the AD model (23.64 ± 0.88) and without treatment group (23.02 ± 1.32) demonstrated a significant decrease in the mean fluorescence intensity of calbindin+ immunoreactive area ($p < 0.05$) when compared to the control group (38.28 ± 2.65) (Figure 6).

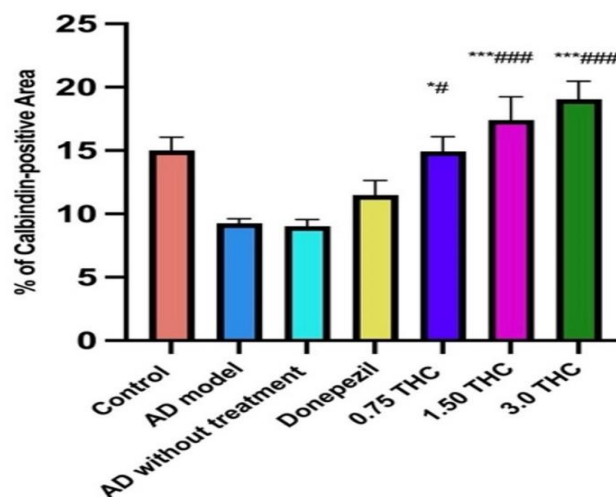


Figure 6. Mean fluorescence intensity of calbindin-positive area in pyramidal cells at the CA1 region. Data were expressed as mean \pm SEM ($n=10$). * $p < 0.05$ vs AD model, # $p < 0.05$ vs without treatment group, x $p < 0.05$ vs control.

4. Discussion

Alzheimer's disease (AD) is a common type of dementia. This broadly spreading neurodegenerative disorder is distinctively defined as the progression of loss of cognitive

functions to the extent of deterioration of intellect and disability. It is alarming, especially in an aging population, as it starts with mild cognitive malfunctions and progressively becomes worse over the years. This neurological disorder is characterized by the presence of plaques and neurofibrillary tangles in the brain, causing gradual degeneration and death of cortical cells [29]. The current FDA-approved pharmacotherapies that have a positive impact on the majority of people living with dementia (PLWD) are symptomatic and predominantly based on the cholinergic hypothesis that lies on the approach to inhibit enzymes cleaving the neurotransmitter acetylcholine (ACh), acetylcholinesterase (AChE), and butylcholinesterase (BChE) as treatments for AD [30]. However, upon clinical use of cholinergic inhibitor therapy, there is still a critical need to evaluate its efficiency in curing the cause of AD. This is because the major cause of cognitive decline is neuronal death, which is related to disturbance in the regulation of beta-amyloid (AB) peptides. In AD, neuronal survival depends on the regulation of AB production and clearance, which must be in equilibrium [31].

Beta amyloid, also known as A β , is a short peptide that is an abnormal proteolytic by-product of Amyloid Precursor Protein (APP). APP is a single-pass transmembrane protein that is expressed at high levels in the brain, mainly in neuronal and glial cells. A β fragments exist due to a set of enzymes that cleave this protein sequentially at different sites [32], forming a variety of fragments that are involved in synaptic plasticity, regulate the excitability of neurons, and protect neurons from oxidative and metabolic stress [33]. APP can be cleaved by two pathways: non-amyloidogenic via α -secretases and γ -secretases, or via β - and γ -secretases, producing several species of A β fragments. Neurotoxic forms of A β are formed by cleavage of APP initially by β -Site APP-cleaving enzyme 1 (BACE1), a major β -secretase in the brain [34], followed by cleavage via γ -secretase, which is carried out by an aspartyl proteolytic enzyme complex composed of presenilin 1 (PSEN1) and presenilin 2 (PSEN2). Mutations in the proteins alter γ -secretase activity and increase the A β ratio in early-onset forms of AD [35, 36]. The mutations will form aggregates and senile plaques in the brains of the AD patients. Accumulation of AB aggregates and plaques will hinder neural signal transmission, triggering a brain immune response that activates the inflammation cascade, which eventually damages neuronal cells and leads to apoptosis [37, 38].

Δ^9 THC can be used therapeutically, as it has analgesic, antiemetic, and anticonvulsant effects, which may be neuroprotective and anti-inflammatory via stimulation of the CB1 receptor [39]. This may be due to the nature of Δ^9 THC acting as a partial agonist at cannabinoid receptors, with a very high binding affinity for CB1 receptors that enables regulation of its psychoactive properties, such as changes in mood or consciousness, memory processing, and motor control [40]. Δ^9 THC was shown to interact effectively with AB peptides in an in vitro study, demonstrating dose-dependent anti-AB aggregation activity [41,42,43]. The mechanism lies in the fact that when Δ^9 THC stimulates the removal of intraneuronal A β , the inflammatory response is blocked by activating cannabinoid receptors, interrupting AB fibril formation and aggregation, and stimulating the removal of intracellular AB [44]. Δ^9 THC binds to the anionic region of the enzyme, a critical region for amyloidogenesis that competitively inhibits acetylcholinesterase and subsequently interacts with AB peptides, preventing their aggregation. In rat models, administration of Δ^9 THC improved neurogenesis and protected against cognitive damage induced by lipopolysaccharide neuroinflammation [45]. The inhibition of neurotoxicity induced by high levels of the excitatory neurotransmitter glutamate by the neuroprotective actions of Δ^9 THC is evidently shown in a cannabinoid receptor-independent manner. This shows that cannabinoid receptor antagonists did not disrupt the

mechanism of action of Δ^9 THC in modulating neurotoxicity and neuroinflammation, which may lead to neuronal apoptosis [46]. Thus, Δ^9 THC has shown strong therapeutic values in affecting the endocannabinoid system (ECS), specifically the CB1 receptor.

Findings from this study demonstrate that Δ^9 THC administration is potent in improving neuronal survival. Morphological observation in the CA1 region of the rats' hippocampus across multiple comparisons among all treatment groups demonstrated that neuronal viability increased with higher Δ^9 THC administration, specifically 3.0 mg/kg. A low dose of Δ^9 THC at 0.75 mg/kg has been shown to be insufficient to alleviate neuronal degeneration and restore brain function. On the contrary, there is no significant difference in proving the positive effects of self-recovery in AD. This is evident between Group 2 and Group 3, which are both AD models without treatment; however, Group 3 serves to investigate the potential for neuronal self-recovery in an AD model. The results showed that without treatment, AD progressively worsens, with fewer viable and more apoptotic pyramidal cells. A previous study by [47] found that prolonged exposure to Δ^9 THC at a dose of 3.0 mg/kg daily for 4 weeks improved cognitive performance in old mice, on par with that of young control mice. From this, it was evident that Δ^9 THC stimulates CB1 receptor, the main component of inhibitory synaptic feedback processes and synaptic plasticity [48]. However, Δ^9 THC as a monotherapy in treating AD has not been fully evaluated clinically. Despite the findings showing positive implications post-administration, there is a diminishing return of high doses, which is the psychoactive side effect of Δ^9 THC.

Interestingly, in another recent study [49], A β 1-42 upregulated genes that contribute to neuronal apoptosis were modulated by the neuroprotective role of a Δ^9 THC derivative, delta-8-tetrahydrocannabinol (Δ^8 THC). The pre-treatment of Δ^8 THC in an in vitro model of AD (retinoic acid-differentiated neuroblastoma SH-SY5Y cells treated with A β 1-42) has restored cell viability and reduced neuronal apoptosis, indicating that Δ^8 THC might be a potential neuroprotective agent in AD. The findings from this study also showed no statistically significant differences between donepezil and Δ^9 THC; both increased cell survival and reduced apoptotic cells; however, donepezil showed a lower effect than Δ^9 THC. These were shown through medium to high doses of Δ^9 THC administration. Hence, the results suggest that Δ^9 THC may have potent neurotherapeutic properties, improving cell survival rates and thereby benefiting the preservation of communication between adjacent neurons and signal transmission from one neuron to another, while also modulating inflammatory cascades that can cause apoptosis.

Donepezil is one of the current drugs that are available for AD treatment, and it is widely used commercially to treat mild to moderate stages of AD compared to past treatment, tacrine [50, 51]. Other cholinesterase inhibitors (ChEIs) primarily used for AD treatment include tacrine, donepezil, rivastigmine, galantamine, and memantine. Donepezil is a reversible acetylcholinesterase inhibitor and the main purpose of this drug is to use it as a symptomatic treatment whose main physiological function is to amplify the action of acetylcholine (ACh) by preventing its hydrolysis as acetylcholine is a direct indicator of cholinergic neurotransmission that plays essential role in cognitive functions in enhancing afferent input to cortical structures at which memory is encoded, increased levels of synaptic modifications and maintenance of novel information in cortical neurons [52,53]. However, pre-clinical studies in rats proved that Δ^9 THC is competitive in inhibiting acetylcholinesterase and diminishing acetylcholinesterase-induced A β aggregates on par with anti-dementia drugs like donepezil. Aside from the cholinesterase inhibitors, there is another drug that relieves the moderate to severe stage of AD,

which is memantine, an N-methyl-D-aspartic acid (NMDA) antagonist. N-methyl-D-aspartate receptor (NMDAR) is a calcium-gated channel that requires interaction with G protein-coupled receptors (GPCRs) to stimulate cellular signaling pathways and regulate one another [54, 55]. Reduced NMDAR activity compromises cell viability; hence, activation of CB1 receptors is crucial to re-establish the function of dysregulated NMDARs [56, 57].

However, the existing literature mostly reported combined treatments of Δ^9 THC and cannabidiol (CBD) rather than a synergistic effect of Δ^9 THC with donepezil [58]. There is a possibility that cannabinoids may provide a synergistic or enhanced effect among the other acetylcholinesterase inhibitors to confer neuroprotection. Results from a previous study have shown that a combination of donepezil and memantine as an AD treatment has positive effects on spatial memory in mouse models of AD. Unlike donepezil, memantine, to avoid neuronal excitotoxicity, binds preferentially to NMDA receptors, which regulate Ca^{2+} channels with low to moderate affinity, thereby preventing excessive influx of Ca^{2+} [59]. Prolonged excitotoxicity has been postulated to cause neuronal degeneration and the loss of functional synapses by overactivating NMDA receptors [60]. Based on both *in vivo* and *ex vivo* rodent studies, Δ^9 THC has been shown to activate the extracellular signal-regulated kinase (ERK) pathway and to depend on glutamate activity at NMDA receptors [61].

In addition, cannabinoids have the potential to alleviate the pathogenesis of AD by interrupting its underlying pathophysiological processes, such as inducing neurogenesis. Calbindin expression is upregulated in AD-induced rats in the treatment groups. The effects were impactful, and calbindin was expressed the most when a high dose of Δ^9 THC was administered. This was evident in this research, as Δ^9 THC significantly increased the percentage area of calbindin-positive cells and their mean fluorescence intensity. The upregulation of calbindin expression is indicated by the increasing percentage area of calbindin-positive stained cells and by the subsequent increase in the mean fluorescence intensity of calbindin-positive stained cells. Calbindin used in this study is one of the neurogenesis protein markers and is a promising candidate biomarker in the early stage of AD. Calbindin is vital for neurogenesis, as it is involved in synaptic integration and the development of functional, mature neurons during the last stage of neurogenesis [62]. As shown in the results, this was further supported by previous studies that correlated relevance for processing learning and memory with the presence of neurogenic sites and functional neuronal relations and integration during neurogenesis [63, 64]. Hence, if calbindin expression is upregulated, this indicates that a series of neurogenesis stages are being undergone to produce calbindin. Higher calbindin expression indicates stronger synaptic integration between neurons and their functional synaptic pathways, and vice versa. Based on the current results, a marked increase in the number of calbindin+ cells in Δ^9 THC treatment groups compared to the donepezil and no-treatment groups suggests that Δ^9 THC can reverse the neurodegenerative processes associated with AD, as evidenced by an increase in the survival cell count in morphology. The improved survivability of neuronal cells would lead to enhanced neuronal function and corresponding cognitive performance. Furthermore, calbindin correlates with learning and memory, as familial AD pathology has been reported to reflect depleted levels of calbindin in the brain [65]. Depletion of calbindin will elicit significant alterations in neuronal cell death pathways, synaptic transmission, and cytoskeleton organization, leading to apoptosis. The early neurodegenerative process of AD is accompanied by calcium dysregulation, neuronal death, and mitochondrial and synaptic dysfunction.

Calbindin is one of the major calcium-binding proteins that serve as a buffer for Ca²⁺ levels and as transporters of calcium-binding proteins, modulating its homeostasis. As in brain development, newly generated cells in the adult brain progress through a series of lineage commitment stages before adopting mature phenotype markers and demonstrating functional evidence [66]. Neurogenesis is a multi-step process that generates functional neurons, occurring in two main regions: the subventricular zone (SVZ), which contributes to olfactory neural circuitry and is limited to early childhood, and the subgranular zone (SGZ) of the hippocampus. In SGZ, stage 1 neurogenesis occurs when neural progenitors proliferate as radial glia-like cells (RGLs), commonly known as radial astrocytes or Type 1 cells, which express GFAP, nestin, and SOX2 and later give rise to intermediate progenitors, which eventually generate neuroblasts [67]. Stage 2 is responsible for differentiation into intermediate progenitors or Type 2 cells, which are highly proliferative and express doublecortin (DCX) and the polysialylated neural cell adhesion molecule (PSA-NCAM). Next, in stage 3, neuroblasts (Type 3 cells) form, and immature neurons migrate and differentiate into granule cells in the hippocampus before they begin forming dendrites towards the cornu ammonis (CA). NeuN and calbindin are highly expressed when neurons are in their mature stage, facilitating synaptic integration of new neurons into the existing circuitry of mature neurons [68]. Thus, neurogenesis contributes to hippocampal learning and memory consolidation.

5. Conclusion

In conclusion, this research shows that Δ^9 THC is a potential neurotherapeutic agent that can be considered as an alternative pharmacotherapy for AD. Δ^9 THC acts against hippocampal alterations by improving cell survivability and promoting neurogenesis to develop new functional neurons and synaptic transmissions. In our study, reduced neuronal apoptosis and improved neuronal survival suggest an early indication that Δ^9 THC is effective against AD. The promotion of neurogenesis subsequently leads to increased calbindin expression in pyramidal cells in the rat hippocampus. Interestingly, levels of calbindin in the brain correlate with learning and memory during neurodevelopment. All these findings might represent an important aspect in modulating neuroprotective mechanisms and in developing new pharmacotherapies for AD targeting cognitive functions in learning and memory. This could be beneficial in providing scientific databases that may slow down and halt AD progression by lowering or preventing cognitive decline, which supports the neurodevelopment of interventions as a reliable approach for neurodegenerative diseases, specifically AD. However, while there was scientific evidence that highlighted Δ^9 THC being used in treating cognitive impairment of AD, it is still premature to conclude that Δ^9 THC has any effects on other major progressive dementia symptoms. On the contrary, administration of Δ^9 THC has resulted in improvements in pre-clinical AD rodent models, suggesting the need for deeper investigation to clarify its potential clinical utility, not limited to animal models but also to clinical evidence. More double-blind, randomized, placebo-controlled human trials are needed to evaluate the efficacy and safety of cannabinoids in a therapeutic context. The findings are a significant milestone for researchers to continue investigating potential modifications, leading to a promising therapeutic tool in the future. A few main challenges to take into account include: (1) the correct amount of dosage/concentration to be used; (2) duration of drug exposure; and (3) suitable analytical instrumentation that covers the major concerns when cannabinoids are to be determined. It is vital to address these concerns in a dose-dependent manner that may vary with age. Δ^9 THC might be effective in older age groups, but in young people it may have

opposing effects that could lead to cognitive impairments. Thus, the age range needs to be clearly defined before implementation in clinical settings. In addition, cannabis legalization is important to emphasize in future studies. With the current situation, there are still many countries that have ambiguous legislation towards regulated access for cannabis use in medical situations. Thus, there is no doubt that thorough investigations are needed to ascertain the potential benefits that compensate for the risks, both short-term and long-term adverse effects, which are poorly understood.

Author Contributions

Experimental methodology, A. A. M.; data collection, A. A. M. and F. N. Z.; data analysis, A. A. M. and F. N. Z.; result calculation, A. A. M. and F. N. Z.; formal analysis, R. D. and S. M. C.; original draft preparation, A. A. M.; writing manuscript, A. A. M.; experimental supervision, F. N. Z. and Z. A.; project administration, Z. A.; funding acquisition, F. N. Z. and M. A. M. M.; reviewing and editing, F. N. Z., S. M. C. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

The animal study protocol was conducted in accordance with approval from the Institutional Review Board of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, and the Animal Ethical Committee of Universiti Putra Malaysia (UPM/IACUC/AUP-R094/2019), with the approval date on 17th February 2020. All experimental protocols involving behavioral animal studies were conducted in the Animal Behavioural Laboratory at Universiti Putra Malaysia. The following laboratory treatments and data analysis were done in Universiti Teknologi MARA (Puncak Alam).

Informed Consent Statement

Not applicable. This research did not involve humans.

Data Availability Statement

The data obtained in this research are available from both main and corresponding authors upon reasonable request.

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Conflicts of Interest

The authors declare no conflict of interest.

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