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RESEARCH

Neuroprotective Mechanisms of *Ficus deltoidea* in an Alzheimer's Disease-Like Rat Model: Targeting Tau Hyperphosphorylation Through Glycogen Synthase Kinase-3 Beta and Protein Phosphatase 2A Regulation

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterised by memory loss, neurodegeneration, amyloid plaque accumulation and tau hyperphosphorylation. Dysregulation of glycogen synthase kinase-3β (GSK-3β) and protein phosphatase 2 A (PP2A) plays a pivotal role in tau pathology, contributing to synaptic dysfunction and memory impairment. Current AD medications offer limited palliative care, underscoring the need for multifaceted therapeutic strategies. *Ficus deltoidea* (FD), a medicinal plant renowned for its antioxidant and anti-inflammatory properties, has demonstrated neuroprotective effects, however, its specific role in modulating tau-associated proteins in AD remains underexplored. Thus, this study investigated the neuroprotective properties of FD on the spatial learning and memory, hippocampal histology and the levels of GSK-3β and PP2A in an AD-like rat model. Male rats were administered D-galactose (60 mg/kg) and aluminum chloride (200 mg/kg) for 11 weeks to induce AD-like characteristics. Rats were divided into six groups: control, AD model, donepezil-treated (1 mg/kg), and FD-treated groups receiving 50, 100 and 200 mg/kg of FD extract. Behavioural performances were assessed using the open field test (OFT) and modified elevated plus maze (mEPM). FD administration significantly improved spatial learning and memory in AD-like rats. Nissl staining revealed an increase in viable hippocampal granule neurons in FD-treated rats. Immunoblot analysis reported a reduction in GSK-3β and an increase in PP2A levels, suggesting reduced hippocampal tau phosphorylation. These findings indicate that FD confers neuroprotection by restoring the kinase-phosphatase balance, which in turn enhances hippocampal neuronal survival and memory, thereby supporting its potential as a phytotherapeutic agent for AD intervention.

Keywords Alzheimer's disease · *Ficus deltoidea* · Protein phosphatase 2A · Glycogen synthase kinase-3 beta · Tau · Neuroprotective

Introduction

Alzheimer's disease (AD) is a progressive neurological condition associated with neurodegeneration and the leading cause of dementia, characterised by memory loss, most commonly affecting the elderly population [1]. The progressive, irreversible degeneration of neurons is commonly described in AD, further impacting behavioural functions, including cognition and memory. About 60–80% of dementia cases are attributed to AD, making AD the most prevalent cause of dementia [2, 3]. Epidemiological studies reported that approximately 50 million individuals are diagnosed with AD worldwide currently and this figure is expected to increase to 152 million by 2050, which raises concerns about public health [4, 5]. In Malaysia, estimates indicate a high prevalence of dementia, approximately 8.5% among the older generations, with projections estimating 590,000 cases by 2050 [6, 7]. Statistics on the percentage

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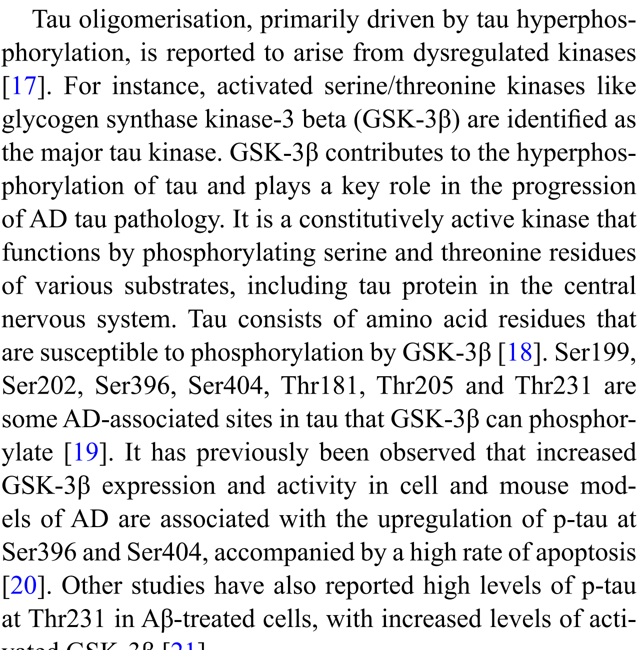
of the older population (65 years and above) showed an increase in this age group every year, from 6.7% in 2019 to 7.0% in 2020, and is estimated to reach 15% by 2030, thus marking Malaysia as an ageing nation. This is undoubtedly an additional concern as ageing is a risk factor for AD and the prevalence of dementia is set to rise as the worldwide population ages [7, 8].

Besides cognitive and memory loss, complications of AD include executive dysfunction, language impairment, personality changes, depletion in socialisation skills, physical disability and mental disorder symptoms [5, 7]. AD may progress from an asymptomatic phase to mild cognitive impairment, followed by mild AD dementia, moderate AD dementia and ultimately to the severe stage of AD dementia [3]. The defining hallmarks of AD, which were characterised as early as 1906 by Alois Alzheimer [9], are familiarly known as the formation and deposition of amyloid beta (Aβ) plaques and accumulation of neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau [10]. The Aβ peptides, which form senile plaques, are found extracellularly between neurons in the brain and are formed through the amyloidogenic pathway when the amyloid precursor protein (APP) undergoes abnormal sequential cleavage by β- and γ-secretases. Overproduction or decreased clearance of the toxic Aβ peptide aggregates leads to aberrant accumulation of Aβ plaques in the brain, subsequently triggering inflammation, oxidative stress and the formation of NFTs [4, 10–12].

The intracellular accumulation of NFTs is generated from the abnormally phosphorylated tau (p-tau) proteins in the neurons. From the transentorhinal and entorhinal regions of the AD brain, NFTs spread to the hippocampus and neocortex in the later stages of AD [13, 14]. The tau protein, also known as a microtubule-associated protein or axon-associated protein, binds to the microtubules in the axon and is involved in microtubule assembly, stabilisation, and axonal transport. Mutations or abnormal post-translational modifications, such as tau phosphorylation, can lead to conformational changes in tau, altering its affinity for microtubules and ultimately causing it to detach from the axonal microtubules. The disassembled tau further aggregates into paired helical filaments, leading to the accumulation and formation of NFTs, impairing synaptic function and neuronal integrity [15]. Synaptic loss in the hippocampus is well associated with cognitive and memory decline in individuals with AD. Besides, the soluble forms of tau oligomers, which are formed through binding between individual tau proteins prior to the NFT formation, are more neurotoxic and could be transmitted between neurons, increasing neuroinflammation, apoptosis, neurodegeneration, neuronal dysfunction and cognitive disability [16, 17].



Tau oligomerisation, primarily driven by tau hyperphosphorylation, is reported to arise from dysregulated kinases [17]. For instance, activated serine/threonine kinases like glycogen synthase kinase-3 beta (GSK-3β) are identified as the major tau kinase. GSK-3β contributes to the hyperphosphorylation of tau and plays a key role in the progression of AD tau pathology. It is a constitutively active kinase that functions by phosphorylating serine and threonine residues of various substrates, including tau protein in the central nervous system. Tau consists of amino acid residues that are susceptible to phosphorylation by GSK-3β [18]. Ser199, Ser202, Ser396, Ser404, Thr181, Thr205 and Thr231 are some AD-associated sites in tau that GSK-3β can phosphorylate [19]. It has previously been observed that increased GSK-3β expression and activity in cell and mouse models of AD are associated with the upregulation of p-tau at Ser396 and Ser404, accompanied by a high rate of apoptosis [20]. Other studies have also reported high levels of p-tau at Thr231 in Aβ-treated cells, with increased levels of activated GSK-3β [21].

Irregular phosphatase activity also significantly contributes to abnormal levels of p-tau in the AD brain. Primary tau phosphatase, for instance, protein phosphatase 2 A (PP2A), is responsible for 70% of tau dephosphorylation in the brain [22]. PP2A is a widely expressed, ubiquitous and conserved enzyme that originates from the serine/threonine phosphatase family [23, 24]. Hassan et al. have stated that PP2A dysfunction is present in neurodegenerative diseases and is one of the causal factors leading to tau pathology in AD [24]. According to Theendakara et al., the total PP2A activity was decreased in cortical and hippocampal brain homogenates of AD [25]. In a study using a D-galactose (D-gal) and aluminium chloride ($AlCl_3$)-induced AD rat model, PP2A activity was decreased in the hippocampus, leading to increased levels of p-tau. Besides, there was an increased level of GSK-3β expression in these AD-induced rats, which further contributed to the tau hyperphosphorylation [26, 27]. Cell models exposed to $AlCl_3$, characterised by elevated p-tau levels, exhibited reduced levels of PP2A and high levels of GSK-3β [28]. Taken together, research evidence has shown that an imbalance in the tau kinase and tau phosphatase activities can result in hyperphosphorylation of tau, leading to its aggregation [29, 30].

For decades, researchers have sought to develop a suitable in vivo AD model to study the pathological mechanisms of AD. A non-transgenic rat model of AD developed through the co-administration of D-gal and $AlCl_3$ is a practical and inexpensive technique for gaining deeper insights into AD pathogenesis and exploring potential therapeutic targets. D-gal, commonly known as reducing sugar, functions by reacting with free amines on amino acids in proteins, producing advanced glycation end-products [26].

Aluminium (Al) is one of the most common and abundant elements found on Earth [28]. Both D-gal and $AlCl_3$ are neurotoxins that cause toxic effects on the brain [26]. The conventional therapy developed for AD primarily focuses on pharmacological interventions that alleviate symptoms and slow disease progression [31]. They include medications like memantine and acetylcholinesterase inhibitors (AChEIs), namely rivastigmine, donepezil and galantamine [32]. Unfortunately, these drugs do not provide long-term effects by altering the fundamental neurodegenerative processes [33]. A monoclonal antibody drug, aducanumab, was also approved by the Food and Drug Administration (FDA) as the first disease-modifying therapy targeting Aβ plaques [34]. However, the clinical efficacy and safety of aducanumab remain a matter of debate [1, 35, 36]. Thus, developing effective alternative therapies that can function through different pathogenesis pathways associated with AD is highly crucial. Natural products have been predicted as a promising alternative to synthetic drugs for AD since they possess lower toxicity and fewer side effects. Recent experimental therapies, including both in vitro and in vivo studies, have been extensively conducted to investigate the pharmacological properties of natural compounds [37, 38].

Mas cotek or mistletoe fig, scientifically known as *Ficus deltoidea* (FD), is an epiphytic shrub locally characterised for its traditional benefits. Besides Malaysia, FD is native to Southeast Asian nations, including Indonesia and Thailand [39]. Every component of the FD plant, including its leaves, stem, fruit and root, has traditional medicinal applications that have been proven to be due to the existence of phytochemicals in this plant. FD leaves serve as a postpartum tonic for uterine and vaginal muscular contractions [39–43]. The powdered FD roots and leaves were applied to wounds, sores and joints for relief from rheumatism, while its stem is beneficial in treating pneumonia and diabetes mellitus [39, 40, 44, 45]. FD encompasses various phytochemical compounds, particularly those derived from leaves and figs, which exhibit significant biological activities and contribute to their pharmacological properties. For instance, the leaves contain compounds from the flavonoid, phenol, saponin, tannin, terpene, and steroid classes [43, 46]. Pharmacological studies of FD have revealed that this plant has anti-inflammatory [42], antinociceptive [43], antioxidative [39], antidiabetic [47, 48], anticancer [49, 50] and wound-healing [51], properties.

Two bioactive flavonoids in FD, vitexin and isovitexin, have been shown to have neuroprotective effects. A literature search revealed few studies that showed that vitexin and isovitexin from FD leaves exhibit neuroprotective properties by suppressing pro-inflammatory proteins in microglial cells and attenuating brain oxidative stress in diabetic rats [45, 52]. Despite these studies, very little attention has been paid to the neuroprotective properties of FD against AD and its mechanistic role in AD, particularly in regulating proteins associated with tau phosphorylation. Since recent evidence has suggested that tau pathology is highly associated with the severity of AD and cognitive decline, it is necessary to discover alternative therapeutic targets for the development of anti-tau drugs to mitigate and slowly diminish tau progression [53]. Therefore, the present research explores, for the first time, the neuroprotective effects of FD on the levels of two tau-related proteins, GSK-3β and PP2A. This study also aimed to investigate whether GSK-3β and PP2A, therapeutic targets of FD, can reduce hippocampal neurodegeneration and enhance spatial learning and memory in an AD-like rat model.

Materials and Methods

Reagents and Chemicals

The reagents used for the drug administration for the rats include D-galactose (D-gal) and aluminium chloride ($AlCl_3$) from R&M Chemicals (Essex, United Kingdom), donepezil hydrochloride from European Pharmacopoeia (Strasbourg, France) and standardised ethanol extract powder of *Ficus deltoidea* (FD) leaves from Platinum Herbs Sendirian Berhad (Kuala Lumpur, Malaysia). The chemicals of analytical grade were purchased from Thermo Fisher Scientific (Waltham, MA, USA), Nacalai Tesque (Kyoto, Japan), and Bio-Rad (California, USA). Antibodies used in the Western blot were purchased from Abcam (Cambridge, United Kingdom).

Animals

The research obtained ethical approval from the Institutional Animal Care and Use Committee of Universiti Putra Malaysia (UPM) on August 2, 2022 (UPM/IACUC/AUP-R051/2022). The experimental procedures were conducted in accordance with the guidelines provided by the UPM Animal Ethics Committee. Seventy-two male Albino Wistar rats, 2–3 months old, weighing approximately 150–200 g, were purchased in batches from Takrif Bistari Enterprise. The rats were allowed to acclimate for one week under constant temperature (25 ± 2 °C) and a 12-hour light/dark cycle. They were given food and water ad libitum.

Experimental Design & Drug Administration

The rats were categorised into six groups ($n = 12$). After one week of acclimatisation, the rats were administered different treatments according to their assigned groups, as shown in

the experimental design illustrated in Fig. 1. A rat model of AD was developed by administering D-gal and $AlCl_3$ for 11 consecutive weeks. Donepezil and FD treatments were co-administered concurrently with D-gal and $AlCl_3$ throughout the 11-week period to represent a protective (co-treatment) approach. The donepezil treatment group serves as the positive control in this study. D-gal, $AlCl_3$ and donepezil were dissolved in distilled water, while the ethanol extract powder of FD leaves was dissolved in 0.9% normal saline before administering to the rats. The ethanol extract of FD was commercially obtained from a single production batch and standardised by the supplier according to the established phytochemical markers. High-performance liquid chromatography was employed by the supplier to quantify and standardise flavonoids (vitexin and isovitexin) in the FD extract to ensure consistency in the concentration of bioactive compounds throughout the extract.

The control group of rats was administered distilled water orally and 0.9% normal saline intraperitoneally. D-gal (60 mg/kg) was administered intraperitoneally, while $AlCl_3$ (200 mg/kg), donepezil (1 mg/kg) and FD (50 mg/kg, 100 mg/kg and 200 mg/kg) were administered orally to the rats. The doses for the chemicals and FD extract were determined based on prior studies conducted by Chiroma et al. and Fazliana et al., respectively [26, 54]. During the 9th to 10th week of the drug administration period, behavioural tests were performed using the open field test (OFT) and the modified elevated plus maze (mEPM) one hour after the drugs were administered. A brief interval occurred between the completion of behavioural testing and

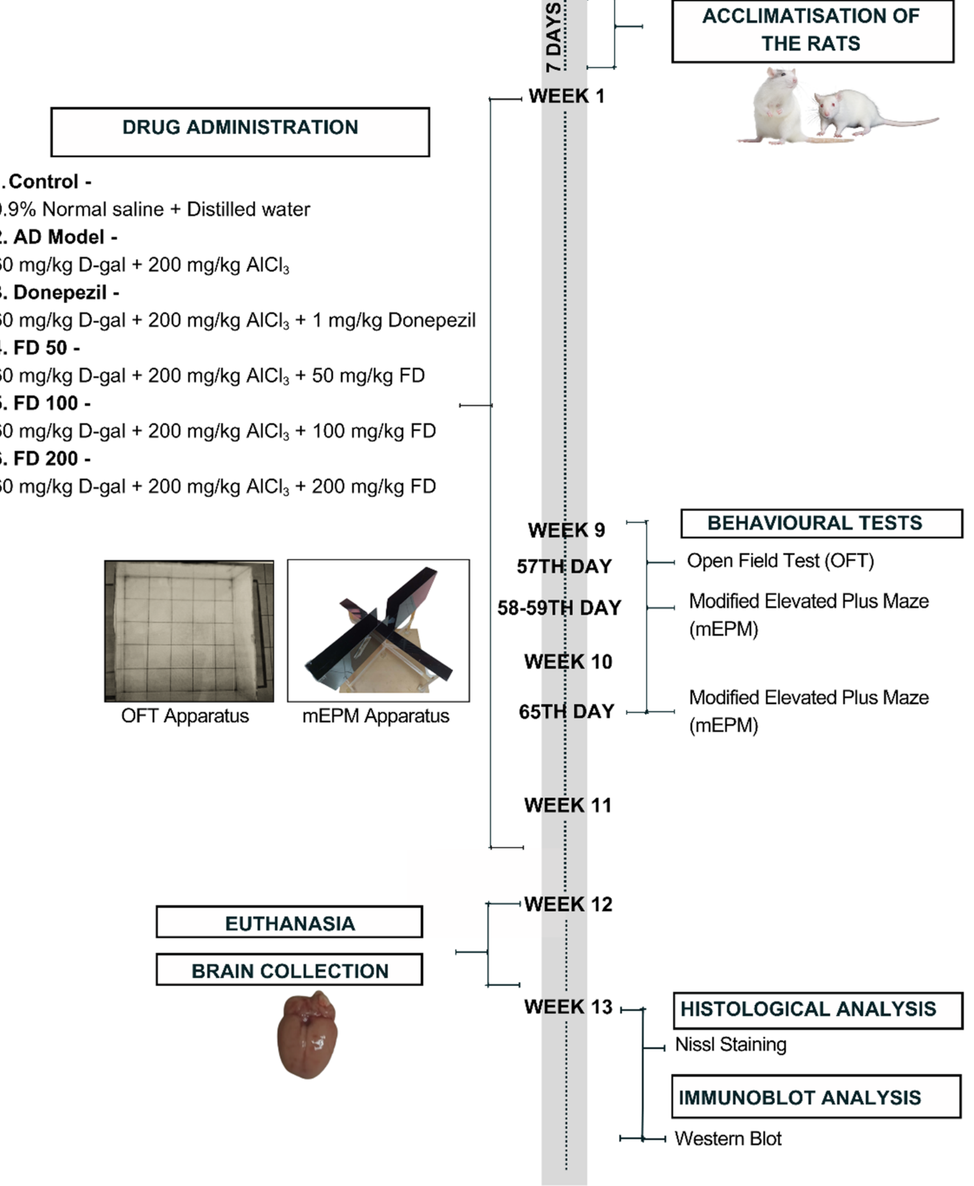


Fig. 1 Experimental timeline for evaluating the neuroprotective effects of *Ficus deltoidea* (FD) in a D-galactose (D-gal) and aluminium chloride ($AlCl_3$)-induced rat model of Alzheimer's disease (AD). Rats were acclimatised for 7 days and divided into six groups, including a control, AD Model, Donepezil and three FD-treated groups (50, 100 and 200 mg/kg). AD induction and treatment lasted for 11 weeks. The behavioural tests (OFT and mEPM) were performed in the 9th to the 10th week of the AD induction and treatment period. Brain tissues were collected after euthanasia on the 12th week for histological (Nissl staining) and immunoblot (Western blot) analyses

euthanasia due to additional imaging procedures performed at the end of the 11th week as part of a separate investigation. The drug administration was stopped one day before the imaging. The imaging outcomes are not reported in the present manuscript as they are currently unpublished and retained for future publication. On the 12th week, the rats were euthanised by decapitation to prevent the use of anaesthetic drugs and gases that could contaminate brain tissues. The brains were harvested and rinsed in ice-cold 1× phosphate-buffered saline before tissue fixation in 10% neutral buffered formalin for histological testing. The hippocampi were harvested from the rats' brains and stored at −80 °C for molecular studies.

Open Field Test

The locomotor functions of the rats were evaluated using the open-field test (OFT). The equipment utilised for conducting OFT consisted of a square plexiglass box with 75 cm in length, 75 cm in width and 40 cm in height. The box's base was partitioned into 25 smaller square sections. Following the acclimatisation process, the experiment commenced by placing a rat into the centre of the box and granting it unrestricted access to explore the empty box for 5 min. Upon each evaluation session, the box was cleaned with a 70% alcohol solution to avoid any olfactory cues for the subsequent rats. The rats' locomotion was monitored using a camera connected to a video-tracking system software (ANY-maze version 7.44, USA). The software then recorded the number of lines traversed by the rats within the enclosure and the velocity at which the rodents moved. This test was performed based on the methods used by Firdaus et al. and Chiroma et al. [55, 56].

Modified Elevated Plus Maze

To study the spatial learning and memory of the rodents, the rats were allowed to participate in a test known as the modified elevated plus maze (mEPM). A plexiglass device in a plus-shaped with two open arms and two enclosed arms was used for this test. Each of the arms has a length of 50 cm, a width of 10 cm, and a height of 40 cm for the enclosed ones. The plus maze device was elevated to a height of 50 cm above the ground level and all four arms were connected through a central square. On the first day of the test (acquisition session), the rats were gently placed on one end of the open arm facing away from the centre. Once the rats entered either of the enclosed arms, they were allowed to stay in the enclosed arm for 20 s. The time taken by each rat to enter either one of the enclosed arms (initial transfer latency, ITL) was tracked and recorded using the ANY-maze software. The retention session was conducted 24 h and 7 days after the acquisition session, in which the first transfer latency (TL1) and second transfer latency (TL2) were recorded, respectively. During the retention phase, the rats were again positioned at the open arm end before recording their transfer latency. The device was thoroughly cleaned with a 70% alcohol solution to prevent any olfactory cues for the subsequent rats. The mEPM was executed using similar methods detailed in the studies by Chiroma et al. and Yildiz Akar et al. [56, 57].

Nissl Staining

Nissl staining was performed to evaluate the extent of neurodegeneration and quantify the total number of viable granule neurons in the dentate gyrus (DG) subregion of the rat hippocampus. The brain tissues collected were fixed in 10% neutral buffered formalin and desiccated using a tissue processing machine. The processed brain tissues were subsequently embedded in paraffin blocks and sectioned to obtain 5 µm coronal sections of the tissue. The brain tissue sections were first deparaffinised in xylene. Subsequently, the sections were rehydrated in a series of alcohol solutions with decreasing concentrations, ranging from 95% to 70% alcohol. After that, the sections were stained in a 0.1% cresyl violet acetate solution, followed by washing, dehydration, clearing and mounting with dibutyl phthalate xylene (DPX) medium. The stained sections were viewed using a brightfield microscope (Olympus BX51TRF-CCD) at magnifications of 100× and 400×. The morphology of the DG subregion was initially examined to identify any histological signs of neurodegeneration. The total number of viable granule neurons in the mid-portion of the DG subregion was quantified at 400× magnification using ImageJ software (version 1.54 g, Java 1.8.0_345; NIH, USA) for three rats per group. For each rat, three hippocampal sections were selected for analysis. Neuronal cell quantification was performed in a blinded manner to minimise potential observer bias. The protocol adhered to the procedures employed by Mahdi et al., with certain adjustments applied [58].

Preparation of Hippocampal Tissue Homogenates and Protein Quantification

The hippocampal tissue was supplemented with 1× lysis buffer and phosphatase inhibitor. The lysis buffer was prepared by diluting 10× radioimmunoprecipitation (RIPA) buffer containing protease inhibitors and sodium dodecyl sulfate (SDS) with deionised H_2O. The tissue was then homogenised using an electric homogeniser and centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant produced after centrifugation was collected for further protein quantification and immunoblot analysis. The total protein

concentration in the hippocampal tissues was determined using the Pierce BCA Protein Assay kit. After quantifying the total protein in the tissue samples, 20 µg of protein samples were diluted in 2× Laemmli sample buffer supplemented with β-mercaptoethanol in a ratio of 950:50 µL. The samples were vortexed and heated in a water bath at 95 °C for 5 min [59].

Western Blot

The Western blot technique was applied to determine the levels of GSK-3β and PP2A in the hippocampus. Gel electrophoresis was initiated by preparing a 10% resolving gel and a 4% stacking gel for the separation of proteins (20 µg per sample). Once the proteins were separated in the gel, they were transferred to a 0.45 µm polyvinylidene fluoride (PVDF) membrane (1 h, 100 V). The protein transfer to the membrane was confirmed by performing Ponceau S staining. The membrane was incubated with 5% non-fat dry milk for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies against GSK-3β (1:1000), PP2A alpha + beta (1:1000), and β-actin (1:10,000). After washing with Tris-buffered saline-Tween 20 (TBST), the membrane was probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:20,000) for 1 h at room temperature. The membrane was incubated with WesternBright™ Sirius chemiluminescent HRP substrate before being visualized. The images of the protein bands on the membrane were captured and viewed via a Gel Documentation machine. Further analysis of the protein band expression levels was performed using ImageJ software. The loading control protein (β-actin) levels were used to normalize the GSK-3β and PP2A protein levels. The protocol was followed as outlined in the Western blot protocol by Abcam, with specific modifications applied [59].

Statistical Analysis

Statistical analyses were performed using one-way and two-way analysis of variance (ANOVA) to evaluate behavioural test results, depending on the experimental design. One-way ANOVA was used to analyse Nissl staining and Western blot data. After the ANOVA results were determined, Tukey's post hoc test was used to make subsequent comparisons between the different rat groups. A difference of $p < 0.05$ was reported as statistically significant between the groups and all data were presented as mean ± SEM. All the analyses were performed using the statistical analysis software GraphPad Prism (version 9.3.1; MA, USA).

Results

Effects of *Ficus deltoidea* on Locomotor Function in D-gal and $AlCl_3$-induced Rats: Open Field Test

OFT was conducted to determine the rats' locomotor functions after the FD administration to the D-gal and $AlCl_3$-induced AD rats. The parameters measured in this test were the number of lines crossed by the rats and their speed during their exploration time in the open field box. The number of lines crossed by the rats was then analysed by one-way ANOVA, which showed statistically significant differences between the different groups of the rats [F (5, 30) = 10.23, $p < 0.0001$]. Tukey's post hoc test was performed to determine whether the number of lines crossed by the AD model group differed significantly from the other groups. The results of this test confirmed that there is a significant decrease in the number of lines crossed by the D-gal and $AlCl_3$-induced AD model group (25.23 ± 2.536) compared to the control (42.43 ± 2.247, $p < 0.0001$), donepezil (43.80 ± 1.508, $p < 0.0001$), FD 50 mg/kg (41.33 ± 2.837, $p = 0.0001$), FD 100 mg/kg (38.57 ± 1.174, $p = 0.0014$) and FD 200 mg/kg (39.97 ± 1.942, $p = 0.0004$), groups [Fig. 2 (A)].

Additionally, the rats' speed was also analysed by one-way ANOVA and the result successfully reported a statistically significant difference [F (5, 30) = 4.893, $p = 0.002$] between the different cohorts of the rats. Tukey's post hoc test revealed that there is a significant decrease in the speed of the D-gal and $AlCl_3$-induced AD model group (6.683 ± 0.6760) compared to the control group (10.28 ± 0.6150, $p = 0.003$). The rats from the FD 50 mg/kg (10.28 ± 0.2880, $p = 0.003$), FD 100 mg/kg (9.650 ± 0.5602, $p = 0.021$) and FD 200 mg/kg (9.867 ± 0.5970, $p = 0.011$) groups exhibit significantly higher speed when compared with the D-gal and $AlCl_3$-induced AD model group [Fig. 2 (B)]. Since the FD-treated rats significantly crossed more lines at a higher speed in the open field box, these results indicated better locomotion among these FD-treated rats, unlike the D-gal and $AlCl_3$-induced AD model rats.

Effects of *Ficus deltoidea* on the Spatial Learning and Memory of D-gal and $AlCl_3$-induced Rats: Modified Elevated Plus Maze

The mEPM test was conducted to assess the effects of FD on rats' spatial learning and memory. The experiment lasted three days and the rats' time to enter the enclosed arm was measured and analysed using one-way ANOVA. On the initial day of the test, the one-way ANOVA result showed a statistically significant difference in the initial transfer latency (ITL) (time taken to enter the enclosed arm) between

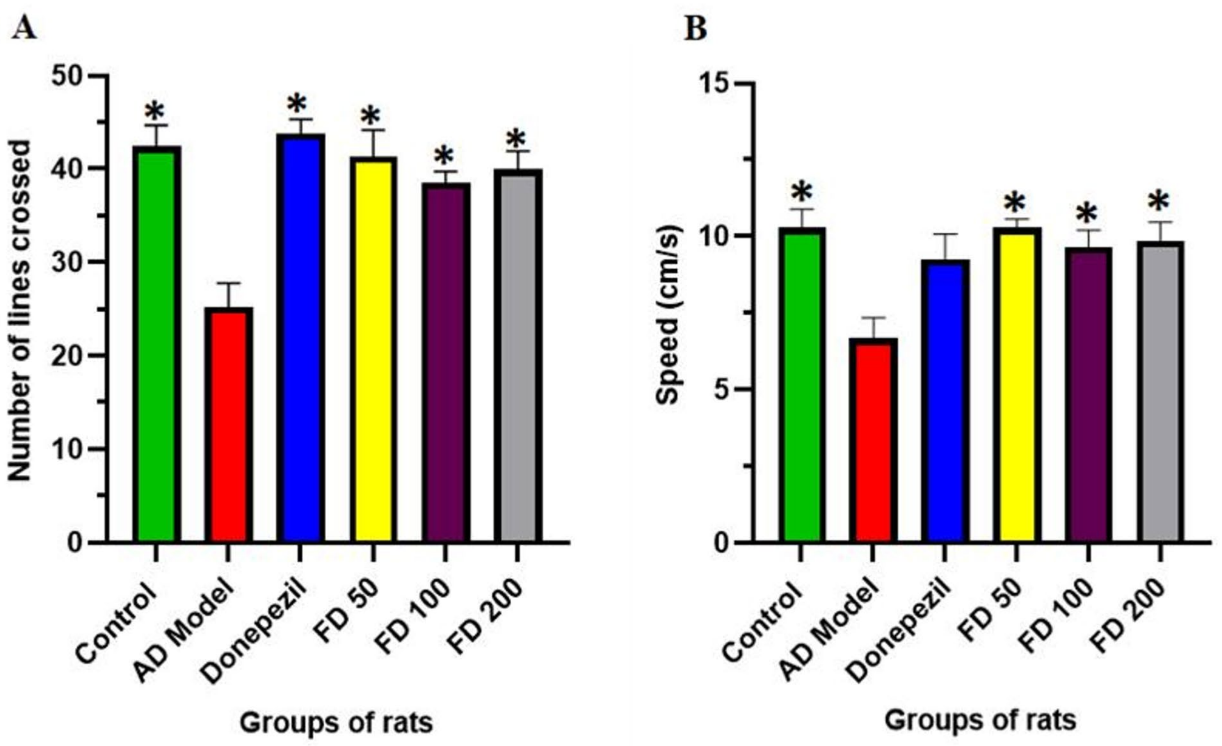


Fig. 2 The effects of FD on the locomotor function in D-gal and $AlCl_3$-induced AD rats in the OFT. **A** Graph representing the number of lines crossed by the different groups of rats in the open field box. **B** Graph representing the speed of the rats during their exploration time in the open field box. Data were expressed as mean ± SEM ($n=6$). * signified $p<0.05$ in comparison with the AD model group. [*Control (Normal saline + distilled water)*, *AD Model (60 mg/kg D-gal + 200 mg/kg $AlCl_3$)*, *Donepezil (60 mg/kg D-gal + 200 mg/kg $AlCl_3$ + 1 mg/kg donepezil)*, *FD 50 (60 mg/kg D-gal + 200 mg/kg $AlCl_3$ + 50 mg/kg FD)*, *FD 100 (60 mg/kg D-gal + 200 mg/kg $AlCl_3$ + 100 mg/kg FD) and FD 200 (60 mg/kg D-gal + 200 mg/kg $AlCl_3$ + 200 mg/kg FD)*]

the different groups of rats [$F(5, 30)=8.519$, $p<0.0001$]. Tukey's post hoc test further confirmed that there is a significant increase in the ITL of the D-gal and $AlCl_3$-induced AD model group (61.47 ± 9.642) than the control (27.78 ± 3.849, $p=0.0021$), FD 50 mg/kg (17.72 ± 2.588, $p<0.0001$), FD 100 mg/kg (26.47 ± 1.573, $p=0.0013$) and FD 200 mg/kg (31.42 ± 3.639, $p<0.0072$), groups [Fig. 3 (A)].

On the second day of the test, the results also reported a statistically significant difference in the transfer latency 1 (TL1) (time taken to enter the enclosed arm after 24 h) between the different groups of rats [$F(5, 30)=13.09$, $p<0.0001$]. Similarly, Tukey's post hoc test demonstrated that the D-gal and $AlCl_3$-induced AD model group (26.28 ± 1.648) revealed a significantly longer TL1 than the control (17.380 ± 1.859, $p=0.0065$), donepezil (18.500 ± 1.379, $p=0.0221$), FD 50 mg/kg (14.720 ± 1.303, $p=0.0003$), FD 100 mg/kg (12.320 ± 1.505, $p<0.0001$) and FD 200 mg/kg (9.317 ± 1.949, $p<0.0001$), groups [Fig. 3 (B)].

After 7 days, the test was repeated to measure how well the rats remembered the enclosed arm area. All the rat groups reported a statistically significant difference in the transfer latency 2 (TL2) (time taken to enter the enclosed arm after 7 days) based on one-way ANOVA [$F(5, 30)=33.82$, $p<0.0001$]. Tukey's post hoc test affirmed that the D-gal and $AlCl_3$-induced AD model group (26.97 ± 1.967) again showed a significantly longer TL2 compared to the control group (13.03 ± 1.394, $p<0.0001$). Meanwhile, donepezil (12.60 ± 1.027, $p<0.0001$), FD 50 mg/kg (8.683 ± 0.5873, $p<0.0001$), FD 100 mg/kg (8.400 ± 0.6445, $p<0.0001$) and FD 200 mg/kg (10.18 ± 0.9871, $p<0.0001$), groups showed significant decrease in their TL2 than the AD model group [Fig. 3 (C)]. The TL2 measurement in D-gal and $AlCl_3$-induced AD rats is slightly higher than their TL1 reading. In contrast, rats from the other groups displayed a declining trend in TL2 after 7 days following the TL1 assessment on the second day of the test.

The detailed results of the analysis above showed that the D-gal and $AlCl_3$-induced AD model rats demonstrated decreased spatial learning and memory, as they took longer to learn and remember the location of the enclosed arm. However, administration of FD at 50, 100 and 200 mg/kg

Fig. 3 The effects of FD on the spatial learning and memory of the D-gal and $AlCl_3$-induced AD rats in the mEPM test. **A** Graph representing the initial transfer latency (ITL) of the different groups of rats on the first day of the test. **B** Graph representing the transfer latency 1 (TL1) of the different groups of rats after 24 h of the first test. **C** Graph representing the transfer latency 2 (TL2) of the different groups of rats after 7 days from the initial day of the test. Data were expressed as mean ± SEM ($n = 6$). * signified $p < 0.05$ in comparison with the AD model group. **D** The representative images of track plots recorded by the ANY-maze software during the retention session on the 8th day of the mEPM test. The AD model group's trajectory is longer since they spent more time in the centre of the apparatus before entering the enclosed arm, reflecting an increased transfer latency than the FD-treated groups. The FD-treated groups took a shorter and direct route to enter the enclosed arm, similar to the control and donepezil group, indicating a shortened transfer latency. [*Control (Normal saline + distilled water), AD Model (60 mg/kg D-gal + 200 mg/kg $AlCl_3$), Donepezil (60 mg/kg D-gal + 200 mg/kg $AlCl_3$ + 1 mg/kg donepezil), FD 50 (60 mg/kg D-gal + 200 mg/kg $AlCl_3$ + 50 mg/kg FD), FD 100 (60 mg/kg D-gal + 200 mg/kg $AlCl_3$ + 100 mg/kg FD) and FD 200 (60 mg/kg D-gal + 200 mg/kg $AlCl_3$ + 200 mg/kg FD)*]

improved spatial learning and memory. The track plots of the rats recorded by the ANY-maze software during the retention session on the 8th day of the mEPM test are depicted in Fig. 3 (D). Since the D-gal and $AlCl_3$-induced AD rats showed increased TL2, their trajectory to the enclosed arm shown in their track plot is longer. Unlike the AD rats, the FD-treated rats took a shorter and more direct route to enter the enclosed arm, similar to the control and donepezil groups, indicating a shorter TL2.

Effects of *Ficus deltoidea* on the Number of Viable Granule Neurons and Level of Neurodegeneration in the Dentate Gyrus Hippocampal Subregion in the D-gal and $AlCl_3$-induced Rats: Nissl Staining

The DG subregion of the hippocampus was examined using Nissl staining to quantify the overall population of viable granular neurons. The extent of neuronal degeneration in

the DG subregion was also studied. The results evidently showed that the control group of rats displayed densely arranged granular neurons marked by intact nuclear membranes and distinguishable nucleoli. However, the D-gal and $AlCl_3$-induced AD model rats showed only a thin layer of granular neurons, partly shrunken and darkly stained neurons, indicating significant neuronal loss. Meanwhile, the administration of FD at 100 mg/kg and 200 mg/kg yielded positive results, as the rats showed numerous live and healthy granular neurons arranged in thick layers, with only a few degenerated neurons. Figure 4 below shows the photomicrographs of the DG subregion in the different rat groups.

These histological observations were supported by one-way ANOVA results, which showed statistically significant differences in the total number of viable granular neurons across groups [F (5, 12) = 3.842, p = 0.0261]. Tukey's post hoc test further reported a significant decrease in the total number of viable granular neurons, specifically in the AD model group (81.00 ± 6.658) than in the control (206.70 ± 12.02, p = 0.0483), FD 100 mg/kg (218.00 ± 14.57, p = 0.0289) and FD 200 mg/kg (210.00 ± 5.774, p = 0.0416), groups. Although no significant changes were observed in the donepezil and low-dose FD (50 mg/kg) groups, the total number of viable granular neurons in these groups showed an increasing trend compared with the AD model group (Fig. 5).

Effects of *Ficus deltoidea* on the Levels of GSK-3β and PP2A in the D-gal and $AlCl_3$-induced Rats: Western Blot

An immunoblotting approach was used to assess the impact of FD on the levels of tau-related proteins, GSK-3β and PP2A, in the hippocampus of rats induced with D-gal and $AlCl_3$. One-way ANOVA was used to analyse the levels of each protein. Analysis of the level of GSK-3β protein revealed a statistically significant difference [F (5, 18) = 6.178, p = 0.0017] between groups. A significant increase in GSK-3β levels was observed in the hippocampus of D-gal and $AlCl_3$-induced AD model rats (1.743 ± 0.240) compared with control rats (1.000 ± 0, p = 0.0451). There is significant reduction in GSK-3β levels exhibited in the hippocampus of the FD 50 mg/kg (0.942 ± 0.136, p = 0.0272), FD 100 mg/kg (0.697 ± 0.112, p = 0.0029) and FD 200 mg/kg (0.720 ± 0.220, p = 0.0036) rats compared to the AD

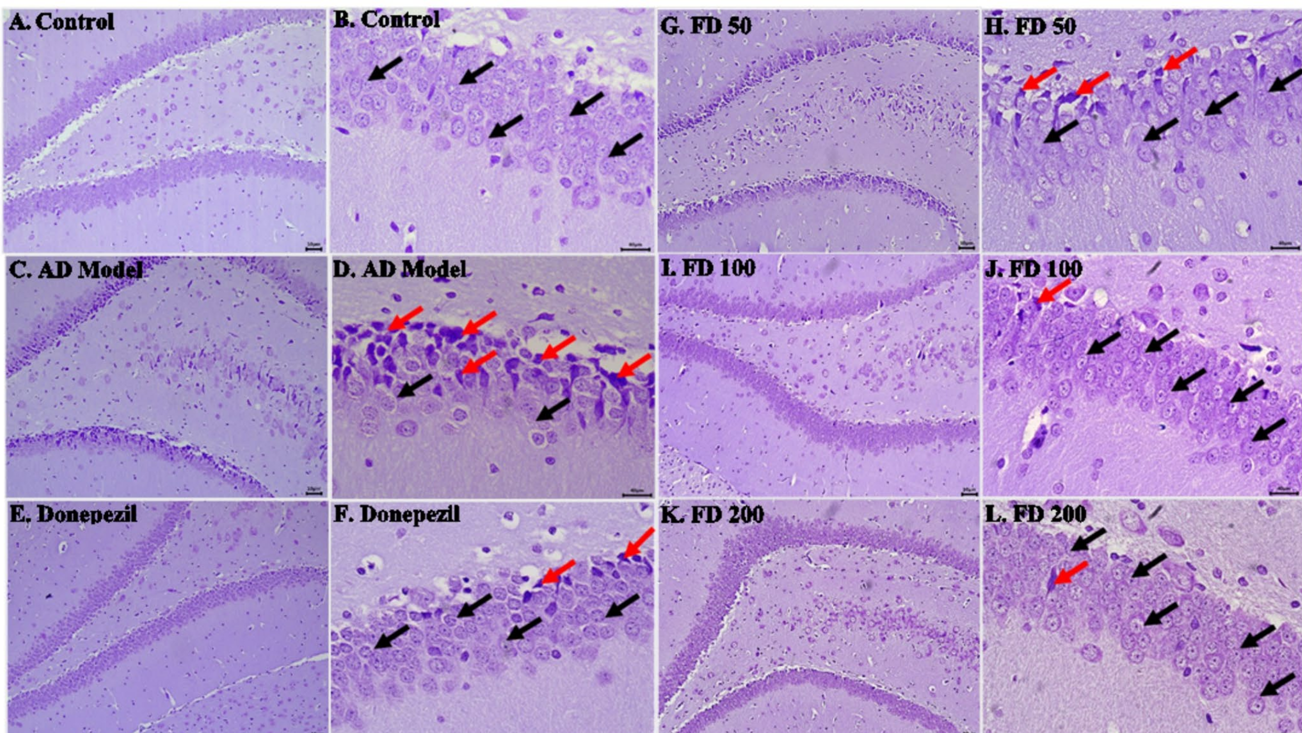


Fig. 4 Representative photomicrographs of the granule cells in the DG subregion of the hippocampus in the different groups of rats. A, C, E, G, I and K were taken at a magnification of 100×, while B, D, F, H, J and L were taken at a magnification of 400×. (**B**) Control, (**F**) Donepezil, (**H**) FD 50, (**J**) FD 100 and (**L**) FD 200 groups have numerous live and healthy granular cells arranged in thick layers. (**D**) The AD model group showed more distorted and degenerated granular cells than viable healthy cells. Only a few deteriorated cells were found in the (**F**) Donepezil, (**H**) FD 50, (**J**) FD 100 and (**L**) FD 200 groups. The black arrows show normal, viable and healthy granular neurons, while the red arrows show degenerated neurons. **A** and **B**: Control; **C** and **D**: AD model; **E** and **F**: Donepezil; **G** and **H**: FD 50; **I** and **J**: FD 100; **K** and **L**: FD 200, groups

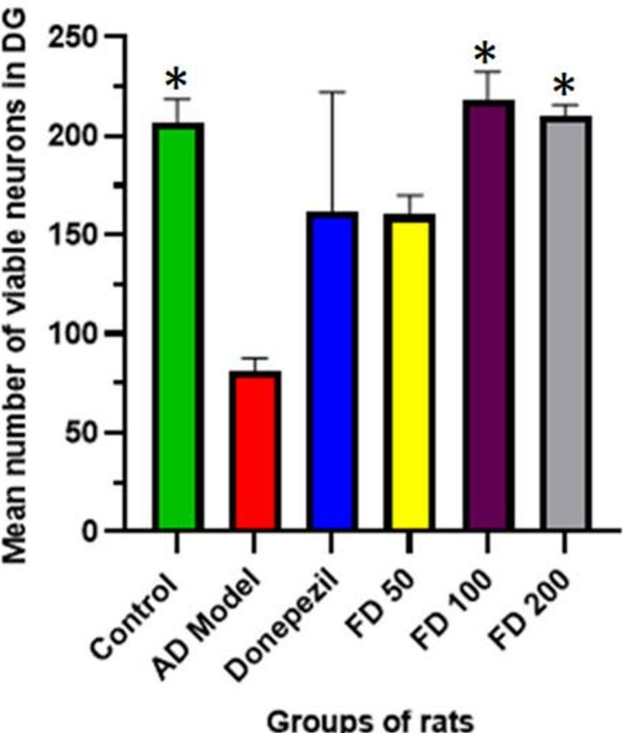


Fig. 5 The effects of FD on the number of viable granule neurons in the hippocampal DG subregion in the D-gal and $AlCl_3$-induced AD rats. The graph above represents the mean number of viable granular neurons in the DG subregion across different groups of rats. Data were expressed as mean ± SEM ($n = 3$). * refers to $p < 0.05$ compared to the AD model group. [*Control (Normal saline + distilled water), AD Model (60 mg/kg D-gal + 200 mg/kg $AlCl_3$), Donepezil (60 mg/kg D-gal + 200 mg/kg $AlCl_3$ + 1 mg/kg donepezil), FD 50 (60 mg/kg D-gal + 200 mg/kg $AlCl_3$ + 50 mg/kg FD), FD 100 (60 mg/kg D-gal + 200 mg/kg $AlCl_3$ + 100 mg/kg FD) and FD 200 (60 mg/kg D-gal + 200 mg/kg $AlCl_3$ + 200 mg/kg FD)*]

model rats (1.743 ± 0.240). The donepezil-treated rats (1.356 ± 0.146) also exhibit lower GSK-3β levels than the AD model rats, although no significant difference was observed between the groups.

In addition, PP2A levels showed a statistically significant difference [F (5, 18) = 8.826, $p = 0.0002$] among the groups. Tukey's post hoc test indicated that the AD rats induced with D-gal and $AlCl_3$ (0.647 ± 0.040, $p = 0.0373$) exhibit significantly lower levels of PP2A in the hippocampus than the control rats (1.000 ± 0). There is significant increase in the PP2A levels in the hippocampus of rats treated with donepezil (1.055 ± 0.107, $p = 0.0128$), FD 50 mg/kg (1.228 ± 0.120, $p = 0.0004$), FD 100 mg/kg (1.138 ± 0.061, $p = 0.0024$) and FD 200 mg/kg (1.260 ± 0.051, $p = 0.0002$) compared to the AD rats induced with D-gal and $AlCl_3$ (0.647 ± 0.040). The images of the protein bands and the levels of each protein are illustrated in Fig. 6. The complete image of the blot, which includes the molecular weight marker lane and protein bands, is presented in the supplementary material (Online Resource 1).

Discussion

AD is an irreversible, progressive neurodegenerative disease characterised by impaired memory function in affected individuals [3]. Although commercial drugs provide symptomatic alleviation for AD, researchers have given considerable attention to alternative therapeutic strategies utilising natural products that may halt disease progression [37]. FD, a traditionally recognised plant, has been extensively studied for its phytochemical compounds, as these compounds abundantly exhibit pharmacological properties such as antioxidative, anti-inflammatory, and anti-diabetic benefits [45, 60]. Research on the properties of FD that could effectively hinder AD progression, particularly tau pathology, is still uncertain. Thus, in this study, the neuroprotective effects of FD on AD have been investigated by assessing the spatial learning and memory, histological changes in the hippocampal subregion and the levels of tau-related proteins in D-gal and $AlCl_3$-induced rat models of the disease. Overall, the findings of this study suggest that FD significantly ameliorates spatial learning and memory, attenuates neurodegeneration and increases the number of viable granule neurons in the DG subregion of D-gal and $AlCl_3$-induced AD rats. It was also confirmed that FD administration resulted in a decline in the level of GSK-3β and an upsurge in the level of PP2A in D-gal and $AlCl_3$-induced AD rats.

The initial phase of this study focused on conducting neurobehavioural assessments for the rats. Firstly, OFT was conducted to assess the locomotion capabilities of the rat models before they were assessed for their learning and memory behaviour. Motor impairment is a prevalent sign in the early occurrence of AD [61] and has been discovered in AD patients as well as in vivo studies. Previous studies on the transgenic mouse model of AD reported deterioration in motor function, as evidenced by reduced movement, decreased exploratory activity, and gait alterations [62]. These results are consistent with clinical studies, which also found similar motor behavioural deficits. According to a preliminary study by Pottorf et al., the locomotor adaptation magnitude, for instance, the ability to adjust stepping movements during split-belt treadmill walking, was evaluated to determine whether it is an influencing factor in high fall risk and cognitive decline in older adults with AD. Their findings indicated that individuals with AD experienced decreased gait speed and increased risk of falling, indicating that those with greater cognitive impairment demonstrated poor locomotor adaptation [63]. This study by Pottorf et al. asserted a significant correlation between locomotor performance

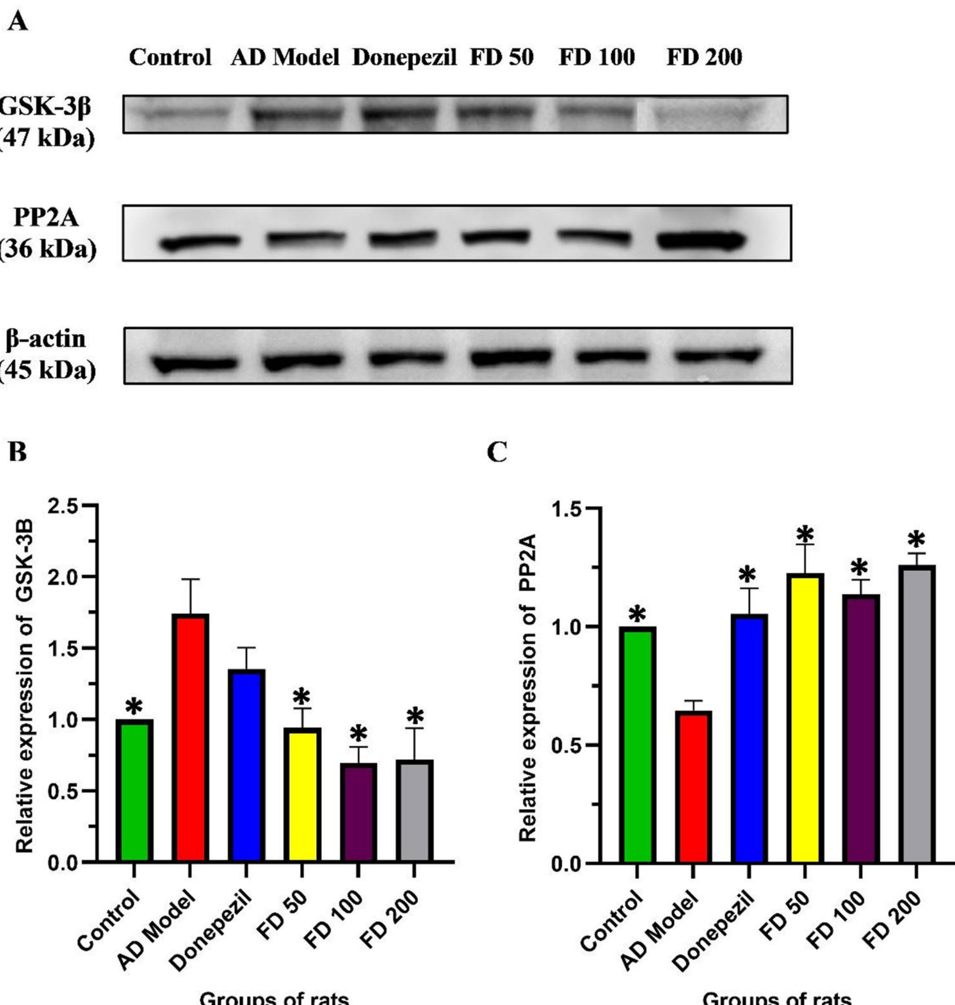


Fig. 6 The effects of FD on the levels of GSK-3β and PP2A in the D-gal and $AlCl_3$-induced AD rats. **A** Images of the GSK-3β, PP2A and β-actin protein bands are shown across the groups. The distinct molecular weights of the GSK-3β, PP2A, and β-actin bands were verified using a protein marker (9–180 kDa). The graphs represent the relative expressions of GSK-3β (**B**) and PP2A (**C**) across the various groups. β-actin was used as the loading control protein to normalise the total protein in the samples. Data were expressed as mean ± SEM ($n = 4$). * refers to $p < 0.05$ compared to the AD model group. [*Control (Normal saline + distilled water)*, *AD Model (60 mg/kg D-gal + 200 mg/kg $AlCl_3$)*, *Donepezil (60 mg/kg D-gal + 200 mg/kg $AlCl_3$ + 1 mg/kg donepezil)*, *FD 50 (60 mg/kg D-gal + 200 mg/kg $AlCl_3$ + 50 mg/kg FD)*, *FD 100 (60 mg/kg D-gal + 200 mg/kg $AlCl_3$ + 100 mg/kg FD) and FD 200 (60 mg/kg D-gal + 200 mg/kg $AlCl_3$ + 200 mg/kg FD)*]

and cognitive ability, hence validating the use of the OFT to assess locomotor function in the AD-like rat model. Consequently, the results of the OFT could support the evaluation of other behavioural aspects in the AD-like rat model, specifically spatial learning and memory behaviour.

In the present study, the number of lines crossed by the rats and their speed in the open-field apparatus were used as indices of locomotor activity in the OFT. D-gal and $AlCl_3$-induced rats administered with FD demonstrated increased locomotion compared to the untreated rats induced with D-gal and $AlCl_3$, as evidenced by more lines crossed at a higher speed in the open field area by the FD-treated rats. In contrast, the rats induced with D-gal and $AlCl_3$ exhibited prolonged movement, as confirmed by a significantly reduced number of lines crossed at a lower speed. It could also be implied that the FD-treated rats showed enhanced exploratory behaviour, unlike the D-gal and $AlCl_3$-induced rats. The study's results broadly support those of similar studies, despite differences in the therapeutic agents or natural plant extracts used to assess their locomotor effects in AD. For instance, the locomotion and the exploratory behaviour of the D-gal-induced rats treated with plant extract (*Centella asiatica*) were found to be significantly better than those of the untreated D-gal-induced rats [55]. Similarly, a significant improvement in locomotor activity and motor coordination was observed in the tauopathy mouse model treated with glimepiride [64]. Yang et al. reported reduced grid-crossing in the OFT in D-gal-treated mice, indicating their reduced exploration in a novel environment. However, the D-gal-treated mice administered with ferulic acid compound showed substantially improved exploratory ability as indicated by increased grid-crossing activity [65]. Taken together, these previous behavioural results of OFT showed that the AD-like rodent models have poor adaptation to normal locomotion and this impairment could be successfully reversed by various other plant extracts or their bioactive compounds. In contrast to earlier findings, the present study specifically demonstrated that administering FD plant extract could increase locomotion and exploratory behaviour in D-gal- and $AlCl_3$-induced AD rats.

After the rats' locomotor function was assessed, the results of the mEPM test were reported to determine the effects of

FD on spatial learning and memory. The EPM is frequently utilised to assess rats' learning and memory functions and evaluate anxiety levels. In the mEPM, the time required for rats to transition from the open arm to the enclosed arm was used to assess spatial learning and memory. Rodents naturally avoid open arms due to their natural aversive behaviour to open areas and high spaces, thus triggering them to choose a safer location: the enclosed arms. A shorter transfer latency during the retention session (time taken to move from the open arm to the enclosed arm) can be observed if the rats have been previously exposed to the open arms and this could be correlated to the consolidation of memory function [57, 66]. According to Yildiz Akar et al., the mEPM test is hippocampal-dependent and the transfer latency during the retention test is reported as notably shorter than that on the first training day, denoting that transfer latency can be an indicator of learning and memory [57]. The present study has shown that the TL1 and TL2 of the FD-treated rats induced with D-gal and $AlCl_3$ were comparable to those of the control rats, indicating that these rats navigated to the enclosed arm more easily after the initial day of the test, as evidenced by the reduced time taken to enter the enclosed arm. However, D-gal and $AlCl_3$-induced AD model rats took significantly longer to enter the enclosed arm, as they could not recall the positions of the open and enclosed arms, indicating poor spatial learning and memory. These differences were also evident in the track plots: the AD model rats spent more time in the centre of the apparatus before finding their way to the enclosed arm, whereas the FD-treated rats took a more direct route to enter the enclosed arm. Consistent with the present results, previous studies have also demonstrated that AD-like model rats exhibited a significant increase in transfer latency at 1 and 24 h after the first training day, compared to AD-like rats pre-treated with the compound naringenin [67]. Chiroma et al. utilised the mEPM to evaluate the D-gal and $AlCl_3$-induced memory loss and their findings showed that the administration of plant extracts, such as *Centella asiatica*, could positively restore memory function in AD rats [56]. Apart from these studies, there has been limited research on utilising the mEPM test to assess spatial learning and memory, since most studies have employed the EPM to measure anxiety-like behaviour in AD rats [68–70]. Hence, in the current study, the mEPM test has demonstrated a marked increase in spatial learning and memory in D-gal and $AlCl_3$-induced AD rats administered with FD, as evidenced by the progressive reduction in transfer latency.

A significant inference from the behavioural data is that the amelioration of spatial learning and memory by FD in D-gal and $AlCl_3$-induced AD rats could be further augmented by the improved locomotion observed in the OFT. This is justified in previous investigations that have proposed that their adaptive behaviour in locomotion could reflect cognitive ability in AD [63]. Nevertheless, increased locomotor activity alone is unlikely to fully account for the substantial cognitive improvements observed in the FD-treated rats. Together, these findings suggest that the enhanced cognitive outcomes more likely reflect direct neuroprotective effects of FD, as supported by the histological and molecular findings rather than secondary influences of locomotor changes. It may be implied that the improved spatial learning and memory, coupled with the enhanced locomotion contributed by FD, are mechanistically related to hippocampal function and alteration in the AD pathophysiology. Moreover, in accordance with previous findings, it was reported that motor dysfunction and cognitive impairment are directly linked to increased amyloid burden and hippocampal atrophy [61]. Firdaus et al. also reported that reduced hippocampal neurodegeneration and modulation of AChE activity and oxidative pathways reversed the memory loss and cognitive decline in D-gal-induced aged rats treated with a plant extract [55]. Although the current study has not addressed the impact of FD on AD pathologies, such as increased brain oxidative stress and AChE activity, in rat models, FD may play a mechanistic role in exhibiting neuroprotective effects against D-gal and $AlCl_3$-induced AD-like symptoms, including poor locomotion and impairments in learning and memory.

The following section of this study focused on histological examination of the hippocampus using the Nissl staining technique. The hippocampus is crucial for memory processing, learning and spatial navigation. It converts short-term memory into long-term memory [71]. The cornu ammonis (CA) and dentate gyrus (DG) subfields are the interlinked structures contributing to hippocampal formation. The DG subfield of the hippocampus consists mainly of densely packed granule cells, which form the granular cell layer between the molecular and polymorphic cell layers. The granular neurons in DG and the pyramidal neurons distributed across the CA1 and CA3 subregions form the intrinsic hippocampal neuronal circuit, which is necessary for memory processing [72–74]. The present study reported an extensive degeneration of granular neurons in the DG subfield in D-gal and $AlCl_3$-induced AD rats. A notably greater number of viable granular neurons was apparent in the control and FD-treated rats, particularly in the 100 mg/kg and 200 mg/kg FD groups. In the same vein, Haider et al. noted significant neurodegenerative alterations induced by D-gal and $AlCl_3$ in the rat hippocampus, while Hassan et al. also reported similar hippocampal neuronal morphology in the $AlCl_3$-induced AD model [67, 75]. Numerous studies on various natural compounds have demonstrated protective effects against neuronal degeneration. For instance, ginsenoside Rb1, a component of Panax ginseng root extract, exhibited increased survival of CA1 and CA3 pyramidal

neurons and DG granular neurons from degeneration, as evidenced by the extensive distribution of neuron cell bodies with round nuclei in these hippocampal subregions [76]. In addition, the fact that natural compounds, such as flavonoids derived from various sources, can traverse the blood-brain barrier (BBB) and exert neuroprotective properties provides compelling evidence supporting the present finding of hippocampal cell viability. Flavonoids, such as vitexin, commonly originate in FD [45] and other plants, including mung bean, mosses, bamboo, chaste berries and pigeon peas [77]. Vitexin contributes to neuroprotection and cognitive function by inhibiting targets that drive neurodegeneration, such as abnormal protein aggregation, oxidative stress and neuroinflammation [77]. Interestingly, vitexin also significantly attenuated neurodegeneration and chromatolysis, thereby improving cognitive functions, as reported by Amedu & Omotoso [77]. Vitexin could reduce neuronal loss and slow neuronal ageing in the hippocampus, improving neuronal cell structure and function [78, 79]. These results align with an in vitro study by Zolkiffly et al., which found that treatment with vitexin and isovitexin from FD extract increased cell viability to approximately 80% in microglial cells. This result may be attributed to the potential of vitexin and isovitexin to cross the BBB, as in silico results showed they have high permeability across the BBB [45]. Another study found that FD may promote cellular growth in a neuroblastoma cell line after treatment with FD extract, resulting in a marked increase in cell viability [39]. All the evidence presented thus far supports our results, which showed that co-administration of FD protects hippocampal neurons from D-gal and $AlCl_3$-induced neurodegeneration, signifying the remarkable neuroprotective role of FD.

Since the hippocampus has a central role in many complex functions linked to memory, any alteration in its function caused by diseases could lead to cognitive impairment and memory disruption [72]. For instance, severe degeneration of pyramidal cells and disorientation of the cell layers due to the toxic administration of D-gal and $AlCl_3$ hampered hippocampal function, according to Zakaria et al. [80]. As noted by Haider et al., the abnormalities in behavioural performances can serve as valuable evidence to indicate neurodegeneration in the early stages of AD [67]. In the present study, we believe that the massive neurodegeneration observed in the DG subregion of the hippocampus in D-gal and $AlCl_3$-induced AD rats may have affected their behavioural performances in the OFT and mEPM. However, co-administration of FD significantly protected the rats' spatial learning and memory, possibly by protecting neurons from D-gal and $AlCl_3$-induced neurodegeneration. The protective role of FD against neurodegeneration is evidenced by the markedly higher number of viable granule neurons in the DG subfield observed in the FD-treated rat groups.

Apart from behavioural studies and histological analysis of the hippocampus, the neuroprotective effects of FD were evaluated by measuring levels of tau-related proteins, specifically GSK-3β and PP2A, in the hippocampus of D-gal and $AlCl_3$-induced rat models. Several studies have shown that dysregulation in the expression or activation of tau kinases and phosphatases significantly contributes to tau hyperphosphorylation. Major proteins involved in tau phosphorylation and dephosphorylation include GSK-3β and PP2A, respectively [81]. As mentioned in the literature review, the inhibition of PP2A leads to increased hyperphosphorylated tau levels and, consequently, is associated with spatial memory loss [82]. Additionally, overexpressed GSK-3β could trigger tau hyperphosphorylation and increase neuronal loss, leading to spatial learning impairment [83]. GSK-3β is also reported to strongly target Thr181, an evident epitope in tau pathology, as observed in human brain samples and mouse models of AD [84]. The current experimental evidence revealed that PP2A was substantially expressed at higher levels, while GSK-3β was distinctly expressed at very low levels in the hippocampus of all three dose groups of FD-treated AD rats, similar to the control group. In contrast, rats with AD induced by D-gal and $AlCl_3$ were found to have reduced levels of PP2A and elevated levels of GSK-3β in their hippocampus. It can therefore be assumed that the elevated GSK-3β and reduced PP2A levels are associated with AD-like characteristics in the rat model, particularly in the progression of tau pathology. These further lead to increased neurodegeneration and impaired spatial learning and memory in the AD rat model. Previous observations by Chiroma et al. have reported that hyperphosphorylation and aggregation of tau in rat models induced by D-gal and $AlCl_3$ were due to the increased GSK-3β activity and depleted PP2A activity in the hippocampal region [27]. A study by Xiong et al. also confirmed that PP2A inactivation prevented dephosphorylation of p-tau in neuroblastoma N2a cell lysates [85]. Prior studies have also noted that several compounds from natural plant extracts have the potential to function as tau phosphatase activators and tau kinase inhibitors [27, 86–88]. Flavonoids such as naringin have been reported to modulate the GSK-3β signalling pathway. This further leads to inhibition of tau phosphorylation, as observed in PC12 cells and mouse models exposed to Aβ neurotoxicity [21]. The existing literature indicates that modulation of GSK-3β and PP2A activity is closely associated with changes in tau phosphorylation levels in AD models. However, it is important to note that only total GSK-3β and PP2A levels were influenced by FD in this study. Since the enzymatic activity of GSK-3β and PP2A is regulated by site-specific phosphorylation [89, 90] rather than total protein levels,

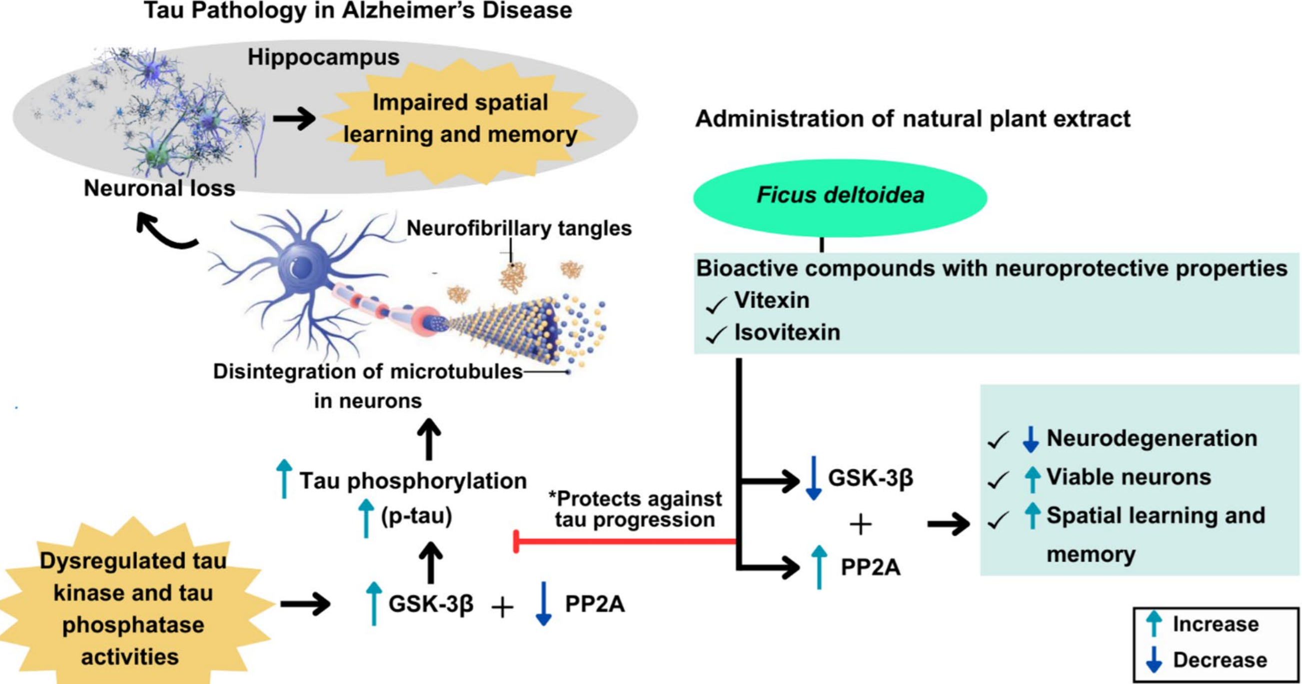


Fig. 7 Proposed potential neuroprotective pathways of *Ficus deltoidea* (FD) against Alzheimer's disease (AD) tau pathology. FD administration is associated with altered total protein levels in the hippocampus, as evidenced by reduced glycogen synthase kinase-3 beta (GSK-3β) and increased protein phosphatase 2 A (PP2A) levels. This may lead to reduced neurodegeneration, increased hippocampal neuronal viability and enhanced spatial learning and memory. Although GSK-3β and PP2A are known regulators of tau phosphorylation, the present study assessed only total protein levels; therefore, direct effects on enzymatic activity and phosphorylated tau (p-tau) cannot be inferred. The potential effects of GSK-3β/PP2A regulation on the protection against tau progression warrant further investigation

conclusions regarding kinase and phosphatase activities, as well as downstream effects of FD on the p-tau levels, cannot be drawn from the present data alone. Further studies incorporating phosphorylated GSK-3β (p-GSK-3β), phosphorylated PP2A (p-PP2A) and site-specific p-tau markers will be essential to confirm whether changes in kinase/phosphatase levels complement functional modulation of enzymatic activity. Thus, this would establish a mechanistic effect of the flavonoid-rich FD in regulating GSK-3β and PP2A levels to inhibit the progression of tau pathology.

Since the literature lacks information on GSK-3β and PP2A as potential therapeutic targets for FD, the present study is the first to reveal the neuroprotective effects of FD against tau pathology, as evidenced by significant decreases in GSK-3β levels and increases in PP2A levels in the hippocampus of D-gal- and $AlCl_3$-induced rats. These results contribute to reduced neurodegeneration and increased healthy, viable neurons in the hippocampus. This preserves the stability and integrity of hippocampal neurons, hence improving spatial learning and memory in the AD model rats. All of this evidence suggests that FD may play a mechanistic role in mediating neuroprotective function by modulating GSK-3β/PP2A in the AD rat model. A schematic representation of the potential neuroprotective pathways associated with FD through modulation of GSK-3β and PP2A levels and the subsequent effects on hippocampal neuronal cell viability and spatial learning and memory performance is illustrated in Fig. 7.

Several lines of evidence have highlighted the significance of other pathogenic mechanisms, such as neuroinflammation and oxidative stress, which are also closely associated with dysregulated levels of GSK-3β and PP2A and contribute to neurodegeneration [91–93]. Moreover, previous research on the FD plant and its natural compounds has primarily focused on their antioxidative, anti-inflammatory and anti-diabetic properties [39, 42, 52, 94]. These properties of FD are positively associated with its neuroprotective effects in treating AD, as exemplified in the works of Zolkiffly et al., Nurdiana et al. and Dzolin et al. [39, 45, 52]. Therefore, it could conceivably be hypothesised that the role of balanced GSK-3β and PP2A levels may indirectly result from the clearance of neuroinflammation and oxidative stress by FD. The assessments of specific oxidative stress markers such as superoxide dismutase (SOD), catalase and glutathione, or inflammatory markers such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and cyclooxygenase-2 (COX-2) [4, 95, 96] in future studies may show a mechanistic link between the attenuation of oxidative and

inflammatory damage and the neuroprotective role of FD in GSK-3β/PP2A modulation.

This research highlights that all three doses of FD (50 mg/kg, 100 mg/kg and 200 mg/kg) effectively improved spatial learning and memory in D-gal and $AlCl_3$-induced AD rats, as evidenced by the mEPM test. All FD-treated cohorts exhibited increased numbers of viable granule neurons in the hippocampal DG subregion relative to the AD model group, although no significant increase was evident, particularly in the FD 50 mg/kg cohort. Comparison of the protein abundance results confirms that GSK-3β is expressed at low levels, whereas PP2A is expressed at high levels in all FD-treated rats compared to the AD model rats. Overall, the study unexpectedly revealed no significant differences between the low-dose FD group, the medium-dose FD group and the high-dose FD group. Although no clear dose-response relationship was observed, the medium dose (100 mg/kg) and high dose (200 mg/kg) revealed more profound effects and may be preferable for future preclinical work. Nevertheless, detailed pharmacokinetic, pharmacodynamic and safety profiling are required to define these doses as optimal for protection against AD. Consistent with the literature, the subchronic toxicity effects of FD has been previously investigated, revealing no significant haematological or biochemical alterations in rats treated with doses up to 100 and 300 mg/kg of FD [54]. In 2013, Farsi et al. reported that acute and subchronic oral administration of the FD extract to rats was safe at doses of 750, 1250 and 2500 mg/kg. No substantial unfavourable effects on the rats' body weight or organ weights were observed, verifying the absence of toxicity signs [97]. These findings comply with the current results, which confirmed that the FD at 50, 100 and 200 mg/kg doses exhibited neuroprotective effects, with no adverse reactions observed in the rat models.

The current investigation showed that donepezil, utilised as a positive control, not only significantly enhanced spatial learning and memory but also increased PP2A levels in D-gal and $AlCl_3$-induced AD rats, similar to the FD-treated AD rats. Despite this, it is worth noting that no significant differences were found in hippocampal granule cell viability and GSK-3β levels between the donepezil-treated AD rats and the AD model rats. This suggests that the effects of donepezil treatment, as deduced from the experimental investigations, are not as pronounced as those seen in the FD-treated groups. According to Salomone et al., donepezil and other AChEIs, including rivastigmine and galantamine, have often been used for symptomatic relief and short-term benefits rather than for altering the pathogenic mechanisms of AD, despite suggestions that they may exhibit potential neuroprotective effects [98]. Consequently, a natural pharmaceutical product such as FD may act as a potential disease-modifying agent with neuroprotective advantages against GSK-3β/PP2A imbalance associated with tau pathology, as evidenced both symptomatically and pathologically.

Despite the promising results, this study had certain limitations since the effects of FD on upstream tau regulators other than GSK-3β and PP2A were not discussed. For instance, p-GSK-3β at Ser 9 can deactivate GSK-3β and decrease p-tau levels, since GSK-3β activity is regulated by site-specific phosphorylation [90]. Meanwhile, the increase in activation of tau kinases like cyclin-dependent kinase-5 (CDK5) also contributes to the tau hyperphosphorylation [99]. Thus, measuring proteins such as CDK5, p-GSK-3β and p-PP2A can strengthen the neuroprotective mechanism of FD. Additionally, site-specific p-tau markers, particularly AT8 and PHF-1, which can recognise abnormally phosphorylated tau at serine and threonine residues [100], can be usefully explored to study the modulation of the kinase/phosphatase effects on specific tau phosphorylation sites. Although hyperphosphorylated tau is a central hallmark of AD pathology, p-tau analysis is not included in the present manuscript because it is part of a separate, dedicated study currently under independent peer review. As a result, the current work focuses primarily on upstream tau regulatory pathways, including GSK-3β and PP2A, which nonetheless provide meaningful insight into the neuroprotective effects of FD. We acknowledge that the absence of p-tau data here limits the ability to fully delineate tau-specific mechanisms in a single report. However, these forthcoming findings will complement the present results and provide a more comprehensive understanding of FD's influence on tau pathology.

Conclusion

In toto, the findings of this study confirmed the neuroprotective effects of FD by demonstrating that this plant significantly enhances spatial learning and memory, attenuates neurodegeneration and increases the number of viable granule neurons in the hippocampal subregion in D-gal and $AlCl_3$-induced AD rats. The results of this investigation also demonstrated elevated levels of PP2A and decreased levels of GSK-3β in the hippocampus in D-gal and $AlCl_3$-induced AD rats. Thus, these data suggest that modulation of the kinase/phosphatase balance contributes to the significant reduction in hippocampal neurodegeneration, thereby improving spatial learning and memory in AD-like rats. Nevertheless, this study lacks information on other upstream tau regulators besides GSK-3β and PP2A. Notwithstanding these limitations, the study certainly offers some insight into the neuroprotective potential of FD. Further research should focus more broadly on elucidating the specific molecular mechanisms of FD against different upstream tau regulators and site-specific p-tau markers. Furthermore, additional

information on the neuroprotective effects of FD against various AD pathologies in an in vivo model will be necessary to achieve greater accuracy on this matter. Hence, it is hoped that these recommendations will help strengthen the translational potential of FD as a neuroprotective agent in clinical studies. Notably, we assert that FD will make a significant contribution to the development of a drug that can hinder the progression of tau pathology in AD, offering a novel perspective on alternative therapeutic strategies for this condition.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11064-026-04702-0

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Data Availability The data supporting the findings of this research will be made available by the corresponding author upon reasonable request. The data are not publicly available due to privacy and ethical restrictions.

Declarations

Competing Interests The authors declare no competing interests.

Ethical Approval Humans were not used in this study. All animal experiments were performed in full compliance with the Institutional Animal Care and Use Committee at Universiti Putra Malaysia (UPM) [UPM/IACUC/AUP-R051/2022].

Consent to Participate Not applicable.

Consent for Publication Not applicable.

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