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Phenylethanoid Glycosides (AIE2)-Enriched *Cistanche tubulosa* Extract Attenuates Oxidative Stress and Amyloid Pathology in an Aβ_{1-40}-Induced Rat Model of Alzheimer's Disease

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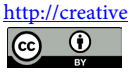

Abstract

The mechanisms underlying accelerated cognitive decline in Alzheimer's disease (AD) remain incompletely understood. Accumulating evidence suggests that AD is a complex, multifactorial neurodegenerative disorder in which oxidative stress plays a central role. Oxidative stress, often triggered by amyloid-β (Aβ)-induced reactive oxygen species (ROS), leads to severe cellular damage and contributes to cognitive deterioration. This study investigated the effects of phenylethanoid glycosides (AIE2)-enriched *Cistanche tubulosa* extract (CTE) on oxidative stress, cognitive impairment, and AD-like pathology in an Aβ_{1-40}-induced rat model. The AD-like model was established by hippocampal infusion of Aβ_{1-40}. Antioxidant enzyme activities, including superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, were evaluated. CTE treatment significantly attenuated Aβ_{1-40}-induced oxidative stress, enhanced antioxidant enzyme activities, and improved cognitive performance in the AD-like animal model. Toxicological evaluation revealed no adverse effects, as evidenced by stable body weight and unchanged liver and kidney function parameters. Additionally, CTE reduced acetylcholinesterase activity as well as Aβ_{1-40} and apolipoprotein E deposition without inducing toxicity. These findings suggest that CTE exerts antioxidant and neuroprotective effects and may represent a promising therapeutic candidate for the treatment of Alzheimer's disease.

Subject Areas

Biotechnology, Neuroscience

Keywords

Cistanche tubulosa, Phenylethanoid Glycosides, Alzheimer's Disease, Oxidative Stress, A$\beta_{1\text{-}40}$

1. Introduction

Alzheimer's disease (AD), the most common form of dementia, is pathologically characterized by extracellular amyloid-β (Aβ) plaque deposition and intracellular tau-containing neurofibrillary tangles. These pathological features lead to a progressive decline in memory, cognitive function, speech, performance, and orientation, predominantly affecting the elderly population [1]. Managing behavioral symptoms in patients with AD presents a substantial challenge for caregivers, highlighting the importance of preserving cognitive function and slowing disease progression to alleviate this burden. Consequently, increasing research efforts have focused on the development of therapeutic agents to improve cognitive and memory impairments associated with AD. However, despite the approval of several drugs by the U.S. Food and Drug Administration (FDA), effective disease-modifying treatments remain unavailable [2].

Oxidative stress is widely recognized as a critical contributor to the pathogenesis and progression of AD, playing a pivotal role in neuronal damage and cognitive decline [3] [4]. Although several studies have demonstrated the antioxidant and neuroprotective properties of glycosides derived from *Cistanche tubulosa* and their potential to attenuate Aβ-induced AD-like symptoms in animal models [5]-[7], their specific effects on oxidative stress in Aβ-induced AD-like models remain to be fully elucidated.

Although the precise mechanisms underlying AD remain incompletely understood, accumulating evidence identifies Aβ-induced neurotoxicity and oxidative stress as key pathological events [8]. Aβ accumulation is widely accepted as a central factor in AD development [9]. Impaired degradation and clearance of Aβ proteins promote amyloid fibril formation and plaque deposition, leading to cholinergic neuron degeneration, synaptic loss, and ultimately neurodegeneration and cognitive impairment [10]. In parallel, oxidative stress has been implicated as a major driver of AD pathogenesis, initiating and exacerbating disease progression [11]. An imbalance between reactive oxygen species (ROS) production and antioxidant defense mechanisms results in excessive ROS accumulation, leading to oxidative stress. In the brain, this process induces neuroinflammation, damages DNA, RNA, and proteins, impairs mitochondrial function, and ultimately contributes to neurodegeneration and cognitive dysfunction [12].

Aβ accumulation further exacerbates neuronal oxidative stress by inducing excessive ROS production [13], thereby triggering apoptotic signaling pathways and neuronal cell death [14]. Notably, oxidative stress may precede Aβ plaque deposition and tau phosphorylation [15] and can further promote Aβ generation and

aggregation.[16] Given the central role of oxidative stress in AD pathogenesis, increasing attention has been directed toward therapeutic strategies aimed at mitigating oxidative damage [11] [16].

Cistanche tubulosa (Schrenk) R. Wight, a parasitic plant primarily distributed in the Taklamakan Desert, has been used in traditional Chinese medicine for thousands of years, as documented in *Shennong's Materia Medica* and the Pharmacopeia of Chinese mainland and Taiwan region [17] [18]. Traditionally, *C. tubulosa* has been employed to treat various conditions, including kidney deficiency, impotence, female infertility, morbid leucorrhea, profuse metrorrhagia, and senile constipation.

C. tubulosa extract (CTE), which is rich in phenylethanoid glycosides [19] [20], has been reported to possess a broad spectrum of pharmacological activities, including antioxidant, neuroprotective, anti-inflammatory, immunomodulatory, anti-aging, and cognition-enhancing effects, as well as potential therapeutic benefits for AD [21]. Increasing evidence further supports the role of phenylethanoid glycosides in the treatment of neurodegenerative diseases such as AD, Parkinson's disease, and vascular dementia [22]-[24]. Several studies have identified the major glycosidic constituents of *C. tubulosa* [25] [26], among which acteoside and echinacoside are the predominant compounds. These constituents have demonstrated neuroprotective effects and the ability to alleviate memory impairment [27]-[29]. Moreover, *C. tubulosa* extracts exhibit significant antioxidant activity [30] [31] and have been shown to enhance intracellular antioxidant defenses by increasing the activities of enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) [32]. However, whether CTE effectively attenuates oxidative stress—a critical factor in AD pathogenesis—remains unclear.

As increasing attention has been directed toward the neuroprotective potential of phenylethanoid glycosides, numerous studies have reported their beneficial effects in AD models. These compounds have been shown to alleviate neurological deficits and reduce neuronal apoptosis in AD animal models [7] [33]. Furthermore, phenylethanoid glycosides have demonstrated the capacity to enhance learning and memory by promoting the expression of nerve growth factors [34]. Our previous studies also revealed that acteoside and echinacoside effectively inhibit Aβ accumulation and aggregation in both *in vitro* cellular systems and in vivo animal models [7] [35]. Despite evidence supporting the antioxidant properties of isolated glycosides from *C. tubulosa* and the established role of oxidative stress in AD, the effects of CTE on oxidative stress in AD remain insufficiently characterized. Therefore, the present study aimed to investigate the effects of CTE on oxidative stress, key antioxidant enzyme activities, and cognitive and memory impairments in an Aβ_{1-40}-infused AD-like rat model [36] [37].

2. Materials and Methods

2.1. Experimental Materials

Powdered stems of *Cistanche tubulosa* were extracted by water reflux and subse-

quently filtered to obtain the filtrate. Ethanol was added to the filtrate, after which the supernatant was collected and subjected to resin separation using macroporous adsorption resin. The resulting extract was spray-dried to obtain the aqueous *C. tubulosa* extract (CTE), with a yield of approximately 10%. The extract was manufactured by Sinphar Tian-Li Pharmaceutical Co., Ltd. (Hangzhou, Sinphar Group, China).

CTE primarily consists of phenylethanoid glycosides, including acteoside, isoacteoside, echinacoside, and 2′-acetylechinacoside (AIE2). A cholinesterase inhibitor (0.52 mg/kg) was used as a positive control. Synthetic human Aβ_{1-40} peptide was purchased from Tocris Bioscience (Ellisville, MO, USA).

2.2. Animals

A total of 63 male Sprague-Dawley rats (250 - 300 g) were obtained from BioLASCO Taiwan region Co., Ltd. Animals were housed under controlled environmental conditions (25˚C ± 1˚C, 60% relative humidity) with free access to standard laboratory chow and tap water and maintained on a 12-h light/dark cycle (light phase: 09:00 - 21:00). All rats were acclimated for one week prior to experimentation, and body weight was recorded daily throughout the study.

Rats were randomly assigned to seven groups (n = 9 per group):

1) Control group (sham-operated): saline infusion and oral administration of distilled water

2) AC group: Aβ_{1-40} infusion and oral administration of distilled water

3) AM group: Aβ_{1-40} infusion and oral administration of a cholinesterase inhibitor (0.52 mg/kg)

4) AS0.5 group: Aβ_{1-40} infusion and oral administration of CTE (14.6 mg/kg/day)

5) AS1 group: Aβ_{1-40} infusion and oral administration of CTE (29.2 mg/kg/day)

6) AS2 group: Aβ_{1-40} infusion and oral administration of CTE (58.4 mg/kg/day)

7) AS6 group: Aβ_{1-40} infusion and oral administration of CTE (175.2 mg/kg/day)

CTE and the cholinesterase inhibitor were administered once daily via gastric gavage for 28 days. Following behavioral assessments, rats were fasted for 12 h prior to sacrifice for biochemical, histological, and safety evaluations.

The CTE doses were extrapolated from a previous clinical trial using a standardized human dose of 300 mg/day (AS1) [21]. Additional doses of 150 mg/day (AS0.5), 600 mg/day (AS2), and 1800 mg/day (AS6) were calculated and converted to rat-equivalent doses using body surface area normalization, resulting in doses of 14.6, 29.2, 58.4, and 175.2 mg/kg/day, respectively.

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of NTU (Approval No. 12, Year 98) and were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals (Protocol No. 99-127-B).

2.3. Aβ_{1-40}-Infused AD-Like Rat Model and Experimental Schedule

The AD-like rat model was established by continuous hippocampal infusion of Aβ_{1-40}, as previously described [36] [37]. Briefly, rats were anesthetized via intra-

peritoneal injection of sodium pentobarbital (45 mg/kg) and placed in a stereotaxic apparatus (S724791, Narishige, Tokyo, Japan). An infusion cannula was implanted into the lateral ventricle, and a mini-osmotic pump (Alzet 2002; Alza, Palo Alto, CA, USA) was implanted subcutaneously in the posterior neck region.

Aβ_{1-40} was dissolved in 35% acetonitrile containing 0.1% trifluoroacetic acid to a concentration of 250 pmol/μL and loaded into the mini-osmotic pumps. Beginning one day after implantation (day 0), pumps delivered Aβ_{1-40} at a rate of 300 pmol/day for 28 days. Control animals received the same volume of vehicle solution.

From day 1 onward, CTE, cholinesterase inhibitor, or vehicle (distilled water) was administered intragastrically once daily throughout the infusion period. On behavioral testing days, treatments were administered 1 h prior to testing, including the passive avoidance test (days 19 - 21) and the Morris water maze (days 22 - 27). On day 28, rats were sacrificed 1 h after the final administration for biochemical and histological analyses, including measurements of malondialdehyde (MDA), antioxidant enzyme activities (SOD, catalase, GPx, GRd), acetylcholinesterase (AChE) activity, and serum biochemical parameters.

2.4. Oxidative Stress Measurements

Lipid peroxidation was assessed using a thiobarbituric acid–reactive substances (TBARS) assay kit (Cayman Chemical, #10009055). Samples were reacted with thiobarbituric acid (TBA), and absorbance was measured at 540 nm using a microplate reader. Lipid peroxidation levels were expressed as malondialdehyde (MDA) equivalents based on a standard curve.

Oxidative DNA damage was evaluated by measuring 8-hydroxy-2′-deoxyguanosine (8-oxo-dG), a commonly used biomarker of oxidative stress. DNA oxidation was quantified using a commercial ELISA kit (Trevigen, #4380-096-K) according to the manufacturer's instructions. Absorbance was measured at 450 nm, and 8-oxo-dG concentrations were calculated using a standard curve.

Protein oxidation was assessed by measuring protein carbonyl content using a commercial kit (Cayman Chemical, #10005020). Absorbance was measured at 360 - 385 nm, and results were expressed as nmol carbonyl per mg protein.

2.5. Antioxidant Enzyme Activity Assays

Superoxide dismutase (SOD) activity was measured using a commercial assay kit (Ransod-SD125, Randox Laboratories, UK). Absorbance was measured at 505 nm, and activity was expressed as U/mg protein. Catalase activity was determined using a commercial kit (Cayman Chemical, #707002) by measuring formaldehyde production at 540 nm and expressed as mM/min/mL. Glutathione peroxidase (GPx) activity was measured using a coupled enzymatic assay (Cayman Chemical, #703102), with activity expressed as nmol NADPH/min/mg protein. Glutathione reductase (GRd) activity was assessed using a commercial kit (Cayman Chemical, #703202), with enzyme activity expressed as nmol NADPH/min/mg protein.

2.6. Cortical and Hippocampal AChE Activity

AChE activity was determined using a modified Ellman method [38]. Samples were incubated with 5,5′-dithiobis(2-nitrobenzoic acid), followed by acetylthiocholine. Absorbance was measured at 412 nm, and enzyme activity was expressed as U/mg protein.

2.7. Hematological Analysis

Serum biochemical parameters related to lipid metabolism, liver function, and kidney function were analyzed, including triglycerides, total cholesterol, HDL-C, LDL-C, AST, ALT, alkaline phosphatase, total protein, albumin, globulin, blood urea nitrogen, uric acid, electrolytes (Na^+, K^+, Cl^-), calcium, and phosphorus.

2.8. Immunohistochemistry

Brain tissues were fixed in 10% formalin, embedded in paraffin, and sectioned at 3 - 5 μm thickness. Sections were dewaxed, rehydrated, and subjected to antigen retrieval. After blocking with 10% goat serum, sections were incubated overnight at 4˚C with antibodies against $A\beta_{1-40}$ or ApoE (1:100). Detection was performed using an HRP-conjugated system with DAB chromogen, followed by hematoxylin counterstaining.

Protein expression was quantified using mean integrated optical density (IOD) from three randomly selected fields per section at 400× magnification. $A\beta$ plaque counts were performed in at least 20 fields per section at 40× magnification using an image analyzer (Leica Q500MC).

2.9. Behavioral Tests

Passive Avoidance Test:

The passive avoidance test was conducted as previously described. Retention latency times were recorded at 24, 48, and 72 h post-training.

Morris Water Maze Test:

Spatial learning and memory were assessed using reference memory, probe, and working memory tasks in a Morris water maze, as previously described. Escape latency, swimming distance, and quadrant occupancy were recorded using EthoVision® XT tracking software.

2.10. Statistical Analysis

All data were analyzed using SPSS version 17.0. Group differences were evaluated by one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test. Data are presented as mean ± SD (n = 9). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of *C. tubulosa* Extract (CTE) on Oxidative Stress in the Hippocampus and Serum of $A\beta_{1-40}$-Induced AD Rats

Oxidative stress is a critical contributor to the development and progression of

Alzheimer's disease (AD), leading to neuronal damage and cognitive decline [3]. In contrast, *C. tubulosa* extract (CTE) has been reported to possess antioxidant properties [30] [31]. To evaluate the antioxidant potential of CTE against Aβ aggregation–induced oxidative stress, malondialdehyde (MDA)—a lipid peroxidation end product and a commonly used marker of oxidative stress [37]—was measured in hippocampal tissues of A$\beta_{1\text{-}40}$-induced AD rats. As shown in **Figure 1(a)**, hippocampal MDA levels were significantly elevated in the A$\beta_{1\text{-}40}$-infused control group compared with the non-infused group. CTE administration significantly and dose-dependently attenuated the A$\beta_{1\text{-}40}$-induced increase in MDA levels relative to the A$\beta_{1\text{-}40}$-infused control group. Systemic oxidative stress was further assessed by measuring plasma markers of lipid peroxidation (MDA), protein oxidation (protein carbonyls), and DNA oxidation (8-hydroxy-2′-deoxyguanosine, 8-OHdG) [39]. Plasma MDA levels exhibited a pattern similar to that observed in hippocampal tissues (**Figure 1(b)**), with significantly higher levels in

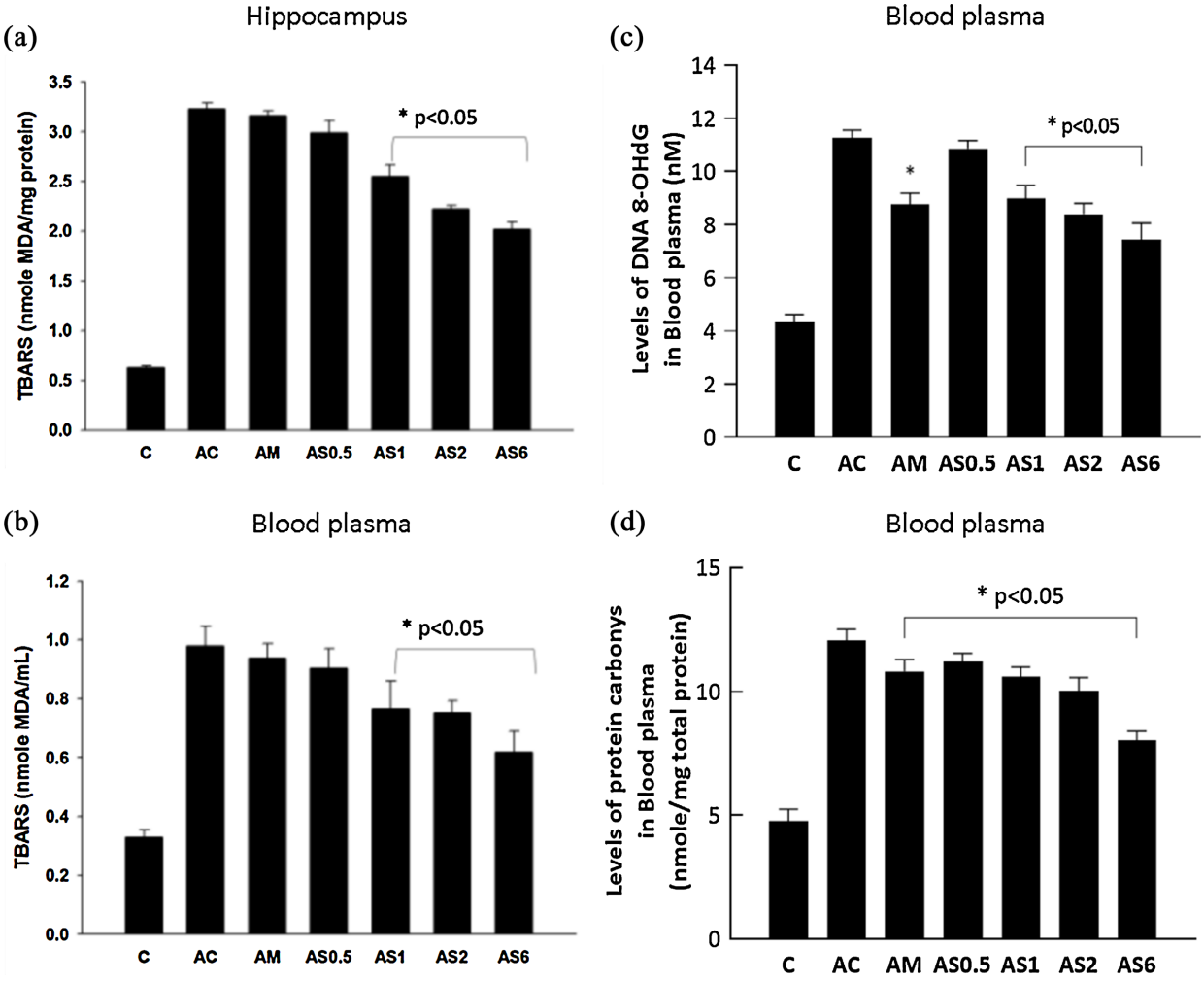

Figure 1. CTE effects on oxidative stress in A$\beta_{1\text{-}40}$-infused rats. MDA levels in the hippocampus (a) and blood plasma (b) were measured to evaluate lipid peroxidation. 8-OHdG (c) and protein carbonyl (d) levels were measured to assess oxidative stress on DNA and proteins, respectively. The results are presented as mean ± SD (n = 9). *$p < 0.05$ indicates statistical significance compared to the AC control group.

the A$\beta_{1\text{-}40}$-infused control group compared to the non-infused group. CTE treatment resulted in a dose-dependent reduction of plasma MDA levels relative to the A$\beta_{1\text{-}40}$-infused control group. Similarly, plasma 8-OHdG levels were significantly increased following A$\beta_{1\text{-}40}$ infusion compared with the non-infused group (**Figure 1(c)**), whereas CTE administration dose-dependently decreased 8-OHdG levels. In addition, protein carbonyl levels—a marker of protein oxidation—were markedly elevated in the A$\beta_{1\text{-}40}$-infused control group compared with the non-infused group and were dose-dependently reduced by CTE treatment (**Figure 1(d)**).

3.2. Effects of CTE on Antioxidant Enzyme Activities in the Hippocampus of A$\beta_{1\text{-}40}$-Induced AD Rats

To further elucidate the relationship between the antioxidant effects of CTE and the reduction of A$\beta_{1\text{-}40}$-induced oxidative stress, the activities of key antioxidant enzymes—including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase (GRd)—were assessed. SOD catalyzes the conversion of superoxide radicals into hydrogen peroxide, which is subsequently decomposed into water and oxygen by catalase and peroxidases. GRd plays a critical role in maintaining GPx activity by regenerating reduced glutathione from glutathione disulfide [40]. As shown in **Figure 2(a)**, SOD activity was significantly

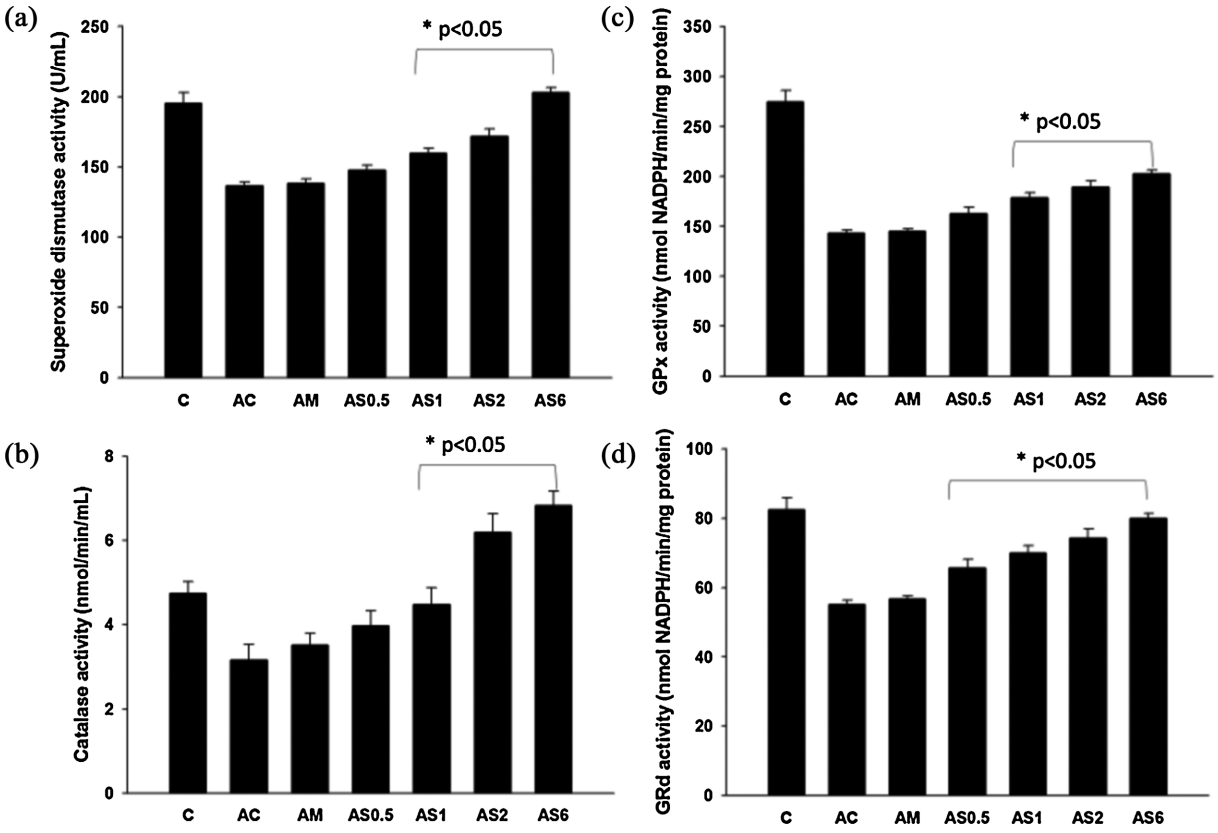

Figure 2. CTE effects on antioxidant enzyme activities (SOD, catalase, GPx, and GRd) in the hippocampus of A$\beta_{1\text{-}40}$-infused rats. (a) Superoxide dismutase (SOD); (b) Catalase; (c) Glutathione peroxidase (GPx); and (d) Glutathione reductase (GRd) activities in hippocampal tissue are shown. The results are presented as mean ± SD (n = 9). *$p < 0.05$ indicates statistical significance compared to the AC control group.

reduced in the A$\beta_{1\text{-}40}$-infused control group compared with the non-infused group. CTE administration significantly and dose-dependently increased SOD activity relative to the A$\beta_{1\text{-}40}$-infused control group. Notably, the highest CTE dose (AS6) restored SOD activity to levels comparable to those observed in the non-infused group. Similarly, catalase, GPx, and GRd activities were significantly decreased in the A$\beta_{1\text{-}40}$-infused control group compared with the non-infused group. CTE treatment resulted in dose-dependent increases in the activities of these enzymes (**Figures 2(b)-(d)**). Interestingly, higher doses of CTE (AS2 and AS6) increased catalase activity beyond the levels observed in the non-infused group. Taken together, these results indicate that CTE enhances the activities of major antioxidant enzymes in a dose-dependent manner, supporting its potential therapeutic role in mitigating A$\beta_{1\text{-}40}$-induced oxidative stress in AD.

3.3. Effects of CTE on Learning and Memory Assessed by the Passive Avoidance Test in A$\beta_{1\text{-}40}$-Induced AD Rats

To assess the effects of CTE on A$\beta_{1\text{-}40}$-induced learning and memory impairment, a passive avoidance test was performed. As shown in **Figure 3**, the A$\beta_{1\text{-}40}$-infused control group exhibited significantly reduced retention latency times to enter the dark chamber compared with the non-infused group on days 1, 2, and 3. CTE administration significantly and dose-dependently increased retention latency times relative to the A$\beta_{1\text{-}40}$-infused control group at all testing time points. Notably, rats receiving the highest CTE dose (AS6) exhibited retention latencies comparable to those of the non-infused group. These findings indicate that CTE effectively ameliorated A$\beta_{1\text{-}40}$-induced deficits in learning and memory as assessed by the passive avoidance test.

3.4. Effects of CTE on Spatial Memory in A$\beta_{1\text{-}40}$-Induced AD Rats Assessed by the Morris Water Maze

Spatial learning and memory were further evaluated using the Morris Water Maze

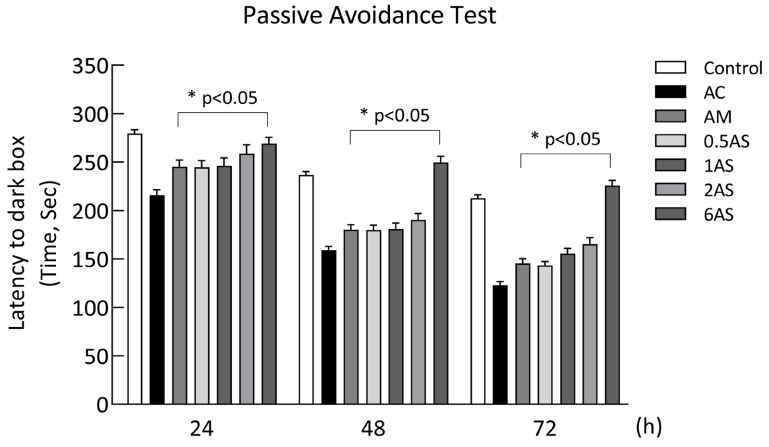

Figure 3. CTE effects on passive avoidance task in A$\beta_{1\text{-}40}$-infused rats. The latency of entering the dark compartment was recorded. The results are presented as the mean ± SD (n = 9). *$p < 0.05$ indicates statistical significance.

(MWM). In the reference memory task, the A$\beta_{1\text{-}40}$-infused control group displayed significantly prolonged escape latencies compared with the non-infused group on days 1, 2, and 3 (**Figure 4(a)**). In contrast, CTE treatment significantly and dose-dependently reduced escape latencies relative to the A$\beta_{1\text{-}40}$-infused control group. In the probe test, the A$\beta_{1\text{-}40}$-infused control group spent a significantly lower percentage of time in the target platform area compared with the non-infused group (**Figure 4(b)**). CTE administration dose-dependently increased the time spent in the target area relative to the A$\beta_{1\text{-}40}$-infused control group. No significant differences were observed in total swimming distance among groups (**Figure 4(c)**), indicating that locomotor activity was not affected and did not confound the cognitive outcomes. To further assess working memory, the MWM working memory task was conducted. The A$\beta_{1\text{-}40}$-infused control group exhibited significantly prolonged escape latencies compared with the non-infused group on days 1, 2, and 3 (**Figure 4(d)**). CTE-treated groups showed dose-dependent reductions in escape latency relative to the A$\beta_{1\text{-}40}$-infused control group, indicating improved working memory performance.

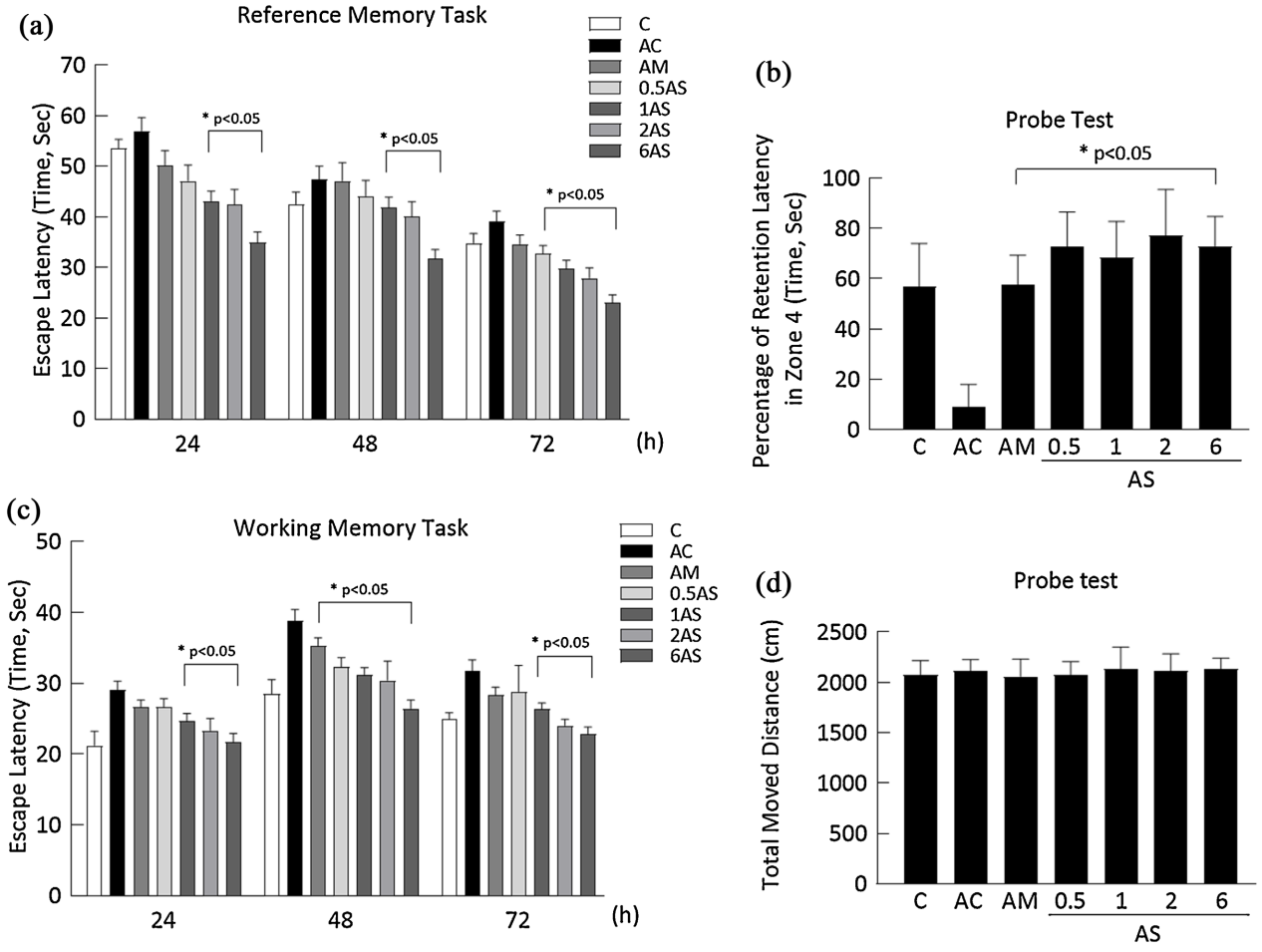

Figure 4. CTE effects on cognitive performance in A$\beta_{1\text{-}40}$-infused rats. (a) Escape latency in the reference memory task. (b) Percentage of time spent in quadrant 4 during the probe test, where the escape platform was previously located. (c) Total swimming distance during the probe test. (d) Escape latency in the working memory task. Results are expressed as mean ± SD (n = 9).

3.5. Effects of CTE on Body Weight and Serum Biochemical Parameters in A$\beta_{1\text{-}40}$-Induced AD Rats

Potential toxicity associated with CTE administration was evaluated throughout the experimental period. No significant differences in body weight were observed among the experimental groups (Table 1). Additionally, serum biochemical parameters related to lipid metabolism, liver function, and kidney function—including AST, ALT, alkaline phosphatase, total protein, albumin, globulin, A/G ratio, BUN, creatinine, uric acid, and electrolytes (sodium, potassium, chloride, calcium, and phosphorus)—did not differ significantly among groups compared with the non-infused controls (Table 2 and Table 3). These findings indicate that neither A$\beta_{1\text{-}40}$ infusion nor CTE administration produced detectable hepatic or renal toxicity during the experimental period.

Table 1. Effect of the CTE on body weight.

Treatment	Body weight (g)				
	Week 0	Week 1	Week 2	Week 3	Week 4
C	316.4 ± 6.3	334.6 ± 5.7	353.6 ± 5.7	386.2 ± 4.2	417.6 ± 1.6
AC	313.8 ± 5.1	336.1 ± 7.5	352.2 ± 5.0	386.6 ± 3.1	414.7 ± 3.7
AM	316.3 ± 8.1	333.9 ± 6.7	355.3 ± 8.1	388.4 ± 5.9	417.2 ± 8.5
AS0.5	309.8 ± 5.4	335.8 ± 6.2	348.8 ± 5.4	382.6 ± 6.6	413.3 ± 5.4
AS1	316.4 ± 3.7	332.9 ± 2.4	355.4 ± 3.7	389.3 ± 8.0	415.1 ± 8.3
AS2	313.4 ± 4.2	335.2 ± 6.6	352.4 ± 4.2	386.7 ± 3.1	414.7 ± 5.7
AS6	311.3 ± 6.9	331.3 ± 6.9	350.3 ± 6.9	386.0 ± 5.9	415.9 ± 6.2

Data were presented as means ± SD (n = 9). Abbreviations: C: sham with regular diet; AC: A$\beta_{1\text{-}40}$-infused with regular diet; AM: A$\beta_{1\text{-}40}$-infused with cholinesterase inhibitor drug at 0.52 mg/day/kg bw; AS0.5: A$\beta_{1\text{-}40}$-infused with 14.6 mg/day/kg bw of CTE; AS1: A$\beta_{1\text{-}40}$-infused with 29.2 mg/day/kg bw of CTE; AS2: A$\beta_{1\text{-}40}$-infused with 58.4 mg/day/kg bw of CTE; AS6: A$\beta_{1\text{-}40}$-infused with 175.2 mg/day/kg bw of CTE.

Table 2. Summary of hematology and biochemistry parameters for rats' lipid metabolism and liver function.

Treatment	Triglyceride (mg/dL)	Cholesterol (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)	Cholesterol/HDL-C	CPK (U/L)	AST (U/L)	ALT (U/L)	Alk-p (IU/L)	Total protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Albumin/Globulin
C	72.6 ± 3.1	71.7 ± 4.1	57.5 ± 5.3	11.9 ± 1.8	1.2 ± 0.1	780.3 ± 59.8	113.3 ± 8.4	45.4 ± 2.9	130.4 ± 5.1	7.1 ± 0.3	4.7 ± 0.2	2.4 ± 0.2	2.0 ± 0.3
AC	74.0 ± 6.9	68.9 ± 5.3	58.9 ± 5.0	9.0 ± 1.9	1.2 ± 0.1	745.2 ± 81.0	113.0 ± 6.0	47.2 ± 3.9	131.4 ± 7.2	7.2 ± 0.2	4.9 ± 0.2	2.2 ± 0.1	2.2 ± 0.2
AM	73.0 ± 4.1	72.9 ± 4.6	60.5 ± 6.4	10.4 ± 1.5	1.2 ± 0.1	722.6 ± 42.0	116.3 ± 6.1	47.1 ± 5.0	135.6 ± 7.3	7.0 ± 0.3	4.7 ± 0.2	2.3 ± 0.3	2.0 ± 0.3
AS0.5	72.7 ± 5.1	70.8 ± 5.1	60.9 ± 6.3	11.1 ± 1.5	1.2 ± 0.1	720.2 ± 71.6	113.6 ± 6.4	48.0 ± 5.8	130.4 ± 9.2	7.2 ± 0.3	4.9 ± 0.2	2.2 ± 0.2	2.2 ± 0.2

Continued

AS1	72.1 ± 3.9	70.0 ± 5.3	58.9 ± 6.8	10.8 ± 1.9	1.2 ± 0.2	726.3 ± 56.7	110.8 ± 6.8	45.7 ± 6.6	132.2 ± 6.8	7.0 ± 0.4	4.5 ± 0.3	2.5 ± 0.4	1.9 ± 0.3
AS2	72.1 ± 3.0	73.6 ± 5.6	59.2 ± 10.9	10.6 ± 2.2	1.3 ± 0.2	733.6 ± 80.9	115.4 ± 4.3	45.6 ± 3.3	129.7 ± 4.9	6.9 ± 0.5	4.8 ± 0.3	2.1 ± 0.3	2.3 ± 0.4
AS6	70.0 ± 6.3	70.8 ± 4.6	61.1 ± 7.0	9.4 ± 1.9	1.2 ± 0.1	739.8 ± 41.7	116.3 ± 8.3	47.4 ± 4.2	133.0 ± 8.2	6.9 ± 0.3	4.6 ± 0.3	2.3 ± 0.2	2.1 ± 0.3

Table 3. Summary of hematology and biochemistry parameters for electrolytes and kidney function in rats.

Treatment	BUN (mg/dL)	Uric acid (mg/dL)	Sodium (mg/L)	Potassium (mg/L)	Chloride (mg/L)	Calcium (mg/dL)	Phosphorus (mg/dL)
C	22.6 ± 3.0	5.7 ± 1.5	150.1 ± 1.1	7.6 ± 1.0	91.9 ± 1.7	12.5 ± 0.6	17.3 ± 3.4
AC	20.6 ± 4.0	6.6 ± 1.6	148.9 ± 1.0	7.0 ± 0.6	91.1 ± 1.6	12.7 ± 0.3	15.4 ± 2.7
AM	21.0 ± 4.0	6.3 ± 2.3	148.2 ± 1.9	7.5 ± 1.8	91.9 ± 1.7	12.6 ± 0.8	15.6 ± 3.7
AS0.5	20.9 ± 2.3	6.0 ± 1.9	149.9 ± 1.6	7.1 ± 0.9	91.8 ± 1.8	12.8 ± 0.6	16.3 ± 2.1
AS1	17.9 ± 4.3	6.2 ± 1.5	148.4 ± 1.8	7.2 ± 1.2	93.4 ± 1.8	12.5 ± 0.6	13.7 ± 2.7
AS2	17.1 ± 2.0	6.3 ± 2.5	148.0 ± 2.2	7.3 ± 1.4	92.5 ± 1.9	12.5 ± 0.9	13.7 ± 2.5
AS6	20.2 ± 2.5	5.0 ± 1.7	148.0 ± 1.8	7.3 ± 1.0	93.9 ± 1.3	12.4 ± 0.6	14.4 ± 2.1

Data were presented as means ± SD (n = 9). Abbreviations: C: sham with normal diet; AC: A$\beta_{1\text{-}40}$-infused with normal diet; AM: A$\beta_{1\text{-}40}$-infused with cholinesterase inhibitor drug at 0.52 mg/day/kg bw; AS0.5: A$\beta_{1\text{-}40}$-infused with 14.6 mg/day/kg bw of CTE; AS1: A$\beta_{1\text{-}40}$-infused with 29.2 mg/day/kg bw of CTE; AS2: A$\beta_{1\text{-}40}$-infused with 58.4 mg/day/kg bw of CTE; AS6: A$\beta_{1\text{-}40}$-infused with 175.2 mg/day/kg bw of CTE. Blood urea nitrogen, BUN. High-Density Lipoprotein-Cholesterol, HDL-C; Low-Density Lipoprotein-Cholesterol, LDL-C; Creatine Protein Kinase, CPK; Aspartate aminotransferase, AST; Alanine aminotransferase, ALT; Alkaline Phosphatase, Alk-p.

3.6. Effects of CTE on Acetylcholine Levels, A$\beta_{1\text{-}40}$ Deposition, and ApoE Deposition in the Hippocampus of A$\beta_{1\text{-}40}$-Induced AD Rats

Reduced acetylcholine (ACh) levels are a hallmark of AD pathology, largely due to excessive hydrolysis by acetylcholinesterase (AChE) [41]. The effects of CTE on brain AChE activity are shown in **Figure 5**. A$\beta_{1\text{-}40}$ infusion significantly increased AChE activity in both hippocampal and cortical tissues compared with the control group. CTE administration significantly attenuated this A$\beta_{1\text{-}40}$-induced hyperactivation of AChE. The effects of CTE on A$\beta_{1\text{-}40}$ deposition were evaluated using immunohistochemical staining of hippocampal sections. As shown in **Figure 6(a)** and **Figure 6(b)**, A$\beta_{1\text{-}40}$-infused rats exhibited significantly higher percentages of A$\beta_{1\text{-}40}$-immunopositive areas compared with controls. CTE treatment dose-dependently reduced A$\beta_{1\text{-}40}$ deposition in hippocampal tissues relative to the A$\beta_{1\text{-}40}$-infused group. Given the role of apolipoprotein E (ApoE) in Aβ aggregation and AD pathogenesis, as well as its reported co-deposition with Aβ in amyloid plaques [42], ApoE deposition was also assessed. The A$\beta_{1\text{-}40}$-infused group showed significantly increased ApoE-immunopositive areas in both hippocampal and cortical tissues compared with controls. CTE administration resulted in dose-

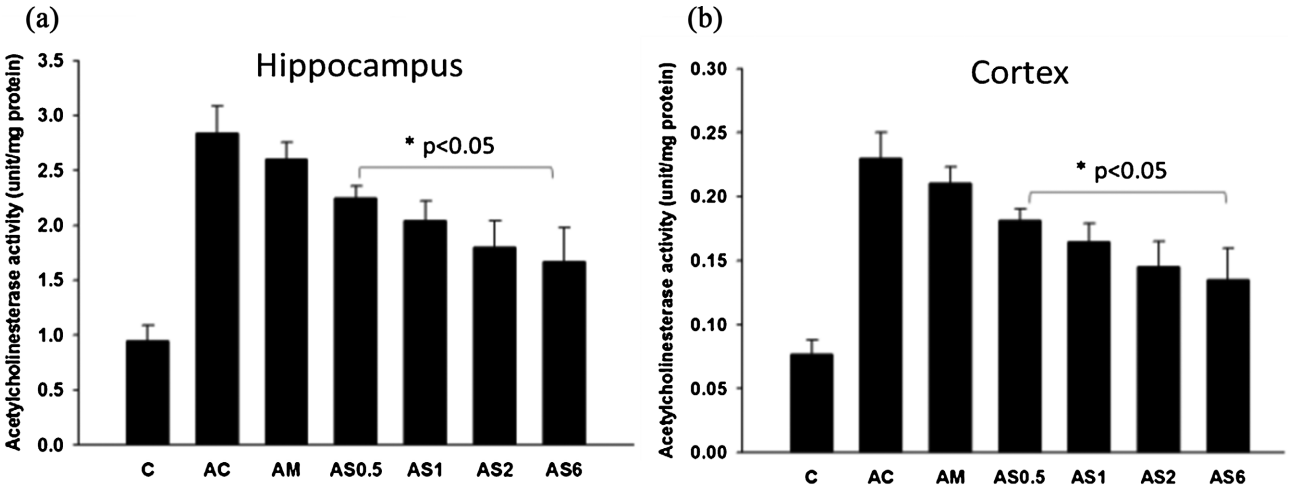

Figure 5. CTE effects on AChE activity in the hippocampus and cortex of A$\beta_{1\text{-}40}$-infused rats. AChE activity was measured in the hippocampus (a) and cortex (b). The results are expressed as mean ± SD (n = 9).

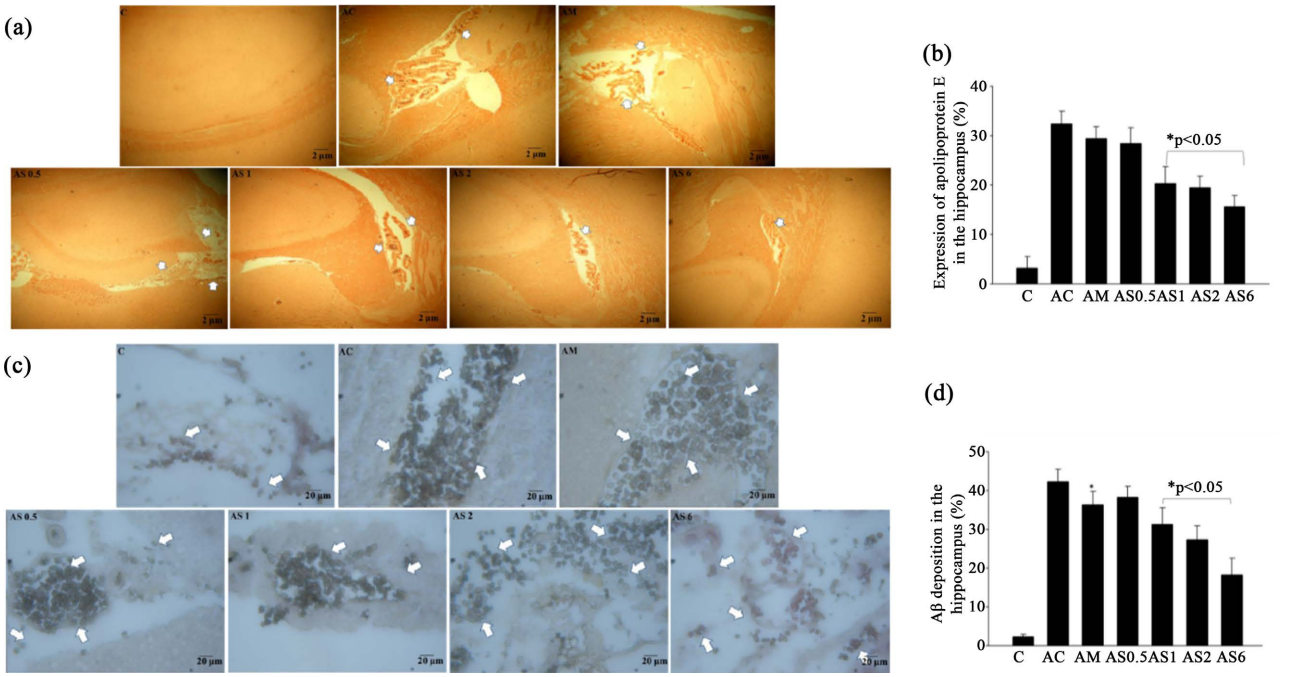

Figure 6. CTE effects on A$\beta_{1\text{-}40}$ and ApoE depositions in A$\beta_{1\text{-}40}$-infused rats. Brain sections were stained with anti-A$\beta_{1\text{-}40}$ (a) and anti-ApoE (c) antibodies using immunohistochemistry. A$\beta_{1\text{-}40}$-stained plaques were counted in at least 20 fields per brain section under 40X magnification with an image analyzer (Leica, Q500MC, Nussloch, Germany). The percentages of A$\beta_{1\text{-}40}$ (b) and ApoE (d) depositions were quantified. Results are expressed as mean ± SD (n = 9).

dependent reductions in ApoE deposition in both regions (Figure 6(c) and Figure 6(d)). Overall, these results demonstrate that CTE effectively reduced A$\beta_{1\text{-}40}$ accumulation, ApoE deposition, and AChE activity in A$\beta_{1\text{-}40}$-induced AD rats, supporting its neuroprotective potential.

4. Discussion

Oxidative stress plays a pivotal role in the development and progression of Alz-

heimer's disease (AD), contributing to neuronal damage and cognitive decline [3]. Elevated oxidative stress has been reported in both central and peripheral biological systems in AD-like animal models [4] [43] as well as in patients with AD [43] [44]. Given its central involvement in AD pathogenesis, therapeutic strategies aimed at mitigating oxidative stress warrant further investigation. In the present study, we evaluated the neuroprotective potential of a phenylethanoid glycosides–enriched *Cistanche tubulosa* extract (CTE) against oxidative stress and memory impairment in A$\beta_{1\text{-}40}$-infused rats.

To better model the chronic nature of AD pathology, we modified the conventional A$\beta_{1\text{-}40}$-induced AD-like animal model by administering continuous daily A$\beta_{1\text{-}40}$ infusions throughout the experimental period, rather than a single injection. Previous studies have commonly employed a single Aβ injection to induce AD-like pathology, including oxidative stress and cognitive impairment [36] [45]. However, continuous daily Aβ infusion more closely mimics the sustained accumulation of Aβ observed in the AD brain. Using this modified model, we observed pronounced oxidative stress, evidenced by elevated MDA levels in hippocampal tissue and blood plasma, increased plasma 8-OHdG levels, and enhanced protein carbonyl formation (**Figure 1**). In parallel, the activities of key antioxidant enzymes—SOD, catalase, GPx, and GRd—were significantly reduced in the hippocampus (**Figure 2**), accompanied by marked impairments in learning and memory (**Figure 3** and **Figure 4**) compared with sham-operated rats. These findings are consistent with previous reports showing that intra-hippocampal Aβ injection increases hippocampal ROS and MDA levels, reduces antioxidant defenses, and induces cognitive deficits [46].

The temporal relationship between oxidative stress and Aβ deposition in AD remains a topic of debate. Accumulating evidence suggests a bidirectional interaction between oxidative stress and Aβ accumulation. Oxidative stress may precede Aβ plaque formation and tau phosphorylation [15] [47], thereby promoting Aβ generation and aggregation [11]. Conversely, Aβ accumulation induces neuronal oxidative stress through excessive ROS production [13], leading to apoptotic signaling and neuronal cell death [14]. Indeed, Aβ-injected mice display AD-like features, including oxidative stress, neuronal apoptosis, and cognitive impairment [48]. This oxidative burden contributes to neuro inflammation, synaptic dysfunction, and neuronal loss, ultimately manifesting as memory and cognitive decline in AD patients [49]. Our findings support this reciprocal relationship, demonstrating that prolonged Aβ accumulation induces substantial oxidative stress accompanied by diminished antioxidant enzyme activities. Collectively, these results suggest that continuous daily hippocampal infusion of A$\beta_{1\text{-}40}$ represents a robust model for investigating Aβ-driven oxidative stress, hippocampal degeneration, and associated cognitive deficits.

Importantly, our results demonstrate that CTE effectively attenuated several key pathological features induced by A$\beta_{1\text{-}40}$, including Aβ deposition (**Figure 6**), elevated acetylcholinesterase (AChE) activity (**Figure 5**), and impairments in

learning and memory (**Figure 3** and **Figure 4**). Notably, CTE administration significantly reduced oxidative stress markers while restoring antioxidant enzyme activities in a dose-dependent manner (**Figure 1** and **Figure 2**). Consistent with our findings, previous studies have shown that CTE enhances antioxidant capacity by upregulating enzymes such as SOD and GPx [32], and its antioxidant properties have been widely documented [30] [31]. These observations suggest that the protective effects of CTE against A$\beta_{1\text{-}40}$-induced AD-like pathology are mediated, at least in part, through its ability to suppress oxidative stress and reinforce endogenous antioxidant defenses.

Lipids, particularly polyunsaturated fatty acids (PUFAs), are highly susceptible to oxidative damage due to the lipid-rich composition of the brain [50]. Lipid peroxidation has been proposed as an early event in AD pathogenesis [51], and the hippocampus—rich in PUFAs—is especially vulnerable to oxidative injury. In the present study, hippocampal lipid peroxidation was significantly increased in A$\beta_{1\text{-}40}$-infused rats and markedly attenuated by CTE treatment (**Figure 1(a)** and **Figure 1(b)**). Furthermore, ApoE-bound lipid particles enriched in PUFAs are particularly prone to peroxidation [52]. Our results revealed that increased lipid peroxidation in A$\beta_{1\text{-}40}$-infused rats coincided with enhanced ApoE deposition, both of which were significantly reduced following CTE administration. Emerging evidence suggests that oxidative stress may impair ApoE and its receptor ApoER2 via lipid peroxidation of critical functional domains, thereby disrupting the entorhinal-hippocampal memory system in sporadic AD [47]. Nevertheless, the causal relationship between oxidative stress and pathological ApoE accumulation remains controversial [53]. Given ApoE's established role in Aβ clearance, plaque formation, and co-deposition [54], further studies are required to clarify the interplay among oxidative stress, ApoE dysfunction, and Aβ pathology.

Our previous work demonstrated that CTE can cross the blood-brain barrier and chelate redox-active iron ions [35]. Increasing evidence implicates brain iron dyshomeostasis in AD pathogenesis, with oxidative stress and iron imbalance being closely intertwined [55]. Aβ peptides can bind redox-active metal ions, including iron, thereby promoting oxidative damage and neuronal death [13] [56]. Dysregulated iron metabolism can also drive free radical generation via the Fenton reaction, resulting in extensive damage to lipids, proteins, and nucleic acids [55]. Moreover, disturbances in redox homeostasis and iron availability can induce severe lipid peroxidation and trigger ferroptotic cell death [57]. Together with our findings, these studies suggest that oxidative stress in AD arises from multiple interconnected mechanisms involving Aβ accumulation and iron dysregulation, forming a deleterious feedback loop that exacerbates disease progression [58]-[60].

AD is characterized by progressive cognitive decline and memory loss, ultimately leading to severe dementia and imposing substantial caregiving, economic, and societal burdens. Although several FDA-approved drugs are available for AD treatment [2], their clinical benefits remain limited, reflecting the complex and

multifactorial nature of AD pathology. In the present study, CTE administration significantly ameliorated A$\beta_{1\text{-}40}$-induced cognitive and memory impairments, at least in part through its antioxidant effects. While the therapeutic efficacy of antioxidants in AD remains controversial—given the lack of FDA-approved antioxidant-based therapies [11]—the present findings, together with our previous studies [7] and those of others [34], highlight the multi-targeted neuroprotective properties of CTE.

The diverse bioactive components of CTE, particularly echinacoside and acteoside, have been shown to exert neuroprotective effects through multiple mechanisms, including the attenuation of AD-related pathology [7] [20] [21] [26]. Notably, echinacoside has been reported to inhibit Aβ deposition and improve cognitive function in APP/PS1 transgenic mice via activation of the PI3K/Akt/Nrf2/PPARγ signaling pathways [39]. Moreover, *C. tubulosa* aqueous extract has been approved in China as a botanical drug for vascular dementia [35]. Although limited by small sample size, an open-label, non-placebo-controlled clinical study suggested that CTE may stabilize cognitive function and daily living activities in patients with mild to moderate AD, with efficacy comparable to long-term acetylcholinesterase inhibitor therapy [21]. While the precise mechanisms underlying the protective effects of phenylethanoid glycosides from *C. tubulosa* remain to be fully elucidated, their ability to target multiple pathological pathways simultaneously positions CTE as a promising candidate for the development of alternative or adjunctive therapeutic strategies for AD.

Nonetheless, this study has certain limitations. First, the oxidative stress markers, Aβ/ApoE deposition, and behavioral outcomes were evaluated as individual endpoints, and the causal relationships among these parameters were not established. Second, the antioxidant pathway and anti-amyloid effects were interpreted as parallel outcomes, as no mechanistic linkage between them was directly examined.

5. Conclusion

The findings of this study demonstrate that CTE significantly attenuated oxidative stress in A$\beta_{1\text{-}40}$-induced AD rats, accompanied by enhanced activities of key antioxidant enzymes. These results support the hypothesis that the mitigation of A$\beta_{1\text{-}40}$-induced AD-like pathology by CTE is mediated, at least in part, through the suppression of oxidative stress and the reinforcement of endogenous antioxidant defenses. In addition, CTE reduced acetylcholinesterase activity as well as A$\beta_{1\text{-}40}$ and apolipoprotein E deposition, without inducing detectable toxicity. Collectively, these findings indicate that CTE exerts robust antioxidant and neuroprotective effects and may represent a promising therapeutic candidate for the prevention or treatment of Alzheimer's disease.

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Authors' Contributions

Conceptualization, M.-H.S.; methodology, H.-C.L; formal analysis, C.-T.C and C.-L.C and Y.-H.H; investigation, Y.-T.W; Critical revision of manuscript, C.-T.C and C.-L.C; writing—original draft preparation, C.-T.C and W.-F.H; writing—review and editing, M.-H.S. All authors have read and agreed to the published version of the manuscript.

Availability of Data and Materials

Data will be made available on request.

Ethical Approval

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of NTU (Approval No. 12, Year 98).

Statement of Human and Animal Rights

All of the experimental procedures involving animals were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals (Protocol No. 99-127-B) and approved by the Institutional Animal Care and Use Committee (IACUC) of NTU.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Statement of Consent

The authors give their consent for personal information related to the subject to be published by OALib publishing.

Conflicts of Interest

The authors declare no conflicts of interest.

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