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Effect of *Tacca Chantrieri* André Rhizome Extract on Alleviating Memory Deficit in Scopolamine-Injected Rats by Protecting Against Oxidative Damage and Enhancing Cholinergic Function

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Abstract

This study aims to investigate the neuroprotective effects of *Tacca chantrieri* André rhizome extract (TCE) on memory, oxidative stress and cholinergic function. The animals were divided into six groups: A control group receiving no treatment and five experimental groups administered either a vehicle, donepezil (a positive control), or TCE at doses of 50, 100 and 200 mg/kg body weight (BW) for 14 consecutive days. On day 8, scopolamine was also intraperitoneally injected into the animals daily for the remaining 7 days. At the end of the experiment, the hippocampus was utilized for biochemical assays and histological studies. TCE administration in scopolamine-injected animals resulted in a significantly higher discrimination index than in animals treated with scopolamine alone. TCE treatment also decreased acetylcholinesterase activity, resulting in elevated acetylcholine levels and an increased quantity of surviving neurons in the CA3 region of the hippocampus. Additionally, TCE administration reduced hippocampal lipid peroxidation, as indicated by lower malondialdehyde levels. Furthermore, TCE treatment significantly enhanced the activity of antioxidant enzymes, which protect cells from oxidative stress. Qualitative analysis of compounds in TCE was conducted using LC-QTOF-MS/MS, which identified saponins, flavonoids and phenolic compounds as the predominant chemical constituents. Therefore, these compounds present in the plant extract may confer protection against memory impairment and oxidative stress in animal models of Alzheimer's disease.

Keywords: *Tacca chantrieri*, Scopolamine, Memory impairment, Oxidative stress, Cholinergic function

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder, marked by progressive declines in cognition, behavior and memory. Its key histopathological features include extracellular senile plaques, intracellular neurofibrillary tangles and cholinergic neuronal loss in the brain [1]. Increasing evidence suggests that oxidative stress plays a crucial role in the early stages of AD, preceding cytopathological changes [2]. This stress primarily results from the accumulation of reactive oxygen species (ROS) within cells [3]. Elevated ROS levels in neuronal cells can lead to DNA and protein damage, trigger lipid peroxidation and ultimately cause cell death [4]. The brain is particularly vulnerable to oxidative stress due to its high oxygen consumption, abundance of unsaturated lipids and relatively low levels of endogenous antioxidant defenses [5]. As a result,

oxidative stress-induced neuronal death is considered a significant factor in AD development [6].

To counteract oxidative damage, cells utilize both enzymatic and non-enzymatic defense systems [7]. Key antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT), help neutralize oxidative stress [7]. Studies have shown that increasing the levels of these enzymes can mitigate oxidative stress associated with AD [1,2,8]. Additionally, the cholinergic system plays a crucial role in memory processing and is a major focus of AD research related to cognitive decline [9]. Cholinergic dysfunction in AD is typically characterized by reduced acetylcholine (ACh) levels, increased acetylcholinesterase (AChE) activity and cholinergic neuronal loss, leading to impaired neurotransmission essential for memory function [9,10]. These findings highlight the strong link between cholinergic dysfunction, oxidative stress and cognitive deficits.

Scopolamine, an anticholinergic agent, impairs learning, memory acquisition and consolidation by blocking muscarinic acetylcholine receptors (mAChRs) in the hippocampus [9]. These receptors are crucial for cognitive function, as they regulate synaptic plasticity, neuronal excitability and neurotransmitter release [9]. Their activation facilitates neuronal communication and supports long-term potentiation (LTP), particularly in the hippocampus - a brain region vital for memory formation and retrieval [9,11]. By antagonizing ACh at muscarinic receptors across both the central and peripheral nervous systems, scopolamine disrupts cholinergic neurotransmission, leading to sedative, antiemetic and amnesic effects [9,11]. Impaired muscarinic signaling has been closely associated with cognitive decline seen in aging and neurodegenerative disorders such as AD [9,11]. Beyond cholinergic dysfunction, scopolamine also induces oxidative stress and neuroinflammation in the brain. It increases malondialdehyde (MDA) levels - an indicator of lipid peroxidation - and impairs the activities of key antioxidant enzymes, including SOD, GSH-Px and CAT [8,11]. This imbalance between the production and elimination of ROS compromises the brain's antioxidant defense system and contributes to neuronal damage. Due to its capacity to reproduce multiple aspects of AD pathology - including memory impairment, oxidative stress, elevated AChE activity and disrupted cholinergic signaling - scopolamine is widely used in preclinical models to study AD-related cognitive decline [11,12]. Given the involvement of oxidative stress and neuroinflammation in scopolamine-induced cognitive dysfunction, plant-based interventions with antioxidant and anti-inflammatory properties are gaining attention as promising therapeutic strategies. Medicinal plants rich in phytochemicals such as saponins, flavonoids and phenolic acids have shown potential to counteract these damaging effects, making them attractive candidates for managing neurodegenerative disorders like AD with fewer adverse effects than conventional drugs [13].

Tacca chantrieri André, commonly known as the bat flower, is a traditional medicinal plant native to northern Thailand and belongs to the Dioscoreaceae family. The plant's rhizomes are notably abundant in bioactive constituents, including saponins, glycosides, flavonoids and phenolic compounds [14-16]. These phytochemicals are associated with a range of pharmacological activities, notably antioxidant, neuroprotective and anti-inflammatory properties [15,16]. Oxidative stress plays a critical role in the progression of neurodegenerative diseases like AD, as excessive free radical production leads to neuronal damage, mitochondrial dysfunction and synaptic loss. *Tacca chantrieri* André rhizome extract (TCE) has demonstrated strong antioxidant capacity in various *in vitro* assays, including 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging and ferric ion reducing antioxidant power (FRAP) assays [15,17,18]. These assays confirm its ability to neutralize ROS, reduce oxidative damage, and maintain redox homeostasis. In addition to its antioxidant potential, TCE has shown neuroprotective effects in experimental models. TCE has demonstrated promising effects in enhancing memory and alleviating cognitive impairment, likely through its neuroprotective and anti-inflammatory properties, as observed in lipopolysaccharide (LPS)-induced rat models [19]. Emerging evidence suggests that TCE can protect hippocampal neuronal damage, suppress neuroinflammatory responses and improve cognitive performance in animal models. These findings indicate that TCE may serve as a potential therapeutic candidate for neurodegenerative disorders such as AD [16,19]. Moreover, TCE exhibits anti-inflammatory activity in

LPS-stimulated murine microglial BV-2 cells by reducing the production of proinflammatory cytokines, which are known to exacerbate oxidative stress and neuronal damage [20]. Notably, TCE has been found to prevent neuronal cell death induced by amyloid-beta peptide 25-35 ($A\beta_{25-35}$), a key contributor to AD-related neurodegeneration [20]. Recent studies have demonstrated that TCE enhances cognitive function by increasing the percentage of alternation in the Y-maze test. It also attenuates neuroinflammation by elevating serotonin levels, reducing pro-inflammatory cytokines and astroglial activation, and promoting neuronal survival in a scopolamine-induced rat model [16]. Saponins, a key component of TCE, have shown potential in improving memory and cognitive function, particularly in the context of AD. They may enhance memory through various mechanisms, including reducing amyloid-beta protein deposition, inhibiting tau phosphorylation, modulating oxidative stress and reducing inflammation [21]. Saponins have been shown to enhance synaptic plasticity - the capacity of synapses to strengthen or weaken in response to activity - which is fundamental to learning and memory processes [22]. Moreover, flavonoids, naturally occurring plant compounds found in TCE, have been linked to improved memory and cognitive function. Several studies suggest that a higher dietary intake of flavonoids correlates with a reduced risk of cognitive decline and memory impairment. The cognitive benefits of flavonoids are believed to arise through multiple mechanisms, including the suppression of neuroinflammation, enhancement of cholinergic function and upregulation of brain-derived neurotrophic factor (BDNF), a key protein involved in neuronal survival, synaptic plasticity and memory formation [23]. Given its potent antioxidative, anti-neuroinflammatory and neuroprotective properties, TCE may attenuate cognitive deficits and neuronal cell loss in animal models of AD by reducing oxidative stress, suppressing neuroinflammation, supporting cholinergic function and promoting neuronal survival. Consequently, TCE has the potential to slow disease progression and enhance cognitive function. This study aims to investigate the effects of TCE on memory performance, oxidative stress markers and cholinergic neurotransmission in a scopolamine-induced AD animal model.

Materials and methods

The TCE preparation from *Tacca chantrieri* André rhizomes

Tacca chantrieri André rhizomes were harvested from Chiang Rai, Thailand, in September 2023. Plant identification was verified by Dr. Chaiyong Rujjanawate and a voucher specimen (No. 168-M) was deposited at the School of Medicine, Mae Fah Luang University. The rhizomes were carefully washed, dried and ground into coarse powder. Ethanol extraction was then performed on the powdered material. The resulting mixture was filtered to remove solid residues and the filtrate was concentrated using a rotary evaporator (Heidolph Hei-VAP Core, Schwabach, Germany) under reduced pressure at 50 - 55 °C. The concentrated extract was subsequently freeze-dried (lyophilized) using a laboratory freeze dryer (Labconco Freezone 4.5 Liter Benchtop, Kansas, USA) to obtain a solid powder, which was stored at –20 °C for further use.

Analysis of TCE by liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS)

The chemical profiling of TCE was carried out using Agilent MassHunter software (Agilent Technologies Inc., USA). Chromatographic separation was achieved on a Poroshell EC-C18 column (2.7 µm, 2.1×150 mm^2) with a binary mobile phase system consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Instrumental parameters were set as follows: Gas temperature at 300 °C, gas flow rate of 10 L/min, nebulizer pressure at 40 psi and sheath gas flow rate of 12 L/min. The capillary voltage and nozzle voltage were adjusted to 3,500 V and 3.5 kV, respectively. A sample volume of 0.5 µL from a 1 mg/mL solution (diluted in distilled water) was injected for analysis, with the mobile phase delivered at a constant flow rate of 0.2 mL/min. Sampling speeds for draw and ejection were set at 100 µL/min and 400 µL/min, respectively. Mass spectral data were acquired across a mass range of 50 - 1,100 amu, using a scan rate of 4 spectra/s for both MS and MS/MS modes. Fragmentation during MS/MS analysis was performed automatically using collision energies of 10, 20 and 40 eV.

Animal study

A total of 36 healthy male Wistar rats were obtained from Nomura Siam International Co., Ltd. (Bangkok, Thailand). The animals were 7 weeks old and weighed between 220 and 240 g. They were randomly divided into 6 groups, with 6 rats in each group. Throughout the experiment, three animals were accommodated per cage, kept under a 12-h light/dark cycle and supplied with unrestricted access to water and food. Animal health was recorded daily, including observations on general appearance, behavior and body weight monitoring. The study adhered to humane endpoints to minimize unnecessary suffering and no animal deaths occurred during the experiment. The research was designed to obtain meaningful and reproducible data while using the minimal number of animals required. Additionally, humane handling and housing conditions met established welfare standards. All experimental procedures were conducted following Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes and the ARRIVE guidelines for reporting experiments using live animals. The ethical guidelines for the handling and care of laboratory animals were strictly approved by the Ethics Committee of Mae Fah Luang University's Laboratory Animal Research Center on July 21, 2023, under approval number AR03/66.

Prior to the start of the experiment, animals were randomly assigned to various treatment groups. They underwent a three-day acclimation period to the behavioral apparatus before testing. Behavioral evaluations were conducted on day 0 (baseline) and day 14. Baseline assessments revealed no significant differences in locomotor activity or cognitive performance among the groups, confirming their equivalence. Behavioral assessments on day 14 comprised the novel object recognition test (NORT) and the open field test (OFT). Upon completion of these assessments, all animals were humanely euthanized. The right hippocampus was collected for biochemical analyses, while the left hippocampus was reserved for histological examination. **Figure 1** depicts the procedure and design of the experiment.

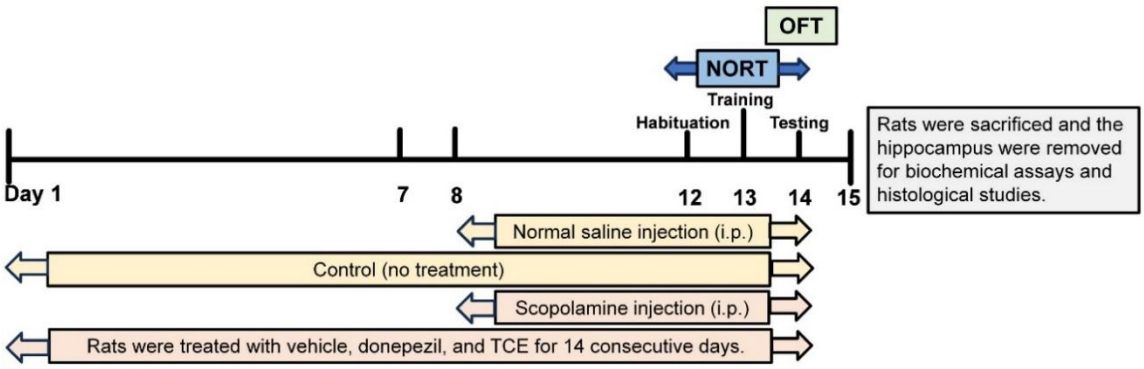

Figure 1 The experimental design and protocol. Animals were administered either vehicle, donepezil, or TCE (at doses of 50, 100 and 200 mg/kg BW) once daily for 14 days. On day 8th, scopolamine was injected intraperitoneally into the animals daily for the subsequent 7 days. Behavioral assessments, including the NORT and OFT, were conducted 1 h after scopolamine administration on day 14 of the experimental protocol.

Drug administration

The animals were randomly allocated into six groups and received the following treatments: (1) control, (2) vehicle, (3) donepezil (DPZ) at 3 mg/kg BW, (4) TCE at 50 mg/kg BW, (5) TCE at 100 mg/kg BW and (6) TCE at 200 mg/kg BW. The doses of DPZ and TCE used in this study were selected based on previous research demonstrating their efficacy in enhancing cognitive function and exerting neuroprotective effects in rodent models [16,19,24]. Specifically, prior studies have shown that these doses of TCE significantly ameliorate memory deficits and modulate neuroinflammatory pathways in models of scopolamine- and LPS-induced cognitive impairment [16,19]. The selected concentrations also fall within the range considered pharmacologically active but non-

toxic, supporting their suitability for investigating therapeutic potential. The control group received normal saline without any additional treatment. Other groups were administered either distilled water (vehicle), DPZ (a positive control), or TCE. All treatments were given once daily for 14 days. Scopolamine hydrobromide (SCO; Sigma-Aldrich, USA) was used to induce cognitive deficit. Starting on day 8, scopolamine was administered intraperitoneally at a dose of 3 mg/kg BW daily for the remaining 7 days. The scopolamine dose was selected based on a prior study demonstrating that seven days of 3 mg/kg BW scopolamine injections increased AChE activity and lipid peroxidation, correlating with memory deficits [8,25].

Behavior test of animals

Two behavioral tests were employed to assess the animal's behavioral performance. The OFT was used to investigate changes in locomotor behavior. In contrast, the NORT was conducted to evaluate cognitive performance before and after scopolamine administration.

Novel object recognition test (NORT)

For the evaluation of cognitive performance, it was assessed using the NORT. The test was conducted in a clear plexiglass cage under constant lighting conditions of 40 lux. The NORT was divided into three phases: Habituation, training, and testing. During the habituation period, the animals were allowed to investigate the clear box for 5 min. During the training period, the animals were given 5 min to examine two familiar objects placed within the box. On the test day, 24 h after the training session, the animals were presented with one familiar object and one novel object. The duration each animal spent examining each object, defined as sniffing and directing its nose towards an object within a distance of less than 2 cm, was recorded for 5 min. The discrimination index (DI), a measure of object recognition memory, was calculated using the following formula [26]:

$$\text{DI}\ (\%) = \left(\frac{T_{novel} - T_{familiar}}{T_{novel} + T_{familiar}}\right) \times 100$$

Open field test (OFT)

To evaluate the locomotion and exploration behaviors of animals, the OFT was utilized [27]. The device comprised a floor-mounted clear plexiglass box. Each animal was put in the field and given the opportunity to freely investigate the box. During 5 min, the number of instances of spontaneous rearing behavior, wherein the animals stood on their hind legs to investigate the box, was recorded.

Biochemical assessments

Following euthanasia, the right hippocampal tissue was immediately extracted and stored at –80 °C for subsequent biochemical analysis. The right hippocampal tissue was weighed and then homogenized in a glass homogenizer using either ice-cold phosphate-buffered saline (PBS) or a lysis buffer solution. Following homogenization, samples were centrifuged at 10,000× g for 10 min at 4 °C, and the resulting supernatant was collected for biochemical analyses. The supernatant was subsequently stored at –80 °C until further use for the determination of MDA levels, antioxidant enzyme activities, ACh concentration, AChE activity and total protein content.

Determination of MDA levels

Oxidative stress was assessed based on the levels of MDA, a byproduct of lipid peroxidation. The levels of MDA were measured using a colorimetric assay kit (Thiobarbituric acid (TBA) method) from Elabscience (Catalog No. E-BC-K025-M, Houston, Texas, USA) following the manufacturer's instructions [28].

Determination of scavenging enzyme activities

Scavenging enzymes, including SOD, GSH-Px and CAT, catalytically remove free radicals and other ROS. The activities of SOD, GSH-Px and CAT were assessed using colorimetric assay kits (Elabscience, Catalog No. E-BC-K020-M; E-BC-K096-M; E-BC-K031-M, Houston, Texas, USA) following the manufacturer's instructions [28].

Determination of ACh levels and AChE activity

The concentration of ACh levels was measured using Enzyme-Linked Immunosorbent Assay (ELISA) kits from Elabscience (Catalog No. E-EL-0081, Houston, Texas, USA), while the activity of AChE was investigated using a colorimetric assay kit (Catalog No. E-BC-K174-M, Houston, Texas, USA) following the manufacturer's instructions [28].

Determination of protein concentration

The total protein concentration of the whole-cell lysate was determined using a Bradford protein assay kit (Catalog No. SK3031; Ontario, Canada), with bovine serum albumin (BSA) employed as the calibration standard.

Processing of tissues and Nissl staining

The left hemispheres of the brains were promptly excised and fixed in 4% paraformaldehyde prepared in 0.1 M PBS, pH 7.4. After fixation, the tissues were embedded in paraffin and sectioned into 5 µm-thick slices using a Leica rotary microtome (RM 2245; Leica, Wetzlar, Germany). The brain sections were deparaffinized in xylene, rehydrated through a graded ethanol series, and rinsed in distilled water. They were then stained with 1% cresyl violet solution for 5 min. After staining, the sections were rinsed twice with distilled water and subsequently dehydrated through an ethanol series (70% to 100%), with each step lasting 5 min. Finally, the slides were cleared in xylene and coverslipped using a mounting medium (Sigma-Aldrich, USA) [8,16].

Cell counts analysis

Hippocampal images were acquired at approximately –3.14 mm from Bregma using an Olympus EP50 microscope at 20× magnification, with image capture performed via EP View software (Olympus, Tokyo, Japan). Neuronal counting was conducted by a blinded investigator to minimize bias. Two hippocampal subregions, CA1 and CA3, were selected for analysis. Within each image, a standardized area of 200 μm^2 was designated, and neurons within this region were counted. Neuronal density was then calculated as the number of cells per 200 μm^2 [8,16].

Statistical analysis

The results were presented as mean ± standard error of the mean (S.E.M.). Analytical statistics were conducted using the SPSS Statistics 25 software program. One-way analysis of variance (ANOVA) followed by a post hoc Tukey test was employed for the analysis. A significance level of p-value < 0.05 was used to determine statistical significance.

Results and discussion

Effects of TCE on behavioral study, neuronal density and cholinergic function

The NORT is a highly validated method for assessing recognition memory, which involves the functioning of the hippocampus [29]. To assess the impact of TCE on scopolamine-induced memory impairment, all animals were allowed to examine familiar and novel objects as observed by NORT. Animals treated with scopolamine demonstrated memory impairments, evidenced by a significant decrease in the discrimination index (p-value < 0.05) compared to untreated controls. However, treatment with DPZ and TCE (100 and 200 mg/kg BW) significantly alleviated cognitive impairment, resulting in an improved discrimination index compared to the vehicle plus scopolamine group (p-value < 0.05 for all groups), as shown in **Figure 2(A)**. These findings highlight the detrimental impact of scopolamine injection on cognitive functions. Our results demonstrated that intraperitoneal injection of scopolamine at a dose of 3 mg/kg BW did not affect locomotor behavior in the animals (**Figure 2(B)**), indicating that their motor function remained unaffected. A prior study reported that a single dose of scopolamine at 20 mg/kg induced hyperactivity in animals [30]. In our study, we administered low doses of scopolamine to gradually impair cognition, similar to the effects observed in AD patients. The NORT results confirm that scopolamine impairs recognition memory without affecting locomotor activity and that TCE treatment effectively reverses these cognitive deficits in animal models induced by scopolamine.

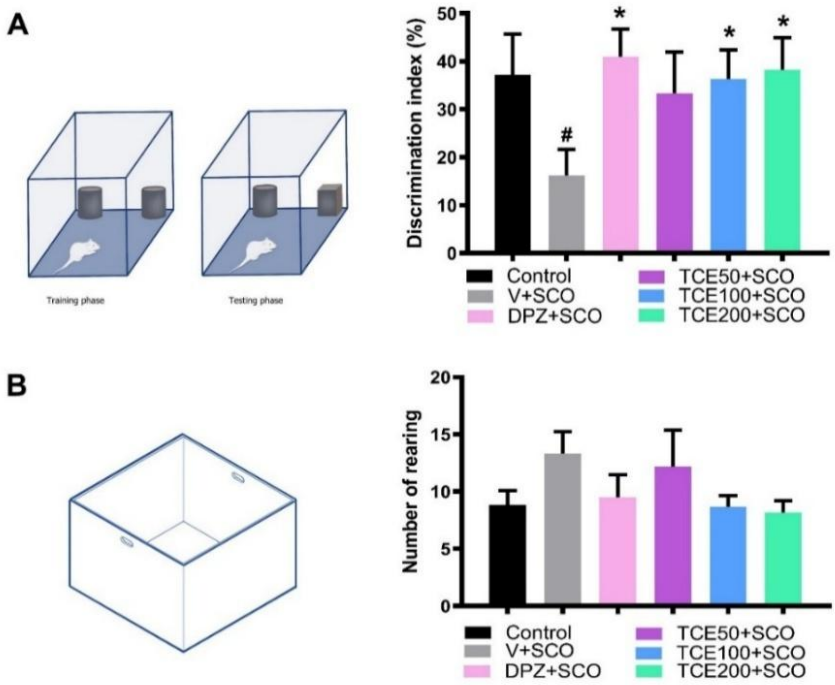

Figure 2 Effects of TCE on scopolamine-induced memory impairment. (A) Diagram of the NORT and graph showing the results of the discrimination index (%). (B) Diagram of the OFT and graph showing the results of the number of rearing behaviors. One-way ANOVA was utilized for data analysis, and the outcomes were displayed as mean ± SEM (n = 6). $^{\#}p$-value < 0.05, vs. the control group; $^{*}p$-value < 0.05, vs. the vehicle with scopolamine group.

The hippocampus is essential for learning and memory functions. Its primary function is to store short-term memories and facilitate their transfer to long-term storage [31]. The hippocampus's neuronal circuit for memory encoding, storage and retrieval involves signals from pyramidal neurons in the CA1 and CA3 regions [31]. Therefore, these regions play a primary role in controlling the memory-related neuronal circuit. The results of neuronal survival in the CA1 and CA3 regions of the hippocampus are presented in **Figures 3(A**) and **3(B)**, respectively. Our findings demonstrated that animals subjected to scopolamine injection exhibited significantly lower neuronal survival in the CA3 area than the control animals (p-value < 0.01). However, animals subjected to scopolamine and subsequently administered with DPZ or TCE at doses of 100 and 200 mg/kg BW showed a significantly greater number of surviving neurons in the CA3 region compared to those receiving the scopolamine vehicle treatment (p-value < 0.01, p-value < 0.05 and p-value < 0.01, respectively). Furthermore, there were no significant differences in the number of neuronal survivals in the CA1 area of the hippocampus among the groups. Our findings are consistent with previous studies showing that scopolamine-injected animals exhibit neuronal loss in the CA3 region of the hippocampus [32]. The hippocampal CA3 region is more susceptible to oxidative damage than the CA1 region, with vulnerability influenced by factors such as the experimental method, age, species and observation period [32]. Our results suggest that scopolamine selectively impairs neuronal survival in the CA3 region of the hippocampus and that both DPZ and TCE treatments confer neuroprotective effects by preserving neuronal integrity in this vulnerable region.

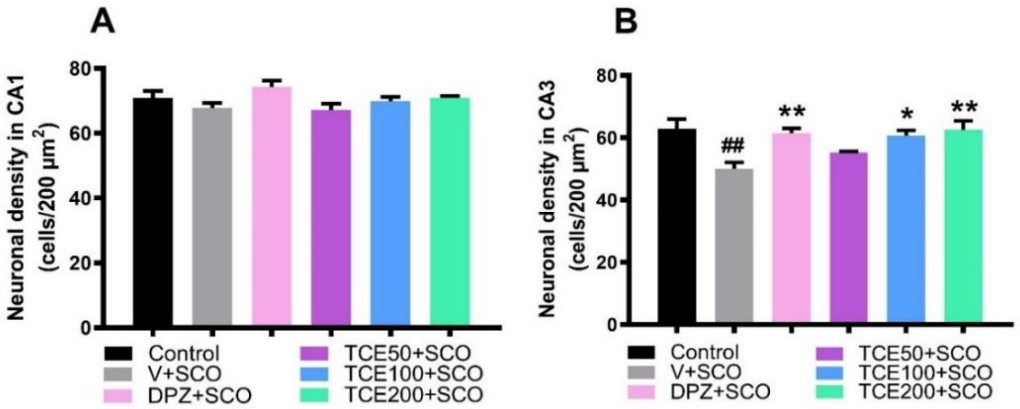

Figure 3 Effect of TCE on neuronal survival in the hippocampus. (A) Neuronal density in the CA1 region. (B) Neuronal density in the CA3 region. One-way ANOVA was utilized for data analysis, and the outcomes were displayed as mean ± SEM (n = 6). $^{\#\#}p$-value < 0.01, vs. the control group; $^{*}p$-value < 0.05, $^{**}p$-value < 0.01, vs. the vehicle with scopolamine group.

Figure 4 presents photomicrographs of the CA1 and CA3 areas of the hippocampus, captured at a 20× magnification and stained with cresyl violet. These images are accompanied by scale bars of 20 µm in length.

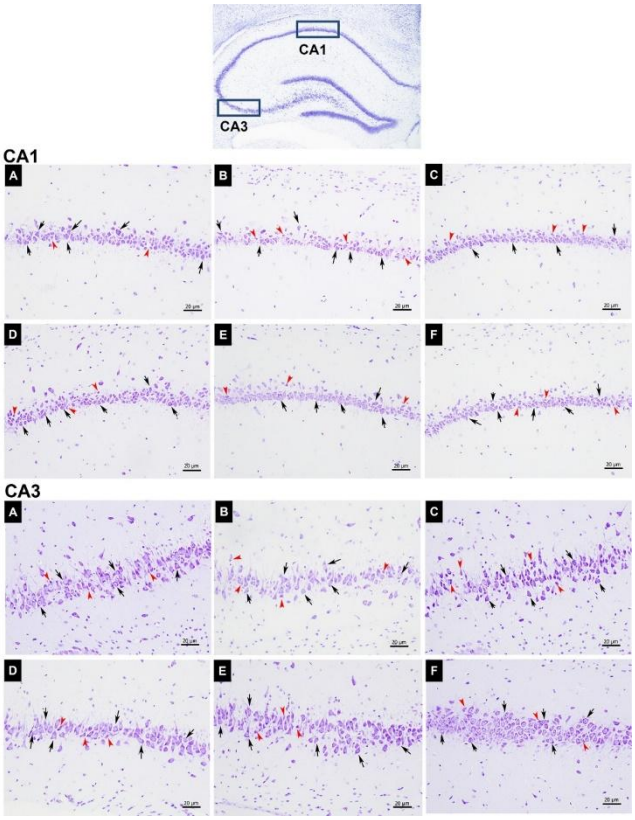

Figure 4 Photomicrographs of the CA1 and CA3 regions of the hippocampus, captured at a 20× magnification and stained with cresyl violet. (A) control, (B) V + SCO, (C) DPZ + SCO, (D) TCE50 + SCO, (E) TCE100 + SCO and (F) TCE200 + SCO. The black arrow represented the survival neurons; the red arrowhead represented the dark, shrunken and destroyed neurons.

The cholinergic system is essential for learning and memory. According to the cholinergic hypothesis of AD, memory impairment arises from the loss of cholinergic neurons in the basal forebrain and hippocampus, resulting in a decline in cholinergic function [9]. ACh, a neurotransmitter released by cholinergic neurons, plays a vital role in signal transduction processes associated with memory and learning abilities. AChE is an enzyme that catalyzes the hydrolysis of acetylcholine into choline and acetate, thereby terminating impulse transmission within the cholinergic system [10]. Hence, cholinergic dysfunction, characterized by alterations in ACh and AChE levels, plays a crucial role in the pathogenesis of AD [9,10]. Scopolamine injection-induced cholinergic neuronal loss leads to a reduction of ACh levels within the cholinergic pathway [33]. A previous study has also reported that scopolamine-induced mitochondrial dysfunction contributes to damage in hippocampal neurons [34]. Our results demonstrated that animals subjected to scopolamine injection exhibited improved AChE activity (p-value < 0.01) while showing reduced hippocampal ACh concentration (p-value < 0.01) compared to the control group, as illustrated in **Figures 5(A)** and **5(B)**. Conversely, animals subjected to scopolamine and administered with DPZ and TCE at doses of 100 and 200 mg/kg BW exhibited significantly decreased AChE activity (p-value < 0.01 for all groups), which corresponded with an increase in hippocampal ACh concentration compared to the vehicle-treated scopolamine group (p-value < 0.001, p-value < 0.05 and p-value < 0.01, respectively).

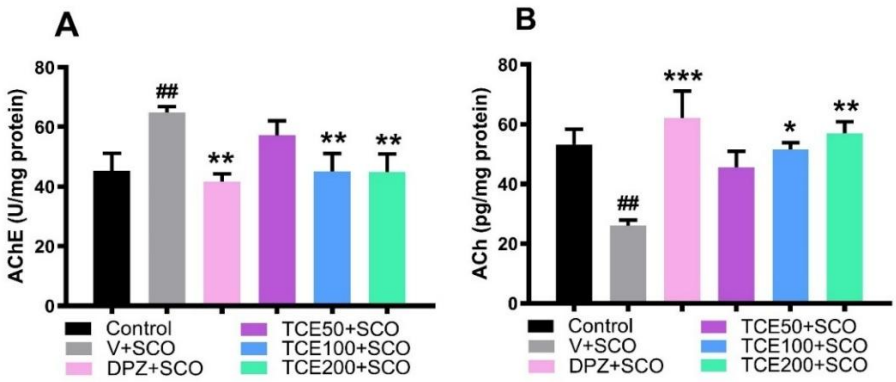

Figure 5 Effect of TCE on the activity of AChE and ACh levels. Graphs of the effect of TCE on (A) AChE activity and (B) ACh levels. One-way ANOVA was utilized for data analysis and the outcomes were displayed as mean ± SEM (n = 6). $^{\#\#}p$-value < 0.01, vs. the control group; $^{*}p$-value < 0.05, $^{**}p$-value < 0.01 and $^{***}p$-value < 0.001, vs. the vehicle with scopolamine group.

Interestingly, animals treated with TCE showed a significantly greater neuronal density in the CA3 region compared to those receiving vehicle plus scopolamine. Moreover, our results revealed elevated ACh levels in the hippocampus of TCE-treated animals, correlating with decreased AChE activity. This was accompanied by increased hippocampal neuronal survival and a higher discrimination index in the NORT compared to animals receiving vehicle plus scopolamine. Therefore, TCE treatment may protect against memory deficits and cholinergic neuronal loss induced by scopolamine, which mimics the AD-like condition in an animal model.

Effect of TCE on oxidative stress and antioxidant activities

Oxidative stress plays a crucial role in the pathogenesis of AD [2]. The brain is particularly vulnerable to oxidative stress. In AD, critical neuronal components - such as lipids, proteins and nucleic acids - undergo oxidative damage due to mitochondrial dysfunction, chronic inflammation and the accumulation of amyloid-beta peptides [1,2]. In the AD model of animals, scopolamine administration leads to cholinergic dysfunction, increased amyloid-beta deposition and induced oxidative stress, as evidenced by elevated MDA levels - a marker of lipid peroxidation.

These effects replicate key pathological features of AD [35-37]. Animals subjected to scopolamine injection exhibited higher MDA levels compared to the control group (p-value < 0.05), as illustrated in **Figure 6(A)**. Conversely, animals treated with DPZ or TCE (200 mg/kg BW) showed reduced MDA levels compared to animals receiving the vehicle plus scopolamine (p-value < 0.01 and p-value < 0.05, respectively). Our results corroborate prior research demonstrating increased MDA levels in animals administered scopolamine [38]. The brain is particularly vulnerable to oxidative stress due to its high oxygen utilization, elevated lipid content, and low levels of antioxidants [5]. The primary enzymatic protectors, such as SOD, GSH-Px and CAT, play a crucial role in protecting cells and membrane lipids from oxidative damage [39]. SOD facilitates the conversion of superoxide radicals into hydrogen peroxide and oxygen. GSH-Px catalyzes the detoxification of hydrogen peroxide and lipid peroxides by utilizing reduced glutathione. CAT further breaks down hydrogen peroxide into water and oxygen, thereby reducing oxidative stress within cells [39]. In **Figures 6(B) - 6(D)**, the results demonstrate that scopolamine-injected animals exhibited reduced activities of SOD (p-value < 0.05), GSH-Px (p-value < 0.01) and CAT (p-value < 0.05) compared to the untreated group. However, animals given DPZ or TCE at doses of 100 and 200 mg/kg BW displayed improved levels of SOD (p-value < 0.05, p-value < 0.05, p-value < 0.01, respectively) and GSH-Px (p-value < 0.01, p-value < 0.05, p-value < 0.01, respectively). Furthermore, the results of CAT showed a positive effect in animals administered DPZ and TCE at a dose of 200 mg/kg BW compared to the vehicle receiving scopolamine group (p-value < 0.05 and p-value < 0.05, respectively). These results suggest that TCE mitigates oxidative stress by reducing lipid peroxidation and enhancing endogenous antioxidant defenses. Our findings are consistent with earlier studies demonstrating TCE's potent antioxidant activity and its ability to scavenge free radicals generated by lipid peroxidation [15,17,18,40]. These results show that TCE effectively attenuated scopolamine-induced oxidative stress by lowering MDA levels and restoring antioxidant enzyme activities, thereby highlighting its neuroprotective potential in an AD-like animal model.

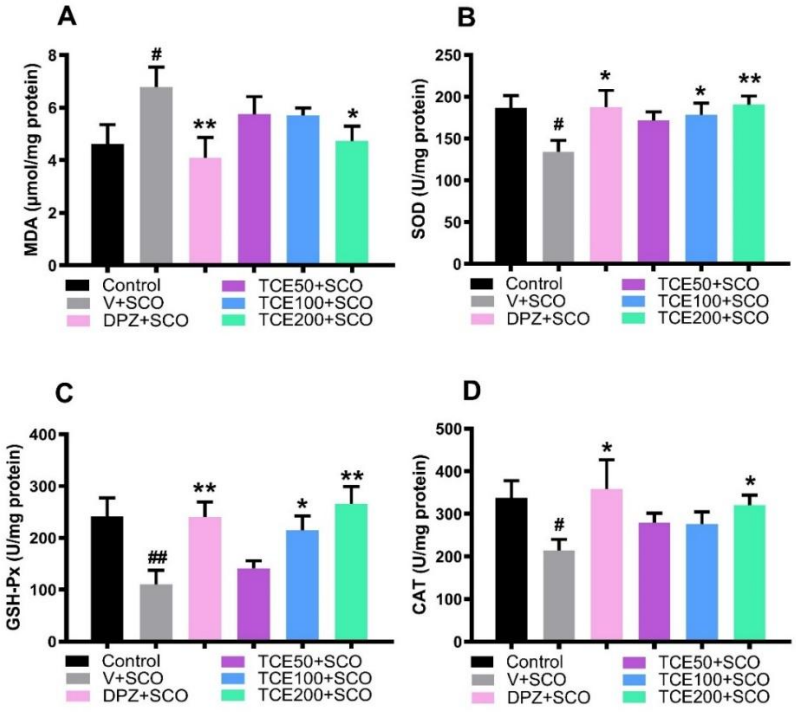

Figure 6 Effect of TCE on oxidative stress and antioxidant activities. Graphs of the effect of TCE on levels of (A) MDA, (B) SOD, (C) GSH-Px, and (D) CAT. One-way ANOVA was utilized for data analysis, and the outcomes were displayed as mean ± SEM (n = 6). $^{\#}p$-value < 0.05, $^{\#\#}p$-value < 0.01, vs. the control group; $^{*}p$-value < 0.05, $^{**}p$-value < 0.01, vs. the vehicle with scopolamine group.

The qualitative identification of compounds in TCE by LC-QTOF-MS/MS analysis

The qualitative analysis of compounds in TCE was performed using LC-QTOF-MS/MS in both positive and negative ionization modes, revealing that saponins, flavonoids, and phenolic compounds are the major chemical constituents, with match scores exceeding 80%, as presented in **Table 1**.

Table 1 The qualitative identification of compounds in TCE was performed using LC-QTOF-MS/MS analysis.

Proposed compounds	Molecular Formula	Molecular weight	Ionization (ESI^+/ESI^-)	Score (DB)
Saponins				
Tragopogonsaponin G	$C_{56}H_{82}O_{21}$	1090.53	$[M-H]^-$	80.47
Basellasaponin A	$C_{47}H_{70}O_{21}$	970.44	$[M-H]^-$	87.85
Kudzusaponin SA4	$C_{47}H_{74}O_{20}$	958.48	$[M-H]^-$	86.39
Achyranthoside C	$C_{47}H_{72}O_{20}$	956.46	$[M-H]^-$	91.89
Quillaic acid	$C_{30}H_{46}O_5$	486.70	$[M-H]^-$	88.22
Ampeloside Bf2	$C_{45}H_{76}O_{21}$	952.49	$[M-H]^-$	80.23
Torvoside G	$C_{34}H_{56}O_9$	608.29	$[M-H]^-$	87.45
Flavonoids				
Catechin 5-O-beta-D-glucopyranoside	$C_{21}H_{24}O_{11}$	452.13	$[M+H]^+$	81.58
Quercetin 5,7,3',4'-tetramethyl ether 3-rutinoside	$C_{31}H_{38}O_{16}$	666.22	$[M+H]^+$	81.39
6-methoxyflavone	$C_{16}H_{12}O_3$	252.08	$[M-H]^-$	94.73
6,8-Di-C-alpha-L-arabinopyranosylapigenin	$C_{25}H_{26}O_{13}$	534.13	$[M+H]^+$	85.89
Epiafzelechin (2R,3R)(-)	$C_{15}H_{14}O_5$	274.08	$[M+H]^+$	92.68
Wanepimedoside A	$C_{33}H_{42}O_{15}$	678.25	$^*[M+H]^+$	83.40
Anastatin B	$C_{21}H_{14}O_7$	378.06	$[M-H]^-$	81.76
Phenolic compounds				
Glucocaffeic acid	$C_{15}H_{18}O_9$	342.10	$^*[M+H]^+$	93.19
Lucidenic acid B	$C_{27}H_{38}O_7$	474.26	$[M-H]^-$	94.25
[6]-Gingerdiol 3,5-diacetate	$C_{21}H_{32}O_6$	380.22	$[M+H]^+$	81.17
Mukoenine A	$C_{18}H_{19}NO$	265.14	$[M+H]^+$	98.91
Fagopyrine	$C_{40}H_{34}N_2O_8$	670.22	$[M+H]^+$	84.46
Glycobismine A	$C_{37}H_{34}N_2O_6$	602.24	$[M+H]^+$	89.82
Ligustroside	$C_{25}H_{32}O_{12}$	524.19	$[M-H]^-$	83.99
Beta-hydroxyacteoside	$C_{29}H_{36}O_{16}$	640.20	$[M+H]^+$	92.67

Note: ESI, electrospray ionization; min, minute.

The symbol * denotes the detection of the compound in both the positive $[M+H]^+$ and negative $[M-H]^-$ modes of ionization.

Our findings suggest that TCE attenuates the neurotoxic effects of scopolamine, potentially through the synergistic action of its bioactive constituents - such as saponins, quercetin and catechin - as presented in **Table 1**. These components exert neuroprotective effects through multiple mechanisms, including reducing oxidative stress, preserving cholinergic function, preventing neuronal loss and enhancing cognitive performance. Saponins have been shown to inhibit oxidative stress by lowering MDA levels in

hydrogen peroxide (H_2O_2)-induced cell damage while enhancing the activity of antioxidant enzymes [41]. Additionally, saponins protect cholinergic neurons by maintaining choline acetyltransferase (ChAT) levels and preserving neuronal integrity, which could explain the cognitive improvements observed in our study [42]. Quercetin has been reported to reverse memory impairment in the NORT and reduce scopolamine-induced neuronal degeneration in the hippocampus [43]. This aligns with our findings of improved cognitive function and preserved neuronal density in the hippocampal CA3 region following TCE treatment. Catechin contributes to cholinergic system enhancement by reducing oxidative stress and inhibiting AChE activity, thereby preventing the breakdown of ACh. Their ability to enhance antioxidant enzyme activity further supports TCE's role in counteracting scopolamine-induced neurotoxicity [35]. Several studies have explored the beneficial effects of these bioactive compounds on cognitive enhancement and neuroprotection [35,41-43]. Accordingly, our results suggest that TCE counters the detrimental effects of scopolamine by mitigating oxidative stress, increasing antioxidant enzyme activity, preserving neuronal density and improving cognitive performance.

To strengthen the translational relevance of our findings, it is important to consider the bioavailability and pharmacokinetic properties of TCE and its individual bioactive constituents. TCE contains a diverse range of phytochemicals - including saponins, flavonoids and phenolic compounds - which are believed to contribute synergistically to its neuroprotective effects. However, the therapeutic efficacy of these compounds can be significantly influenced by factors such as poor water solubility, metabolic instability and limited gastrointestinal absorption. Previous studies have shown that certain saponins and flavonoids in TCE possess moderate oral bioavailability and are capable of crossing the blood-brain barrier (BBB), a key requirement for treating neurodegenerative diseases [44,45]. Therefore, comprehensive pharmacokinetic profiling - including assessment of plasma and brain concentration-time curves, metabolic pathways and BBB permeability - is essential for optimizing the dosing regimen and advancing TCE toward clinical use.

Despite the promising neuroprotective outcomes observed in this study, several limitations warrant consideration. First, although three doses of TCE (50, 100 and 200 mg/kg BW) were tested, the results did not establish a clear dose-response relationship. The absence of a consistent or linear correlation between dose and effect hinders the identification of an optimal therapeutic window. Further pharmacodynamic studies are needed to determine whether a threshold or saturation point exists. Second, long-term safety and toxicity were not evaluated. While no acute adverse effects were observed during the treatment period, chronic toxicity studies are crucial to assess potential cumulative risks, particularly if TCE is intended for prolonged use in conditions such as AD. Specific toxicological endpoints - including hepatotoxicity, nephrotoxicity, and behavioral changes - should be systematically investigated. Additionally, this study did not examine the pharmacokinetics or brain distribution of TCE's active compounds, both of which are vital for understanding their therapeutic potential. Addressing these limitations in future studies will provide a more robust foundation for evaluating the clinical applicability of TCE as a treatment for neurodegenerative disorders like AD.

Conclusions

This study evaluated the therapeutic potential of TCE in an animal model of AD. The findings indicate that TCE exerts neuroprotective effects by mitigating scopolamine-induced neuronal injury and oxidative stress within the hippocampus. TCE administration enhanced neuronal survival, increased ACh levels and reduced AChE activity, suggesting an improvement in cholinergic function. These benefits may be attributed to the presence of multiple bioactive constituents in TCE, including saponins, flavonoids and phenolic compounds, which likely act synergistically on oxidative and cholinergic pathways. However, the precise bioactive components responsible for these effects remain unidentified and their individual pharmacological contributions are yet to be fully elucidated. Future studies should aim to isolate and characterize the active metabolites of TCE *in vivo* and assess their pharmacokinetic properties, including BBB permeability. Additionally, evaluating TCE in transgenic AD models - such as APP/PS1 or 3xTg-AD

mice - would provide more clinically relevant insights into its long-term efficacy, mechanism of action and disease-modifying potential. Such approaches will strengthen the translational value of TCE as a candidate for AD therapy.

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Declaration of Generative AI in Scientific Writing

No content generation or data interpretation was performed by the AI. The authors take full responsibility for the content and conclusions of this work.

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