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Neuroprotective effect of *Tiliacora triandra* (Colebr.) Diels leaf extract on scopolamine-induced memory impairment in rats

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ABSTRACT

Alzheimer's disease is characterized by progressive memory loss caused from alterations in the central cholinergic system. While existing medications often have adverse effects, traditional use of *Tiliacora triandra* in Thailand shows its potential as a revitalizing neurotonic agent. This study explores the impact of *T. triandra* leaf extract on cognitive behaviors, neuronal density, and oxidative stress in male rats with scopolamine-induced cognitive impairment. Experimental groups composed of a control, vehicle, positive control meditation, and *T. triandra* extract-treated groups (100, 200, and 400 mg/kg BW) over 14 days, with scopolamine administration (i.p.) between days 8 and 14. Results showed significant enhancements in the discrimination ratio and spontaneous alteration behavior percentage during novel object recognition (NORT) and Y-maze tests for scopolamine-administered rats treated with *T. triandra* extract or donepezil. In contrast, open field test (OFT)-assessed spontaneous locomotor activity displayed no significant difference. Notably, acetylcholinesterase (AChE) activity and malondialdehyde (MDA) levels reduced significantly in scopolamine-treated rats with *T. triandra* extract or the positive control. Moreover, neuronal density in the hippocampal CA3 region, superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) activities increased significantly. However, catalase (CAT) activity exhibited no significant difference. In conclusion, *T. triandra* leaf extract shows promise in mitigating scopolamine-induced memory deficits, potentially attributed to increased neuronal density, inhibited AChE activity, reduced MDA levels, and enhanced antioxidant activities. This extract has potential as a therapeutic agent for Alzheimer's disease-associated memory impairment.

1. Introduction

Alzheimer's disease (AD), a progressive neurodegenerative condition, stands as the most prevalent form of dementia. AD is characterized by a memory deficit accompanied by behavioral disturbances, which result in a detrimental impact on daily activities [1]. Pathophysiological changes in AD encompass the alteration of cholinergic neurons, buildup of tau protein, and accumulation of

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the β-amyloid peptide [2]. The cholinergic system represents a primary neurotransmission system in the brain, integral to memory and cognitive processing. Therefore, a significant pathophysiological element of AD involves a decrease in cholinergic activity [3,4]. Acetylcholinesterase (AChE), an enzyme responsible for breaking down acetylcholine (ACh) into choline and acetate, has been found to be elevated in individuals with AD. AChE contributes to the aggregation of β-amyloid peptides into insoluble plaques observed in the AD brain [5].

Moreover, age-related neurodegeneration and cognitive decline are thought to be influenced by oxidative stress, which is defined by an imbalance in the radical production of reactive oxygen species (ROS) and antioxidative defense [6]. The hippocampus is essential for acquisition, consolidation, and retrieval of declarative memories. However, it is vulnerable to oxidative stress [7]. The development of spatial memory depends on hippocampal formation, which is composed of the dentate gyrus (DG) and the cornu ammonis 1 and 3 (CA1 and CA3), situated in the medial temporal lobe of the brain. To complete the neural circuit in the hippocampal formation, axons from the dentate granule cell transmit information to the CA3 pyramidal cells, whose axons project to the CA1 pyramidal cells. The CA1 pyramidal cells then send the information back to the deeper layers of the entorhinal cortex [8]. Hence, hippocampal damage leads to an inability to form new memories [9].

Numerous animal models have been developed to assess the various pathophysiological bases of dementia. At muscarinic receptors, scopolamine functions as a competitive antagonist, impairing central cholinergic functioning in rodents and causing problems with learning and memory [10]. Since scopolamine is an anticholinergic drug, it disrupts ACh binding sites and prevents ACh from attaching to its receptor. As a result, there is an impairment of hippocampal cholinergic transmission, which eventually causes memory loss and learning difficulties [11]. Scopolamine injections can be used to evaluate the cholinergic hypothesis because they cause cognitive impairment similar to that seen in AD. The treatment for AD aims to restore cholinergic activity by blocking the AChE enzyme.

Tiliacora triandra (Colebr.) Diels, also known as Ya-nang in Thai, is a member of the Menispermaceae family. It was a natural Southeast Asian plant that was extensively utilized in northeast Thai cuisine. *T. triandra* is one of the traditional Thai remedies. This plant is used as a revitalizing and neurotonic agent. Research suggests that *T. triandra* possesses a range of potential therapeutic properties, including anti-bacterial, anti-malarial, anti-cancer, anti-inflammatory, anti-fever, anti-diabetes, and antioxidant activities, as well as the ability to prevent neuronal damage in the hippocampus, improve cholinergic function, and inhibit AChE [12–17]. Specifically, *T. triandra*'s antioxidant and AChE-inhibiting properties may be particularly useful in treating disorders related to free radicals and hippocampal damage.

2. Materials and methods

2.1. Plant material and preparation of the aqueous extract

To prepare the leaf extract of *T. triandra*, fresh leaves were harvested from the Kantharawichai district in Mahasarakham province, Thailand. The leaves underwent a thorough washing process using distilled water, repeating the wash five times. Subsequently, the fresh leaves were subjected to drying at 60 °C for a period of 72 h in a hot air oven. Once dried, the leaves were finely ground using a blender. The finely ground leaves were then subjected to a boiling process with distilled water, maintaining a ratio of 1:5 (leaves to water), for a duration of 10 min. The resulting solution from the extraction was carefully filtered through a Whatman No.1 filter paper. The solution was subjected to evaporation through distillation in order to preconcentrate the solution. This preconcentration step was facilitated using a rotary evaporator. Following this step, the extract underwent freeze-drying utilizing a lyophilizer. The resulting freeze-dried extract was stored at a temperature of −20 °C, to be readily available for future use.

2.2. LC-ESI-QTOF-MS/MS characterization of T. triandra leaf extract

The identification of phenolic compounds in plants has long been done using LC-MS [18]. A qualitative analysis of phenolic compounds from *T. triandra* leaf extract was performed by LC-ESI-QTOF-MS/MS analysis in both negative and positive ionization modes (Fig. S1 and Table S1–Supplementary Material) with the following parameters: column: Poroshell-EC-C18, 2.7 μm, 2.1 × 150 mm; mobile phase A: 0.1 % formic acid in water; mobile phase B: 0.1 % formic acid in acetonitrile (ACN); capillary voltage, +3500 V; gas temperature, 300 °C; gas flow, 11 L/min; nebulizer pressure, 45 psig; MS scan rate, 2 spectrum/s; and MS/MS scan rate, 3 spectrum/s. The flow rate was kept at 0.2 mL/min, and 1 μL of the sample solution was injected for analysis.

2.3. Animals

Young adult male Wistar rats (8 weeks old and weighing between 240 and 280 g) were obtained from Nomura Siam International Co., Ltd. in Bangkok, Thailand. Three animals were housed in each cage during the experiment, and a 12-h light/dark cycle was maintained. The rats were provided with ad libitum access to food and water. Prior to the experiment, the animals were randomly assigned to different experimental groups (n = 6 per group) and there were no significant differences between the groups in behavioral tests. The rats were given three days to acclimate to the behavior testing apparatus before being evaluated.

2.4. Drugs and administration

The animals were divided into six groups. The animals in each group were given substances as follows: (I) control, (II) vehicle, (III) donepezil, (IV) extract from *T. triandra* leaf dosed at 100 mg/kg BW, (V) extract from *T. triandra* leaf dosed at 200 mg/kg BW, and (VI)

extract from *T. triandra* leaf dosed at 400 mg/kg BW. Animals in the control group had no treatment and were intraperitoneally injected (i.p.) with normal saline (0.9 % NaCl) on days 8–14. The animals received once-daily oral gavage administrations of distilled water used as a vehicle or donepezil HCl (a cognitive enhancer drug, manufactured and distributed by Eisai Co., Ltd., Tokyo, Japan) at a dose of 5 mg/kg BW or *T. triandra* leaf extract at doses of 100, 200, and 400 mg/kg BW (0.5 mL/300 g BW) between 9.00 and 10.00 a.m. for 14 consecutive days. Scopolamine hydrobromide obtained from Sigma-Aldrich (St. Louis, MO, USA) was used to induce memory deficits. Scopolamine, at a dose of 3 mg/kg BW (i.p.), was administered to the animals from day 8 to day 14 to induce cognitive impairment. Oral doses of vehicle, donepezil, and *T. triandra* leaf extract were given 30 min before the scopolamine injection. Behavioral tests were carried out 1 h after the treatment on days 7 and 14. At the end of the experiment, the rats were intraperitoneally injected (i.p.) with thiopental sodium at a dose of 80 mg/kg BW. Subsequently, the right hippocampus was collected to measure the activity of acetylcholinesterase (AChE), malondialdehyde (MDA) levels, and antioxidant enzymes activities, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). The left hippocampus was obtained to evaluate neuronal density.

2.5. The behavioral test of the rats

The behavioral tests, which comprised the novel object recognition test (NORT), Y-maze test (YMT), and open field test (OFT), were conducted on days 7 and 14. The experimental design and protocol are illustrated in Fig. 1.

2.5.1. Novel object recognition test (NORT)

To evaluate cognitive function, NORT was employed. The test was carried out in a transparent plexiglass box with a consistent light condition (40 lux), measuring 40 cm in length, 40 cm in width, and 40 cm in height. The NORT consisted of three phases: habituation, training, and testing [19,20]. During the habituation phase, which took place on the first day, the animals were permitted to explore the empty box for 5 min before being returned to their initial cage.

During the training phase, the animals were placed in the box for 5 min to explore two familiar objects. On the testing day, which occurred 24 h after the training, the animals were exposed to one object with which they were familiar (object A) and another object with which they were unfamiliar (object B). The rats directed their noses towards the objects and sniffed them from a distance of less than 2 cm. Each rat investigated the objects, and afterward, the box and objects were cleaned with a 70 % ethanol solution. The discrimination ratio (DR) was determined using the following formula:

$$\mathrm{DR} = \frac{\text{Time spent exploring object B} - \text{Time spent exploring object A}}{\text{Time spent exploring object B} + \text{Time spent exploring object A}}$$

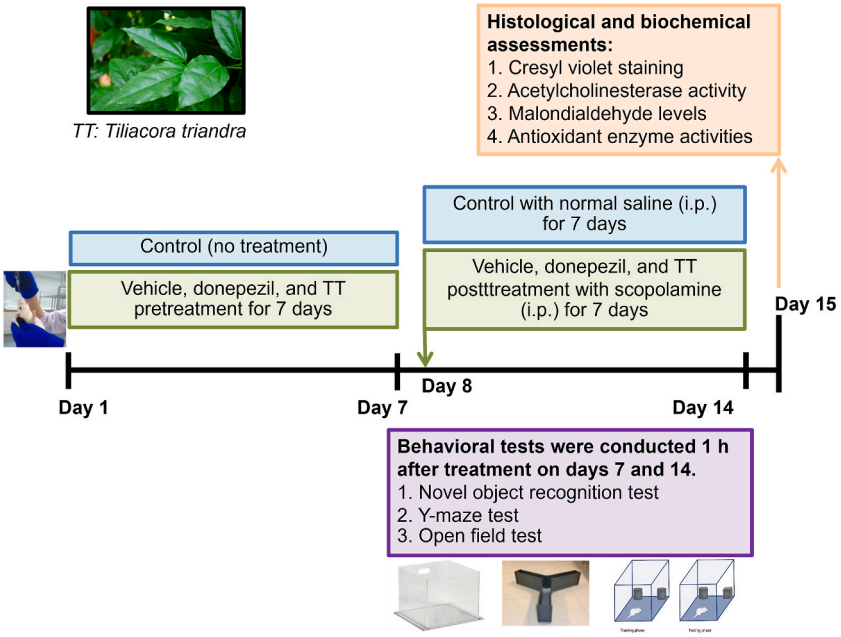

Fig. 1. The experimental design and protocol. Thirty-six rats were randomly assigned to each group and orally administered either distilled water, donepezil, or *T. triandra* (TT) leaf extract at doses of 100, 200, and 400 mg/kg BW once daily for 14 consecutive days. On days 8–14, scopolamine was intraperitoneally injected in all groups, except for the control group, which received normal saline injection. The behavioral tests, including the novel object recognition test (NORT), Y-maze test (YMT), and open field test (OFT), were conducted 1 h after treatment on days 7 and 14. The experimental design followed standard procedures and adhered to ethical guidelines [10].

2.5.2. Y-maze test (YMT)

The Y-maze test was conducted according to the methods described by Conrad and colleagues [21]. As previously mentioned, the Y-maze test was used to evaluate short-term working memory by measuring spontaneous alternation behavior [22]. Spontaneous alternation behavior (SAB) was used as a measure of exploratory behavior and the rats' ability to explore a novel area. Typically, rats tend to explore a different arm of the maze than the one they previously visited. After being placed in the center of the maze, the rats were allowed to freely explore the three arms of the maze for 8 min. The number of arm entries and the number of triads were recorded to determine the percentage of spontaneous alternation behavior. An arm entry was counted when all four paws of the rat were within the arm. The following formula was used to calculate the percentage of spontaneous alternation behavior [SAB (%)]:

$$\text{SAB}\left(\%\right) = \frac{\text{Number of alternations}}{\text{Total arm entries minus two}} \times 100$$

2.5.3. Open field test (OFT)

The exploratory behaviors of rats were frequently assessed using the open field test (OFT) [23,24]. The apparatus consisted of a clear plexiglass box with a floor that was equally divided into 16 squares and measured 40 cm in length, width, and height. Each rat was placed in the center of the field and allowed to freely explore the box. The number of squares crossed with all four paws was used to determine the number of crossings.

2.6. Biochemical assessments

2.6.1. Measurement of acetylcholinesterase (AChE) activity

On day 14 following behavioral testing, rats were sacrificed and the right hippocampus was swiftly dissected and stored at −80 °C for subsequent biochemical analysis. The hippocampal tissues, each weighing 0.025 g, were homogenized on ice in 225 mL of lysis buffer solution using a glass homogenizer. The homogenates were then centrifuged at 10000 ×g for 10 min at 4 °C to obtain the supernatant. The activity of acetylcholinesterase (AChE) was measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Elabscience Biotechnology Co., Ltd., Catalog No. E-BC-K174-M, Houston, USA). The intra-assay coefficient of variation was 4.7 %, while the inter-assay coefficient of variation was 9.3 %. The sensitivity of the assay was 1.225 U/mL. The samples were analyzed within 1 month of storage.

2.6.2. Measurement of malondialdehyde (MDA) activity

The right hippocampus was weighed to 0.025 g and homogenized in 225 mL of ice-cold phosphate buffered saline (PBS) using a glass homogenizer. The homogenates were then centrifuged at 10,000 ×g for 10 min at 4 °C to obtain the supernatant. The malondialdehyde (MDA) levels were measured using an ELISA kit (Elabscience Biotechnology Co., Ltd., Catalog No. E-BC-K025-M, Houston, USA). The intra-assay coefficient of variation was 4.1 %, while the inter-assay coefficient of variation was 7.2 %. The sensitivity of the assay was 1.13 mol/L.

2.6.3. Measurement of antioxidant enzyme activities

The supernatant obtained from the right hippocampal homogenates prepared with PBS was used to determine the activities of antioxidant enzyme. ELISA kits were employed to evaluate the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) (Elabscience Biotechnology Co., Ltd., Catalog No. E-BC-K020-M; E-BC-K031-M; E-BC-K096-M, Houston, USA). The intra-assay coefficient of variation for SOD, CAT, and GSH-Px was 2.9 %, 3.9 %, and 2.4 %, respectively. The inter-assay coefficient of variation for SOD, CAT, and GSH-Px was 3.7 %, 7.7 %, and 6.2 %, respectively. The sensitivities of the assays for SOD, CAT, and GSH-Px were 0.2, 1.12, and 17.17 U/mL, respectively.

2.6.4. Total protein concentration

The supernatant obtained from the right hippocampal homogenate prepared with lysis buffer solution was used to prepare a whole-cell protein lysate. The total protein concentration of the lysate was measured using a commercially available protein assay kit and the colorimetric Bradford method (Bio-Rad, USA), with bovine serum albumin (BSA) as a standard control.

2.7. Tissue processing and cresyl violet staining for neuronal density determination

The left brain hemisphere was preserved in 4% paraformaldehyde in PBS (1X, pH 7.4) for 24 h. Then, the brains were placed into 12.5 % sucrose for cryoprotection. Using a cryostat microtome, the brains were divided into coronal pieces with a 30 μm thickness (AST500, Amos Scientific, Australia). The brain slices were stained for 5 min with 0.2 % cresyl violet stain solution (Sigma-Aldrich, St. Louis, MO, USA), followed by two rinses with distilled water and 5 min of dehydration in ethanol concentrations of 70 %, 95 %, and 100 %. The slides were coated with Dibutylphthalate Polystyrene Xylene (DPX) after being washed with xylene three times.

2.8. Cell count analysis

Using an Olympus EP50 microscope (The Science and Education Co., Ltd., Model U-LHLEDC, Olympus Corporation Tokyo, Japan)

in conjunction with the EP view program, hippocampus images (−3.14 mm from Bregma) were captured at a magnification of 20×. Neuronal counting was conducted by an investigator who remained blind to the experimental conditions. The hippocampus images were further divided into three distinct subregions: cornu ammonis 1 (CA1), cornu ammonis 3 (CA3), and dentate gyrus (DG). The individual cellular layers were examined, focusing on the granule cell layer that encompasses neuronal cells within the CA1, CA3, and DG regions. The criteria employed for distinguishing CA1, CA3, and DG neurons encompassed distinctive characteristics. CA3 neurons were recognized by their triangular shape, substantial size, closely arranged disposition, and vivid staining, displaying features of pyramidal neurons. In contrast, CA1 pyramidal neurons exhibited a more loosely organized pattern and a paler staining. DG granule neurons, on the other hand, presented as diminutive entities with round to oval profiles, forming a densely packed layer of granule cells.

An area of 200 μm^2 was fixed, and the neuron count within this designated area was conducted to determine the average neuron density. The resulting data were represented as neuronal density (cells/200 μm^2). For each rat, cresyl violet staining was assessed using three photomicrographs per designated area.

2.9. Statistical analysis

The data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by a least significant difference (LSD) post hoc test using SPSS Statistics 22 software. Differences between groups were considered statistically significant at probability values less than 0.05 ($P < 0.05$).

3. Results

3.1. Effect of T. triandra leaf extract on recognition memory

The NORT was used to measure their capacity to memorize the examined objects, as determined by the increasing discrimination ratio in the behavioral test. On day 7, there were no appreciable differences in the rats' recognition memories among the groups. However, on day 14, the rats that received vehicle and scopolamine injections demonstrated memory impairment, as shown by a poorer discrimination ratio ($F_{(5, 30)} = 1.854$, $P < 0.05$), compared to the control rats. The scopolamine-injected rats that were treated with donepezil and *T. triandra* leaf extract at doses of 200 and 400 mg/kg BW showed better recognition memory compared to untreated rats ($F_{(5, 30)} = 1.854$, $P < 0.05$ for all groups). Fig. 2 illustrates the results.

3.2. Effect of T. triandra leaf extract on spatial working memory

To assess the rats' short-term memory, the Y-maze test was employed. Spontaneous alternation behavior, which measures spatial working memory and is based on rats' natural exploratory behavior to investigate new environments [22], was used as an indicator. On day 7, rats treated with donepezil or *T. triandra* leaf extract at doses of 200 and 400 mg/kg BW exhibited a significant increase in the percentage of spontaneous alternation behavior compared to the vehicle group ($F_{(5, 30)} = 4.331$, $P < 0.01$, $P < 0.05$, $P < 0.05$, respectively). On day 14, rats injected with scopolamine and given vehicle displayed memory impairment compared to the control group, as evidenced by a lower percentage of spontaneous alternation behavior ($F_{(5, 30)} = 1.883$, $P < 0.05$). Treatment with donepezil or *T. triandra* leaf extract at doses of 200 and 400 mg/kg BW in scopolamine-injected rats resulted in higher spontaneous alternation behavior than those of the scopolamine-injected rats that given vehicle ($F_{(5, 30)} = 1.883$, $P < 0.05$ for all groups), as depicted in Fig. 3.

3.3. Effect of T. triandra leaf extract on spontaneous locomotor activity

The open field test (Fig. 4) did not show any significant differences in the number of crossings among the rats treated with vehicle,

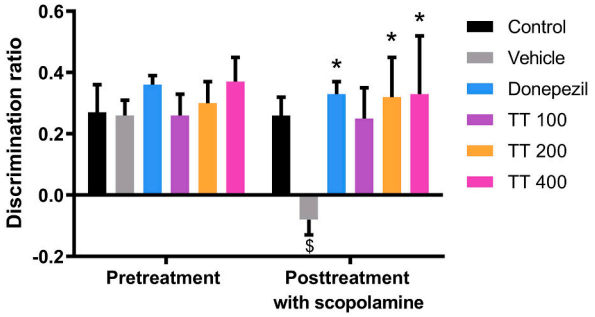

Fig. 2. The effects of donepezil and *T. triandra* (TT) leaf extract at doses of 100, 200, and 400 mg/kg BW on recognition memory in rats, using the novel object recognition test. Data analysis was performed using one-way analysis of variance, and the results are presented as mean ± S.E.M ($n = 6$ per group). $^{\$}P < 0.05$, in comparison to control receiving normal saline injection; $^{*}P < 0.05$, in comparison to the group receiving vehicle with scopolamine injection.

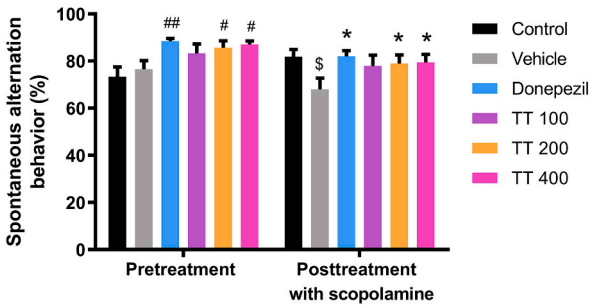

Fig. 3. The effects of donepezil and *T. triandra* (TT) leaf extract at doses of 100, 200, and 400 mg/kg BW on percentage of spontaneous alternation behavior in Y-maze test in rats. One-way analysis of variance was used for data analysis. Data are presented as mean ± S.E.M. ($n = 6$ per group). $^{\#}P < 0.05$, in comparison to vehicle group; $^{\#\#}P < 0.01$, in comparison to vehicle group; $^{\$}P < 0.05$, in comparison to control receiving normal saline injection; $^{*}P < 0.05$, in comparison to the group receiving vehicle with scopolamine injection.

donepezil, and *T. triandra* leaf extract at doses of 100, 200, and 400 mg/kg BW, and the control group ($F_{(5, 30)} = 1.010$). Furthermore, scopolamine-injected rats that received vehicle, donepezil, or *T. triandra* leaf extract at all doses used in this experiment did not exhibit any significant differences in their crossing behavior compared to the control group that received saline injections ($F_{(5, 30)} = 0.279$).

3.4. Effect of T. triandra leaf extract on acetylcholinesterase (AChE) activity

The results of AChE activity are depicted in Fig. 5. AChE activity was found to be significantly higher in scopolamine-injected rats that received vehicle compared to the control group that received normal saline injections ($F_{(5, 30)} = 4.555$, $P < 0.001$). However, treatment with donepezil or *T. triandra* leaf extract at doses of 200 and 400 mg/kg BW significantly reduced AChE activity in scopolamine-injected rats compared to those given a vehicle ($F_{(5, 30)} = 4.555$, $P < 0.05$, $P < 0.05$, $P < 0.01$, respectively).

3.5. Effect of T. triandra leaf extract on malondialdehyde (MDA) levels

The results showing the effect of *T. triandra* leaf extract on MDA levels are presented in Fig. 6. Rats administered with scopolamine injections and provided with a vehicle displayed a higher level of MDA compared to the control rats that received standard saline injection ($F_{(5, 30)} = 2.135$, $P < 0.05$). Conversely, rats that received donepezil or *T. triandra* leaf extract at doses of 200 and 400 mg/kg BW in combination with scopolamine injections exhibited decreased MDA levels in the hippocampus when compared to the scopolamine-injected rats provided with a vehicle ($F_{(5, 30)} = 2.135$, $P < 0.05$, $P < 0.01$, $P < 0.05$, respectively).

3.6. Effect of T. triandra leaf extract on antioxidant enzyme activities

The results depicted in Fig. 7 showed that rats treated with scopolamine injections and vehicle displayed reduced activities of SOD ($F_{(5, 30)} = 4.690$, $P < 0.001$) and GSH-Px ($F_{(5, 30)} = 3.061$, $P < 0.01$) when compared to the control group receiving conventional saline injections. However, rats treated with donepezil or *T. triandra* leaf extract at doses of 200 and 400 mg/kg BW showed increased activities of SOD ($F_{(5, 30)} = 4.690$, $P < 0.01$, $P < 0.05$, $P < 0.05$, respectively) and GSH-Px ($F_{(5, 30)} = 3.061$, $P < 0.01$, $P < 0.05$, $P < 0.05$, respectively) when compared to the scopolamine-injected rats given a vehicle. Nonetheless, the activity of CAT results did not reveal any significant differences between the groups ($F_{(5, 30)} = 0.694$).

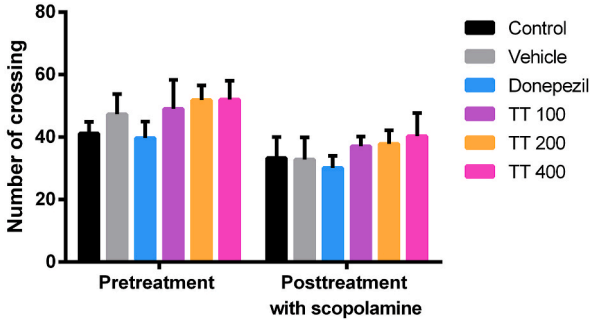

Fig. 4. The effects of donepezil and *T. triandra* (TT) leaf extract at doses of 100, 200, and 400 mg/kg BW on the number of crossings in rats, using the open field test. Data analysis was performed using one-way analysis of variance, and the results are presented as mean ± S.E.M ($n = 6$ per group).

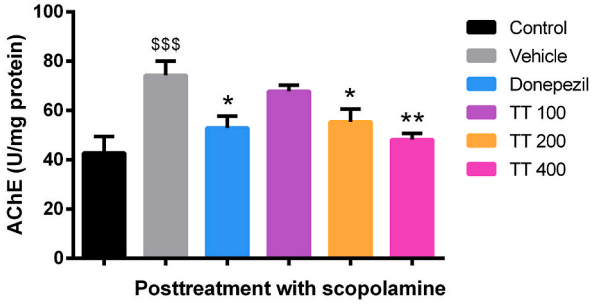

Fig. 5. The effects of donepezil and *T. triandra* (TT) leaf extract at doses of 100, 200, and 400 mg/kg BW on acetylcholinesterase (AChE) activity in the hippocampus of rats. Data analysis was performed using one-way analysis of variance, and the results are presented as mean ± S.E.M. ($n = 6$ per group). $^{\$\$\$}P < 0.001$, in comparison to control receiving normal saline injection; $^{*}P < 0.05$, in comparison to the group receiving vehicle with scopolamine injection; $^{**}P < 0.01$, in comparison to the group receiving vehicle with scopolamine injection.

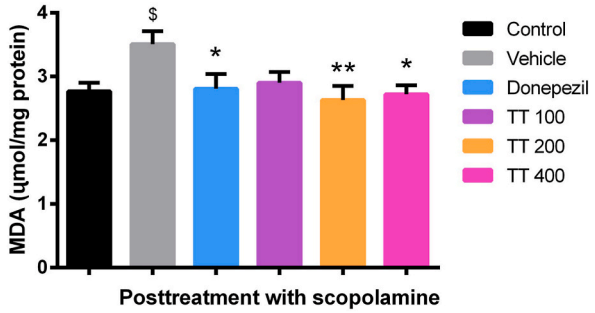

Fig. 6. The effects of donepezil and *T. triandra* (TT) leaf extract at doses of 100, 200, and 400 mg/kg BW on malondialdehyde (MDA) levels in rat's hippocampus. One-way analysis of variance was used for data analysis. Data are presented as mean ± S.E.M. ($n = 6$ per group). $^{\$}P < 0.05$, in comparison to control receiving normal saline injection; $^{*}P < 0.05$, in comparison to the group receiving vehicle with scopolamine injection; $^{**}P <$ 0.01, in comparison to the group receiving vehicle with scopolamine injection.

3.7. Effect of T. triandra leaf extract on neuronal density in the hippocampus

The effect of *T. triandra* leaf extract on neuronal density was investigated using cresyl violet staining in the hippocampus. The results were showed in Fig. 8. When compared to the control rats, rats administered with scopolamine injections and provided with a vehicle had significantly lower neuronal density in the CA3 region of hippocampus ($F_{(5,\ 30)} = 3.706$, $P < 0.01$). The scopolamine-injected rats administered with donepezil and *T. triandra* leaf extract at doses of 200 and 400 mg/kg BW had higher neuronal density in the CA3 region of hippocampus ($F_{(5,\ 30)} = 3.706$, $P < 0.01$ for all groups) compared to the scopolamine-injected rats treated with a vehicle. However, the results of neuronal density in the CA1 and DG regions of hippocampus did not exhibit any significant differences between groups (CA1: $F_{(5,\ 30)} = 1.315$; DG: $F_{(5,\ 30)} = 1.578$).

Fig. 9 showed pictures of the hippocampus (CA1, CA3, and DG) magnified at 4× and 20× and histologically stained with cresyl violet. The scale bars of these images were 20 μm. The rat brain atlas illustration was taken from Paxinos and Watson [25]. A plate of photomicrographs in the Supplementary Material (Fig. S2) illustrates the CA3 region of the hippocampus, which was histologically stained with cresyl violet and captured at a magnification of 40×. The scale bars accompanying these images correspond to a length of 10 μm.

4. Discussion

Medicinal herbs have demonstrated effectiveness in managing memory loss and Alzheimer's disease. The current study aimed to investigate the effect of *T. triandra* leaf extract on memory function in amnesic rats using the NORT and the YMT. In our study, chronic administration of scopolamine reduced the discrimination ratio in the NORT and the percentage of spontaneous alternation behavior in the YMT. However, treatment with *T. triandra* leaf extract at doses of 200 and 400 mg/kg BW mitigated memory impairment by increasing both the discrimination ratio and the percentage of spontaneous alternation behavior. An increase in the discrimination ratio indicates memory improvement [20,26], while a significant increase in the percentage of spontaneous alternation behavior reflects improved memory functioning [27]. Additionally, the OFT was used to evaluate locomotor activity and exploring behavior of rats in a novel environment [28]. There were no significant differences in the number of crossings among the groups of rats receiving scopolamine injections, indicating no differences in locomotor activity and exploratory behavior.

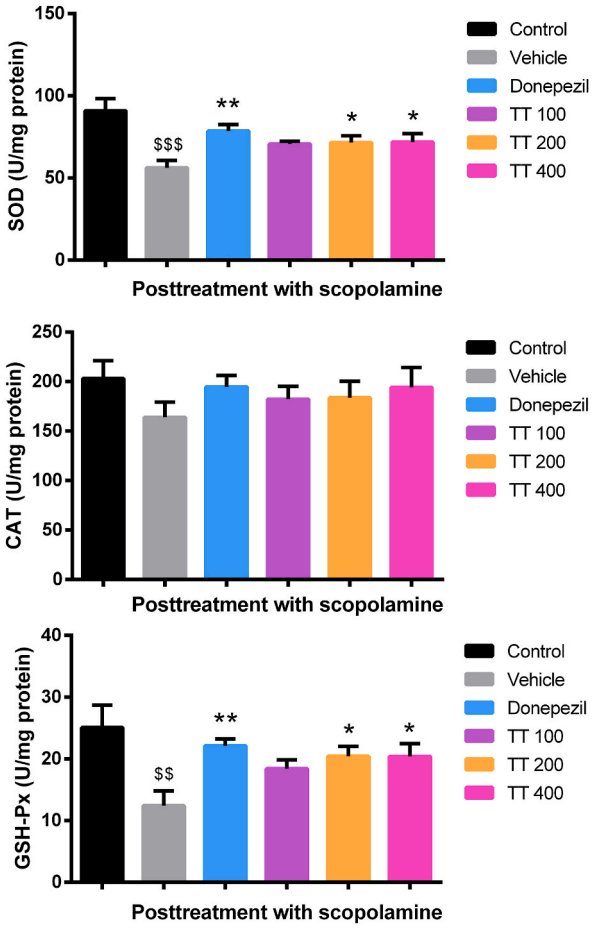

Fig. 7. The effects of donepezil and *T. triandra* (TT) leaf extract at doses of 100, 200, and 400 mg/kg BW on the activity of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), in the hippocampus of rats. Data analysis was performed using one-way analysis of variance, and the results are presented as mean ± S.E.M. ($n = 6$ per group). $^{\$\$}P < 0.01$, in comparison to control receiving normal saline injection; $^{\$\$\$}P < 0.001$, in comparison to control receiving normal saline injection; $^{*}P < 0.05$, in comparison to the group receiving vehicle with scopolamine injection; $^{**}P < 0.01$, in comparison to the group receiving vehicle with scopolamine injection.

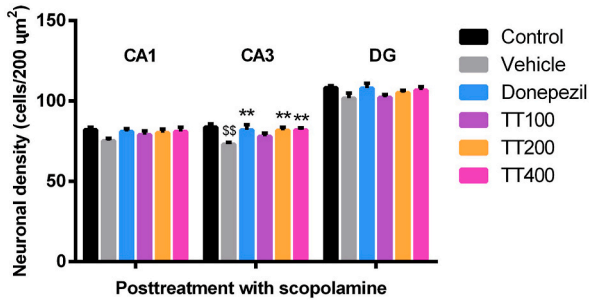

Fig. 8. The effects of donepezil and *T. triandra* (TT) leaf extract at doses of 100, 200, and 400 mg/kg BW on neuronal density in the hippocampus. One-way analysis of variance was used for data analysis. Data are presented as mean ± S.E.M. ($n = 6$ per group). $^{\$\$}P < 0.01$, in comparison to control receiving normal saline injection; $^{**}P < 0.01$, in comparison to the group receiving vehicle with scopolamine injection.

The central cholinergic system significantly influences the mechanisms of memory [3]. Our findings revealed that rats injected with scopolamine and given a vehicle had reduced density of surviving neurons but elevated AChE activity in the hippocampus when compared to the control group. Our results are consistent with previous studies that have reported that scopolamine causes severe cholinergic deficits and increased AChE activity in the hippocampus, contributing to neurodegeneration in the brain [29–31]. Treatment with *T. triandra* leaf extract at doses of 200 and 400 mg/kg BW significantly increased neuronal density while reduced AChE

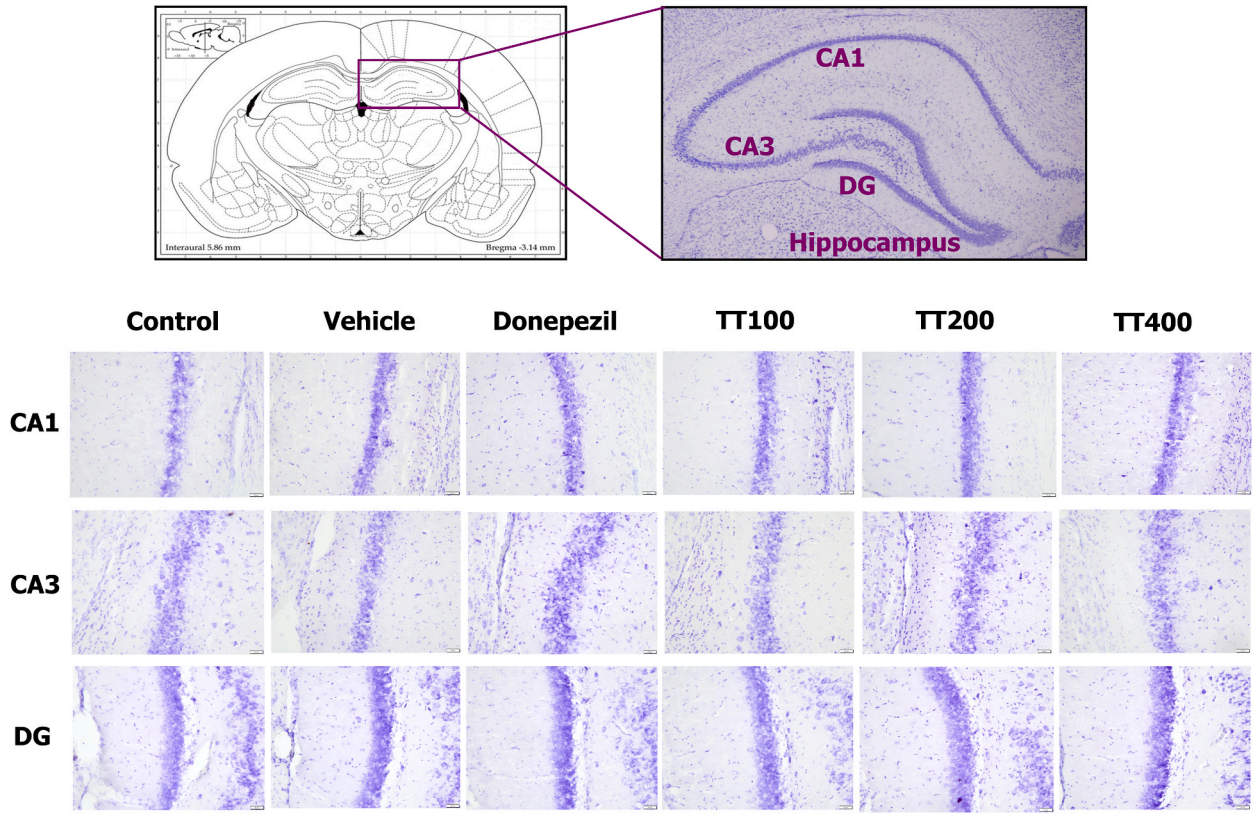

Fig. 9. Images of the cornu ammonis 1 (CA1), cornu ammonis 3 (CA3), and dentate gyrus (DG) of the hippocampus histologically stained with cresyl violet at 4× and 20× magnifications. Scale bar: 20 μm.

activity in the hippocampus compared to the scopolamine-injected rats given a vehicle. These results align with a previous study by Ingkaninan et al. which reported that *T. triandra* leaf extract possesses AChE inhibitory and antioxidant properties [32]. Furthermore, Wachiryah and Hathaipat have reported that polyphenols presented in *T. triandra* leaf extract improves spatial memory and protects against neuronal damage by maintaining choline acetyltransferase (ChAT) activity in the hippocampus [33]. Therefore, this plant could be advantageous for cognitive abilities. The observed effects could be due to increased ACh release into the synaptic cleft, making it available for binding to postsynaptic receptors, and the suppression of AChE activity in the hippocampus. Additionally, the neuronal density of surviving neurons in the CA3 region of hippocampus was improved. As a result, the hippocampus's memory-enhancing neural circuit was strengthened.

Since lipids comprise the bulk of the brain, reactive oxygen species' effect leads to lipid peroxidation, directly affecting brain damage in AD [34]. Our findings show that scopolamine treatment causes a collapse in the brain's antioxidant defense system compared to the control group, as demonstrated by higher MDA levels but lower SOD and GSH-Px activities in the hippocampus. Administration of *T. triandra* leaf extract at doses of 200 and 400 mg/kg BW exhibited significant improvements in the activities of SOD and GSH-Px, concurrently leading to a reduction in MDA levels within the hippocampus. These findings are consistent with a study by Phunchago et al. which demonstrated that polyphenols sourced from *T. triandra* leaf extract ameliorate memory deficits in alcoholic rats by decreasing MDA levels and simultaneously enhancing SOD and GSH-Px activities in the hippocampus [13]. However, in contrast to the results reported by Phunchago and colleagues, the CAT activity observed in our investigation did not display notable differences among the experimental groups [13].

The results presented in this study demonstrate that *T. triandra* leaf extract has antagonistic effects on scopolamine's action, likely due to the extract's bioactive components, including polyphenols such as verbasoside, caffeic acid, catechin, piperine, and 6-gingerol, and both essential and non-essential amino acids such as L-arginine, L-histidine, L-valine, L-leucine, D-tryptophan, L-tyrosine, L-glutamine, and choline, as shown in the Supplementary Material. These substances mitigate scopolamine-induced memory loss. In a previous study, Suzuki et al. reported that the intake of essential amino acids, such as leucine, phenylalanine, and lysine, supplemented with isoleucine, histidine, valine, and tryptophan, improved attention and cognitive function in middle-aged and older individuals [35]. Glutamine supplementation prevented chronic stress-induced moderate cognitive impairment [36], while tyrosine supplementation enhanced cognitive control processes such as task switching, response inhibition, and working memory [37]. Furthermore, L-arginine administration attenuated impaired memory and synaptic plasticity caused by lipopolysaccharide (LPS) [38], and choline, a precursor of acetylcholine, improved cognitive performance in elderly adults [39]. The antioxidant properties of polyphenols (verbasoside, caffeic acid, catechin, piperine, and 6-gingerol) have been found to scavenge free radicals and improve cognitive function

[40–44]. Therefore, several studies have investigated whether these polyphenols and amino acids can cross the blood-brain barrier (BBB) to scavenge free radicals and protect against oxidative damage to the brain and the nervous system [45–48]. The current investigation's results show that medium and high doses of *T. triandra* leaf extract can prevent scopolamine-induced neuronal and oxidative damages and have positive effects on cognitive function. However, low dose of this extract did not appear to have any cognitive-enhancing effects, likely due to the decreasing amount of active components in low dose of *T. triandra* leaf extract.

The present study has certain limitations, outlined as follows: (1) The potential influence of the active ingredient within the *T. triandra* leaf extract on cognitive abilities was not explored in this study. While the identification of phenolic components within the *T. triandra* leaf extract was achieved through LC-ESI-QTOF-MS/MS analysis, determining the specific bioactive substance responsible for cognitive enhancements remained uncertain; (2) The study did not comprehensively investigate ChAT activity or quantify cholinergic neurons within the hippocampus, which could have provided additional support for the observed positive findings.

5. Conclusion

The administration of *T. triandra* leaf extract demonstrated a significant reduction in scopolamine-induced memory impairment, potentially attributed to its ability to reduce oxidative stress, inhibit AChE activity, increase neuronal density, and enhance antioxidant enzyme activities. These findings provide initial insight into the cognitive-enhancing properties of *T. triandra* leaf extract in an Alzheimer's rat model induced by scopolamine and can be used as a basis for future research in this area.

Ethics approval

This project was approved by the Ethics Committee of the Laboratory Animal Research Center at Mae Fah Luang University (approval number AR02/65).

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Data available statement

Data will be made available on request.

CRediT authorship contribution statement

Thaneeya Hawiset: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Resources, Writing - original draft, Writing - review & editing. **Napatr Sriraksa:** Methodology, Resources, Writing - original draft. **Utcharaporn Kamsrijai:** Methodology, Software, Visualization. **Siwaporn Praman:** Investigation, Methodology, Validation. **Prachak Inkaew:** Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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