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Effect of *Centella asiatica* on cognitive deficits caused in trimethyltin-induced neurotoxicity model mice

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Abstract:

***Centella asiatica* (L.) Urb. is a nootropic that has been used medicinally for centuries. The present study investigates the anti-dementia effect of an n-butanol extract of *Centella asiatica* in a mouse model of neurodegeneration caused by trimethyltin (TMT). Mice were administered *Centella asiatica* extract (150 and 540 mg/kg) or a reference drug, tacrine (2.1 mg/kg), daily for one week before TMT injection and during tests to analyse spatial short-term and spatial working memory. The treatment of mice with TMT caused deficits in short-term spatial working memory and decreased the number of pyramidal cells in the hippocampal CA1 and CA3 regions elucidated by Nissl staining at 28 d post-TMT treatment. The administration of *Centella asiatica* extract or tacrine significantly attenuated the behavioural and histochemical changes caused by TMT. These results suggest that *Centella asiatica* extract improved memory deficits partly by protecting hippocampal neurons from TMT-induced damage.**

Keywords: ***Centella asiatica*, dementia, hippocampus, neuroprotection, trimethyltin.**

Classification number: **3.3**

Introduction

Alzheimer's chronic neurodegenerative disease (AD) causes memory loss and cognitive deficits, which develop slowly for 20-30 years until symptoms become apparent in patients at 65 years of age and over. AD is the most common dementia with the number of AD patients reaching more than 40 million worldwide [1, 2].

For AD patients with a severely damaged brain, health care costs are estimated to be hundreds of billions of dollars each year. Currently, no cure for AD is available, but medicines such as acetylcholinesterase inhibitors are employed to temporarily reduce the symptoms of AD patients although they have significant side effects. Development of effective treatments/drugs with fewer undesirable effects is critically important.

Centella asiatica (L.) Urb. is used in Ayurvedic medicine [3] to treat depression, anxiety, convulsion, and cognitive deficits [4, 5]. However, studies on the potential benefits of this plant for memory loss associated with AD are limited. Therefore, this study aims to evaluate the effects of *Centella asiatica* collected in Vietnam on memory deficits caused in an animal model of trimethyltin-induced neurodegeneration to explore possible mechanisms underlying the effects.

Materials and methods

Preparation of Centella asiatica extract

Centella asiatica (L.) Urban was collected in the Thanh Hoa province in August 2019 and identified by the staff (Department of Resource Medicinal Materials) of the National Institute of Medicinal Materials, Hanoi, Vietnam (NIMM). *Centella asiatica* extract was prepared by cutting aerial parts into small pieces and drying at 55°C. Plant material (650 g) was extracted with 80% alcohol (1:7 w/v) at room temperature for 24 h, filtered, and dried in vacuo at 50°C. The residue was suspended in H_2O and then partitioned three times with n-butanol, successively. The

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combined n-butanol extracts were concentrated *in vacuo* at 60°C and yielded 40 g dried extract.

Centella asiatica extract was analysed for candidate secondary metabolites by UPLC (with diode detection) - TOF-MS plus MS/MS (with lockspray ionization operated in the ESI negative mode). Tentative identification of the peaks was performed by comparing data with published values.

Chemicals and reference drug

Reference drug: Tacrine (Sigma-Aldrich, USA) was dissolved in a phosphate-buffered saline (PBS) for peritoneal injection (i.p.) in mice at a dose of 2.1 mg/kg body weight.

Animal and drugs treatment

Male *Swiss albino* mice 6-7 weeks old were obtained from the National Institute of Hygiene and Epidemiology, Hanoi, Vietnam. Food and water were provided *ad libitum*. The animals were habituated for at least one week in quarters that were thermostatically maintained at 22±1°C with constant humidity (65%) and a 12-h light-dark cycle (lights on: 07:00-19:00). The behavioural experiments were performed during the light phase from 9:00 to 18:00 according to experimental schedules shown in Fig. 1.

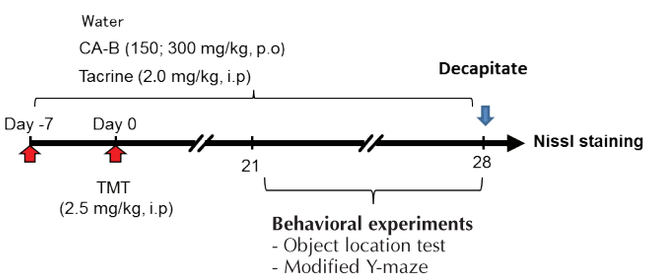

Fig. 1. Experimental schedule.

Behavioural study

The TMT-induced memory deficits were induced as previously described [6]. Briefly, mice were randomly divided into 5 groups (9-12 animals/group), namely, Groups 1 through 5. As shown in Fig. 1, Groups 2-5 received TMT injection on day 0.

Group 1 (naïve group) and Group 2 (TMT-group) were administered distilled water (p.o.) daily during the experimental period.

Groups 3-4 (TMT+*Centella asiatica* extract) and Group 5 (TMT+Tacrine) were administered *Centella asiatica* extract (150 and 300 mg/kg, p.o.) and tacrine (2.1 mg/kg, i.p.), respectively, at a volume of 0.1 ml/10 g body weight from day 7 to day 28.

After completion of the behavioural assays, the animals were decapitated under anaesthesia (pentobarbital, 50 mg/kg, i.p.) on day 28 to isolate brains for Nissl staining.

Object location test (OLT): the object location test was performed from 09:00 to 17:00 at 4-5 lux, without noise from day 21 to day 23, as previously described [6, 7]. Briefly, after acclimatization to an observation box (35×35×50 cm) for 10 min, a sample phase trial was conducted. Each mouse was placed in the observation box for 5 min and exposed to two identical objects. The total time spent to explore each object was measured and the mouse was then returned to the home cage. The test phase trial was conducted 30 min after the sample phase trial. In the test trial, objects were replaced by identical copies. One was placed in the same position and the other was placed diagonally on opposite corners. The animals were exposed to the objects for 5 min and the total time spent exploring each object was measured using Any Maze system® (Stoelting Co., IL, U.S.A.).

Modified Y-maze: the modified Y-maze test was performed from 9:00 to 17:00, at 8-9 lux, without noise on days 27 and 28, as previously described [8, 9]. This test consisted of a sample trial and a test trial, which were separated by an interval. In the sample trial, each mouse was placed in the maze with one arm closed. The animal was allowed to explore the other two arms freely for 5 min. In the test trial conducted 30 min after the sample trial, the animal was placed in the maze with 3 arms open and allowed to explore the arms freely. The previously closed arm was defined as the novel arm, and the time the animals spent in the new arm was measured using an Any Maze system® (Stoelting Co., IL, U.S.A.).

Nissl staining

The Nissl staining was conducted according to Pham, et al. (2019) [6] and Kádár, et al. (2009) [10]. After completing behavioural tests, mice were anesthetized with a peritoneal injection of pentobarbital (50 mg/kg; i.p) and then perfused intracardially with heparinized saline followed by 10% formalin saline. The brains were excised and post-fixed in 10% formalin saline for 3 h. The brains were soaked in a graded strength of sucrose solutions (12, 15, and 30%) for 48 h and then stored at -80°C until histological analysis. Coronal sections (slice thickness 20 µm) were taken at an appropriate position (Bregma -1.96 to -2.30 mm) using a freezing microtome (Leica CM 1950, Japan) and stained with 0.5% cresyl violet. Three sagittal brain sections prepared from each mouse were used for Nissl staining. The images of CA1 and CA3 regions were captured using a microscope (Olympus PROVIS®, Olympus Corporation, Tokyo, Japan) and the average intensity was analysed by Image-J software (ver. 1.41, NIH; Bethesda, MD, U.S.A.).

Statistical analysis

Data were expressed as the mean±standard error of the mean (S.E.M.). All the data obtained in the present study, except those from the OLT, were analysed by a one-way ANOVA followed with a post hoc comparison test (Student-Newman-Keuls/Dunnett). OLT data were analysed using a paired Student's t-test. Differences of $p<0.05$ were considered significant.

Results and discussion

Centella asiatica extract treatment improved memory deficits in TMT mice using object location test and modified Y-maze

The spatial short-term memory of the mice was evaluated on day 21 using the object location test. In the sample phase trials, no significant differences were observed in time spent exploring each identical object between the animal groups. In the test phase trial, the naïve group spent a significantly longer time exploring the object placed in the new location than the object placed in the familiar location ($p<0.05$). *Centella asiatica* extract (300 mg/kg) and tacrine-treated TMT mice were able to recognize a difference between objects placed in novel and familiar locations ($p<0.05$) (Fig. 2).

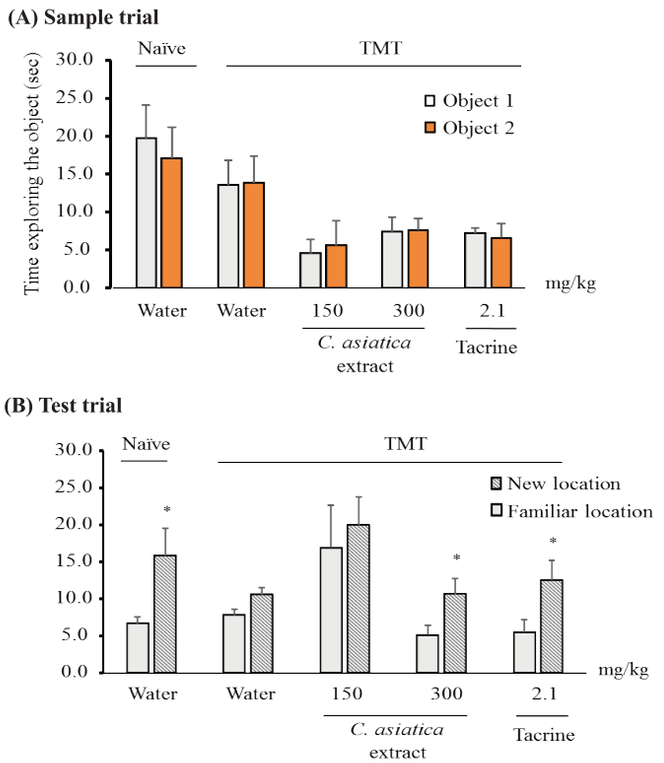

Fig. 2. Effect of *Centella asiatica* extract (150 and 300 mg/kg) on spatial short-term memory deficits in TMT-treated mice. (**A**) Time exploring the object in the sample trial. (**B**) Time exploring the object in the test trial; n=9-12 animals/group; *p<0.05 vs. the time spent exploring the non-displaced object.

To further study the effects of *Centella asiatica* extract on spatial working memory deficits in TMT mice, we conducted the modified Y-maze test on days 27-28 using the same animals.

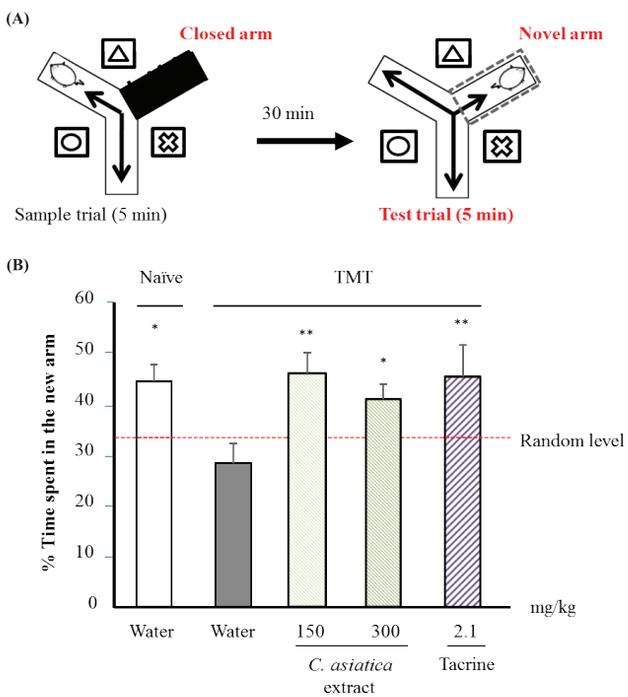

Fig. 3. Effect of *Centella asiatica* extract (150 and 300 mg/kg) on spatial working memory deficits in TMT-treated mice. (**A**) Experiment protocol of the modified Y-maze test. (**B**) Results are expressed as % time animals spent exploring the novel arm in the test trial; n=9-12 animals/group; *p<0.05; **p<0.01 vs. the water-treated TMT group.

As shown in Fig. 3, naïve mice preferred to visit the new arm compared to two familiar arms (higher than the chance level of 33.3%). Water-treated TMT mice significantly spent a shorter time in the new arm than untreated mice ($p<0.05$), indicating TMT-induced spatial working memory deficits. TMT mice treated with *Centella asiatica* extract (150 and 300 mg/kg) or tacrine (2.1 mg/kg) significantly spent a long time exploring the new arm compared to water-treated TMT mice (P values as shown in Fig. 3).

Centella asiatica extract exerts neuroprotective effects on TMT-induced hippocampal cell damage in vivo

Next, we evaluated the effect of *Centella asiatica* extract on the protection of hippocampal neurons in TMT mice. The hippocampal tissues were stained with crystal violet and then morphological changes were observed. The density of cresyl violet-stained cells in CA1 and CA3 regions were analysed by Image-J software. The results in Fig. 4 showed that mice exposed to TMT had a severe loss of pyramidal neurons in the hippocampal CA1 and CA3 regions and that

the administration of *Centella asiatica* extract (150 and 300 mg/kg) prevented the TMT-induced damage in the CA1 and CA3 regions.

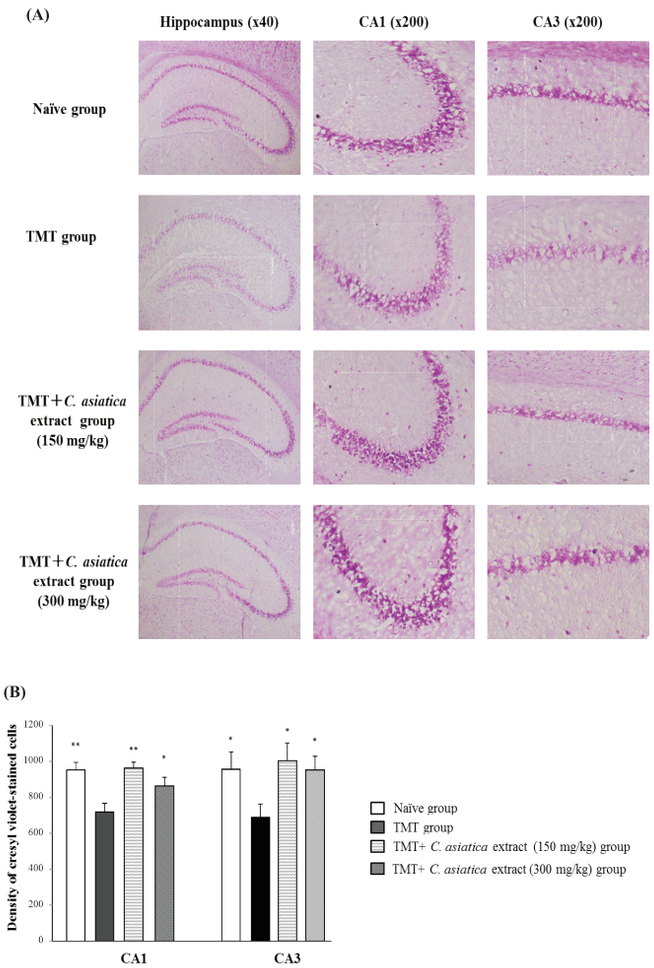

Fig. 4. Effects of Centella asiatica extract (150 and 300 mg/kg) on TMT-induced neuronal cell damage in the CA1 and CA3 of the hippocampus. (**A**) The upper, middle, and bottom panels are representative photos obtained from the naïve group, water-treated TMT group, and Centella asiatica extract-treated TMT groups (150 and 300 mg/kg), respectively. (**B**) The relative percentage values of the cell density in the CA1 and CA3 regions of each animal group. Each data column represents the mean±S.E.M. calculated from 8-10 animals in each group; *p<0.05; **p<0.01 vs. the water-treated TMT group at the same area of the mice hippocampus.

Centella asiatica is consumed in daily meals and used as a nootropic for emotional disorders such as depression, sedation, anxiety, mental weakness, and memory deficits [5, 11] via its neuroprotective, neurogenesis, and antioxidant properties [12]. Clinical studies have shown that *Centella asiatica* attenuates age-related decline in cognitive function and mood disorders in the elderly [13, 14]. Bioactive compounds of *Centella asiatica* include triterpenoids glycosides (asiaticoside and madecassoside) and aglycons (asiatic acid and madecassic acid) [15, 16]. In this study, by analyses with UPLC-TOF-MS plus MS/MS, we determined the constituents of the *Centella asiatica* extract used in these experiments to include madecassoside and asiaticosid (Fig. 5).

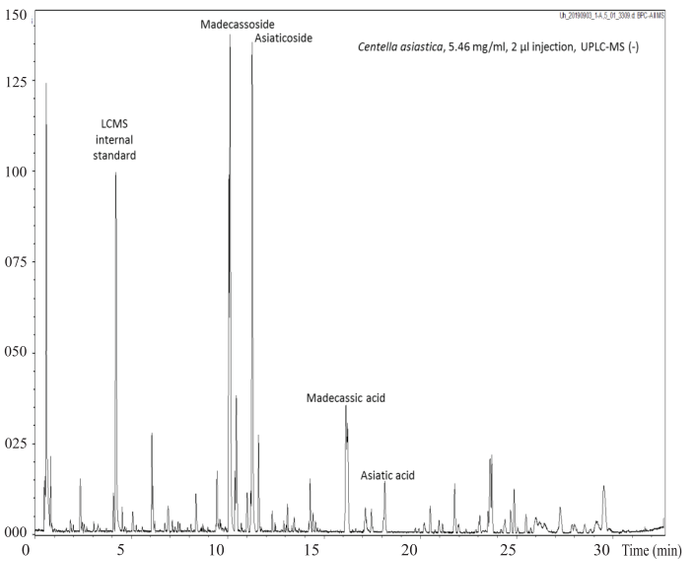

Fig. 5. UPLC-TOF-MS plus MS/MS chromatogram of *Centella asiatica* extract.

The hippocampus is a part of the limbic system and plays important roles in the consolidation process of information from short-term memory to long-term memory and spatial memory that enables navigation. The hippocampus proper consists of Cornu ammonis and dentate gyrus, and Cornu ammonis is divided into CA1, CA2, CA3, and CA4 regions. In dementia patients, the hippocampus is first affected, which induces initial symptoms of short-term memory impairment and disorientation [17]. TMT is a neurotoxicant that damages the hippocampal CA1 and CA3 regions. Thus, TMT-induced neuronal and cognitive impairments have been proposed as an experimental animal model for neurodegeneration in the hippocampus [18, 19]. The present study showed that TMT causes neuronal damage in the hippocampal CA1 and CA3 regions and spatial memory impairment in mice. An important finding in the present study is that *Centella asiatica* extract treatment attenuates memory impairments and prevents neuronal damage in CA1 and CA3 regions in TMT mice. A previous study reported by Gadahad, et al. (2008) [20] demonstrated that the administration of *Centella asiatica* juice stimulated the proliferation and morphological changes of neuronal cells in CA1 and CA3 regions in adult rats. Moreover, Sirichoat, et al. (2015) [21] reported that asiatic acid isolated from *Centella asiatica* improved hippocampus-dependent memory deficits via a mechanism

involved in neuronal proliferation. Taken together, it is likely that *Centella asiatica* extract ameliorates cognitive deficits in TMT-treated mice via preventing neuronal cell damages or stimulating neuronal cell proliferation, or both in the hippocampus.

Conclusions

The present study indicated that *Centella asiatica* extract has a protective effect against TMT-induced neuronal and cognitive impairments in mice suggesting that *Centella asiatica* extract treatment may be beneficial for the prevention/therapy of dementia.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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