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Effects of *Centella asiatica* L. On Spatial Memory and Bcl-2 Gene Expression in the Hippocampus of Rats Injected With Trimethyltin

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ABSTRACT

Cell death (apoptosis) in the hippocampus is related to impaired memory functions. Gotu kola (*Centella asiatica*) contains asiatic acid and asiaticoside, which act as antioxidants and improve memory function. This study was intended to determine the effects of gotu kola extracts on Bcl-2 gene expression and spatial memory in rats with impaired memory functions due to trimethyltin (TMT) injection. The rats were subdivided into six groups, each group consisting of 10 animals. The normal group was given sodium carboxymethyl cellulose (CMC-Na); the TMT group was given CMC-Na; the positive group was given 200 mg/kg bw citicoline; the extract groups were given a variation of gotu kola extract at 100 (P100), 200 (P200), and 400 mg/kg bw (P400) doses, respectively. These treatments were conducted for 35 days. TMT was injected intraperitoneally on Day 8 of treatment at a dose of 8 mg/kg bw in all groups except the normal group. On Days 29-35, the Morris water maze (MWM) spatial memory test was conducted. The rats were sacrificed, and their hippocampi were taken for immunohistochemical observation of Bcl-2 gene expression. The results showed TMT injection could lower the spatial memory of rats in the MWM and cause apoptosis of the pyramidal cells of the hippocampus. Gotu kola extract at 100, 200 and 400 mg/kg bw could increase the percentage of time spent (duration) and frequency of rats staying in the target quadrant in the probe trial of the MWM and increase Bcl-2 gene expression in the pyramidal cells of the hippocampus. Overall, the results have confirmed that gotu kola extract has the potential to prevent hippocampal cell death and improve the spatial memory of rats injected by TMT.

Keywords: *Centella asiatica*, Bcl-2, spatial memory, trimethyltin

INTRODUCTION

Spatial memory is the type of memory related to the ability to remember field space; recognize shape, distance, and area; and identify direction or position. The hippocampus and parts of the cerebrum have a large role in memory functions (JE, 2011). However, the hippocampus is a tissue sensitive to oxidative damage that can cause cell death in the form of apoptosis. The apoptotic process can occur when the amount of antiapoptotic and proapoptotic proteins in cells are not in balance. Apoptosis is regulated by several genes, including Bcl-2, which plays a role in preventing apoptosis (anti-apoptosis) (Anvekar *et al*., 2011).

Organotin trimethyltin (TMT) is known to cause cell death in rat hippocampus through oxidative stress mechanisms (Shuto *et al*., 2009). Cell death is characterized by chromatin condensation, nuclear fragmentation, mitochondrial dysfunction, production of reactive oxygen species (ROS), and activation of genes that play a part in the apoptosis process (Geloso *et al*., 2002; Jenkins and Barone, 2004). Prior scholars have also reported that intraperitoneal injection of TMT decreases the ability of rats to complete the Morris Water Maze (MWM) (Park *et al*., 2011; Kang *et al*., 2016; Yuliani *et al*., 2018). Thus, TMT injection in rats can be used to model memory loss in humans.

Centella asiatica L. or gotu kola is a type of plant often used for traditional medicine. Gotu kola contains asiatic acid and asiaticoside, which have antioxidant and neuroprotective properties. It also has other nutritious compounds, such as centelloside and madecassoside (Irham *et al*., 2019). Rather *et al*. (2018) have reported that asiatic acid can prevent oxidative stress and

apoptosis in rats with aluminum chloride-induced dementia. Asiaticoside is also able to improve spatial memory, inhibit mitochondrial apoptosis, and reduce inflammatory factors in rat models of dementia injected with Aβ 1-42 oligomers by lateral intracerebroventricular route (Zhang *et al*., 2017). Previous research has also proven that the administration of gotu kola extract for 35 days can prevent the expression of the caspase 3 gene in pyramidal cells in the CA2-CA3 areas of the hippocampus (Yuliani and Linar, 2019). The objective of this study was to determine the effects of gotu kola extract on spatial memory and Bcl-2 gene expression in the hippocampus of rats injected with trimethyltin (TMT).

MATERIAL AND METHODS

Extract preparation

Gotu kola powder was acquired from CV Merapi Farma, Yogyakarta, Indonesia. Five hundred grams of this powder was macerated twice with 2.5 L of 70% ethanol (Sigma Aldrich) for 24 hours. The macerate was filtered with a Buchner funnel until separated from the pulp and then concentrated in a rotary evaporator (Heidolph) at 40°C. The extract was then suspended with 1% sodium carboxymethyl cellulose (CMC-Na) before given to the test animals. Every 200g of test animals received 2mL of suspension with a concentration of 10mg/mL for the dose of 100mg/kg bw, 20mg/mL for 200mg/kg bw, and 40mg/mL for 400mg/kg bw.

Treatment of test animals

The test animals used were male Sprague-Dawley (SD) rats (weight=150-200g, age=1.5-2 months old) obtained from the Indonesian Food and Drug Administration (BPOM), Jakarta. The use of animals in the research was approved for preclinical research by the Research Ethics Committee of Universitas Ahmad Dahlan, Yogyakarta, Indonesia (approval number 011804050). A total of 60 rats were acclimatized for seven days and divided into 6 groups of 2 per cage. On Day 8, all groups, except the normal control, were injected with TMT solution (Sigma Aldrich) at 8mg/kg bw intraperitoneally. Rats weighing 200 g received 0.2mL of the solution with a concentration of 8mg/mL.

The normal group and the TMT group were given 1% CMC-Na solution orally, while the citicoline group was given a comparator drug at 200 mg/kg bw. The three treatment groups were given ethanol extract of gotu kola with dose variations of 100 (EC100), 200 (EC200), and 400 mg/kg bw (EC400), respectively. The treatments were carried out once a day from Day 1 until Day 35. Afterward, on Day 36, the rats were sacrificed, then their hippocampus tissue was taken for immunohistochemical observation of the Bcl-2 gene.

Spatial memory test

The spatial memory of the rats was observed using the procedure conducted by Uygur and Arslan (2010) with modifications. This study consisted of two phases of testing, namely the acquisition trial and the probe trial, which used a pool with 1.5m in diameter and 45cm in height. The pool was imaginatively partitioned into four quadrants and given additional cues in the form of posters, doors, light sources, and two observers around the pool, and a video camera (Prolink, Taiwan) was installed above the pool. In one of the quadrants, a platform was placed high below the water surface. Water mixed with coconut milk was poured into the pool and changed every day.

The acquisition trial was carried out for five consecutive days (on Day 29-34), during which the rats were allowed to swim to reach the platform four times per day with a duration of 60 seconds. The rats failing to find the platform for 60 seconds would be guided toward it then placed on it for 15 seconds prior to the next exercise. The time they required to reach the platform was recorded using a stopwatch. The length of their swimming track was manually measured from the video camera recorder using a curvimeter (Silva Sweden AB, Bromma, Sweden). On Day 35, the probe trial started by removing the platform, and then, for 60 seconds, the rats were allowed to swim to remember its location. The duration and frequency of rats staying in the quadrant where the platform was placed previously were recorded.

Immunohistochemistry (IHC) staining of Bcl-2

On Day 36, the rats were sacrificed through CO_2 inhalation. The brains were taken, and the hippocampus was separated and immersed in a mixture of formaldehyde and phosphate-buffered saline (PBS). Indirect immunohistochemical (IHC) staining was performed at the Anatomical Pathology Laboratory of Sardjito Hospital, Yogyakarta, using the indirect method of Starr Trek universal detection system (Biocare Medical, Concord, California, USA), with a primary antibody (monoclonal B-cell lymphoma 2/Bcl-2) and a secondary antibody (Trekkie Universal Link).

The results of the IHC staining were observed using a binocular microscope (Olympus) connected to a digital camera (Optilab Advance, PT Miconos, Yogyakarta, Indonesia) with a magnification of 400x. Observations were made on the hippocampus pyramidal cells in the CA2-CA3 areas of 2 tissue slices per hippocampus. In this staining, the cytoplasm of cells that expressed Bcl-2 protein would appear in brown spots, while that of cells that did not would be in blue.

The percentage of cells expressing Bcl-2 was calculated using the formula below:

$$\frac{\sum cells\ with\ expression}{\sum (cells\ with\ expression + cells\ without\ expression)} \text{ X } 100\%$$

Data Analysis

The study used the Shapiro-Walk test to assess data normality and the Levene test for variance homogeneity. Normal and homogeneous data were analyzed using ANOVA (one-way analysis of variance) then further processed by a post-hoc Tukey HSD test to determine the difference in the mean values of non-identical samples. The significance level was set at $p<0.05$.

RESULTS AND DISCUSSION

Spatial memory test results

The Morris water maze (MWM) is a test to determine spatial memory abilities, where test animals must remember the location of a submerged platform (Ploughman, 2008). This test has several advantages, such as: (1) it does not require a previous training period and the exercise can be conducted in a short time, namely in 10-20 exercises; (2) it is not influenced by the sense of smell; (3) it can identify any visual deficits, motor or motivational; (4) it does not hurt the test animals, as opposed to giving electric shocks or starving them first; (5) it is easy to perform and is cheaper (Terry Jr, 2009; D'Hooge *and* De Deyn, 2001). The latency and path length in 20 exercises for five consecutive days in the acquisition trial show the spatial learning processes, whereas the frequency and time spent in the target quadrant (percentage) during the probe trial show the spatial memory storage ability of the rats (Bizon *et al*., 2009; Purves *et al*., 2004).

In general, the study results indicate that TMT injection can reduce the memory abilities of rats. The speed and length of the rat trajectory showed fluctuations every day. On Day 34 (after 20 exercises), the rats in the TMT group showed almost the same latency as the others (Figure 1) but had the greatest path length compared to the other groups (Figure 2). This could have occurred because the rats swam faster after 20 exercises, but because of decreased memory skills, they took a longer route to find the platform.

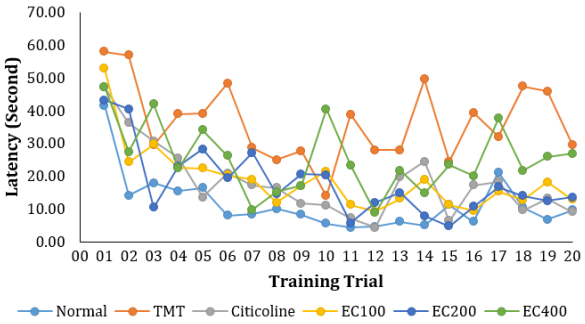

Figure 1. The mean latency (seconds) of rats in all treatment groups during 20 exercises in the MWM acquisition trial

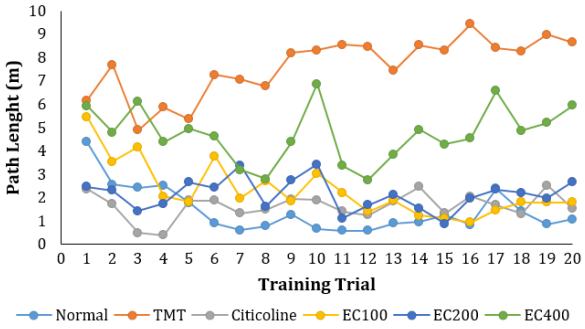

Figure 2. The mean trajectory length (m) of rats in all treatment groups during 20 exercises in the MWM acquisition trial

The statistical test results showed that the TMT group had significantly increased mean latency and path length in 20 exercises ($p<0.05$), as compared to the normal group (Table I). Furthermore, in the probe trial, it showed the lowest duration (percentage) and frequency compared to the other groups (Figures 3 and 4). The probe trial was a test to determine the spatial memory storage ability of the rats. Normal rats would randomly search for hidden platforms, and when the platform was removed, they would use their memory to find the platform and often stay in the quadrant where it was previously located (Vorhees and Williams, 2006).

Table I. Average of latency and path length during 20 exercises in all treatment groups

Group	Latency (second) ± SEM	Path length (m) ± SEM
Normal	11.65±1.90[a]	1.43±0.31[a]
TMT	36.44±2.61	7.62±0.40
Citicoline	18.07±2.38[a]	1.63±0.18[a]
EC100	18.77±2.17[a]	2.30±0.37[a]
EC200	18.05±2.30[a]	2.13±0.21[a]
EC400	25.33±2.34[ab]	4.71±0.37[ab]

Notes: a shows p<0.05 or significant difference from the TMT group, b shows p <0.05 or significant difference from the normal group

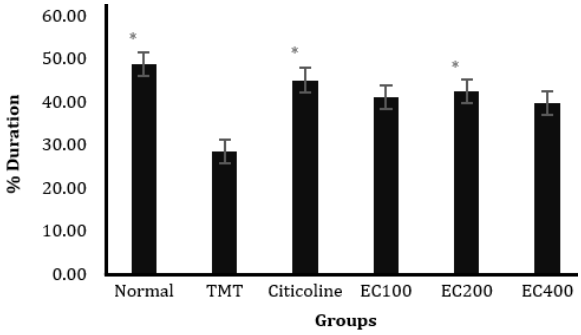

Figure 3. The mean percentage (%) of the time spent in the target quadrant (duration) during the MWM probe trial in all treatment groups. *p<0.05, compared to the TMT group.

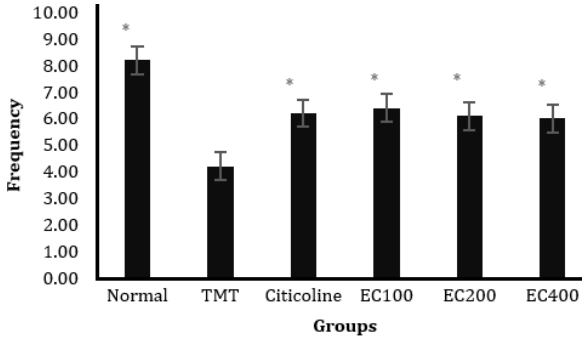

Figure 4. The mean frequency of the rats staying in the target quadrant during the MWM probe trial in all treatment groups *p<0.05, compared to the TMT group.

Trimethyltin (TMT) is an organometal compound that is especially neurotoxic to the hippocampus (Furukawa *et al*., 2011). The toxicity mechanism of TMT is not fully understood, but prior scholars have confirmed that neurotoxic damage due to TMT injection is mediated by derivatives of reactive oxygen species (ROS) and reactive nitrogen species (NOS) (Viviani *et al*., 2001; Wang *et al*., 2008; Park *et al*., 2011). Because TMT intoxication can decrease the memory of the test animals, TMT injection is suitable for neurodegenerative models of memory impairment (Kassed *et al*., 2003; Lee *et al*., 2016).

In this study, the administration of gotu kola extract was able to accelerate the path length and latency of rats during the acquisition trial. Also, at the doses of 100, 200, and 400mg/kg bw, it could substantially increase the mean frequency and the percentage of the duration of rats staying in the target quadrant during the probe trial compared with the ones belonging to the TMT group (p<0.05). There is no significant difference in the memory ability of the rats given gotu kola extract at 100, 200, and 400 mg/kg bw.

Previous studies have reported the activities of compounds contained in gotu kola. Triterpenoid and asiatic acid were proven to increase learning and memory in vivo by modulating the cholinergic and GABAergic systems (Nasir *et al*., 2011). In addition, asiatic acid has antioxidant activity, which can reduce reactive oxygen species (Lee *et al*., 2000) and restore levels of glutathione and superoxide dismutase (SOD) activity in the hippocampus to normal levels (Xu *et al*., 2012), thereby preventing oxidative stress. Another study explains that asiatic acid increases spatial memory in test animals through the regulation of the N-methyl-D-aspartate (NMDA) receptor (Lee *et al*., 2014) and acetylcholine (Ach) synthesis (Kim *et al*., 2004). According to Xing Lin *et al*. (2013), gotu kola contains asiaticoside, which has been proven as a cognitive enhancer in senescence-accelerated rats by up-regulating antioxidant enzyme activities, scavenging free radicals, ameliorating dysfunction in synaptic plasticity, and normalizing Ach level and Ach esterase activity. Asiatic acid and asiaticoside are compounds that dissolve in ethanol (Hashim *et al*., 2011). Therefore, the maceration method in this study also used ethanol as a solvent.

Table II. The mean percentage of cells (±SEM) expressing bcl-2 in the pyramidal cells of the ca2-ca3 regional hippocampus in all treatment groups.

Groups	Mean ± SEM of Bcl-2 expression in pyramidal cells (%)
	CA2-CA3
Normal	23.38±1.03
TMT	11.83±0.75
Citicoline	60.45±0.95*#
EC100	54.33±0.72*#
EC200	62.09±1.18*#
EC400	54.76±0.79*#

Notes: * shows p<0.05 or significant difference from the TMT group, # shows p<0.05 or significant difference from the normal group

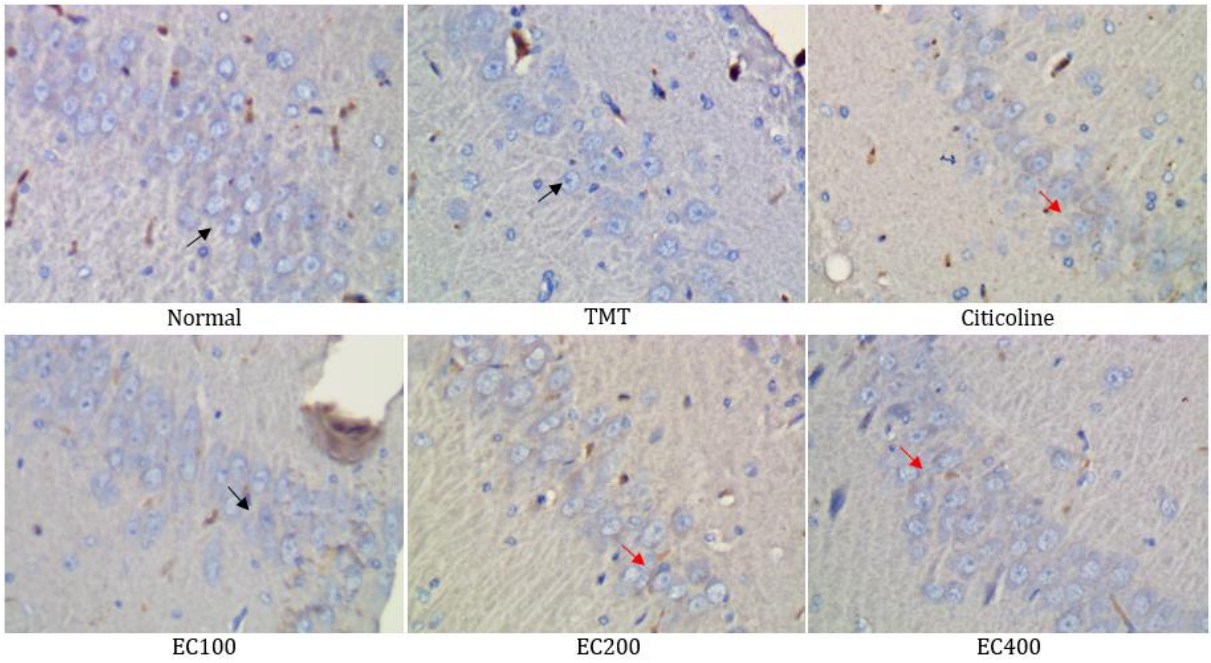

Figure 5. Immunohistochemical staining of Bcl-2 gene in pyramidal cells in the CA2-CA3 hippocampus in all treatment groups. Normal cells are expressed as blue (↗), and the Bcl-2 protein expression in the cytoplasm is presented in brown (↘). Magnification 400x.

IHC staining of Bcl-2

The results of immunohistochemical tests showed that compared with the normal group, TMT injection could decrease the amount of Bcl-2 gene expression (Figure 5 and Table II) in the pyramidal hippocampus cells in the CA2-CA3 regions.

TMT can cause selective neuronal death in the granular neurons of the Fascia Dentata and the pyramidal cells of the Cornu Ammonis (CA) in the hippocampus (Geloso *et al*., 2011). CA2-CA3 are cells in the CA that serve to convert spatial information into a coded form in short-term memory and support its retrieval by spatial pattern completion (Kesner *and* Hunsaker, 2010). Cell death or apoptosis is related to the increased production of ROS by TMT (Chen *et al*., 2011; Zhang *et al*., 2006), which results in mitochondrial dysfunction and the release of cytochrome-c to the cytosol, thereby inducing apoptosis (Yoon *and* Gores, 2002; Youle *and* Strasser, 2008).

The administration of gotu kola extract at 100, 200, and 400 mg/kg bw in this study increased the mean percentage of cells that expressed Bcl-2 in the pyramidal cells of the hippocampus in the CA2-CA3 regions. These pyramidal cells are susceptible to the influence of physical stressors and exposure to chronic stress in the hippocampus, which results in the loss of neurons in the hippocampus and potential reduction in memory function (Suparno, 2008). Zhang Zhou *et al*. (Zhao *et al*., 2014) have

shown that the asiaticoside in gotu kola increases cell viability and the expression level of Bcl-2 apoptosis induced by Aβ 25-35 in PC12 cells. Bcl-2 family and their membrane interactions are proteins that are key to cell death through the mechanism of intrinsic apoptosis. Bcl-2 overexpression can impede the release of cytochrome-c from mitochondria, caspase activation, and DNA fragmentation, thereby preventing apoptosis (Cleary *et al*., 1986). Caspase 3 is a proapoptotic protein, a member of caspases (i.e., the cysteine-aspartic acid protease family), which acts as an executor in the apoptotic cascade (Crawford *and* Wells, 2011). Other studies have shown that asiaticoside has neuroprotective effects on neurons in the hippocampus via redox balance mechanisms and increases Bcl-2 gene expression. (Xu *et al*., 2012). Increased Bcl-2 gene expressions prevent cells from undergoing apoptosis.

In this study, no significant difference was detected between the effects of 100, 200, and 400mg/kg bw of gotu kola extract on spatial memory and the mean percentage of cells expressing Bcl-2 in the pyramidal cells of the hippocampus in the CA2-CA3 regions. This may have been caused by the increase in the dose, which subsequently increases the presence of compounds with opposing mechanisms and, in turn, decreases the pharmacological activity of the extract.

The administration of gotu kola extract at various doses showed memory ability and apoptosis inhibition that were not significantly different from citicoline at 200mg/kg bw. Citicoline serves as a raw material for the synthesis of phospholipids, i.e., components of cell membranes needed to maintain nerve cell structure (Takasaki *et al*., 2011). Citicoline functions as a choline donor in acetylcholine biosynthesis, and in test animals, the release of acetylcholine has been proven to improve attention, learning, and memory performance (Saver, 2008). Citicoline can also inhibit apoptosis in neurodegeneration (Cotroneo *et al*., 2013). In another study, when combined with nimodipine, it can decrease apoptosis and increase Bcl-2 expression (Sobrado *et al*., 2003).

CONCLUSION

Gotu kola extract has the potential to prevent hippocampal cell death and improve the spatial memory of rats injected with TMT. There was no significant difference in activities among the doses of 100, 200, and 400mg/kg bw.

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