Available online on www.ijtpr.com
International Journal of Toxicological and Pharmacological Research 2016; 8(1); 23-28

ISSN: 0975-5160

Research Article

Effect of Ethyl Acetate Fraction of *Morinda citrifolia* (L.) on Ethanol-Induced Impairment of Learning and Memory in Mice Passive-Avoidance Test

Kartikasari A, Adiningsih P, Nurrochmad A*

Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Available Online:12th January, 2016

ABSTRACT

The effect of ethyl acetate (EtOAc) fraction of *Morinda citrifolia* (L.) on learning and memory have been conducted in ethanol-induced impairment by mice passive-avoidance test. Animals were administration orally different treatment for seven days the EtOAc fraction of *M.citrifolia* (100, 200 and 400 mg/kg BW). Mice received training for four days and test trials were performed at day 7 to 10 after test administration and the region of hippocampus were take for histological obeservation. The results showed that the EtOAc fraction of *M.citrifolia* 200 and 400 mg/kg BW (P<0.05) reverses ethanol-induced impairment learning and memory in mice passive-avoidance test. In addition, histological observation showed that the EtOAc fraction of *M.citrifolia* 200 and 400 mg/kg BW restored density of hippocampal CA1 pyramidal cells compare to ethanol group (P<0.05). It conclude that EtOAc fraction of *M.citrifolia* significantly reversed and improved ethanol-induced impairment learning and memory in mice.

Keywords: *Morinda citrifolia* (L.), learning and memory, hippocampus, passive avoidance test, mice

INTRODUCTION

Brain damage caused by ROS often known as oxidative stress. Oxidative stress describes the condition of an imbalance between the production of ROS and the endogenous antioxidants. Brain damage due to oxidative stress can trigger memory loss. This memory loss is associated with damage to the hippocampus, which plays a role in memory consolidation. According to the prevoius report, hippocampus and input of cholinergic, a neurobiological substrates are important in learning and memory[1]. If there is an interruption in the hippocampus, the learning and memory will also be affected, given the hippocampus is a brain region that is vulnerable to toxic compounds. Decrease in the number of hippocampal neurons as a result of oxidative stress can lead to memory loss and cognitive function. Strong evidence supporting the involvement of oxidative stress in degenerative changes within the forebrain cholinergic system has been suggested[2]. This suggests that drugs that have the effect of antioxidants have neuroprotective effect and support brain function. Cognitive deficits such as impaired learning and memory can be due to oxidative stress induced formation of free radicals that cause cell damage and death. Ethanol is able to alter cognitive and behavioral performance in both humans and laboratory animals. One of the principal cognitive effects of ethanol is disruption of learning and memory. Ethanol preferentially impairs hippocampal-dependent learning and memory[3,4]. Both ethanol and hippocampal lesions impair water-maze performance on spatial learning and memory[5]. Acute ethanol administration produces lipid peroxidation, which is an indicator of oxidative stress, in the brain[6-8]. Although ethanol is extensively metabolized in the liver, it has toxic effects in the brain[8]. Several studies indicate that cognitive effects of alcohol are mediated through the dysregulation of the glutamate system in the hippocampus and prefrontal cortex (PFC)[9-11]. Lesions in the hippocampus, frontal lobe, and the limbic system induced by ethanol causing learning problems[12]. Therefore, ethanol administration can used as a model for learning and memory disorder. The nervous system, including the brain, spinal cord and peripheral nerves have the content of unsaturated fatty acids and iron are very high. The high carbon-carbon double bond lipids in nervous tissue and the high metabolic activity causes the tissues sensitive to oxidative stress[13]. The way to protect a network of free radicals or ROS overload is with antioxidants. Antioxidants capable of preventing the oxidation caused by free radicals. Several classes of compounds that have the potential as a class of phenolic antioxidants, such as flavonoids and coumarin[14]. *M. citrifolia* has been used in folk remedies by Polynesians for over 2000 years, and is reported to have a broad range of therapeutic effects, including antibacterial, antiviral, antifungal, antitumor, antihelmin, analgesic, hypotensive, anti-inflammatory, and immune enhancing effects[15]. The aqueous extract of *M. citrifolia* is able to heal the wounds in rats were wounded as well as to reduce the level of lipid peroxidation[16]. *M. citrifolia* leaves have an antioxidant

**Author for Correspondence*

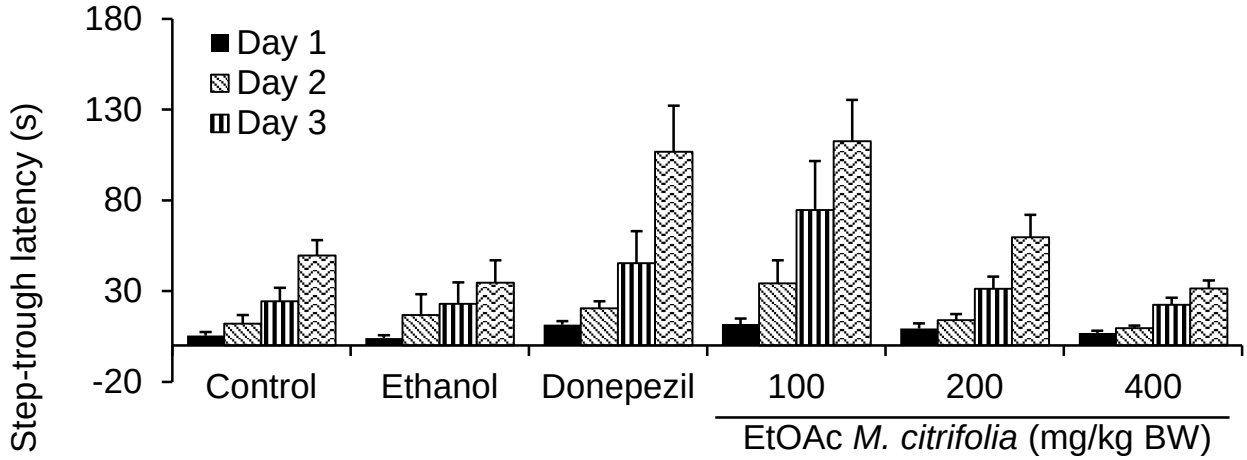

Figure 1: Effects of training on basal the step-through latency in normal mice for days. Mice did not any treatment and training were conducted 4 consecutives days. Each bar represent mean ± SEM.

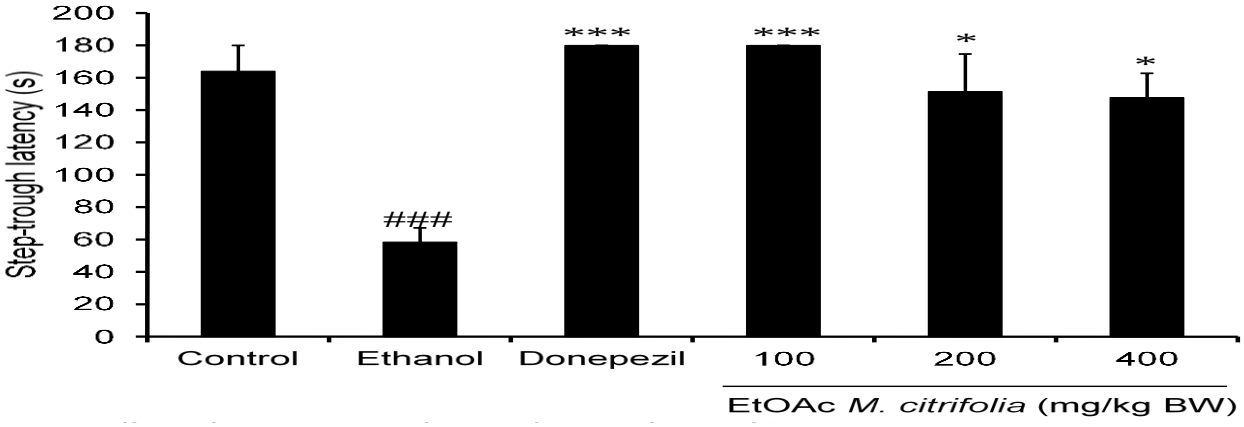

Figure 2: Effects of administration of EtOAc fraction of *M.citrifolia* on the step-through latency in ethanol-induced impairment learning and memory. EtOAc fraction or donepezil were administered for 7 consecutives days and 30 after the last administration mice were test by passive avoidance test. ($^{###}P < 0.01$, , significantly different vs. Control group; $^{*}P<0.05$, $^{*}P < 0.01$, significantly different vs. Ethanol-treated group. Each bar represent mean ± SEM.

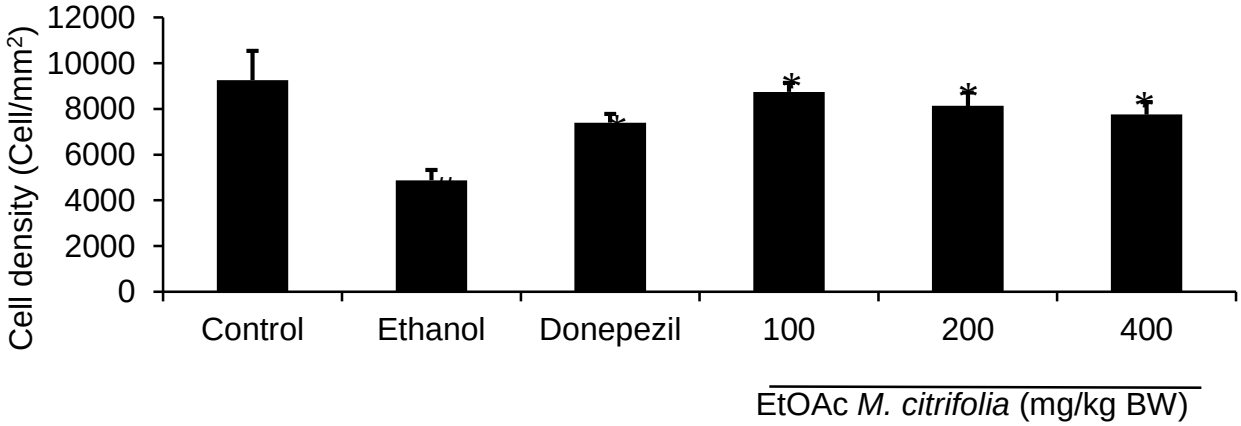

Figure 3: Effects of administration of EtOAc fraction of *M.citrifolia* on hippocampal CA1 neuronal cells density in ethanol-induced damage neuronal cells. EtOAc fraction or donepezil were adminstratrated for 7 consecutives days. After test by passive avoidance test mice was sacrified and the hippocampus CA1 region removed and for histopatological observation. ($^{#}P < 0.05$, significantly different vs. Control group; $^{*}P < 0.01$, significantly different vs. Ethanol-treated group. Each bar represent mean ± SEM.

activity and phenol total of 1.14 times to 0.21 times higher than green tea[17]. The previus study reported that antioxidant activity of *M. citrifolia* is higher than the leaves of blackberry, raspberry, and strawberry-based method of oxygen-radical absorbance capacity (ORAC)[18]. In addition, previous study also reported that *M. citrifolia* leaf cell culture is contain flavonoids extracellular higher than the fruit[19]. Ethyl acetate fraction *M. citrifolia* leaf contains flavonoids that were more soluble in semi-polar solvents

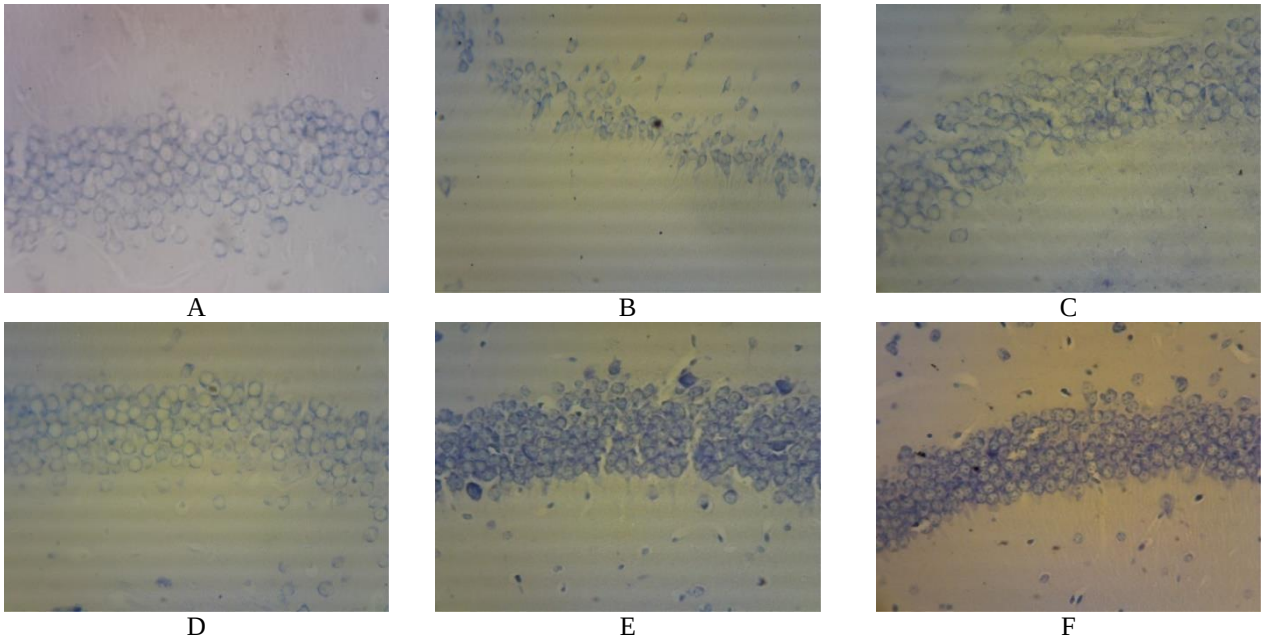

Figure 4: Effects of administration of EtOAc fraction of *M.citrifolia* on histopathological of hippocampal CA1 neuronal cells. Control group (A); Ethanol-treated group (B); Donepezil-treated group (C); EtOAc fraction of *M.citrifolia* 100 mg/kg BW, p.o. (D); EtOAc fraction of *M.citrifolia* 200 mg/kg BW, p.o. (E); EtOAc fraction of *M.citrifolia* 400 mg/kg BW, p.o. (F).

such as ethyl acetate. Based on the previous studies, we carried out investigation to determine the effects of ethyl acetate fraction of *M. citrifolia* on mice passive avoidance test-induced by ethanol. We also investigated the effect of ethyl acetate fraction of *M. citrifolia* on hippocampal neurons based on the density of hippocampal CA1 pyramidal cells in mouse brain.

MATERIALS AND METHODS

Preparing the Plant Extract and EtOAc Fraction.

Stem of *M. citrifolia* obtained from Sleman district and authenticated by Taxonomist from the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada. A specimen has been preserved in the Taxonomic Division of Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada. *M. citrifolia* powder were weighed and extracted with ethanol 96%. Ethanolic extract obtained were liquid-liquid partitioned using hexane and ethyl acetate. Ethyl acetate (EtOAc) fraction obtained was then evaporated. The yield of the EtOAc extracts was calculated as: (weight of crude extract/weight of dried powder) ×100% and used for further investigation. The EtOAc extract of *M. citrifolia* was obtained with the yield of 6.20%.

Animals

Healthy BALB/c mice were obtained from the Animal Experimental Unit, Animal Research and Development Centre, Universitas Gadjah Mada, Yogyakarta. All animals were maintained in the institutional animal facilities. The animals were housed and maintained under the standard conditions of 12-h light/dark cycle, 25 ± 2°C and 60–70% humidity and were fed with standard rat chow and water ad libitum. All the animals were acclimatized quarantined for one week prior to experimentation. The experimental protocol was conducted in accordance with the Guideline for Care and Use of Animals Laboratory and approved by the Institutional Animal Ethics Committee (IAEC) of Universitas Gadjah Mada.

Experimental Protocol

Thirty six normal rats were divided randomly into six groups of six mouse each group, and used in the experiments. Group I, served as normal rats received vehicle (CMC Na 0.5%) once daily for seven consecutive days; group II, served as ethanol-treated mice were administered vehicle and 90 minutes later were given ethanol 10% once daily for seven consecutive days; group III, served as donepezil-treated mice were administered donepezil (0.28 mg/kg BW) and 90 minutes later were given ethanol 10% once daily for seven consecutive days; group IV, IV and VI were administered variable doses of 100, 200 and 400 mg/kg BW of the EtOAc fraction *M. citrifolia*, respectively and 90 minutes later were given ethanol 10% once daily for seven consecutive days.

Passive Avoidance Test

Briefly, the test was divided into a training and test session. Training and test of passive avoidance was performed on two identical compartments that compartment light and dark compartments (Columbus ® PACS Box 30). Training and test each performed for 4 consecutive days. Mice were placed and allowed to explore the environment in the light compartment and 10 s later the door between compartments was opened. When mice completely entered the dark compartment, the door automatically closed and an electrical foot-shock (0.1 mA/10 g body weight) for a time period of 2 s was delivered through the stainless steel rods (one trial training). The step-through latency to enter the dark compartment was measured. If the mice did not enter the dark compartment within 180 s, the experiment

was stopped. Training was conducted for four consecutives days. Mice were administered vehicle or EtOAc fraction of *M. citrifolia* (100-400 mg/kg BW). Ninety (90) minutes later, mice were treated ethanol (10%, 0.5 BB mL/20g, diluted using vehicle, p.o.) to induce oxidative stress. The administration of EtOAc fraction of *M. citrifolia* were conducted for seven consecutives days. The test session was performed 30 minutes after the last administration of EtOAc fraction of *M. citrifolia* or vehicle using the same paradigm, but without applying the foot-shock. The step-through latency for animals to enter the dark compartment was measured. The test was conducted for four consecutives days. After last test mice were sacrified and the brain romoved and fixed in 10% neutral buffered formalin solution for histological observation.

Histological examination

Histological preparations were made by cutting the hippocampal CA1 region by referring to map the brain of mice[20]. Histological preparations were prepare using paraffin blocks method. Brains were fixed using 10% neutral buffered formalin solution. Subsequently, brain drained using running water and dehydrated using alcohol 70%, 80%, 90%, and absolute alcohol for 60 minutes each 2 times. After that the mid sagittal brain was trimmed and put in xylol 3 times each for 30 minutes. Subsequently infiltrated into tissue in paraffin, and continued the process of embedding and slicing tissue using a sliding microtome. The dye used is 0.1% toluidine blue with pH 3.0 to 3.3.

Statistical Analysis

Data from the passive avoidance test and the density of hippocampal CA1 pyramidal lamina was presented as mean ± SEM. The step-through latency of passive avoidance test and the density of hippocampal CA1 pyramidal lamina were analyzed using ANOVA and Bonferroni post hoc test. The data were considered to be statistically significant if the probability had a value of $P<0.05$ or less.

RESULTS

Memory enhancing activity of EtOAc fraction of M. citrifolia on memory impairment induced by ethanol in mice

The data of step-through latency illustrates in figure 1 that indicated the step-through latency in the training for 4 days. The step-through lantency in training demonstrated that the latency was increased compared to the previous day. This suggests that the mice was getting to memorize the presence of electrical shock in the dark compartment. Cognitive function begins to increase. Mice learn that there is a dangerous situation for himself in the dark compartment resulted increasing in latency than previous day. In general, there is a pattern of display memory and cognitive functions are organized every day during training (Fig.1). Figure 2. represents the test session after administration of drug or EtOAc fraction of *M. citrifolia* for 7 days. The result demonstrated that the ethanol-treated mice indicated the impaired memory based on the significantly decreased the step-through latency than control group ($P<0.05$). The administration of donepezil and EtOAc fraction of *M. citrifolia* (100-400 mg/kg BW) indicated maintained and reversed the step-through latency compare to the ethanol-treated group. This result suggest that donepezil and EtOAc fraction of *M. citrifolia* maintained the ethanol-Induced impairment of learning and memory in mice.

Histological examination of CA1 pyramidal cell density Lamina Hippocampus

In this study, cells were counted cells are all contained in the CA1 area of the hippocampus. The density of pyramidal cells in the lamina hippocampal shown in figure 3. The result demonstrated that in the ethanol-treated group showed histological appearance hippocampal CA1 pyramidal lamina with the lowest density of the cells. The ethanol-treated group indicated that cells described atrophy and irregular cell shapes (Fig.4). The administration of Donepezil (0.28 mg/kg BW) and EtOAc fraction of *M. citrifolia* (100-400 mg/kg BW) for 7 days significantly reverse the CA1 pyramidal cell density ($P<0.05$) (Fig. 3 and 4).

DISCUSSION

The present study was investigated the effect of ETOAc fraction of *M. citrifolia* on learning and memory in normal and memory impairment mice-induced by ethanol. The EtOAc fraction of *M. citrifolia* (100-400 mg/kg BW, p.o) capable to reversed and enhanced the learning and memory in mice-induce by ethanol by the passive avoidance test. In addition, the histopatologically observation of CA1 hippocampal neuron cell indicated that the administration of EtOAc fraction of *M. citrifolia* could improved the the hippocampal damage induced by ethanol. Ethanol can increase ROS formation by inducing the enzyme cytochrome P4502E1 widely distributed in brain tissue[21]. Hippocampus and cerebellum part of the brain that is most susceptible to oxidative damage because the part has the lowest concentration of antioxidants[22]. The administration of ethanol can reduce antioxidant enzymes, such as glutathione peroxidase, and can disrupt the homeostasis of glutathione[23]. Improved memory and cognitive function based on the passive avoidance test is best demonstrated by the treatment of EtOAc fraction of *M. citrifolia* 100-400 mg/kg BW compare to the ethanol-treated group ($P<0.05$). An increase in memory and cognitive function is possible due to plasticity in EtOAc fraction of *M. citrifolia* -treated group is similar to the donepezil-treated group. Strong plasticity resulted in an increase in synaptic strength between neurons in the hippocampus region, especially the CA1 hippocampus and gyrus dentatus. The second section has an important role in the formation of spatial information[24]. Flavonoids contained in the EtOAc fraction of *M. citrifolia* is able to provide neuroprotective effects of free radicals caused by ethanol administration. The memory-improving action of donepezil in scopolamine-induced amnesia could be explained, in part, by neurochemical changes in the brain. Donepezil reversed the scopolamine-induced memory deficit in which available synaptic acetylcholine was increased via inhibition of the degradative enzyme, acetylcholine esterase[25]. It suggest that may be at least in part the mechanism of EtOAc fraction of *M. citrifolia* improved

leaning and memory via acetylcholine esterase. The CA1 region of the hippocampus to express the genes that encodes a protein antioxidant nor genes that is associated with an increase in free radicals more than the CA3 region of the hippocampus. Neurons in the CA1 region of the hippocampus are more suceptible to damage by oxidative stress than CA3 region. Levels of superoxide produced by mitochondria in CA1 is also greater than the CA3 region[26].The administration of ethanol lead to form oxidative stress and impaired the density of hippocampal CA1 pyramidal cell. The administration EtOAc fraction of *M. citrifolia* improved the hippocampal CA1 pyramidal cells density. Therefore, it can be concluded that the EtOAc fraction of the stem *M. citrifolia* has a neuroprotective effect due to ethanol administration. In addition, it is possible that the EtOAc fraction of *M. citrifolia* also has a neurotrophic effect. Neurotrophic effect is the ability to increase the density of pyramidal cells through sinaptogenesis lamina and axon regeneration. Natural antioxidants like polyphenols provide neuroprotective effects through a variety of biological actions, such as interaction with transition metals, inactivation of free radicals, modulation in the activity of different enzymes, and effects on intracellular signaling pathways and gene expression[27,28]. The active compounds that act as antioxidants in general are phenolic and polyphenolic compounds that can be flavonoid, cinnamic acid derivatives, coumarin, tocopherol, and polyfunctional organic acids. *M. citrifolia* containing quercetin and kaempferol which are flavonoid compounds[29]. Quercetin was more effectively reduce oxidative stress-induced nerve cell membrane damage than vitamin C[29]. These results is a significant biological advantage of *M. citrifolia* to protect nerve cells from oxidative stress-induced neurotoxicity such as in Alzheimer's disease (AD)[30]. In conclusion, administration of EtOAc fraction of *M. citrifolia* can improve memory and cognitive function and affect the density of hippocampal CA1 pyramidal cells. This study suggest that *T. citrifolia* can be used as neuroprotective supplement to improve learning and memory impairment such as in Alzheimer's disease.

ACKNOWLEDGMENTS

The authors acknowledge the support extended by the Dean, Faculy of Pharmacy, Universitas Gadjah Mada for carrying out the work. We also acknowledge to Dr. Joko Santoso for authenticated the plant material.

AUTHOR CONTRIBUTIONS

AK and PA were involved in plant collection, processing, and carrying out the experimental work. AN supervised the overall study and drafted the manuscript.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper

FUNDING

The authors received no financial support for the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

The study was approved by the Animal Ethical Committee of the Universitas Gadjah Mada, Yogyakarta, Indonesia.

REFERENCES

1. Stoltenburg DG. Neuropathology of The Hippocampus and Its Susceptibility to Neurotoxic Insult. J. Neurotoxic 1994; 15(3):445–450.
2. Mattson MP, Pedersen WA. Effects of amyloid precursor protein derivatives and oxidative stress on basal forebrain cholinergic systems in Alzheimer's disease. Internat J. Develop. Neurosci. 1998; 16:626–642.
3. Melia KR, Ryabinin AE, Corodimas KP, et al. Hippocampal-dependent learning and experience-dependent activation of the hippocampus are preferentially disrupted by ethanol. Neuroscience 1996; 74:313-322.
4. Acheson SK, Ross EL, Swartzwelder HS. Age-independent and dose-response effects of ethanol on spatial memory in rats. Alcohol. 2001; 23:167-175.
5. Matthews DB, Ilgen M, White AM, Best PJ. Acute ethanol administration impairs spatial performance while facilitating nonspatial performance in rats. Neurobiol. Learn. Mem. 1999; 72:169-179.
6. Renis M, Calabrese V, Russo A, et al. Nuclear DNA strand breaks during ethanol-induced oxidative stress in rat brain. FEBS Lett. 1996; 390:153–156.
7. Somani SM, Husain K, Diaz-Phillips L, et al. Trammell GL. Interaction of exercise and ethanol on antioxidant enzymes in brain regions of the rat. Alcohol 1996; 13: 603–610, 1996.
8. Mansouri A, Demeilliers C, Amsellem S, et al. Acute ethanol administration oxidatively damages and depletes mitochondrial DNA in mouse liver, brain, heart, and skeletal muscles: protective effects of antioxidants. J. Pharmacol. Exp. Ther. 2001; 298:737–743, 2001.
9. Fadda F, Rossetti ZL. Chronic ethanol consumption: from neuroadaptation to neurodegeneration. Prog. Neurobiol. 1998; 56:385–431.
10. Stephens DN, Duka T. Review. Cognitive and emotional consequences of binge drinking: role of amygdala and prefrontal cortex. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 2008; 363:3169–3179.
11. Cippitelli A, Damadzic R, Frankola K, et al. Alcohol-induced neurodegeneration, suppression of transforming growth factorbeta, and cognitive impairment in rats: prevention by group II metabotropic glutamate receptor activation. Biol. Psychiatry 2010; 67:823–830.
12. Lundqvist C, Alling C, Knoth R, Volk B. Intermeittent Ethanol Exposure of Adults Rats: Hippocampal Cell Loss After One Month of Treatment. Alcohol 1995; 30(6):737–48.
13. Singh RP, Saharad S, Kapur S. Free Radicals and Oxidative Stress In Neurodegenerative Diseases:

Relevance of Dietary Antioxidants. JIACM 2004; **5**(3):218−225.
14. Kahkonen MP, Hopia AI, Fuorella HC. Antioxidant Activity of Extract Containing Phenolic Compound, J. Agric. Food Chem. 1999; **47**:3954−3962.
15. Wang MY, West BJ, Jensen CJ, Nowicki D, Anderson G, Chen X. *Morinda citrifolia* (noni): A Literature Review and Recent Advances in Noni Research, Acta Pharmacol. Sin. 2002; 23(12): 1127−1141.
16. Rasal VP, Sinnathambi A, Ashok P,Yeshmania S. Wound Healing and Antioxidant Activities of *Morinda citrifolia* Leaf Extract in Rats, IJPT 2007; **7**:49−52.
17. Prior RL, Cao G. Antioxidant Capacity and Polyphenolic Components of Teas: Implications for Altering In vivo Antioxidant Status, Proc. Soc. Exp. Biol. Med. 1999; 220:255−261.
18. Wang SY, Lin HS. Antioxidant Activity in Fruits and Leaves of Blackberry, Raspberry, and Strawberry Varies with Cultivar and Developmental Stage. J. Agric. Food Chem. 2000; 48:140−146.
19. Deshmukh SR, Wadegaonkar VP, Bhagat RP, Wadegaonkar PA. Tissue Specific Expression of Anthraquinones, Flavonoids, and Phenolics in Leaf, Fruit and Root Suspension Cultures of Indian Mulberry (*Morinda citrifolia* L.). POJ 2011; 4(1):6−13.
20. Paxinos G, Watson C. The rat brain in stereotaxic coordinates 5th edition. New York: Academic Press; 2005.
21. Montoliu C, Sancho-Tello M, Azorin I, Burgal M, Valles S, Renau-Piqueras J. et al. Ethanol Incerases Cytochrome P4502E1 and Induces Oxidative Stress in Astrocytes. J. Neurochem. 1995; 65(6):2561–2570.
22. Heaton MB, Mitchell JJ, Paiva M. Amelioration of Ethanol-induced Neurotoxicity in the Neonatal Rat Central Nervous System by Antioxidant Therapy. Alcohol Clin. Exp. Res. 2000; 24(4):512−518.
23. Coleman MD, Eason CR, Bailey JC. The Therapeutic Use of Lipoic Acid in Diabetes : A Current Perspective. Environ. Toxicol. Pharmacol. 2001; 10(4):167–172.
24. Mizumori SJ. Hippocampal Place Fields : Relevance to Learning and Memory, Oxford University Press, New York; 2008.
25. Giacobini E. Cholinesterases: new roles in brain function and in Alzheimer's disease. Neurochem Res. 2003; 28:515–522.
26. Wang X, Pal R, Chen X, Limpeanchob N, Kumar KN, Michaelis EK. High Intrinsic Oxidative Stress May Underlie Selective Vulnerability of The Hippocampal CA1 Region. Mol. Brain. Res. 2005; 140:120–126.
27. Obrenovich ME, Nair NG, Beyaz A, Aliev G, Reddy VP. The role of polyphenolic antioxidants in health, disease, and aging. Rejuvenation Res. 2010; 13:631–43.
28. Soobrattee MA, Bahorun T, Aruoma OI. Chemopreventive actions of polyphenolic compounds in cancer. Biofactors 2006; 27:19–35.
29. Deng S, West JBC, Jensen J. Simultaneous Characterisation and Quantification of Flavonol Glycosides and Aglycones in Noni Leaves Using A Validated HPLC-UV/MS Method. Food. Chem. 2008; 125: 1430−1435.
30. Groteworld E.The Science of Flavonoids, 239-268, Springer Science Business Media, Inc., USA, 2006, 239−268.