Research Article

Tiliacora triandra, an Anti-Intoxication Plant, Improves Memory Impairment, Neurodegeneration, Cholinergic Function, and Oxidative Stress in Hippocampus of Ethanol Dependence Rats

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Oxidative stress plays an important role in brain dysfunctions induced by alcohol. Since less therapeutic agent against cognitive deficit and brain damage induced by chronic alcohol consumption is less available, we aimed to assess the effect of Tiliacora triandra extract, a plant possessing antioxidant activity, on memory impairment, neuron density, cholinergic function, and oxidative stress in hippocampus of alcoholic rats. Male Wistar rats were induced ethanol dependence condition by semivoluntary intake of alcohol for 15 weeks. Alcoholic rats were orally given T. triandra at doses of 100, 200, and 400 mg kg\(^{-1}\) BW for 14 days. Memory assessment was performed every 7 days while neuron density, activities of AChE, SOD, CAT, and GSH-Px and, MDA level in hippocampus were assessed at the end of study. Interestingly, the extract mitigated the increased escape latency, AChE and MDA level. The extract also mitigated the decreased retention time, SOD, CAT, and GSH-Px activities, and neurons density in hippocampus induced by alcohol. These data suggested that the extract improved memory deficit in alcoholic rats partly via the decreased oxidative stress and the suppression of AChE. Therefore, T. triandra is the potential reagent for treating brain dysfunction induced by alcohol. However, further researches are necessary to understand the detail mechanism and possible active ingredient.

1. Introduction

Alcohol (ethanol) consumption in Thailand is dramatically increased. Data obtained from alcohol consumption collected by World Health Organization have demonstrated that Thailand is the top ethanol-consuming country in Asia and 40% of drinkers are in North-East region [1]. Chronic ethanol exposure can produce multiple and durable changes in the central nervous system. It has been reported that chronic ethanol consumption produces a significant loss of brain tissues especially in forebrain and hippocampus [2] together with the neurodegeneration of cholinergic neurons in basal forebrain [3]. Several lines of evidence from animal study have also revealed that a specific neuronal loss in the dentate gyrus, increased arborizations of the dendritic spines of the granule cells, a reduction of the number of spines of the CA3-pyramidal cells, and a reorganization of synaptic formations [4–7] are also presented. In addition, chronic ethanol consumption also induces memory impairment. This impairment has been demonstrated to relate with hippocampus degeneration and cholinergic function [8].

Accumulative lines of evidence have demonstrated that neurodegeneration induced by chronic ethanol consumption is associated with the elevation of oxidative stress [9, 10].
The elevation of oxidative stress induced by chronic ethanol consumption is reported to occur both via the increased free radical formation and via the decreased antioxidant enzyme activities [11]. In addition, these changes can be mitigated by the substances possessing antioxidant activity [10]. Currently, drugs which target at protecting against brain damage and memory impairment in alcoholism are less available. Therefore, the therapeutic benefit of herbal medicine has gained much attention especially in Asian countries [12].

_Tiliacora triandra_ (Colebr.) Diels or Ya-nang in Thai belongs to the family of Menispermaceae. It is the native plant of Southeast Asia and widely used in the cuisines of northeast Thailand and Laos. Ya-nang is used not only as food but also as medicine in traditional folklore. According to the traditional medicine of many countries in Southeast Asia, it has been used as anti-pyretic, detoxication agent, anti-inflammation, anticancer, antibacterial, and immune modulator. In addition, it also possesses antioxidant activity [13]. Recent toxicity study has revealed that water extract of _T. triandra_ leaves shows no toxicity up to 5000 mg kg$^{-1}$ in single administration. Moreover, no adverse effects were observed following the subchronic administration of the water extract of this plant at doses of 300, 600, and 1200 mg kg$^{-1}$ [14]. Based on the antioxidant effect together with the detoxification reputation of this herb and the benefit of substance possessing antioxidant activity against ethanol neurotoxicity mentioned earlier, the health benefit against neurotoxicity of _T. triandra_ extract has been considered. Thus, this study was carried out to determine the effect of water extract of _T. triandra_ on memory impairment, neurodegeneration, cholinergic function, and oxidative stress in hippocampus of ethanol dependence rats.

### 2. Materials and Methods

#### 2.1. Experimental Animals

Adult male Wistar rats, 8 weeks old, were used as experimental animals. They were purchased from National Laboratory Animal Center, Salaya, Nakorn Pathom. All animals were acclimatized for two weeks on normal diet of rat chow, with water given ad libitum at room temperature with a 12-hour light and dark cycle before the commencement of the experiment. The weights of the animals on the first day of experiment were 180–220 g. The experiments were performed to minimize animals’ suffering and the experiment protocols were approved by the Institutional Animal Care and Unit Committee Khon Kaen University, Thailand.

#### 2.2. Plant Material and Extract Preparation

The aerial parts of _T. triandra_ were collected from Khon Kaen province, Thailand. The plant was authenticated by Associate Professor Panee Siri-sa-ard, Faculty of Pharmacy, Chiangmai University, Chiangmai, Thailand (authentication number 023160). They were cleaned dried and ground to fine powder. Then, the ground powder was boiled with distilled water at a ratio of 1:6 (w/v) for 5 minutes with a continuous stirring. After being left at room temperature for 24 hours, the extract was filtered using Whatman No. 1. The filtrate was evaporated under reduced pressure using a rotary evaporator [15]. The percent yield of the extract was 5.5.

#### 2.3. Sample Analysis

The total phenolic compounds content was determined with the Folin-Ciocalteu reagent. Gallic acid was used as a standard and the total phenolics were expressed as mg/g gallic acid equivalents (GAE) [16].

The finger print of _T. triandra_ leaves extract was carried out by using gradient high performance liquid chromatography (HPLC) system. The system consists of 515 HPLC pump and 2998 Photodiode array detector of Waters company, USA. Chromatographic separation was performed using Purospher STAR, C-18 encapped (5 μm), LiChroCART 250-4.6, and HPLC-Cartridge, Sorbet Lot number HX255346 (Merk, Germany). Two mobile phases consisting of methanol and 2.5% acetic acid in deionized (DI) water were used to induce gradient elution. The injection volume was 20 μL and the flow rate was 1.0 mL/min. During HPLC analysis the solvent gradient was programmed as shown in Table 1 and data analysis was performed using Empower 3.

| Times (minutes) | A (methanol) | Solvents (%) |
|----------------|--------------|--------------|
| 0              | 10           | 90           |
| 17             | 70           | 30           |
| 18             | 100          | —            |
| 20             | 100          | —            |
| 20.5           | 10           | 90           |
| 25             | 10           | 90           |

#### 2.4. Experimental Protocol

The animals were induced ethanol dependence by using a semivoluntary intermittent intake method [17]. In brief, rats were exposed to ethanol in drinking water. The ethanol concentration in drinking water was gradually increased in a stepwise fashion from 5% at a rate of 5% per week until reaching a 20% ethanol within 4 weeks in order to allow the animals to get used to the taste of alcohol and mimic the condition of alcohol addiction in human. Then, the ethanol concentration was raised to 30% ethanol and maintained at this concentration between the fifth and the fifteenth weeks. The hyperexcitability symptoms which reflected ethanol dependence condition including the increased response to environmental stimuli such as startle, irritability, and hypervigilance were evaluated [17]. In addition, all ethanol dependence rats used in this study showed the average ethanol concentration at 9.89 ± 0.86 mg/dL prior to the intervention. The ethanol dependence rats were selected for further study and randomly assigned to 7 groups of 6 animals each as follows.

- **Group I:** control group: rats in this group received no treatment.
- **Group II:** ethanol + vehicle: alcoholic rats were orally administered vehicle (distilled water).
- **Group III:** ethanol + Aricept (donepezil): ethanol dependence rats were administered donepezil via oral route at dose of 1 mg/kg$^{-1}$ BW (this group served as positive control because this drug has been used as
standard drug for treating memory deficit patients) [18].

Group IV: ethanol + vitamin C: ethanol dependence rats were administered vitamin C at dose of 250 mg/kg • BW. (This group also served as positive control based on the previous findings that the substances possessing antioxidant effect exert neuroprotective effect against alcohol neurotoxicity of vitamin C [19].)

Group V: ethanol + T. triandra 100: ethanol dependence rats were orally administered T. triandra extract at dose of 100 mg kg • 1 BW.

Group VI: ethanol + T. triandra 200: ethanol dependence rats were orally administered T. triandra extract at dose of 200 mg kg • 1 BW,

Group VII: ethanol + T. triandra 400: ethanol dependence rats were orally administered T. triandra extract at dose of 400 mg kg • 1 BW.

All animals received the assigned treatments at a period of 14 days after the induction of ethanol dependence by a 15-week alcohol treatment. They were assessed spatial memory using Morris water maze test after the single intervention, 7 days and 14 days of treatments. At the end of experiment, they were sacrificed and hippocampi were isolated for the determination of oxidative damage markers including the level of malondialdehyde (MDA) and the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px). In addition, the activity of acetylcholinesterase (AChE) was analyzed based on the basis of malondialdehyde (MDA) using the thiobarbituric acid reaction [22] whereas glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) were determined using a spectrophotometric method [23].

2.6.1. Determination of Malondialdehyde (MDA) Level. Level of malondialdehyde (MDA), a relatively stable lipid peroxidation marker, was determined by using thiobarbituric acid reacting substances (TBARS) assay. MDA which occurred as the result of the breakdown of polyunsaturated fatty acid must react with thiobarbituric acid reacting substances and gives rise to the pink color product. This product could be measured at 532 nm. In this test, 1,3,3-tetra ethoxy propane (TEP) was used as the standard.

2.6.2. Catalase (CAT) Assay. Catalase activity was determined spectrophotometrically by measuring the decrease in H2O2 absorbance at 490 nm. A system devoid of the substrate (hydrogen peroxide) served as the control. The difference in absorbance per unit time was expressed as the activity. One unit was defined as the amount of enzyme required to decompose 1.0 M of hydrogen peroxide per minute at pH 7.0 and 25°C.

2.6.3. Superoxide Dismutase (SOD) Assay. The activity of SOD was performed using a xanthine/xanthine oxidase system for the production of superoxide radical and subsequent measurement of cytochrome c as a scavenger of the radicals. Optical density was determined using a spectrometer (UV-160i, Shimadzu) at 550 nm. A system devoid of enzyme served as control. One unit of enzyme activity was defined as the quantity of SOD required to inhibit the rate of reduction of cytochrome by 50%. SOD activity was presented as units per milligram of protein (U/mg protein).

2.6.4. Glutathione Peroxidase (GSH-Px) Assay. The determination of GSH-Px was performed using t-butyl hydroperoxide as a substrate. The optical density was recorded at 340 nm. One unit of the enzyme was defined as micromole (μmol) of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidized per minute. GSH-Px activity was expressed as U/mg protein.

2.7. Determination of Acetylcholinesterase (AChE) Activity. Acetylcholinesterase (AChE) was analyzed based on the basis that this enzyme catalyzed the hydrolysis of acetylthiocholine (ATCh) which in turn gave rise to the formation of acetate and thiociline. In the presence of the highly reactive 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) ion, thiociline generated a yellow color substance which was quantitatively monitored by spectrophotometric absorption at 405 nm [24].

2.8. Histological Study

2.8.1. Tissue Preparation. At the end of study, all rats were exposed to transcardial perfusion. In brief, the thorax was carefully cut open, the heart was exposed, and a needle
connected to the tubing from the fixative bottle was inserted into the left ventricle. The right atrium was cut open to drain out the blood and fixative. First 20–30 mL of saline was passed transcardially to flush out the blood and then perfuse with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. Fixation was monitored by the gradual discolorations of the tongue and eyeball. After fixation has been established, the brain specimens were further kept in the fixative containing 30% sucrose for 72 h. Regions of hippocampus were then dissected out using Paxinos stereotaxic coordinate method [25]. Serial sections of tissues containing hippocampus area were prepared using a sliding microtome at 20 μm thick. Tissue samples were picked up on slides coated with a 0.01% aqueous solution poly-L-lysine.

2.8.2. Cresyl Violet Staining of Brain Tissue. The sections containing hippocampus of all groups were stained with 0.5% cresyl violet. Analysis of neuronal density was performed on coronal sections of the dorsal hippocampus stained with cresyl violet and corresponding to brain sections located between 3.14 and 4.16 mm posterior to bregma [25]. The densities of living neurons in CA1, CA2, CA3, and dentate gyrus were performed using Olympus light microscope model BH-2 at 40x magnification by an observer who was blind to the treatment at time of analysis.

2.9. Statistical Analysis. Data were presented as mean ± standard error of mean (SEM). Statistical analysis was analyzed using one-way analysis of variance (ANOVA), followed by LSD post hoc test. Probability levels less than 0.05 were accepted as significance.

3. Results

3.1. Sample Analysis. The data obtained from this study showed that the T. triandra leaves extract used in this study contained total phenolic compounds at concentration of 593.33 mg of gallic acid equivalent (GAE)/mg extract. The HPLC fingerprint of T. triandra was shown in Figure 1. We had identified gallic acid, cyanidin, and quercetin according to their retention times and spectral characteristics of their peaks compared with standard. The ultraviolet spectrum of chromatographic bands presented in the fingerprinting of the samples indicated the presence of gallic acid at a concentration of 4.81 ± 0.05 μg gallic/100 mg of extract whereas the concentrations of cyanidins and quercetin were presented at the concentrations of 307.22 ± 4.74 μg Cyn-3-glu/100 mg and 9028.86 ± 695.97 μg QE/100 mg extract, respectively.

3.2. Effect of T. triandra Extract on Spatial Memory. Figure 2 showed that ethanol dependence rats induced by 15-week alcohol consumption which received vehicle or water showed the enhanced escape latency since the first day of treatment until the end of 14-day study period (P value < 0.001 all, compared to control rats). Donepezil, vitamin C, and all doses of T. triandra failed to modulate the enhanced escape latency induced by ethanol at 1-day treatment period. However, when the treatments were prolonged to 7 and 14 days, ethanol dependence rats which received donepezil or vitamin C or T. triandra extract at doses of 100, 200, and 400 mg·kg⁻¹·BW significantly mitigated the elevation of escape latency induced by ethanol (P value < 0.001 all, compared with ethanol dependence rats which received vehicle).

The effect of T. triandra on retention time in Morris water maze test was also investigated and results were shown in Figure 3. It was found that ethanol dependence rats which received vehicle showed the decreased retention time throughout the 14-day study period (P value < 0.001 all, compared to control rats). Ethanol dependence rats which received either donepezil or vitamin C significantly mitigated the decreased retention time induced by alcohol consumption at 7 and 14 days of treatment (P value < 0.001 all, compared with ethanol dependence rats which received vehicle). In addition, ethanol dependence rats which received T. triandra at doses of 200 and 400 mg·kg⁻¹·BW also showed the significant attenuation of the decreased retention time induced by ethanol consumption both at 7 (P value < 0.001 and 0.05, resp., compared with ethanol dependence rats
which received vehicle) and 14 days of treatments (P value < 0.001 all, compared with ethanol dependence rats which received vehicle). However, ethanol dependence rats which received T. triandra at dose of 100 mg·kg⁻¹ BW significantly attenuated the decreased retention time induced by ethanol consumption only at 14 days of treatment (P value < 0.05, compared with ethanol dependence rats which received vehicle).

3.3. Effect of T. triandra on AChE Activity in Hippocampus. Since cholinergic system played an important role on spatial memory, we also investigated the effect of T. triandra on AChE activity in this area. The results were shown in Figure 4. The significant elevation of AChE activity in hippocampus was observed in ethanol dependence rats which received vehicle (P value < 0.001, compared with control rats). Treatment with either donepezil or vitamin C could mitigate the elevation of AChE in hippocampus (P value < 0.01 all, compared with ethanol dependence rats which received vehicle). Ethanol dependence rats which received the extract at dose of 200 mg·kg⁻¹ BW also significantly mitigated an elevation of AChE activity induced by ethanol consumption in hippocampus (P value < 0.01 all, compared with ethanol dependence rats which received vehicle) while no changes were observed in ethanol dependence rats which received low and high doses of extract.

3.4. Effect of T. triandra on Neuron Density in Hippocampus. Effect of T. triandra on neuron density in various subregions of hippocampus including CA1, CA2, CA3, and dentate gyrus was shown in Figures 5(a) and 5(b). Repetitive consumption of alcohol significantly decreased neuron density in CA1, CA2, CA3, and dentate gyrus of hippocampus (P value < 0.001 all, compared with control rats). Ethanol dependence rats which received either donepezil or vitamin C significantly attenuated the decreased neuron density induced by alcohol in all subregions mentioned earlier of hippocampus (P value < 0.01 all, compared with ethanol dependence rats which received vehicle). Interestingly, all doses of T. triandra extract treatment significantly counteracted the decreased neuron density induced by alcohol consumption in hippocampus (P value < 0.01 all, compared with ethanol dependence rats which received vehicle).

3.5. Effect of T. triandra on Oxidative Stress Markers. Table 2 demonstrated the effects of T. triandra on oxidative stress markers including malondialdehyde (MDA) level and the activities of main scavenger enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) in hippocampus. The current data showed that alcohol consumption significantly enhanced MDA level (P value < 0.001, compared with control rats) but decreased SOD, CAT, and GSH-Px (P value < 0.001, 0.01, and 0.01, resp., compared with control rats) enzyme activities. Treatments with donepezil, vitamin C, and T. triandra at doses of 100, 200, and 400 mg·kg⁻¹ BW produced the significant mitigation effect on the elevation of MDA level (P value < 0.01 all, compared with alcoholic rats which received vehicle) in hippocampus of ethanol dependence rats. The significant attenuation of SOD activity induced by ethanol in hippocampus was also observed in ethanol dependence rats which received donepezil or vitamin C or T. triandra at doses of 100, 200, and 400 mg·kg⁻¹ BW (P value < 0.001, 0.001, 0.01, 0.01, and 0.01, resp., compared with ethanol dependence rats which received vehicle). In addition, ethanol dependence rats treated with donepezil showed a significant attenuation effect on the decreased CAT and GSH-Px activities induced by alcohol (P value < 0.01 all, compared with ethanol dependence rats which received vehicle) whereas those which received...
Figure 5: The effect of *T. triandra* extract on the neuron density in CA1, CA2, CA3, and dentate gyrus of hippocampus. (a) Photographs showing the density of the neurons stained with cresyl violet; (b) the bar graph showing density of neurons in various subregions of hippocampus. Data were presented as mean ± SEM, n = 6/group. ## P value < 0.001 compared with control treated group. *** P value < 0.001 compared with ethanol dependence treated group which received vehicle.

Vitamin C showed only the significant change of CAT activity in hippocampus (P value < 0.05, compared with ethanol dependence rats which received vehicle). It was found that *T. triandra* extract at doses of 100, 200, and 400 mg·kg⁻¹ BW significantly mitigated the enhanced MDA level induced by ethanol consumption (P value < 0.001 all, compared with ethanol dependence rats which received vehicle). The significant increased SOD activity in hippocampus was also observed in alcoholic rats which were treated with extract at all doses used in this study (P value < 0.01, 0.001, and 0.01, resp., compared with ethanol dependence rats which received vehicle). However, the significant increased CAT activity in
hippocampus was observed only in ethanol dependence rats which were treated with low and medium doses of extract (P value < 0.01 all, compared with ethanol dependence rats which received vehicle). Treatment with *T. triandra* at the dosage range used in this study also mitigated the decrease of GSH-Px activity in hippocampus of ethanol dependence rats (P value < 0.01, 0.001, and 0.01, resp., compared with ethanol dependence rats which received vehicle).

4. Discussion

The data obtained from this study demonstrated that the ethanol dependence rats used in this study were hyperactive and increased responsiveness to environmental stimuli, such as startled, increased puffing and hair ruffling, irritability, and hypervigilance similar to that observed in the previous study [17]. The average ethanol concentration in the blood of all ethanol dependence rats prior to the intervention was 9.89 ± 0.86 mg/DL. According to this model, it was found that no serious signs of physical dependence such as spasticity and convulsion were observed. Therefore, the animal model used in this study successfully induced mild to moderate ethanol dependence.

It was found that ethanol dependence rats induced by repetitive consumption of alcohol showed the increased escape latency but decreased retention time together with the neurodegeneration of hippocampus and hypocholinergic function. This was in agreement with the previous studies [4–8, 26, 27]. It has been reported that chronic alcohol consumption induces hippocampal damage, spatial memory impairment [28], together with the decreased oxidative stress [19]. Therefore, the results obtained from this study suggested that the elevation of oxidative stress reflected by the increased MDA level in hippocampus was responsible for the hippocampal damage and memory impairment. In addition, the decreased cholinergic function induced by chronic ethanol consumption also plays the important role in the improved hippocampal damage and memory impairment. It was found that donepezil and vitamin C could attenuate an enhanced oxidative stress and a cholinergic impairment which in turn improved memory deficit [10, 29, 30]. In addition, *T. triandra* leaves extract at all doses used in this study could also mitigate both the enhanced escape latency and the decreased retention time. Moreover, it also increased neuron density and improved oxidative stress status and cholinergic function in hippocampus of ethanol dependence rats. Previous studies clearly demonstrated that the neurons in hippocampus play the crucial role in the spatial memory [31]. Therefore, we did suggest that the improved oxidative stress which in turn increased neuron density in hippocampus was responsible for the improved memory impairment in ethanol dependence rats which received *T. triandra* leaves extract. In addition, the improved cholinergic function also played a role in the improved memory deficit in ethanol dependence rats which received a medium dose of *T. triandra* leaves extract. It was found that high dose of the extract failed to show the suppression of AChE in hippocampus. The possible explanation might be associated with the masking effect of the other ingredients in the crude extract of *T. triandra* leaves. Since *T. triandra* contained high concentration of polyphenolic compounds and high concentration of quercetin and these compounds also exerted neuroprotective effect against various insults [32–36], we did suggest that the neuroprotective effect of *T. triandra* extract observed in this study might be partly associated with quercetin. However, this still required further investigation.

5. Conclusions

The current study clearly demonstrates that *Tilia cordata* provides neuroprotective and cognitive enhancing effects in ethanol dependence rats. Therefore, it should be used as functional food and nutraceutical product to improve memory impairment and brain damage induced by ethanol dependence. The possible mechanism of extract may be due to the antioxidant and the suppression of acetylcholinesterase activity in hippocampus. Further studies are required to investigate the possible active ingredients, detail mechanism, and signal pathways.

Conflict of Interests

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