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Curcuma comosa Prevents the Neuron Loss and Affects the Antioxidative Enzymes in Hippocampus of Ethanol-treated Rats

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Abstract: *Curcuma comosa* Roxb. is widely used as a gynaecological traditional medicine in South-East Asia and recent behavioral studies have shown that *C. comosa* extract significantly improved the spatial memory in rats. The present study investigated the protective effects of *Curcuma comosa* hexane extract on the ethanol (EtOH)-induced oxidation in rat brains. Young female Wistar rats were given 20% of EtOH intraperitoneally to induce the oxidative stress. Subsequently, *C. comosa* hexane extract was intraperitoneally co-administered at the doses of 100 and 250 mg kg^{-1} b.wt. to the EtOH-induced rats for 14 days. The neuron densities of CA1, CA3 and CA4 areas of the hippocampus were counted and the activities of hippocampal Catalase (CAT), Glutathione Peroxidase (GPx) and Superoxide Dismutase (SOD) were determined. EtOH significantly decreased the neuron densities in Cornu Ammonis (CA), including CA1 and CA3 areas; however, the decrease was prevented by *C. comosa* co-administration. EtOH administration also increased the CAT and GPx activities in the hippocampus which were reversed by *C. comosa* co-administration. Moreover, *C. comosa* administration increased the SOD activity in a dose-dependent manner in the EtOH treated groups. *C. comosa* prevented the neuron loss in the hippocampus caused by EtOH. The possible neural protective mechanism may involve with the changes in activities of the antioxidant enzymes in the hippocampus.

Key words: *Curcuma comosa*, neuroprotection, superoxide dismutase, catalase, glutathione peroxidase

INTRODUCTION

Oxidative stress is well recognized as an underlying etiology associated with the aging of the brain and many neurodegenerative diseases (Maracchioni *et al*., 2007; Lin and Beal, 2006). The brain is vulnerable to oxidative stress because of its high consumption of oxygen and its structural richness in polyunsaturated fatty acids. Many factors could affect oxidative stress in the brain, especially the presence of some xenobiotics. Alcohol is one of the notable inducers of oxidative stress. In the brain, alcohol is generally oxidized by the enzyme alcohol dehydrogenase and cytochrome P450 enzymes and consequently increases the Reactive Oxygen Species (ROS) concentration. The extra ROS breaks the balance of the oxidative status in the brain and neuronal diseases occur. Alcohol consumers are generally at higher risk of neuron injury associated with cognitive deficits compared to non-alcohol consumer (Parson, 1998; Zeigler *et al*., 2005).

To relieve oxidative stress in the brain, the endogenous ROS scavengers, such as Catalase (CAT), Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx), are generally considered as the essential enzymes to remove excess ROS. The activities of these three enzymes are important in maintaining a normal oxidative status (Bakan *et al*., 2003).

Curcuma comosa Roxb. is widely used as a gynaecological traditional medicine in South-East Asia (Piyachaturawat *et al*., 1995a). The plant's hexane extract has been also reported to have uterotrophic effects and estrogenic activities in rats (Piyachaturawat *et al*., 1995a,b), Other pharmacological effects, such as decreasing the cholesterol (Piyachaturawat *et al*., 1999), enhancing the vascular relaxation (Intapad *et al*., 2009) and preventing the bone loss in the estrogen deficient mice (Weerachayaphorn *et al*., 2011) have also been documented. Recent behavioral studies have shown that *C. comosa* hexane extract significantly improved the spatial memory in the ovariectomized rats

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(Su *et al*., 2010, 2011). The hippocampus is the essential area related to spatial memory. Biological and histological changes can be found in association with behavioral changes. In this study, the effect of *C. comosa* hexane extract on EtOH-induced oxidative stress in rat brains was investigated by measuring hippocampal neuron density and antioxidative enzyme activities to reveal the conceived neural protective mechanism of *C. comosa*.

MATERIALS AND METHODS

Plant material and preparation: *Curcuma comosa* Roxb. rhizome was harvested in January, 2008 from Nakon Pathom Province, Thailand, (identified and provided by Professor Piyachaturawat Pawinee; Mahidol University, Bangkok). A voucher specimen was filed and kept in our laboratory (BS-C-03). The experiment was performed at 2009. Dry *Curcuma comosa* rhizome was crushed to crude powder and extracted in n-hexane Soxhlet apparatus until the outlet hexane was colorless. The hexane fraction was evaporated to a brown-yellow oily extract and then kept at 4°C until use. One of a major pure compounds, 1,7-diphenyl-5-hydroxy-(1E, 3E)-1,3-heptadiene, extracted from this crude extract (kindly provided by Professor Apichart Suksamrarn from Ramkhamhaeng University) was used as a standard reference (Fig. 1). A milligram crude extract was equal to 0.311 mg of 1,7 -diphenyl-5-hydroxy-(1E, 3E)-1,3-heptadiene.

Chemicals: Absolute ethanol was obtained from WNR International Ltd. (England); Potassium thiocyamide (KSCN), ethylenediaminetetraacetic acid (EDTA), nitroblue tetrazolium (NBT), xanthine, xanthine oxidase, glutathione (reduced form) (GSH), glutathione reductase (GR), hydrogen peroxide (H_2O_2), sodium azide (NaN_3) and DL-dithiothreitol (DTT) were obtained from Sigma Ltd. (USA). All other chemicals were analytical grade obtained from local distributors.

Experimental animals: Eighty four female Wistar rats, 8 weeks old, were obtained from the National Animal Center of Mahidol University, Thailand and kept in an environment maintained at 25±2°C, relative humidity of 50~70% and a 12 h light/dark cycle. Food and tap water were provided *ad libitum*. The animals were allowed 1 week to acclimatize this environment prior to the start of

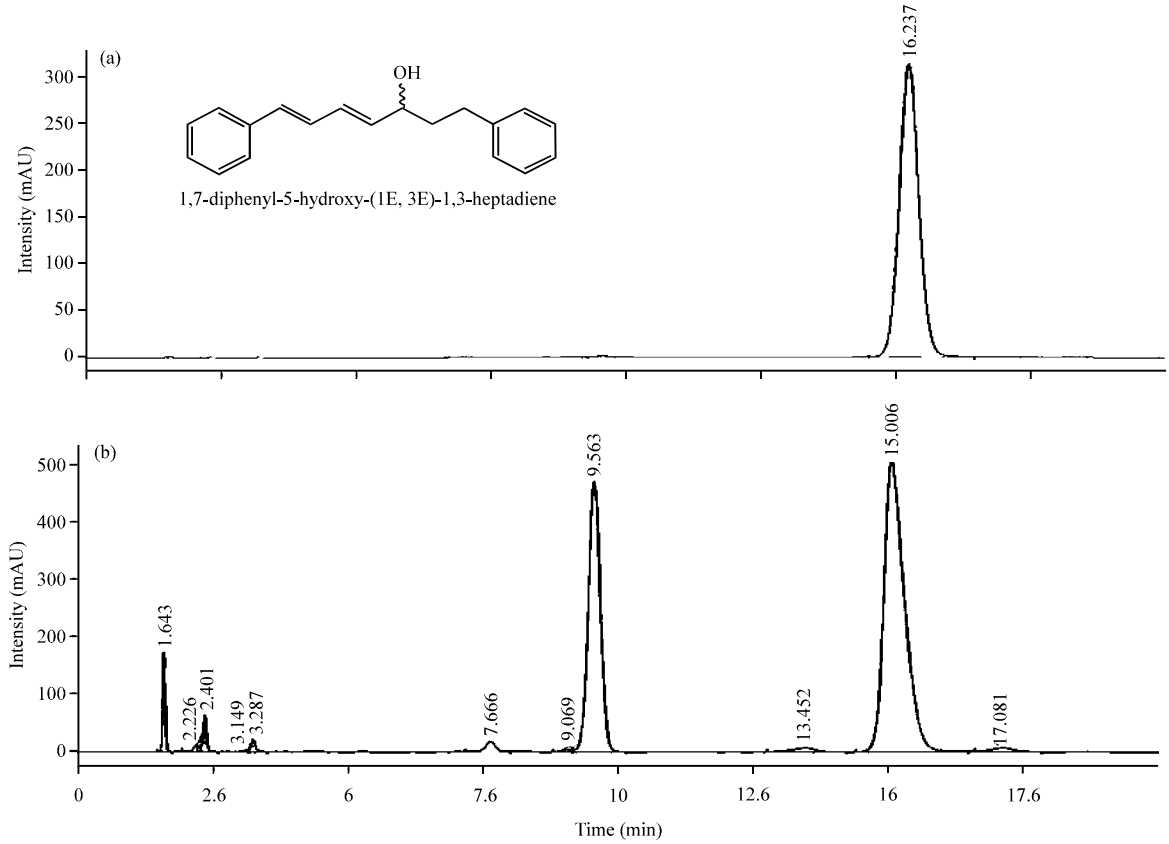

Fig. 1(a-b): The HPLC chromatograph of (a) 1,7-diphenyl-5-hydroxy-(1E, 3E)-1,3-heptadiene (0.2 mg mL^{-1}) and (b) *C. comosa* hexane extract (0.1 mg mL^{-1})

the experiment. All experiments were conducted under the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and approved by the Ethics Committee (Animal Care and Use Committee) of Khon Kaen University (Reference No. 0514.1.12.2/30).

The rats were randomly divided into 6 groups (n = 14) as follows:

- **Group 1:** Control group (control): received normal saline 2 g kg^{-1} b.wt.
- **Groups 2:** Ethanol group (EtOH): received 20% ethanol 2 g kg^{-1} b.wt.
- **Group 3, 4:** *C. comosa* hexane extract treated groups (C1, C2): received *C. comosa* extract at the doses of 100 and 250 mg kg^{-1} b.wt., respectively
- **Group 5, 6:** EtOH and *C. comosa* extract co-treated groups (C1+E, C2+E): received 20% ethanol 2 g kg^{-1} b.wt. plus *C. comosa* extract at the doses of 100 or 250 mg kg^{-1} b.wt., respectively

All the administrations were intraperitoneally injected every day for 14 days. At the 15th day, the rats were sacrificed by cervical dislocation. The brains were immediately removed, rinsed in 0.1 M ice-cold Phosphate Buffer Solution (PBS) and incised longitudinally into two halves. The left halves was used for histological studies and the hippocampi of the right halves were separated on an ice-cold stage and then kept at -80°C for further enzyme assay.

Measurement of the neuron density in the hippocampus
Sample preparation: The left half of the brain used for histological studies was saturated in 0.1 M PBS containing 30% sucrose at room temperature for 5 days. The brain was frozen with dry ice and then sectioned by microtome at 30 μm thickness. Serial one from every six sections was collected and mounted on the glass slide and air dried overnight. Then the collected sections were then stained by 1% cresyl violet.

Neuron counting: The neuron numbers of hippocampus in CA1, CA3 and CA4 areas were counted under a light microscope at 40 times magnification (Fig. 2). The neuron counting began from the section that each area was identified until the longitudinal end of the area and totally 20-30 slides were counted per animal depending on the brain size. All the neuron cells in the 0.075 mm^2 were compared. Overlay neurons were counted twice. The neuron cells at the edges of the field were included if more than 50% of their size were in the counting area. The UTHSCSA Imager Tool computer program (developed at the University of Texas Health Science Center at San Antonio, Texas and available from the Internet by anonymous FTP from maxrad6.uthscsa.edu) was used in the counting process.

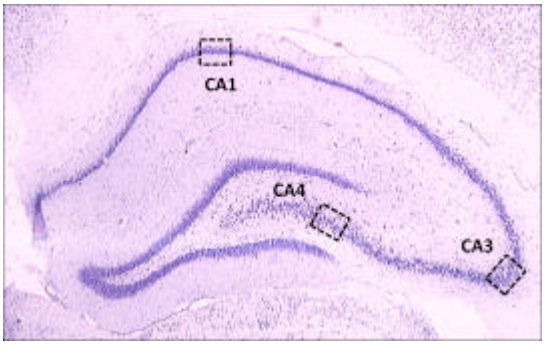

Fig. 2: Morphology of hippocampus demonstrating the CA1, CA3 and CA4 area. The dashed squares show the neuron counting areas

Assay of antioxidant enzymes activity in the hippocampus
Sample preparation: Hippocampi from two animals were pooled together for analysis because of their tiny size so that the sample size for enzymatic study was seven. The pooled hippocampi (around 0.25 g) were separately homogenized with three volume of 0.1 M PBS and then centrifuged at 10,000 g, 4°C for 10 min. The supernatants were taken into aliquots and kept at -20°C until the consecutive analysis. Protein assays were carried out for each sample following the method described in a previous report (Lowry *et al*., 1951).

Assay of enzyme activity: For superoxide dismutase activity, the method was modified from a previous study (Ewing and Janero, 1995). Catalase activity was measured by a modified method of Cohen *et al*. (1996). For glutathione peroxidase activity, the method was modified from Mannervik (1985).

Statistical analysis: The results are shown as Mean±SEM. One-way ANOVA followed by a LSD post hoc test was used to analyze the difference among groups using the SPSS software (version 11.0). The criteria for statistical significance was $p<0.05$.

RESULTS

Quality control of the *C. comosa* extract: One of a major pure compounds, 1,7-diphenyl-5-hydroxy-(1E, 3E)-1,3-heptadiene, extracted from this crude extract was used as

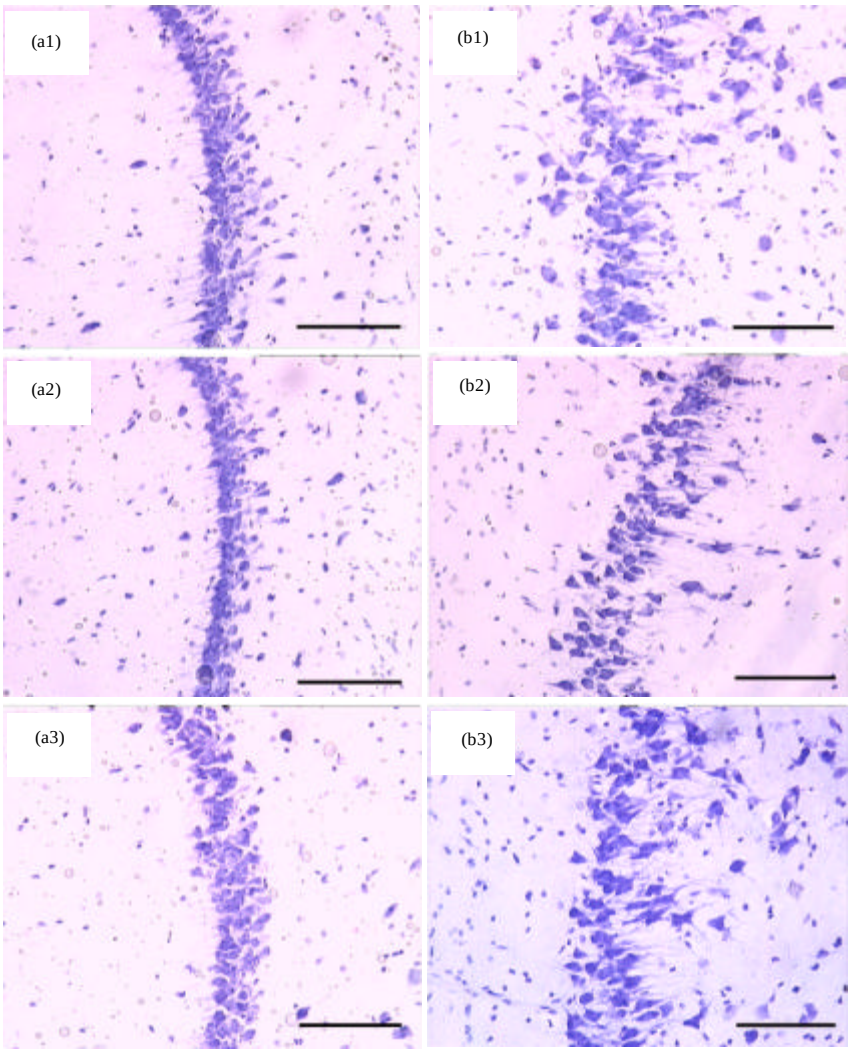

Fig. 3(a-b): The neuron density of (a) CA1 and (b) CA3 areas in hippocampus, (a1) and (b1): Control groups, (a2) and (b2): Ethanol treated groups and (a3) and (b3): Ethanol and *C. comosa* co-treated groups, Bar = 75 μm

a standard reference. Figure 1 showed the HPLC chromatogram of *C. comosa* extract and the reference standard. A milligram crude extract was equal to 0.311 mg of 1,7-diphenyl-5-hydroxy-(1E, 3E)-1,3-heptadiene.

Histological study: A randomly selected area from each area of CA1, CA3 and CA4 in hippocampus were selected for neuron counting and the neuron densities between groups were compared to show the significant difference (Fig. 2). As shown in Fig. 3, a2 and b2 showed the decreased neuron density of CA1 and CA3 in ethanol treated group when compared to that of control (Fig. 3a1, b1) and EtOH plus *C. comosa* treated (Fig. 3a3, b3) groups. As the statistical analyze result (Fig. 4) among the six animal groups, the EtOH-treated group had the significantly lowest neuron density in the CA1 and CA3, but not the CA4 areas. Administration of *C. comosa* in both doses prevented the neuron loss in CA1 and CA3 areas in the EtOH-treated groups. Administration of *C. comosa* alone in the normal rats did not change the neuron density in CA1, CA3 and CA4 areas of the hippocampus.

Antioxidant enzyme activities: As shown in Table 1, the EtOH administration alone slightly increased the SOD activity in hippocampus when compared to the control

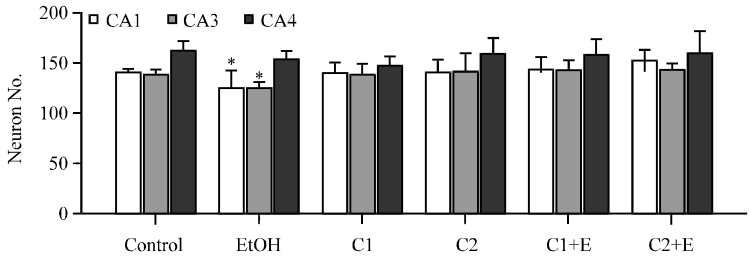

Fig. 4: The neuron density in CA1, CA3 and CA4 areas. The values are expressed in terms of Mean±SEM (n = 14), *Significant decrease of neuron density of the EtOH group when compared to the other 5 groups in the CA1 and CA3 areas

Table 1: The enzyme activities in hippocampus

Enzyme activity (unit/mg protein)	Control	EtOH	C1	C2	C1+E	C2+E
SOD	7.32±1.24	9.40±2.83	9.07±2.69	7.47±2.05	10.94±1.74[1]	11.70±2.22[2]
CAT	1.39±0.35	1.76±0.71[3]	1.46±0.44	1.49±0.45	1.08±0.25	1.21±0.31
GPx	0.039±0.002	0.050±0.008[4]	0.039±0.006	0.039±0.010	0.041±0.007	0.035±0.007

The values are expressed in terms of Mean±SEM (n=7). [1] [1,2] [3] [4] denote the significant difference from Control and C2 groups; Control, C1 and C2 groups; C1+E and C2+E groups; the other 5 groups, respectively ($p<0.05$)

group but with no statistical significance. However, when *C. comosa* hexane extract was administered at the doses of 100 and 250 mg kg^{-1} body weight to the EtOH-treated groups, the SOD activities were significantly higher than the other groups. The EtOH administration slightly increased the CAT activity when compared to the control group. The administration of *C. comosa* significantly reversed the increasing CAT activity. The EtOH administration significantly increased the GPx activity in hippocampus when compared to the other groups. However, the administration of *C. comosa* in the EtOH-treated groups kept the GPx activities at the same level as the control group.

DISCUSSION

The present study used EtOH to induce the toxicity in the central nervous systems. It is well known that EtOH consumption cause neurodegeneration. Numerous studies have provided the histological or the biological evidence to reveal the putative mechanism. Enhanced neuroinflammation and oxidative damage have been observed in animal models after chronic EtOH consumption. As a consequence, neuronal loss is observed in alcoholics from postmortem examination of their brain tissues (Zimatkin and Buben, 2007). In animal study, continually consumption of EtOH was reported to increase the oxidative stress and decrease the brain weight (Jayaraman *et al*., 2008). Similarly, the present study reported the brain damage by the EtOH administration which decreased the neuron densities in CA1 and CA3 areas. Of the sub-regions in hippocampus, CA3 is critical for encoding the information and the CA1 is an important output pathway of the signal from CA3. Lesion of CA3 and CA1 sub-regions resulted in the dysfunction of learning and memory processes (Kesner, 2007). Dentate Gyrus (DG) was another sub-region related to the CA3 output pathway. However, DG was not included in this study because the neuron density of DG in our sections was too high to be clearly identified for counting.

After absorption, more than 90% of EtOH was metabolized while it passed through the ventricular system of the brain (Zimatkin and Buben, 2007) and then in the brain, EtOH was metabolized by the three main ethanol metabolizing enzymes, alcohol dehydrogenase, cytochrome P450 2E1 and catalase (Zimatkin and Deitrich, 1997). Abundant ROSs are generated from this metabolizing process and then followed by the activities changes of the antioxidative enzymes. Several research reported that the EtOH administration decrease the activities of antioxidative enzymes (Pushpakiran *et al*., 2004; Reddy *et al*., 1999). However, in the present study we reported that EtOH increase the CAT and GPx activities while the SOD activity was in the normal level. These might be refer to the different experimental design, such as the use of animal model, doses and duration of EtOH administration or the different body regions observed.

In the animals treated with EtOH and *C. comosa*, the activities of CAT and GPx came back to the normal levels suggesting that *C. comosa* prevented neuron loss. This phenomenon indicated that CAT and GPx may be involved in the neuron loss process. They were more likely to be indicators of neuron loss in this study. The SOD activity responded in a different way. In the

EtOH-induced oxidative rat brain, SOD activity was at the control level but it increased following the administration of *C. comosa* extract which may help to prevent the neuron loss in the CA1 and CA3 areas. It was reasonable to assume that the increasing SOD activity diminished the extra ROS induced by EtOH consumption thus preventing neuron loss.

In the present study, the activities of antioxidative enzymes were changed after the animal receiving the *C. comosa* extract which indicated a modification to the enzyme activity by the plant extract. One explanation for this could be the estrogenic-like effect from the extract. Estrogen has been reported to modulate the antioxidative enzymes in the brain. For example, Schmidt *et al*. (2005) reported that estrogen changed the CAT activity in hippocampus HT22 and C_6 glial cells. Sobocanec *et al*. (2003) found that in the aging female mice, their brain had lower oxidant and higher antioxidant capacity than that of male mice and these differences were mostly related to CAT and GPx activity. Bilateral ovariectomy increased the SOD activity in the rat brain (Pajovic *et al*., 1993). However, whether the effects of *C. comosa* extract on the activities of antioxidative enzymes were related to its unique estrogenic-like effects requires further studies.

In conclusion, neuron loss was found in the rat hippocampus after the ethanol toxicity was induced; and the activities of the antioxidant enzymes were measured. Administration of *C. comosa* extract prevented the neuron loss and the potential mechanism was by modulating the antioxidant enzymes activities of hippocampus.

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