EFFECTS OF *Curcuma comosa* EXTRACTS ON CHOLINE ACETYLTRANSFERASE AND ACETYLCHOLINESTERASE IN MALE RAT'S BRAIN

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ABSTRACT:

Background: *Curcuma comosa*Roxb. (Zingiberazeae) is a well-known medicinal plant in Thailand. Rhizome of this plant has been traditionally used for the treatment of various abnormal symptoms of the uterus. Medicinal indications of *C. comosa* may be associated with its estrogenic-like effects reported by many studies. The objective of this study was to investigate effects of *C. comosa* hexane and fractionated ethanolic extracts on the activities of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) in the rat brain**.**

Methods: Forty male rats were randomly divided into 5 treatment groups, 8 rats per group. In group 1, rats were administered orally with 1 ml/kg/day of corn oil (control group); group 2 and 3 with 250 and 500 mg/kg/day of *C. comosa* hexane extract; group 4 and 5 with 250 and 500 mg/kg/day of *C. comosa* fractionated ethanolic extract for 30 days, respectively. At the end of the treatment, rats were anesthesized and euthanized. Three regions of brain (cerebral cortex, basal forebrain and hippocampus) were dissected out and collected. Each brain region homogenate was prepared for enzyme activity assays.

Results: The results showed that both dosages of *C.comosa* fractionated ethanolic extract caused an increase of ChAT activity in cerebral cortex and hippocampus but not in the basal forebrain. *C. comosa* hexane and fractionated ethanolic extracts did not affect AChE activities in any brain region.

Conclusions: These findings suggest that *C. comosa* fractionated ethanolic extract potentially possesses a beneficial effect on the cholinergic nervous system, via enhancement effect on the activity of ChAT, an enzyme responsible for brain acetylcholine synthesis.

Keywords: *Curcuma comosa*, Acetylcholinesterase, Choline acetyltransferase

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INTRODUCTION

Curcuma comosa Roxb. (Zingiberazeae) is one of the well-known medicinal plant in Thailand. Rhizome of this plant has been traditionally used for treatment of female postpartum uterine inflammation, uterine pain, amenorrhea, peri-menopausal bleeding, and lower abdominal pain in male as well as for the stomachic and choleretic effects [1]. *C. comosa* consists of two groups of chemical constituents: diarylheptanoids and acetophenones [2, 3]. Several studies demonstrated that *C. comosa* extracts exhibited many pharmacological effects in animal models such as *C. comosa* hexane extract possessed uterotrophic effect and estrogenic activity [4, 5]. Butanol and ethyl acetate extracts of *C. comosa* were most effective in choleretic activity [6]. The stimulation of bile secretion and the enhancement of

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biliary excretion of bile salt and cholesterol consequently led to a decrease in plasma cholesterol in rats [6]. Moreover, ethyl acetate extract of *C. comosa* also effectively reduced plasma triglyceride and cholesterol in diet-induced hypercholesterolaemic hamsters [7].

Regarding the Alzheimer's disease (AD), it is found more common and has an earlier onset and progress more rapidly in women than in men. Recent clinical studies found that deficiency of circulating estrogen in women after menopause increases risk of cognitive decline associated with AD. Many epidemiological studies reported the protective potential role of estrogen in AD patients. Estrogen replacement in postmenopausal women has been reported to improve cognitive function and decrease the risk of developing AD, of which the mechanism responsible for these effects is still unclear [8, 9]. In AD patients, acetylcholine (ACh) accompanied with choline acetyltransferase (ChAT), an enzyme catalyzes the biosynthesis of ACh, are reduced in cerebral cortex, basal forebrain and hippocampus. Thus, reduction of cholinergic activity is contributed from the degeneration of neurons in these areas [10]. Current studies in animal model have revealed that estrogen replacement can reverse impairment of ACh and ChAT. It was found that 17β-estradiol caused a restoration of ChAT activity and ChAT mRNA leading to increased ACh levels in ovariectomized (OVX) rats [9, 11, 12]. In addition, raloxifene and estradiol benzoate caused a restoration of hippocampal ChAT activity in OVX rats [13]. While many scientific data indicate that estrogen may increase cognitive function by enhancing ChAT protein or ChAT activity, acetylcholinesterase (AChE) which is an important enzyme of cholinergic neurons was also interested by a number of scientists. Inhibition of AChE is the basic mechanism of most drugs used clinically for symptomatic relief of the early stages of AD. Estradiol exhibited various effects on AChE activity in different brain regions, including a significant decreased AChE activity in thalamus, medulla and hippocampus by the effect of estradiol [14].

Phytoestogens are defined as plant-derived nonsteroidal substances with a diphenolic ring, of which the structure and function are similar to those of 17β-estradiol. Phytoestrogens have been studied for their potential beneficial effects for the prevention of hormone–dependent cancers (e.g., breast cancer, prostate cancer), cardiovascular disease, osteoporosis, symptoms of menopause (e.g., hot flushes) as well as the effect in brain [15]. Dietary soy phytoestrogens have been shown to improve memory in male and female human, postmenopausal women and OVX rats [15-17]. Several animal model studies revealed that phytoestrogen–treated OVX rats were shown to possess an increase of brain derive neurotrophic factor and ChAT in frontal cortex as well as nerve growth factor in frontal cortex and hippocampus [18, 19]. Soy isoflavone caused an increase ChAT in cerebral cortex, basal forebrain and a decrease AChE activity in cerebral cortex, basal forebrain and hippocampus in male rats [20].

According to the reports mentioned above, estrogen and phytoestrogen possess a potential beneficial effect in cholinergic neurons which play an important role in learning and memory process. Induction of ChAT activity and reduction of AChE activity may be one of the mechanism leading to a restoration of ACh in brain. Taken together with the recent studies which have reported that *C. comosa* possesses estrogenic activity, this study aimed to investigate effect of *C. comosa* extracts on ChAT and AChE activity in various region of male rat's brain. Even though *C. comosa* is a traditional medicine mostly used in female, expected benefits of this study focus on the pharmacological effect of *C. comosa* for Alzheimer's disease which is found in both female and male. In addition, *C. comosa* possesses estrogenic effect which could be interfered by estrogenic effect of the natural estrogens produced from ovary of female rat. In this regard, male rats or OVX female rats are normally used to abolish the effect of natural estrogens.

MATERIALS AND METHODS

C. comosa

The rhizomes of *C. comosa* were collected from Nakornpathom, Thailand. The dried rhizome powder (30 kg) was extracted with n-hexane in a Soxhlet extractor to give a pale browish viscous oil (1.01 kg), giving the percent yield of 3.37%. After extraction with n-hexane, it was subsequently extracted with 95% ethanol. The ethanolic fraction was dried under vacuum in rotary evaporator and dried again with high vacuum pump to give a dark reddish-brown viscous oil (1.30 kg), giving the percent yield of 4.33%. Hexane and fractionated ethanolic extracts of *C. comosa* were characterized by thin layer chromatography [3].

Experimental animals

Forty adult male Wistar rats (250-300 g body weight) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand. Two rats were housed in a cage and acclimatized for at least a week prior to the experiment. All animals were in a controlled

humidify room at a constant temperature of 25 ºC and maintained on a 12-hour alternate light-dark cycle at the Faculty of Medicine, Srinakharinwirot University, Bangkok. They were allowed free access to food (C.P. Company, Thailand) and water. Rats were randomly divided into 5 groups. Each group comprised 8 rats. The experimental groups were received *C. comosa* hexane extract or fractionated ethanolic extract orally at doses of 250 and 500 mg/kg/day whereas rats in the control group were given corn oil at 1 ml/kg/day for 30 consecutive days. At the end of the treatment, animals were fasted for 12 hours before anesthesized with diethyl ether and sacrificed by cervical dislocation. The whole rat brain was dissected out and onto ice bucket. It was rinsed with ice-cold 0.9% w/v sodium chloride and stored at -80°C until preparation of brain homogenate and enzyme activity assays.

The protocol of animal housing and treatment used in this study was approved by the Ethic Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University (Approval no. 98/2007).

Brain homogenate preparation

The removed-brain was weighed, thawed. Three regions of brain: cerebral cortex, basal forebrain and hippocampus were dissected on ice. Each brain region was weighed and homogenized with ice-cold 0.1 M sodium phosphate buffer, pH 7.4 to make a concentration of approximately 20 mg/ml of the homogenate. The brain homogenates were kept in microtubes for analysis of protein concentrations and enzyme activity assays.

Determination of choline acetyltransferase activity

ChAT activity was determined according to the method of Chao and Wolfgram [21]. Acetyl CoA and choline chloride were catalyzed by ChAT in brain homogenate yielding acetylcholine and CoA. Activity of ChAT was determined by measuring the rate production of 4-thiopyridone (4-TP), which was a product of the reaction between CoA and 4PDS, using spectrophotometer at 324 nm.

Amount of 4-TP produced in the reaction was calculated using a molar extinction coefficient of 1.98×10^4. Rates of the reaction were calculated by dividing amount of 4-TP in nmol by the time of reaction (20 minutes), and amount of brain homogenate protein used (mg) in the reaction. Unit of ChAT activity was expressed as nmol/mg protein/min. Before using the method for determining ChAT activity in rat brain homogenates, the method was verified for the linearity and the precision of both intraday and interday precision.

Determination of acetylcholinesterase activity

AChE activity was determined using the method of Ellman et al. [22]. The activity was determined by kinetic measuring an increase of the yellow color of the product produced from thiocholine that reacted with 5,5'-dithiobisnitrobenzoic acid. Thiocholine was a degradation product of acetylthiocholine by AChE in the brain homogenate. The absorbance of the product was measured by spectrophotometer and at 412 nm. Amount of 2-nitro-5- mercaptobenzoate produced in the reaction was calculated using a molar extinction coefficient of 1.36×10^4. Rates of the reaction were calculated by dividing amount of 2-nitro-5- mercaptobenzoate in mol by time of reaction (4 minutes) and amount of brain homogenate protein used (mg) in the reaction. Unit of AChE activity was expressed as nmol/mg protein/min. Before using the method for determining AChE activity in rat brain homogenates, the method was verified for the linearity and the precision of both intraday and interday precision.

Determination of protein concentrations in brain homogenates

Brain homogenate protein concentrations were determined according to the method of Lowry et al. [23]. Briefly, protein in the homogenate was reacted with cupric sulfate in an alkaline medium (0.5 M sodium hydroxide solution). The copper-peptide complex was reacted with Folin & Ciocalteu's phenol reagent resulting in a blue color of the product which was measured by spectrophotometer at 500 nm.

Statistical analysis

All numeric quantitative data were presented as mean ± standard error of the mean. One-way analysis of variance and Student-Newman-Keuls test were used for statistical comparison at a significant level of $p < 0.05$.

RESULTS

Verification of the method used for determination of ChAT activity

Linearity of the assay was performed by varying amount of cerebral cortex homogenate protein used in reaction (0.25, 0.5, 1, 1.5 and 2 mg of cerebral cortex homogenate protein / 400 µl of the reaction mixture). Coefficient of determination (R^2) between amounts of cerebral cortex homogenate protein and the corresponding absorbance was 0.9997. Intraday (n =5 experiments/day) and interday (n=4 days) precision of the method were performed using cerebral cortex homogenate of the control rat. Percent CV of the intraday precision of each day was 0.329 (day 1), 3.436 (day 2), 2.980 (day 3), 3.425

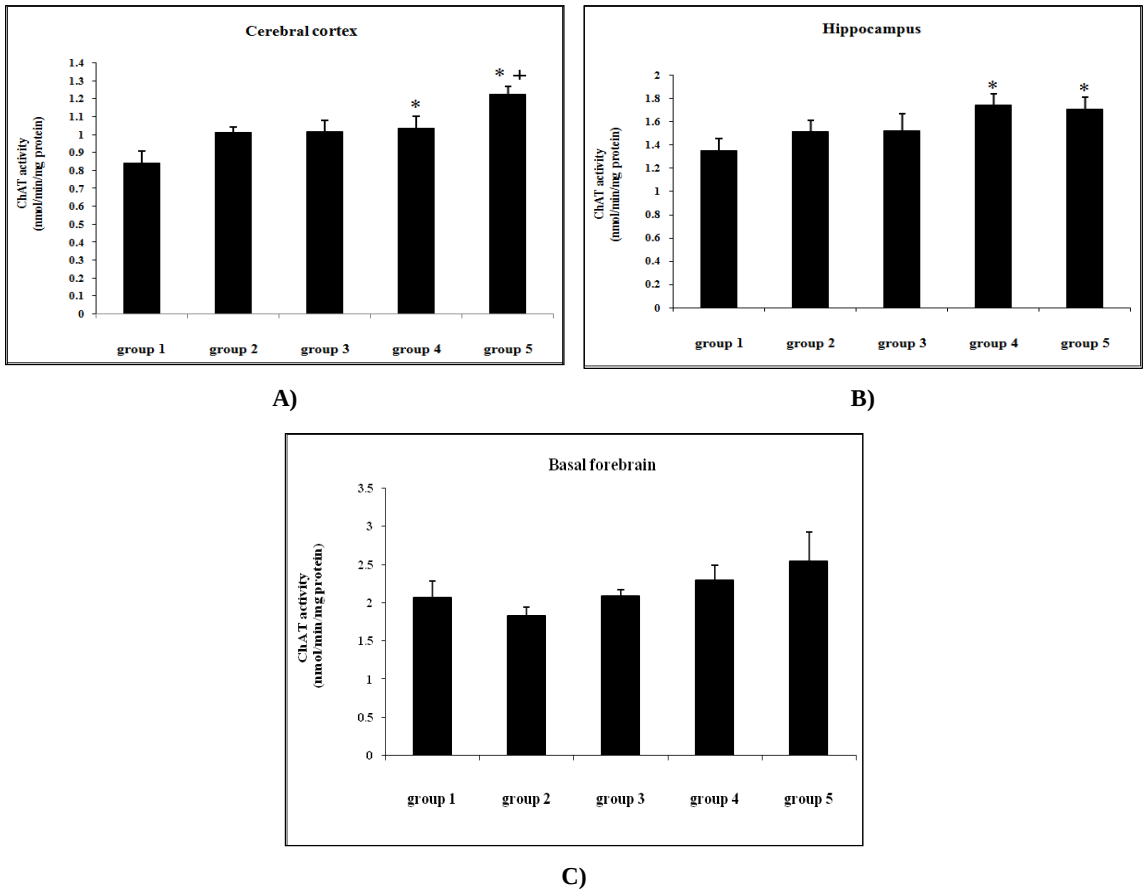

Figure 1 Effects of *C. comosa* extracts on ChAT activity in rat cerebral cortex (A), hippocampus (B) and basal forebrain (C) The individual bar graph represented mean of ChAT activity with standard error of the mean (n=8).

Group 1 = control group receiving 1 ml/kg/day of corn oil; group 2 = rats receiving 250 mg/kg/day of *C. comosa* hexane extract; group 3 = rats receiving 500 mg/kg/day of *C. comosa* hexane extract; group 4 = rats receiving 250 mg/kg/day of *C. comosa* fractionated ethanolic extract; group 5 = rats receiving 500 mg/kg/day of *C. comosa* fractionated ethanolic extract.

* $p<0.05$: *C. comosa* treated group vs control group

+ $p<0.05$: *C. comosa* 500 mg/kg/day vs *C. comosa* 250 mg/kg/day

(day 4), respectively; and % CV of the interday (4 days) precision was 2.357.

Verification of the method used for determination of AChE activity

Linearity of the assay was performed by varying amount of cerebral cortex homogenate protein used in reaction (0.25, 0.5, 0.75, 1, 1.5 and 2 mg of cerebral cortex homogenate protein / 3120 µl of the reaction mixture). Coefficient of determination (R^2) between amounts of cerebral cortex homogenate protein and the corresponding absorbance change was 0.9913. Intraday (n =5 experiments/day) and interday (n=4 days) precision of the method were performed using cerebral cortex homogenate of the control rat. Percent CV of the intraday precision of each day was 3.269 (day 1), 1.698 (day 2), 0.769 (day 3), 1.176 (day 4), respectively; and % CV of the interday precision was 1.538.

Effects of C. comosa extracts on ChAT and AChE activities in rat brains

C. comosa hexane and fractionated ethanolic extracts were given orally to rats at doses of 250 and 500 mg/kg/day once daily for 30 days. The results showed that, *C. comosa* fractionated ethanolic extract caused a significant increase of ChAT activity in cerebral cortex and hippocampus while *C. comosa* hexane extract did not cause any significant change on this enzyme activity (Figure 1A, 1B). In basal forebrain, both hexane and fractionated ethanolic extracts of *C. comosa* did not exhibit any significant effect on ChAT activity (Figure 1C). Hexane and fractionated ethanolic extracts of *C. comosa* at both dosage regimens used in this study did not significant affect AChE activities in cerebral cortex, hippocampus and basal forebrain (Figure 2).

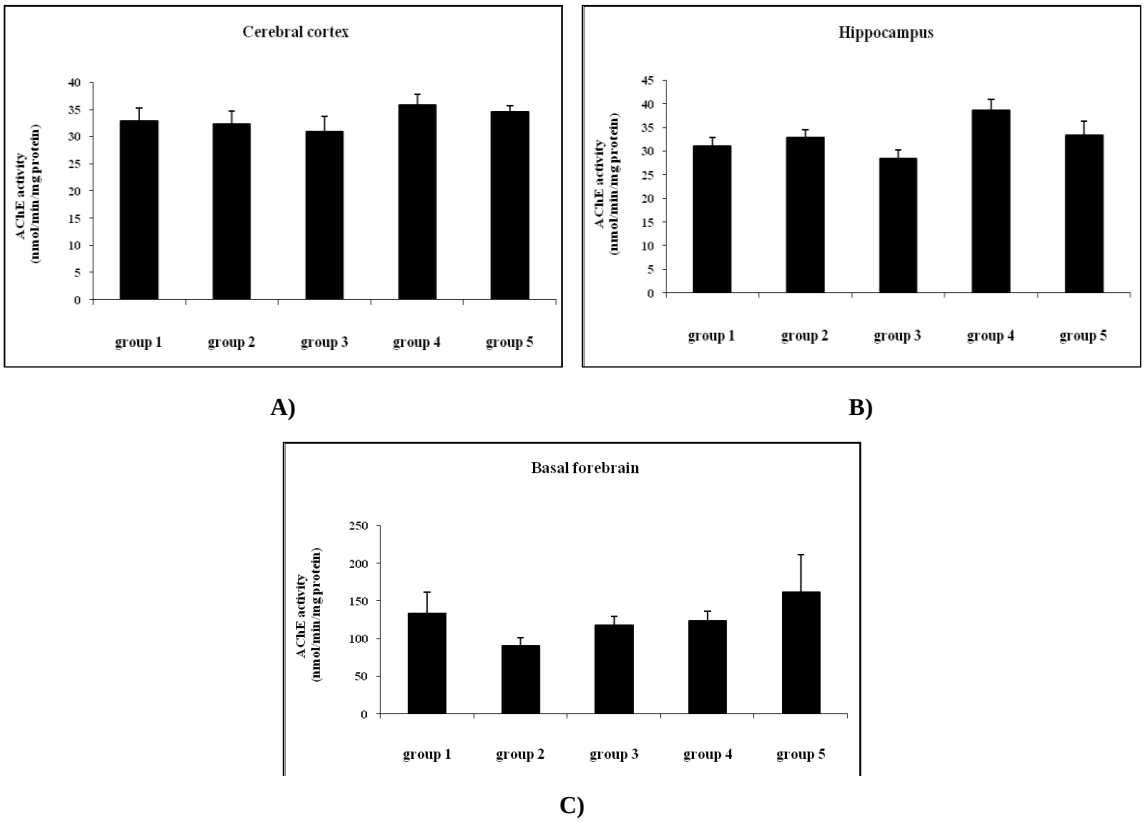

Figure 2 Effects of *C. comosa* extracts on AChE activity in rat cerebral cortex (A), hippocampus (B) and basal forebrain (C) The individual bar graph represented mean of AChE activity with standard error of the mean (n=8).

Group 1 = control group receiving 1 ml/kg/day of corn oil; group 2 = rats receiving 250 mg/kg/day of *C. comosa* hexane extract; group 3 = rats receiving 500 mg/kg/day of *C. comosa* hexane extract; group 4 = rats receiving 250 mg/kg/day of *C. comosa* fractionated ethanolic extract; group 5 = rats receiving 500 mg/kg/day of *C. comosa* fractionated ethanolic extract.

DISCUSSION AND CONCLUSION

This study aimed to investigate effects of *C. comosa* extracts on ChAT and AChE activities in rat's brain. Both enzymes are related to the level of brain ACh, the neurotransmitter which plays an important role in learning and memory process in cholinergic neurons. ACh is synthesized by ChAT and degraded by AChE. Effects of estrogen on the expression of these cholinergic enzymes have been studied for the possible contribution of estrogen on brain beside its reproductive action [8, 9, 11-14]. Several previous studies reported that *C. comosa* exhibited estrogenic-like effects [4, 5]. Whether or not *C. comosa* demonstrates the positive effects on brain, similar to estrogen, is interested. Results from this study would provide a preliminary information of *C. comosa* extracts on the cholinergic enzymes associated to ACh, thus, a potentially beneficial effect of the extracts for neurodegenerative disease.

The doses of the hexane extract of 250 and 500 mg/kg/day exhibited estrogenic effect in rats such as uterotropic effects, while of the alcoholic extract were shown to possess lipid-lowering effects [4, 5, 7]. In this study, effects of *C. comosa* extracts on ChAT and AChE activities were investigated in three brain regions such as basal forebrain, cerebral cortex and hippocampus, the brain regions which normally contain cholinergic neurons. Basal forebrain is a primary source of the cholinergic system that project to the hippocampus and cerebral cortex, the brain regions which associated with ACh that play an importance role in learning and memory [24].

In this study, ChAT and AChE activities were determined using the methods reported earlier [21, 22]. Both methods were verified before using for determination of ChAT and AChE activities in rat brain tissues in this study. Linearity and precision assays were performed. The results showed that linearity between amount of the brain tissue protein and the absorbance of the product produced from the reaction of ChAT and AChE activity assays were highly correlated with the satisfactory R^2. Regarding the precision assay, high precision of both ChAT and AChE activity assays were shown by the small % CV indicating the high precision of the methods

for both enzyme assays.

Results from this study showing that *C. comosa* fractionated ethanolic extract caused an increase of ChAT activity at the dosages of 250 and 500 mg/kg/day in cerebral cortex and hippocampus but not in basal forebrain. No effects of *C. comosa* hexane extract on ChAT activity were seen in all three regions of rat brain. Different constituents in fractionated ethanolic extract and hexane extract were most reasonably contributed to these different results. Even though this is the first report of positive effect of *C. comosa* extract on ChAT activity, other phytoestrogen-containing plants also demonstrated this effect. Soy isoflavone giving to male rats for 16 weeks caused an increase of ChAT activity in cerebral cortex and basal forebrain [20]. Increases of ChAT activity in cerebral cortex and hippocampus by *C. comosa* fractionated ethanolic extract need to be confirmed at the level of enzyme protein via immunoblotting. The mechanism(s) underlying the effect of *C. comosa* on ChAT activity in rat was not explored in the present study. Some chemical constituents in *C. comosa* or their metabolites may directly promote ChAT gene expression or they may possess a similar effect as estrogen which exhibit an increase of brain derived neurotrophic factor [25] or enhances ChAT activity post-translationally by itself [26].

In basal forebrain, both hexane and fractionated ethanolic extracts of *C. comosa* did not exhibit a significant increase effect on ChAT activity. Normally basal forebrain consists of various regions such as NBM, HDB, MS and substantia innominata. Several studied found that estrogen caused an increase of ChAT mRNA in MS and NBM but not the HDB [27, 28] In contrast, Luine [11] found that estrogen significant increased ChAT activity in HDB. The reason for these discrepancies is not clear but may be related to the difference of brain dissection in the region of basal forebrain among studies. Previous reports regarding the beneficial effect of estrogen or estradiol on cholinergic function are mostly associated with an increase of ChAT activity in rat's brain [9, 11-14]. Lack of the positive control treated with estradiol is the limitation of our study, thus, we could only assess effect of *C. comosa* extracts by comparing with the non-treated control group.

Both hexane and fractionated ethanolic extracts of *C. comosa* did not cause any significant effect on AChE activity in all three rat brain regions in this study. In contrast, other phytoestrogens such as soy isoflavone added in food for male rats for 16 weeks was found to decrease AChE activity in cerebral cortex, basal forebrain and hippocampus [20]. In the case of *C. comosa*, no effects of this plant extracts on AChE in rat brain indicated that a decrease effect of these extracts on AChE may not contribute to the positive effect on cholinergic system, if *C. comosa* extracts possess this effect.

In conclusion, effects of *C. comosa* hexane and fractionated ethanolic extracts on ChAT and AChE activity in three rat brain regions (cerebral cortex, hippocampus and basal forebrain) were investigated in this study. Both extracts were given orally to rats at the doses of 250 and 500 mg/kg/day for 30 days. *C. comosa* fractionated ethanolic extract significantly increased ChAT activity in cerebral cortex and hippocampus but not in basal forebrain. *C. comosa* hexane extract did not exhibit any significant effect on this enzyme activity. Both hexane and fractionated ethanolic extracts of *C. comosa* did not demonstrate any significant effect on AChE enzyme. An enhancement of ChAT activity in brain by *C. comosa* fractionated ethanolic extract provide a preliminary information that this extract might possess a beneficial effect on cholinergic nervous system associated with learning and memory. Confirmation of the enhancement of ChAT activity by *C. comosa* fractionated ethanolic extract in the level of protein enzyme with immunoblotting should be explored. Behavioral test should be further performed for investigating the overall outcome of *C. comosa* fractionated ethanolic extract on learning and memory associated cholinergic nervous system.

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