

***Coscinium Fenestratum* Protects Against Ethanol-Induced Neurodegeneration in Adult Rat Brain**

¹Wathita Phachonpai, ^{2,3}Jintanaporn Wattanathorn,
^{2,3}Panapakorn Wannanon, ^{2,3}Chonlatip Thipkaew,
⁴Bungorn Sripanidkulchai and ^{2,3}Supaporn Muchimapura

¹Division of Physiology, School of Medical Science,
University of Phayao, Phayao, 56000, Thailand

²Department of Physiology, Faculty of Medicine,

³Integrative Complementary Alternative Medicine Research and Development Group,

⁴Center for Research and Development of Herbal Health Products,
Khon Kaen University, 40000, Thailand

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ABSTRACT

Alcoholism is a serious problem throughout the world. Despite intensive efforts to develop novel therapeutics for strategy efficacy against neuronal loss leading to memory impairment in alcoholism is still limited. In view of the pitfalls of psychological dependence and adverse behavioral effects of synthetic drugs, the development of low toxicity and high efficiency medicines derived from natural herbal antioxidants exhibits expansive market prospects. In this respect, *Coscinium Fenestratum* (*C. Fenestratum*; CF) could be an attractive candidate as a diet supplement for neuroprotectant against ethanol-induced neurodegeneration. Herein, we determined the neuroprotective effects of CF against ethanol-induced neuronal damage in both hippocampus and cerebral cortex. The stems of CF extract (No. HHP-2-462) were dried, refluxed with ethanol and evaporated with lyophilizer. Extract was gave a yield of 8.53%. The rats were pretreated with the extract at various doses ranging from 5, 10 and 20 mg kg⁻¹ once daily per os, 30 min prior to ethanol administration at a dose of 1.8 g kg⁻¹ via i.p for 14 consecutive days and were evaluated the densities of survival and cholinergic neurons in all areas as mention above by immune histological techniques. CF extract at a dose of 5 mg kg⁻¹ showed the attenuation of the neurotoxicity in all brain areas just mentioned except in the temporal cortex. In addition, it also mitigated the degeneration of cholinergic neurons in all areas of hippocampus except in CA3 region. This study indicated that CF extract consumption may be served as a diet supplement protect against neuronal degeneration resulting from excessive continuous consumption of alcohol. However, further researches about possible active ingredients and pharmacokinetic of the extract are still required before moving forward to clinical trial study.

Keywords: *Coscinium Fenestratum*, Alcoholism, Neuroprotective Effect, Memory Impairment, Neurode Generation, Diet Supplement

1. INTRODUCTION

Inspite of the increasing emphasis placed on alcoholism worldwide, to date only a few therapeutic drugs (namely disulfiram, naltrexone and acamprosate) have been approved and are currently available for

clinical use. However, the overall efficacy of these compounds is often viewed as not being completely satisfactory (Krishnan-Sarin *et al.*, 2008; O'Malley and O'Connor, 2011).

Abundant evidence suggests that the memory impairment induced by chronic alcohol consumption was

Corresponding Authors: Supaporn Muchimapura, Department of Physiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand Tel: 66-43-348394 Fax: 66-43-348394

reported to be associated with the decreased cholinergic neurons (Arendt *et al.*, 1989; Nordberg *et al.*, 1982). In addition, chronic alcoholism could also produce the neurodegeneration in hippocampus (Agartz *et al.*, 1999). There are several factors in the mechanism of ethanol-induced brain damage. However, a substantial increase of oxidative stress to be one factor contributing the important role in neurodegenerative action of ethanol (Esterbauer *et al.*, 1991). A series of experimental data have provided confirmation of alcohol consumption to induce oxidative stress both via increased free radicals formation and decreased antioxidant enzymes activities (Wattanathorn *et al.*, 2012; Das and Vasudevan, 2007). In all respects mentioned above, the development of low toxicity and high efficiency medicines derived from natural herbal antioxidants exhibits expansive market prospects.

Coscinium Fenestratum Colebr (CF) (Menispermaceae), grows widely in the Indochina region. This plant has been mainly used for treating diabetes mellitus in the traditional Ayurvedic and Siddha systems of medicine (Warrier *et al.*, 1996). Its stem is used for dyspepsia and as a febrifuge. Its hypotensive (Singh *et al.*, 1990) and hepatoprotective (Venkumar and Latha, 2002) actions have also been reported. An earlier study carried out by the authors established the significant anti-diabetic activity of CF (Shirwaikar *et al.*, 2005). In addition, the extract also exhibited strong antioxidant activity and its derivative has been reported to exert profound influences on the nervous system including the anti-amnesic effect against memory impairment induced by scopolamine (Peng *et al.*, 1997).

The aim of the present study was to investigate the protective effect of CF extract consumption against ethanol-induced neurotoxicity in rats.

2. MATERIALS AND METHODS

2.1. Plant Material

The stems of CF were purchased from Nakorn Panom province during the month of August, 2011. The tree specimen was identified and a voucher specimen from this plant was deposited at the Center of Research and Development of Herbal Health Products, Khon Kaen University under the number HHP-2-462.

2.2. Extraction

Stems of CF were washed, dried at room temperature and minced into small pieces. Plant materials are then extracted with 50% ethanol by reflux

method. Then the extract was centrifuged at 2500 rpm for 10 min to remove residual debris. The clear supernatant was evaporated under reduced pressure and dried by lyophilizer. The percent yield of extract was 8.53%. The extract was stored at -20°C in a dark bottle until used.

2.3. Animals and Treatment

We used fifty male Wistar rats, 180 to 220 g in our study. These rats were kept under standard laboratory conditions with a 12 h light/dark cycle and *ad libitum* food and water throughout the experiments which were approved by Khon Kaen University ethical committee. Experiments were performed to minimize animal suffering in accordance with the internationally accepted principles for laboratory use and care of European Community (EEC directive of 1986; 86/609/EEC).

The animals were randomly divided into five groups, with ten rats in each. One group received saline (control group), the second group was ethanol treated group; ethanol was administered at a dose of 1.8 g kg⁻¹ (40% v/v) via intraperitoneal (i.p.) route for 14 days. The dose of ethanol was selected on the basis of the previous studies (Witte and Bada, 1983), the third-the fifth groups were treated with extract of CF at various dosages ranging from 5, 10 and 20 mg kg⁻¹ once daily per os, 30 min prior to ethanol exposure via i.p for 14 consecutive days and then all rats were sacrificed and the densities of survival neurons in various subregions in both the cortex and hippocampus were determined. The plant extract at a dose of 5 mg kg⁻¹ was selected for further study on the alteration of cholinergic neurons densities in all areas of hippocampus because of this dose attenuated the maximum amount of neuronal degeneration induced by ethanol administration.

2.4. Tissue Preparation and Sectioning

At the end of the experimentations, rats were deeply anesthetized (Sodium pentobarbital, 60 mg kg⁻¹) and perfused transcardially with fixative (4% paraformaldehyde in phosphate buffer, pH 7.3). Brains were removed, kept in paraformaldehyde for one night and then cryoprotected by a 5-day immersion in a 30% sucrose solution in phosphate buffer and kept at 4°C. Brains specimens were frozen rapidly and serial brain sections (30 μM) of the anterior part of the brain were cut at -20°C on a cryostat (Jung). The sections were mounted onto glass slides coated with poly-L-lysine (0.01%) and stored at -20°C. Adjacent sections were collected on 5 series of slides in order to be able to use different stainings on homolog series of sections.

2.5. Cresyl Violet Staining

Brain coronal sections were stained with 0.75% cresyl violet (Lamb; London, UK) for 4 min and dehydrated in 70% alcohol (for 10 min), 95% alcohol (with a few drops of 10% acetic acid; for 2-3 min) and finally 100% alcohol (for 10 min). Slices were cleared in xylene for 5 min before being covered with DPX and a coverslip.

2.6. Choline Acetyltransferase and Immunohistochemistry

For immunohistochemistry, coronal sections of the hippocampus sections were sequentially incubated in 0.5% hydrogen peroxide in PBS (0.01 M; pH 7.4) for 10 min, rinsed with PBS four times and then incubated with a blocking solution containing 5% normal horse serum (Vector Laboratories, Burlingame, CA, USA) and 0.1% Triton-X100 in PBS at room temperature. The sections were then incubated with the primary antibody (anti-PVA monoclonal antibody; Sigma, dilution 1:5000 in PBS containing 1.5% normal horse serum and 0.1% Triton-X100) for 48 h at 4°C and then rinsed four times in KPBS-BT. Thereafter, the sections were incubated for one hour at room temperature with secondary antibody, biotinylated anti-mouse antibody made in horse (Vector) dilution 1:50 in PBS containing 1.5% normal horse serum and 0.1% Triton-X100. After this step, the sections were rinsed with PBS four times and covered with the ABC reagent (Vectastain Kit, Vector) for one hour at room temperature. After rinsing, sections were incubated for 5-7 min with a mixture of 0.025% diaminobenzidine and 0.05% hydrogen peroxide in PBS. Finally, sections were rinsed in running tap water, air dried, coverslipped and examined using light microscopy.

2.7. Morphological Analysis

Five coronal sections of each rat in each group were studied quantitatively. Neuronal counts in both cerebral cortex and hippocampus were performed by eye using a 40x objective with final field 225 μm^2 according to the following stereotaxic coordinates: (a) frontal cortex: AP 0.2 mm, lateral \pm 1-4 mm, depth 1-3 mm; (b) parietal cortex: AP 0.2 mm, lateral \pm 5-7 mm, depth 1-4 mm; (c) occipital cortex: AP 4.8 mm, lateral \pm 3-5 mm, depth 1-3 mm; (d) temporal cortex: AP 4.8 mm, lateral \pm 6-7 mm, depth 3-6 mm; (e) hippocampus: AP 4.8 mm, lateral \pm 2.4-6 mm, depth 3-8 mm. The observer was blind to the treatment at the time of analysis. Viable stained neurons were identified on the basis of a stained soma with at least two visible processes. Counts were made in five

adjacent fields and the mean number extrapolated to give total number of neurons per 225 μm^2 . All data are represented as number of neurons per 225 μm^2

2.8. Statistical Analysis

Data were presented as mean \pm Standard Error of Mean (SEM). Data were analysed by ANOVA followed by the Duncan test. A level of $p < 0.05$ was considered to be significant.

3. RESULTS

3.1. Effect of CF Extract on the Survival Neurons Densities

The neuroprotective effects of CF extract against ethanol neurotoxicity in various areas of cerebral cortex and hippocampus were investigated and the results were shown in **Fig. 1A and 2**. It was found that the mean \pm SEM survival neurons densities in various subregions of hippocampus and cerebral cortex were significantly ($p < 0.05$ all) lower in ethanol treated group when compared to vehicle treated group. Among various areas of hippocampus, dentate gyrus was the most sensitive area to ethanol neurotoxicity. Surprisingly, CF extract consumption at a dose of 5 mg kg^{-1} could reverse the changes of this parameter in all areas of the brain except in the temporal cortex ($p < 0.05$ all; compared to ethanol treated group). Among the various areas of the brain, the dentate gyrus was the most vulnerable area to the plant extract. However, the increasing doses did not produce significant the alteration in densities of survival neurons in all areas as just mentioned.

As shown in **Fig. 1B**, the effects of vehicle, ethanol administration and CF at a dose of 5 mg kg^{-1} on the survival neurons densities were examined using cresyl violet staining. Representative photomicrographs of the CA1 region in the hippocampal formation at 20X magnification.

3.2. Effect of CF Extract on the Cholinergic Neurons Densities

Based on the previous information that cholinergic neurons played the crucial role on the memory impairment induced by chronic alcohol consumption, the CF extract at a dose of 5 mg kg^{-1} was selected for further study on the changes of cholinergic neurons densities in hippocampus, the area playing an important role on learning and memory. Data were shown in **Fig. 3A**.

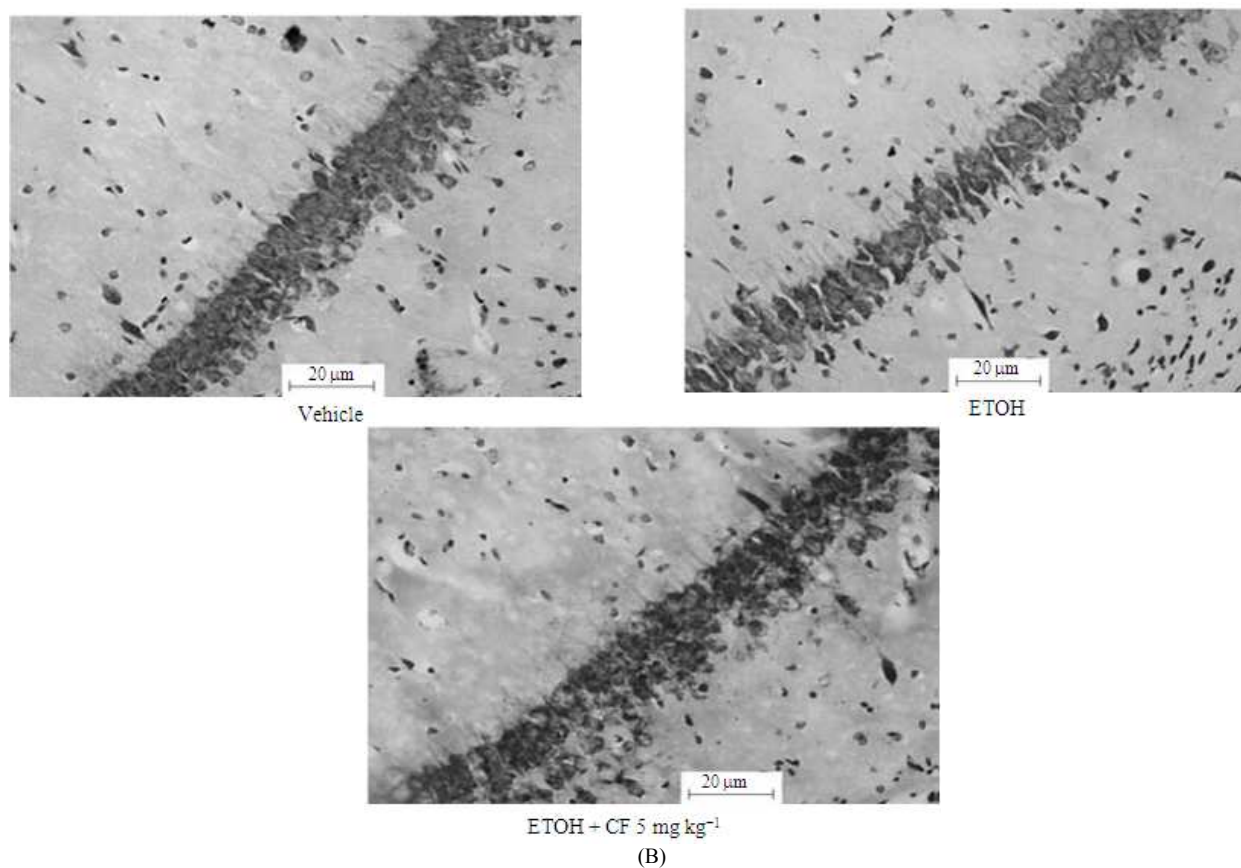
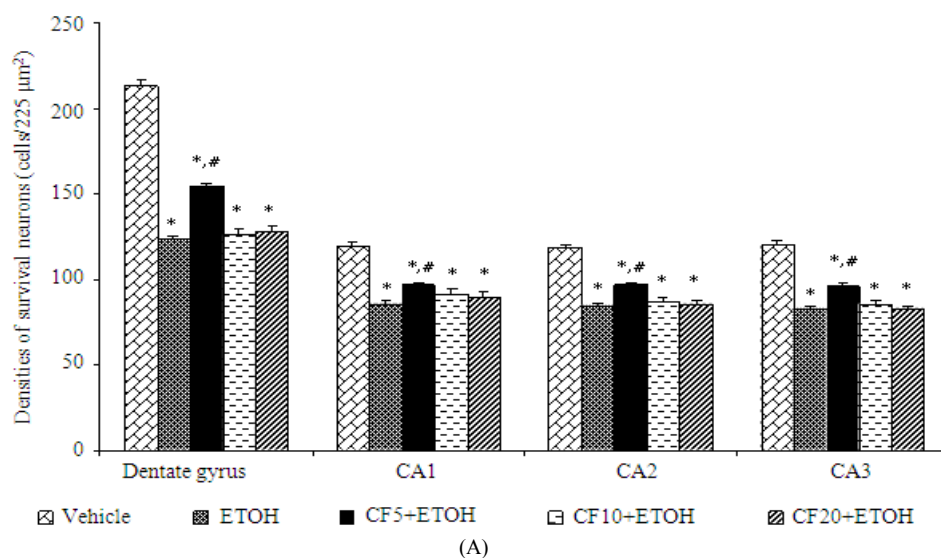


Fig. 1. (A) Effect of CF extract (5, 10 and 20 mg kg⁻¹) on survival neurons densities in various subregions of hippocampus. Values given are the mean ± S.E.M. (n = 10). *p<0.05 as compared to vehicle treated group, # p<0.05 as compared to ethanol treated group; (B) Photomicrographs of Nissl bodies in the CA1 region in the hippocampus at 20X magnification

Ethanol administration produced significant reduction in cholinergic neurons densities in all areas of hippocampus. Interestingly, CF extract treatment could significantly attenuate the decrease of cholinergic neurons densities in all areas of hippocampus except in CA3 region ($p < 0.05$ all. Compared to ethanol treated group).

As shown in **Fig. 3B**, the effects of vehicle, ethanol administration and CF at a dose of 5 mg kg^{-1} on the cholinergic neurons densities were examined using immunohistochemistry with an antibody directed against ChAT. Representative photomicrographs of the CA3 region in the hippocampal formation at $40\times$ magnification.

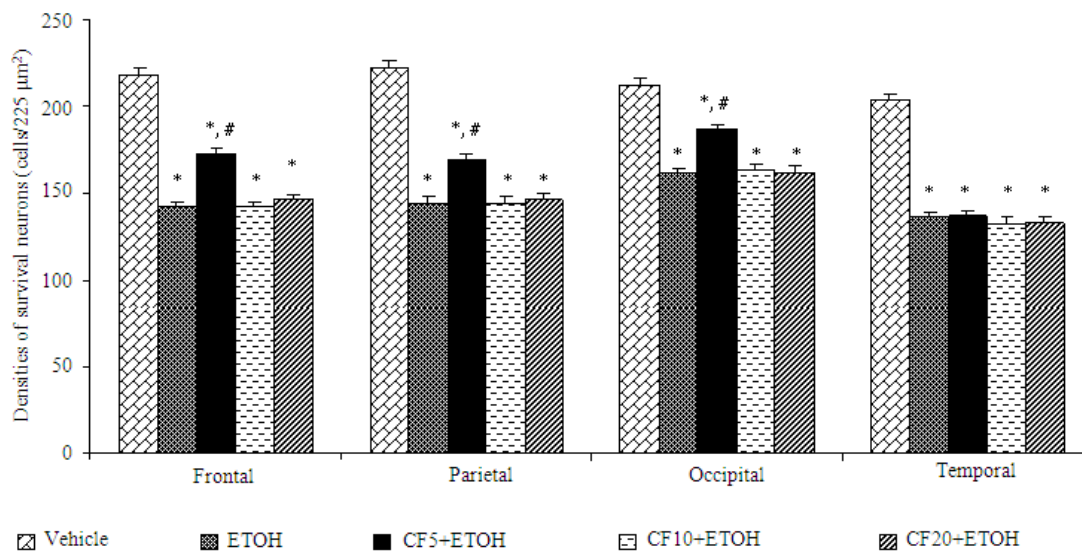
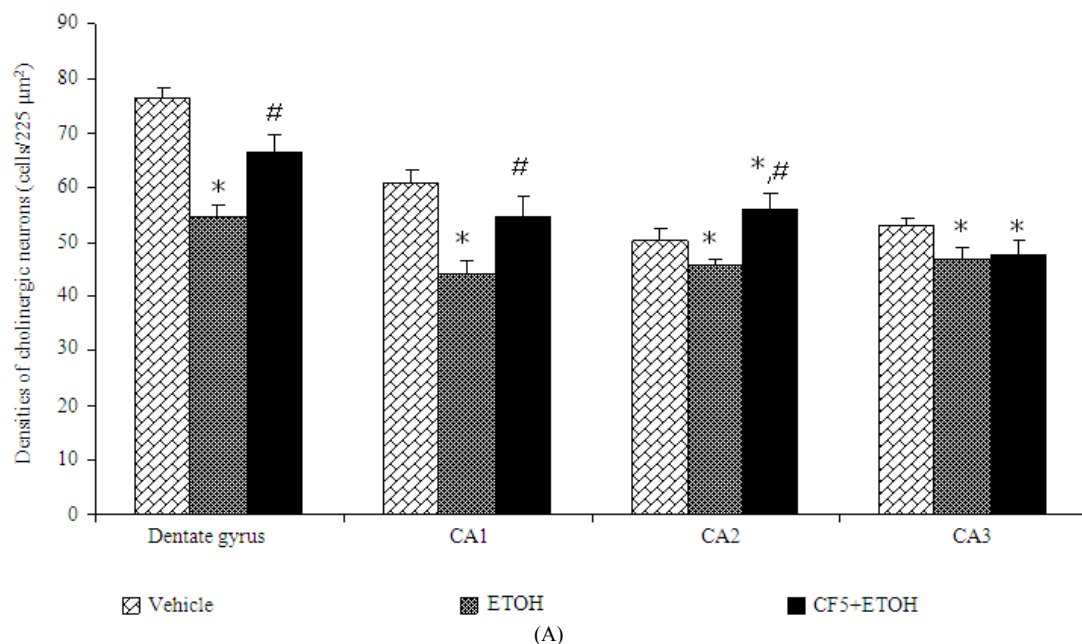


Fig. 2. Effect of CF extract ($5, 10$ and 20 mg kg^{-1}) on survival neurons densities in various subregions of cerebral cortex. Values given are the mean \pm S.E.M. ($n=10$). * $p < 0.05$ as compared to vehicle treated group, # $p < 0.05$ as compared to ethanol treated group



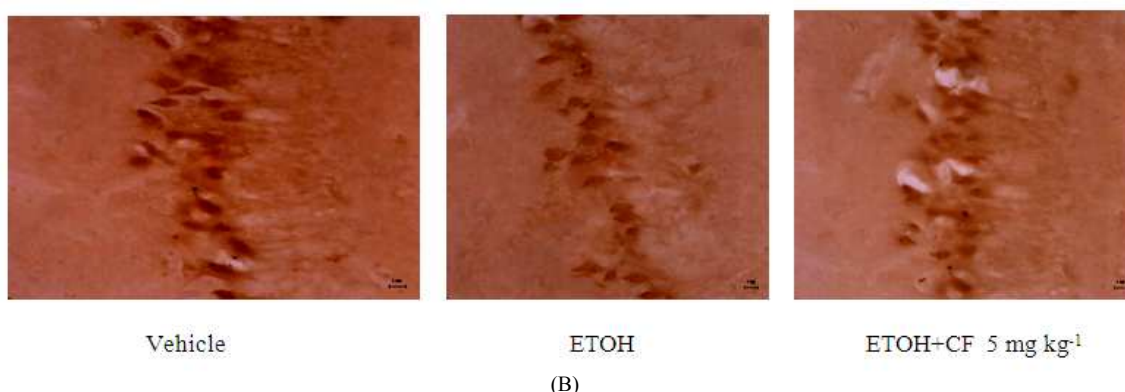


Fig. 3. (A) Effect of CF extracts at dose of 5 mg kg⁻¹ on cholinergic neurons densities in various subregions of hippocampus. Values given are the mean ± S.E.M. (n=10). *p<0.05 as compared to vehicle treated group, # p<0.05 as compared to ethanol treated g group. (B) Photomicrographs of ChAT immunoreactive neurons in the CA3 region in the hippocampus at 40X magnification

4. DISCUSSION

The present study demonstrated that CF extract consumption was able to protect neuronal cells against the harmful effects of chronic alcohol administration. Statistical analysis revealed a significant difference between ethanol treated group and CF extract rats in Nissl staining and immunohistochemistry with an antibody directed against ChAT methods performed to compare survival and cholinergic neurons densities.

Brain tissue contains a large amount of polyunsaturated fatty acids which are particularly vulnerable to free radical attacks (Gutteridge, 1995). Oxidative stress appears to be one key factor inducing the pathogenesis of alcohol related brain damage (Gotz *et al.*, 2001). Ethanol itself is pro-oxidant because it directly generates reactive oxygen species during its metabolism (Koop, 2006). For the same reason, dietary antioxidant supplementation is now regarded as a neuroprotecting measure to counteract alcohol deleterious effects (Phachonpai *et al.*, 2010; 2012).

A large number of growing evidence has showed that chronic ethanol exposure dramatically increased the degeneration of neurons in cortical region and hippocampus (Crews *et al.*, 1999). As expected, the results from our study corresponding with the studied of Collin *et al.* (2005) there were significantly less survival neuronal cells in the ethanol treated group in both cerebral cortex and hippocampus as compared with the vehicles. Interestingly, the brain regions selectivity was also observed in our results. Occipital cortex was the least vulnerability area in cortex and dentate gyrus was the most vulnerability area in

hippocampus. Up to date, there are many possible mechanisms underlying ethanol induced neuronal death. While further study is needed to elucidate the factors which play important role in regulating brain regional sensitivity to ethanol neurotoxicity.

Prophylactic treatment with CF at a low dose of 5 mg kg⁻¹ significantly increased the survival neurons densities in all areas of the brain except in the temporal cortex. Unfortunately, treatment with both medium and high doses of CF extract failed to show the neuroprotective effect in both the cortex and hippocampus. One possible explanation for this phenomenon might occur via the CF extract used in this study was the crude extract; therefore, increasing the dose of the extract might also increase all ingredients concentration and result in the masking effect of active ingredient, leading to the lack of dose dependent response in this study.

Previous studies have shown that the effect of chronic alcohol consumption is associated with cholinergic neuronal damage in hippocampus (Arendt *et al.*, 1989). Our study also show significantly decreased the cholinergic neurons densities in all areas of hippocampus in the ethanol treated group. CF supplementation significantly reduced the degeneration of cholinergic neurons in all area of hippocampus except in CA3. One possible explanation for the selective vulnerability in different hippocampal areas might be associated with various types of neuronal, internal antioxidant defense system, trophic factor particularly Nerve Growth Factor (NGF) and Brain Derived Growth Factor (BDNF) distribution in each area of hippocampus.

It has been reported that, the main constituents secreted from the stem of CF is berberine, a natural isoquinoline alkaloid (Tran *et al.*, 2003). It has been reported that berberine attenuated neuronal damage in ischemia/reperfusion model (Yoo *et al.*, 2006) and enhances neuronal cell survival and differentiation in rat's hippocampus (Lim *et al.*, 2008). In addition, CF extract have been previously reported to exhibit the neuroprotective effect via its antioxidant activity leading to the decreased oxidative stress and finally decreased neurodegeneration in neonatal animal model (Lee *et al.*, 2010). These lines of evidence are in agreement with our finding that CF extract used in this study contained berberine and it could decrease neurodegeneration in rat brain induced by ethanol administration. Moreover, this extract also exerts its cholinoprotective effect in our finding.

Taken all data together, it was possible that the neuroprotective and cholinoprotective effects of CF extract observed in our study might occur partly via its antioxidant effect which in turn gave rise to the decreased of neuronal cells damage. However, other effect related to the neuroprotection induced by this plant extract such as anti-apoptotic effect was also reported (Lee *et al.*, 2010). Thus, the neuroprotective effect of CF related to anti-apoptotic effect still could not be excluded. Anyway, this study did not investigate about the possible active ingredients; this is planned in future studies.

5. CONCLUSION

In a nutshell, present findings indicate that CF extract is a valuable to be candidate as a novel neuroprotective agent against neurodegeneration resulting from excessive continuous consumption of alcohol. However, further researches about possible active ingredients and pharmacokinetic of the extract are still required before moving forward to clinical trial study.

6. ACKNOWLEDGMENT

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7. REFERENCES

- Agartz, I., R. Momenan, R.R. Rawlings, M.J. Kerich and D.W. Hommer, 1999. Hippocampal volume in patients with alcohol dependence. *Arch. Gen. Psychiatry*, 56: 356-363. PMID: 10197833
- Arendt, T., Y. Allen, R.M. Marchbanks, M.M. Schugens and J. Sinden *et al.*, 1989. Cholinergic system and memory in the rat: Effects of chronic ethanol, embryonic basal forebrain brain transplants and excitotoxic lesions of cholinergic basal forebrain projection system. *Neuroscience*, 33: 435-462. PMID: 2636702
- Collin, D., F. Ritort, C. Jarzynski, S.B. Smith and I. Tinoco, 2005. Verification of the Crooks fluctuation theorem and recovery of RNA folding free energies. *Nature*, 437: 231-234. DOI: 10.1038/nature04061
- Crews, F.T., H.G. Waage, M.B. Wilkie and J.M. Lauder, 1999. Ethanol pretreatment enhances NMDA excitotoxicity in biogenic amine neurons: Protection by brain derived neurotrophic factor. *Alcohol.: Clin. Exp. Res.*, 23: 1834-1840. DOI: 10.1111/j.1530-0277.1999.tb04080.x
- Das, S.K. and D.M. Vasudevan, 2007. Alcohol-induced oxidative stress. *Life Sci.*, 81: 177-187. DOI: 10.1016/J.LFS.2007.05.005
- Esterbauer, H., R.J. Schaur and H. Zollner, 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic. Biol. Med.*, 11: 81-128. DOI: 10.1016/0891-5849(91)90192-6
- Gotz, M.E., B. Janetzky, S. Pohli, A. Gottschalk and W. Gsell *et al.*, 2001. Chronic alcohol consumption and cerebral indices of oxidative stress: Is there a link? *Alcohol.: Clin. Exp. Res.*, 25: 717-725. DOI: 10.1111/j.1530-0277.2001.tb02272.x
- Gutteridge, J.M., 1995. Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clin. Chem.*, 41: 1819-1828. PMID: 7497639
- Koop, D.R., 2006. Alcohol metabolism's damaging effects on the cell: A focus on reactive oxygen generation by the enzyme cytochrome P450 2E1. *Alcohol. Res. Health*, 29: 274-280. PMID: 17718406
- Krishnan-Sarin, S., S. O'Malley and J.H. Krystal, 2008. Treatment implications: Using neuroscience to guide the development of new pharmacotherapies for alcoholism. *Alcohol. Res. Health*, 31: 400-407.

- Lee, T., H. Heo and Y.K. Kwon, 2010. Effect of berberine on cell survival in the developing rat brain damaged by MK-801. *Exp. Neurobiol.*, 19: 140-145. DOI: 10.5607/en.2010.19.3.140
- Lim, J.S., H. Kim, Y.S. Choi, H. Kwon and K.S. Shina *et al.*, 2008. Neuroprotective effects of berberine in neurodegeneration model rats induced by ibotenic acid. *Anim. Cells Syst.*, 12: 203-209. DOI: 10.1080/19768354.2008.9647174
- Nordberg, A., C. Larsson, E. Per Dahl and B. Winblad, 1982. Cholinergic activity in hippocampus in chronic alcoholism. *Drug Alcohol. Depend.*, 10: 333-344. DOI: 10.1016/0376-8716(82)90035-7
- O'Malley, S.S. and M.D. O'Connor, 2011. Medications for unhealthy alcohol use. *Alcohol Res. Health*, 33: 300-311.
- Peng, W.H., M.T. Hsieh and C.R. Wu, 1997. Effect of long-term administration of berberine on scopolamine-induced amnesia in rats. *Jpn. J. Pharmacol.*, 74: 261-266. DOI: 10.1254/jjp.74.261
- Phachonpai, W., J. Wattanathorn, S. Muchimapura, T. Tong-Un and D. Preechagoon, 2010. Neuroprotective effect of quercetin encapsulated liposomes: A novel therapeutic strategy against Alzheimer's disease. *Am. J. Applied Sci.*, 4: 480-485. DOI: 10.3844/ajassp.2010.480.485
- Phachonpai, W., S. Maharun, S. Muchimapura, J. Wattanathorn and T. Tong-Un, 2012. Effect of dietary *Kaempferia parviflora* on ischemic brain injury in the rat. *Online J. Biol. Sci.*, 12: 27-33. DOI: 10.3844/ojbsci.2012.27.33
- Shirwaikar, A., K. Rajendran and I.S.R. Punitha, 2005. Antidiabetic activity of alcoholic stem extract of *Coscinium fenestratum* in streptozotocin-nicotinamide induced type 2 diabetic rats. *J. Ethnopharmacol.*, 97: 369-74. DOI: 10.1016/j.jep.2004.11.034
- Singh, G.B., S. Singh, S. Bani and S. Malhotra, 1990. Hypotensive action of a *Coscinium fenestratum* stem extract. *J. Ethnopharmacol.*, 38: 151-155. DOI: 10.1016/0378-8741(90)90004-D
- Tran, Q.L., Y. Tezuka, J.Y. Ueda, N.T. Nguyen and Y. Maruyama *et al.*, 2003. *In vitro* antiplasmodial activity of antimalarial medicinal plants used in Vietnamese traditional medicine. *J. Ethnopharmacol.*, 86: 249-252. PMID: 12738095
- Venukumar, M.R. and M.S. Latha, 2002. Antioxidant effect of *Coscinium fenestratum* in carbon tetrachloride treated rats. *Indian J. Physiol. Pharmacol.*, 46: 223-228. PMID: 12500498
- Warrier, P.K., V.P.K. Nambiar and C. Ramankutty, 1996. *Indian Medicinal Plants: A Compendium of 500 Species*. 1st Edn., Orient Blackswan, Madras, ISBN-10: 8125007636, pp: 592.
- Wattanathorn, J., N. Phunchago, S. Muchimapura, W. Thukhum-Mee and K. Chaisiwamongkol *et al.*, 2012. Mulberry fruit mitigates alcohol neurotoxicity and memory impairment induced by chronic alcohol intake. *Am. J. Applied Sci.*, 9: 484-491. DOI: 10.3844/ajassp.2012.484.491
- Witte, P.D. and M.F. Bada, 1983. Self-stimulation and alcohol administered orally or intraperitoneally. *Exp. Neurol.*, 82: 675-682. DOI: 10.1016/0014-4886(83)90089-4
- Yoo, K.Y., I.K. Hwang, B.O. Lim, T.C. Kang and D.W. Kim *et al.*, 2006. Berberry extract reduces neuronal damage and N-Methyl-D-aspartate Receptor 1 immunoreactivity in the gerbil hippocampus after transient forebrain ischemia. *Biol. Pharm. Bull.*, 29: 623-628. DOI: 10.1248/bpb.29.623