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Exploring Potential Neuroprotective Properties of Aqueous *Centella asiatica* Extract in Chronic Stress-induced Rats

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ABSTRACT

Centella asiatica is one of the traditional herbs consumed by many communities due to its wide range of applications such as treating Parkinsonism, promoting memory enhancement, and preventing oxidative stress. This study was conducted to investigate the neuroprotective potential of aqueous *C. asiatica* extract (CAE) against neurodegeneration induced by chronic stress. Administration of CAE at three different dosages (200 mg/kg/day, 400 mg/kg/day and 800 mg/kg/day) was conducted for a period of 21 days along with exposure to chronic stress using restrainer and forced swimming regimes. The administration of CAE significantly improved the thickness of dentate gyrus and reduced the amount of neuronal cell death at dentate gyrus and CA3 ($p<0.05$). Additionally, administration of CAE significantly alleviated the expression c-fos protein ($p<0.05$). Thus, this study highlighted the neuroprotective effect of CAE against neurodegeneration from chronic stress exposure.

Keywords: Centella asiatica, chronic stress, neuroprotectivity

INTRODUCTION

Stress can be defined as a form of adaptive response towards external demands (Sannino et al., 2016). The adaptive response is primarily mediated by the release of corticosteroids, however, continuous exposure to stress can cause the activation of adaptive response to be severely detrimental (Pinto et al., 2015). Previous studies by McEwen (2012) had shown the relationship

between the hippocampal atrophy due to stress and functional deficits in hippocampal-dependent functions.

The central nervous system (CNS) comprises brain and spinal cord, is made up of intricate and differentiated structures. The structures, particularly the limbic structure however is very susceptible and sensitive to chronic stress. Exposure to chronic stress can lead to hippocampal degradation and cause the hippocampus to be more vulnerable to injuries (Conrad, 2008). The hippocampus has a very important role in cognitive and memory processes, thus exposure to chronic stress can cause severe impacts on cognition and memory (Pinto et al., 2015).

Therefore, neuroprotection from continuous exposure of stress is essential to preserve the hippocampal integrity and function. The spontaneous regeneration of damaged tissues however is protracted and particularly impeded in relation to the rate of neuronal degradation. Numerous therapies and commercial drugs are known to promote neuroprotection, however it is usually accompanied with adverse effects. Levodopa is commonly known as the neuroprotective therapeutic agent that delays the progression of Parkinson's disease. However, the efficacy of levodopa is only apparent in long-term application; and prolonged administration of levodopa was shown to promote dyskinesia and in worse case, accelerates the progression of Parkinson's disease (Jankovic & Aquilar, 2008). Natural herbs on the other hand, have potential as alternative remedy to promote neuroprotectivity and known for its versatility and wide range of benefits. The insubstantial success and latent side effects from modern drugs also contribute to shift of interest towards natural herbs as an alternative option. The consumption of traditional herbs has been a daily practice particularly in South East Asia and India for promoting good health, prevention of ailment or even boosting their energy levels (Misra et al., 2008).

Centella asiatica, synonym *Hydrocotyle asiatica* is well known particularly in South East Asia and India region, has variety of names which include 'pegaga', 'gotu kola', 'Brahmi' and Indian pennywort (Mishra, 2015). *C. asiatica* belongs to family Apiaceae and native to countries with tropical and subtropical climate such as Malaysia, India, and China (Halimi, 2011). The pharmacological benefits of *C. asiatica* have been well documented and proven to promote wound healing, neuroprotectivity, memory enhancement as well as reducing oxidative stress (Somboonwong et al., 2012; Bylka et al., 2014; Nasir et al., 2011; Sainath et al., 2011).

C. asiatica is gaining recognition as an alternative option due to its potential neuroprotective properties. A study by Haleagrahara and Ponnusamy (2010) had shown that administration of aqueous *C. asiatica* extract was effective in preventing neuro degenerative changes from Parkinsonism. The administration of aqueous *C. asiatica* extract on *Sprague Dawley* rats induced to Parkinsonism was shown to promote neuroprotective effect and

antioxidant level in corpus striatum and hippocampus (Haleagrahara & Ponnusamy, 2010). Previous study by Gray et al. (2015) had shown that primary rat hippocampal neurons treated with water extract of *C. asiatica* promoted neuroprotective activities against amyloid-β toxicity.

However, most of previous studies were focused on neuroprotective potential on Alzheimer's disease and Parkinson's disease, and to date, limited knowledge is available on neuroprotectivity against chronic stress, particularly using natural herbs such as *C. asiatica*. Therefore, considering these issues, this study was conducted to investigate the neuroprotective potential of aqueous *C. asiatica* extract (CAE) on the impact of chronic stress condition. To achieve this aim, a chronic stress model was used, followed by potential neurogenesis and histological assessment on the hippocampus.

MATERIALS AND METHODS

Animals

The study was carried out using male Wistar rats weighing between 200 and 220 g. Rats were housed with two animals per cage and maintained in 12:12 h dark and light cycle, constant temperature of $25\pm2°C$, relative humidity of 40% and allowed free access to certified rodent food and water *ad libitum*. All experiments were performed in accordance to protocols reviewed and approved by Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (ACUC No: UPM/IACUC/AUP-R078/2018).

Preparation of Extract

Collected *C. asiatica* leaves were authenticated at Atta-ur-Rahman Institute of Natural Product Discovery (AuRIns), Universiti Teknologi MARA (UiTM) and a sample of the plant was deposited (Voucher No: CA-K017). The leaves were dried in hot air oven for three days at 40°C and coarsely ground using mechanical grinder. The preparation of aqueous extract was done using 25 g of coarse plant powder mixed with 250 mL boiling water for 1 h. The mixture was filtered and concentrated into dried powder under vacuum at 50°C.

Experimental Design

Rats were assigned into five different groups (n=8) and supplemented orally for 21 consecutive days as follows. The positive (PC) and negative control (NC) groups were given distilled water. Three groups were given different dosages of CAE respectively as follows, 200 mg/kg/day (CAE 200), 400 mg/kg/day (CAE 400) and 800 mg/kg/day (CAE 800). The dosage was selected based on previous toxicity studies (Oruganti et al., 2010; Chivapat et al., 2011; Deshpande et al., 2015) that showed lethal dose (LD50) of 2000 mg/

kg and no observed adverse effect level (NOAEL) of 1000 mg/kg. Each rat in respective dosage group was fed through oral gavage with the given amount of CAE daily for 21 days. All rat groups with exception to negative control group were left for one hour before being induced to chronic stress for 30 minutes.

The rats were induced into chronic stress using two types of stressor, the restraint and forced swimming regimes alternatively for 21 days (Abidin et al., 2004; Badowska-Szalweska et al., 2010; Sarjan et al., 2017). In restraint stressor regime, the rats were restrained in a cylindrical open-ended plastic restrainer (7 cm in diameter and 22 cm in length) for 30 minutes per day (Sarjan et al., 2017). In forced swimming stressor regime, the rats were forced to swim in a plastic container (50 cm in diameter and 70 cm in height) filled with 200 litres of water at room temperature (25±2°C) for 30 minutes per day (Sarjan et al., 2017). The study was carried out for 21 consecutive days and rats were allowed free access to food and water.

Following the last day of treatment, rats were sacrificed subsequently by decapitation. Brains were quickly removed, cleaned with saline solution and right hemisphere were stored at -80°C for protein determination and left hemisphere were stored in 10% buffered formalin for histological findings.

Histological Study of Hippocampus

Brain tissues were fixed in 10% buffered formalin for seven days. Paraffin blocks were made in embedding bath and sectioned at 5 microns using rotary microtome. The sections were stained with 0.1% cresyl violet. 0.1 g of cresyl violet acetate was dissolved in 100 mL distilled water and 10 drops of glacial acetate acid was added to give pH of 3.5 (Taib, 2014). The stain was filtered prior to usage. The slide was examined using compound research microscope complete with digital image acquisition system (Leica DM1000, Leica Microsystems, Germany) to observe the thickness of dentate gyrus (DG) and neuronal cell death at DG and CA3. The neuronal cell death was scored according to scoring system as shown in Table 1 (Taib, 2014). The DG and CA3 regions of the hippocampus are as shown in Figure 1.

Table 1
The neuronal cell death scoring system and its descriptions

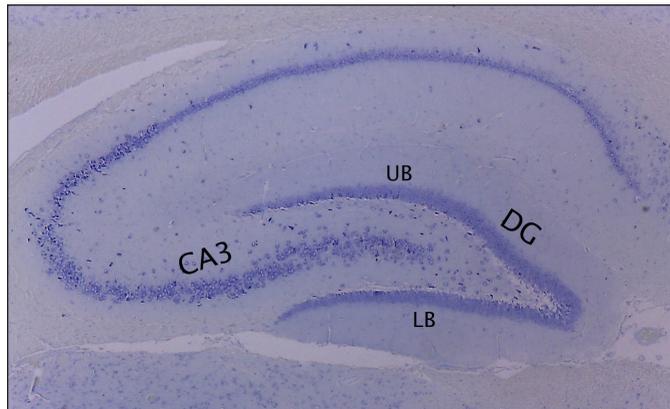| Score	Description
0	No neuronal cell death in field of view or normal
1	A few or sporadically present neuronal cell death in the field of view or normal
2	Group of neuronal cell death in the field of view
3	Group of neuronal cell death in the field of view with minor destruction
4	Massive alteration or all neurons were damaged in the field of view

Figure 1. Cresyl violet-stained section of the hippocampus, containing dentate gyrus (DG) and CA3. The DG comprises of upper blade (UB) and lower blade (LB). The section was observed under 5x total magnification.

Total c-fos Protein Level

Sodium dodecyl sulfate-polyacrylamide (SDS) (10%) gels were used according to molecular weight of c-fos protein, 57 kDa. SDS gel electrophoresis were run at 100 V for 40 minutes followed by 150 V at 60 minutes. The separated protein was transferred onto nitrocellulose membrane at 100 V for 60 minutes. The membrane was incubated for 2 h in blocking solution (5% w/v skimmed milk in Tween20) and overnight in primary anti-c-fos antibody (1:1000 dilution, Santa Cruz Biotechnology, Germany) in similar blocking solution. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies anti-rabbit IgG (1:2000 dilution, Santa Cruz Biotechnology, Germany) for 2 h. Membrane was incubated with chemiluminescent detection reagent (WesternBright[TM] ECL and Peroxide, Advansta, USA) for 5 minutes and exposed to gel documentation (INFINITY System, Vilber Lourmat, Germany) for 10 minutes. The quantification of c-fos protein was done using ImageJ system protocols (Davarinejad, 2013).

Statistical Analysis

The data were analysed using SPSS software (version 21, IBM SPSS, USA) and significant difference between groups was examined using analysis of variance (ANOVA) followed by individual comparison using Student's *t*-test (two-tailed). All data was expressed as mean $\pm$ SD (standard deviation). The values of $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Histological Study of Hippocampus

The thickness of dentate gyrus (DG) of male Wistar rats was measured and the results were as shown in Figure 2. Group CAE 800 recorded the highest thickness of DG, followed by group CAE 400, group CAE 200, group NC and group PC. The thickness of DG in group

PC was observed to decrease significantly with comparison to group NC ($p<0.05$). The thickness of DG in group CAE 200, CAE 400 and CAE 800 was significantly higher when compared to group PC ($p<0.05$). The thickness of the dentate gyrus of group CAE 800 was found to be significantly higher when compared to group CAE 200 ($p<0.05$).

The neuronal cell death assessment was performed at hippocampal structures of DG and CA3. Any abundance or appearance of neuronal cell death or alteration of neurons observed was noted and the results were as shown in Table 2, Figure 3 and Figure 4. For DG region as shown in Figure 3, Group PC recorded the highest amount of neuronal cell death with scoring of 3 (2.79 ± 0.40), followed by group CAE 200 with neuronal cell death score of 2 (2.24 ± 0.53), group CAE 400 with neuronal cell death score of 2 (1.83 ± 0.84), group CAE 800 with neuronal cell death score of 1 (1.43 ± 0.41) and group NC with neuronal cell death score of 1 (0.95 ± 0.73). The amount of neuronal cell death in group PC and group CAE 200 were significantly higher when compared to group NC ($p<0.05$). The amount of neuronal cell death in group CAE 400 and CAE 800 were significantly lower when compared to group PC ($p<0.05$). The amount of neuronal cell death between CAE 400 and CAE 800 however, were not significant when compared to group NC ($p>0.05$). The amount of neuronal cell death in group CAE 200 was significantly higher when compared to group CAE 800 ($p>0.05$).

As for CA3 region as shown in Figure 4, Group PC recorded the highest amount of neuronal cell death with scoring of 3 (2.50 ± 0.74), followed by group CAE 200 with neuronal cell death score of 2 (2.14 ± 0.69), group CAE 400 with neuronal cell death score of 2 (1.88 ± 0.83), group CAE 800 with neuronal cell death score of 1 (1.33 ± 1.00) and group NC with neuronal cell death score of 1 (0.86 ± 0.49). The amount of neuronal cell death in group PC and CAE 200 were significantly higher when compared to group NC ($p<0.05$). The amount of neuronal cell death in group CAE 400 and CAE 800 were significantly lower when compared to group PC ($p<0.05$). The amount of neuronal cell death between CAE 400 and CAE 800 however, were not significant when compared to group NC ($p>0.05$).

Table 2
The neuronal cell death score in DG and CA3 region of the hippocampus

Hippocampal Region	NC	PC	CAE 200	CAE 400	CAE 800
DG	1 (0.95±0.73)	3[a] (2.79±0.40)	2[a] (2.24±0.53)	2[b] (1.83±0.84)	1[b, c] (1.43±0.41)
CA3	1 (0.86±0.90)	3[a] (2.50±0.74)	2[a] (2.14±0.69)	2[b] (1.88±0.83)	1[b] (1.33±1.00)

Note. Values in brackets were expressed as mean ± SD. [a], $p<0.05$, compared to NC; [b], $p<0.05$, compared to PC; [c], $p<0.05$, compared to CAE 200. NC = negative control, PC = positive control, CAE = *Centella asiatica* extract, 200 = 200 mg/kg/day, 400 = 400 mg/kg/day, 800 = 800 mg/kg/day, DG = dentate gyrus.

The brain contains a specific limbic structure known as the hippocampus that plays an essential role in memory and cognitive processes (Fanselow & Dong, 2010). Specific hippocampal structures known as DG and CA3 function as the centre for memory and learning processes (Gao et al., 2007). However, DG and CA3 are very susceptible to neurodegeneration and severe condition of neurodegeneration could lead to functional
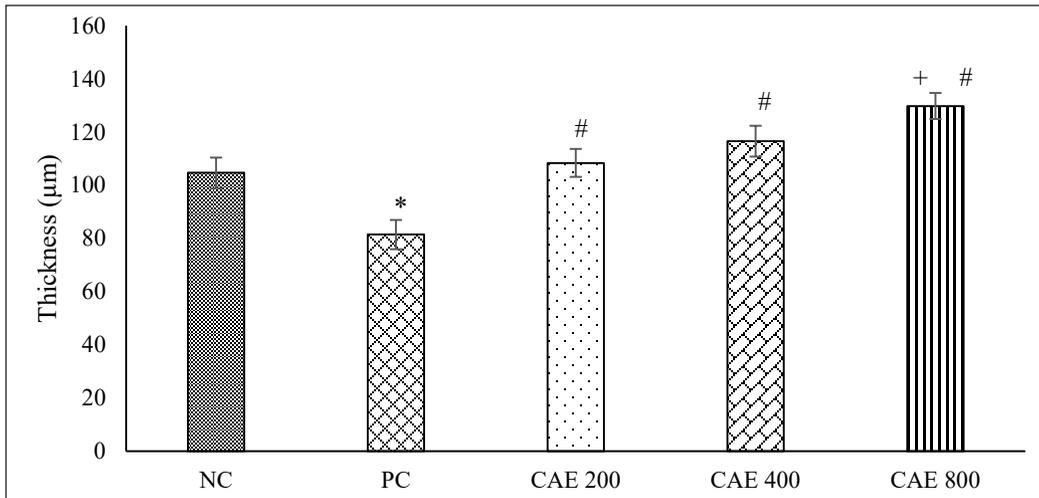

Figure 2. The thickness of dentate gyrus. Data were expressed as mean ± SD. NC = negative control, PC = positive control, CAE = *Centella asiatica* extract, 200 = 200 mg/kg/day, 400 = 400 mg/kg/day, 800 = 800 mg/kg/day. (* indicates $p<0.05$ vs group NC; # indicates $p<0.05$ vs group PC; + indicates $p<0.05$ vs group CAE 200).
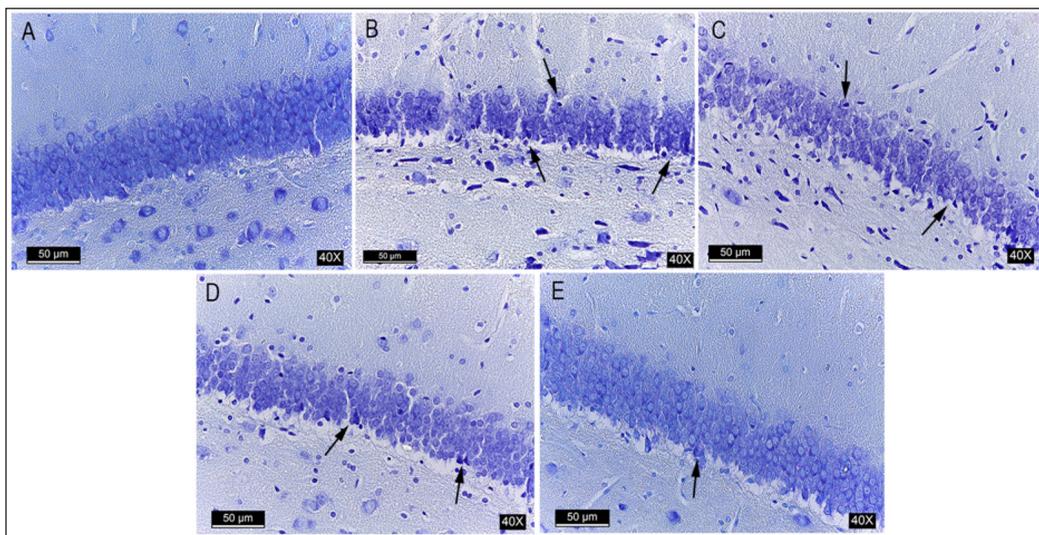

Figure 3. Cresyl violet-stained sections of dentate gyrus. Cells were observed under 40x total magnification. Arrows indicate neuronal cell death. A = negative control, B = positive control, C = CAE 200 (200 mg/kg/day), D = CAE 400 (400 mg/kg/day), E = CAE 800 (800 mg/kg/day).

Figure 4. Cresyl violet-stained sections of CA3. Cells were observed under 40x total magnification. Arrows indicate neuronal cell death. A = negative control, B = positive control, C = CAE 200 (200 mg/kg/day), D = CAE 400 (400 mg/kg/day), E = CAE 800 (800 mg/kg/day).
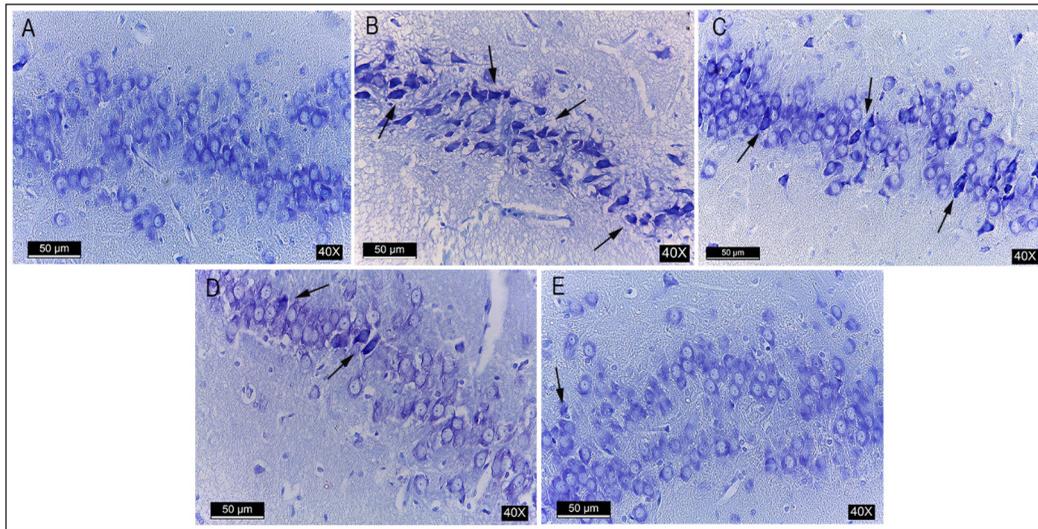
impairment of the hippocampus (Chohan et al., 2009). Continuous exposure to stress can cause the degeneration of hippocampal structures and disrupts the hippocampal functions (Sousa & Almeda, 2012). Despite so, the remission of neurodegeneration of the hippocampus can be achieved through stimulation of neurogenesis or neuroprotection. Therefore, the neuroprotective potential of CAE was studied by assessing its potential shielding effect and neurogenesis upon exposure to chronic stress.

The thickness of DG was observed particularly at the upper blade (UB) and the lower blade (LB), as any fluctuation on its thickness will reflect on the rate of the neurogenesis (Li et al., 2008). Any elevation of neuronal formation activities or neurogenesis can be represented by increasing thickness of the DG and vice versa (Lie et al., 2004). The neuroprotective potential of CAE was further assessed through observation on the amount of neuronal cell death in DG and CA3. The DG and CA3 structures were observed due to their roles in memory and cognitive processes, as well as their susceptibility to neurodegeneration (Evstratova & Tóth, 2014). The observation on the amount of neuronal cell death would translate into potential rate of neuroproliferation at observed hippocampal structures. Surfeited group of neuronal cell death or conspicuous destruction of the prescribed structures are usually accompanied by reduction in size of the hippocampal structures (Langmeier et al., 2003).

The observation on DG as shown in Figure 2 showed a significant decrease in thickness particularly in group PC when compared to group NC ($p<0.05$). The observation on group PC expressed the highest neuronal cell death score in both DG and CA3 as shown in Table 2. The observations postulated the neurodegenerative condition of the hippocampus and

agrees with the study by Hashemet al. (2010). The results indicated that the chronic stress model was successful in exhibiting neurodegenerative condition on hippocampal structures, by which there was apparent neuronal loss after 21 days of exposure to chronic stress.

The administration of CAE in the different dosages (CAE 200, CAE 400 and CAE 800) showed a significant increase in thickness of DG with comparison to group PC as shown in Figure 2 (p<0.05). The evaluation was reflected with the observations in group CAE 200, CAE 400 and CAE 800 that showed a significant decrease of neuronal cell death abundance, particularly DG when compared to group PC (p<0.05). The results were supported by similar observations in previous studies (Hashem et al., 2010; Abdallah et al., 2010) which reflected the evidence of neurogenesis in hippocampal structures by increased neuroproliferation or reduced amount of neuronal cell death as well as improvement on DG thickness. The results showed that the administration of CAE promoted neurogenesis activities in hippocampus upon exposure to chronic stress, indicating a potential neuroprotective effect of the extract.

The administration of CAE in group CAE 200, CAE 400 and CAE 800 respectively showed improving proliferative pattern on DG and CA3, as reflected with lower neuronal cell death score as shown in Table 2. Despite so, the administration of CAE did not show any significant improvement on the neuroproliferation in DG and CA3 when compared to group NC (p>0.05). The observation suggested that the administration of CAE showed a recovery pattern on DG and CA3 upon exposure to chronic stress that corresponded to those in group NC. Similar observations were found in previous study by Madhyasta et al. (2007) which demonstrated neuroprotective effect of fresh *C. asiatica* extract in prenatal stress-induced rats. The administration of fresh *C. asiatica* extract was shown to minimise neuronal loss from prenatal stress and enhanced the neuronal proliferative activities in the hippocampus. Another study by Perederiy and Westbrook (2013) demonstrated that the neuronal regeneration from eventuating injuries on the hippocampal structures was capable of retaining normal hippocampal functions as well achieving structural neuroplasticity. The results supported that continuous supplementation of CAE could reduce or minimise the detrimental consequences from daily chronic stress exposure in human.

Total c-fos Protein Level

The obtained protein bands of the total c-fos protein were quantified and the results were as shown in Figure 5. The percentage changes of total c-fos protein among group PC, CAE 200, CAE 400 and CAE 800 were expressed as percentage over group NC. Group CAE 800 recorded the highest level of total c-fos protein with 175.63 ± 43.60 % changes over group NC, followed by group CAE 400 with 107.25 ± 34.63 % changes over group NC, and group CAE 200 with 75.26 ± 20.43 % changes over group NC. Group PC recorded a reduction of 6.29 ± 19.76 % changes over group NC, however, no significant difference was

recorded between group PC and group NC ($p>0.05$). The expression of total c-fos protein group CAE 200, CAE 400 and CAE 800 showed significant differences in comparison to group NC and PC ($p<0.05$). The expression of total c-fos protein in group CAE 800 is significantly higher with comparison to group CAE 200 ($p<0.05$).

The c-fos is commonly used as a marker in determining the neuronal activity and behavioural response towards acute stimuli exposure (Velazquez et al., 2015a). Another study by Velazquez et al. (2015b) showed that c-fos played a significant role in neurogenesis. Neurogenesis is the formation of new neurons that occurs in constant manner particularly in the hippocampus (Velazquez et al., 2015b). c-fos is made up of two main domains: (1) basic domain (BD) which plays an important role in DNA-binding, and (2) leucine zipper domain (LZ) which plays an important role in heterodimerisation of c-fos with other leucine zipper-containing proteins such as *c-jun* (Curran & Morgan, 1995; Angel & Karim, 1991). The heterodimerisation of c-fos leads to the formation of AP-1 (Activator Protein-1) complex (Vivar & Van Praag, 2013). The AP-1 complex has a significant role in regulating the cellular processes such as differentiation and proliferation (Verma & Graham, 1987).
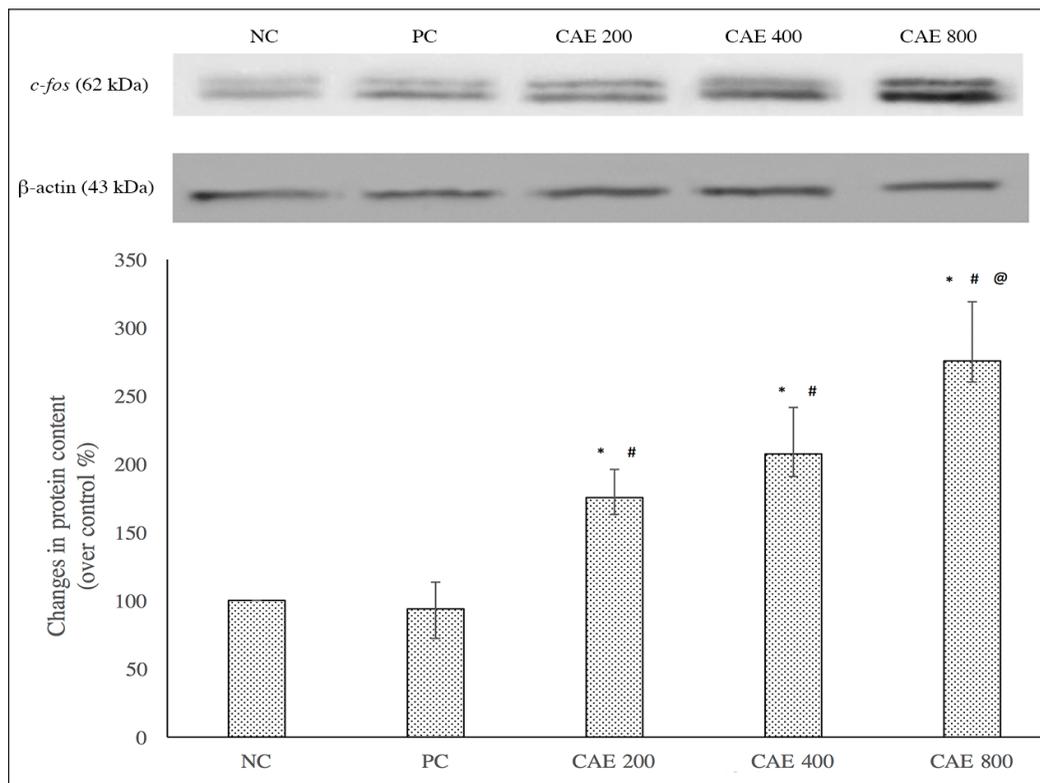

Figure 5. Relative expression of c-fos protein. Data were expressed as percentage change (mean ± SD). Histograms with symbols were significantly different at $p<0.05$ (*, $p<0.05$, compared to NC; #, $p<0.05$, compared to PC; @, $p<0.05$, compared to CAE 200). NC = negative control, PC = positive control, CAE = *Centella asiatica* extract, 200 = 200 mg/kg/day, 400 = 400 mg/kg/day, 800 = 800 mg/kg/day.

Another study by Caubet (1989) demonstrated that the expression of c-fos at various stages of neurodevelopment entailed to the maturation of brain structures. Therefore, elevation of the expression of c-fos protein level may signify an increasing activity of neurogenesis in the hippocampus.

The significant elevation of c-fos expression denotes that CAE promotes the elevation of hippocampal neurogenesis activities in male Wistar rats exposed to chronic stress. The results are supported by previous study by Clark et al. (2010) which suggested that the elevation of neurogenesis activities was in concomitant to an increase in c-fos expression observed in the hippocampus. Clark et al. (2010) concluded that the rate of neurogenesis in the hippocampus was reflected with c-fos expression, by which higher neuronal activities and neurogenesis were parallel to upregulation of hippocampal c-fos. Interestingly, the increasing dosage of CAE demonstrated an increasing pattern of c-fos protein expression. The relative expression of c-fos in group CAE 800 showed the highest expression of c-fos. Therefore, the results denoted that the administration of CAE at high dosage promotes the optimum elevation of neurogenesis activities and was reflected on histological findings on the hippocampus.

CONCLUSION

The administration of CAE at three different dosages exhibited significant neurogenesis activities through the apparent thickening of dentate gyrus and improved neuroproliferation in the hippocampus. The apparent improvement in neurogenesis activities was reflected by significant elevation pattern of c-fos protein expression in groups administered with CAE.

The results of this study have demonstrated the neuroprotective effect of continuous administration of CAE against degeneration of hippocampus structures upon exposure to chronic stress. Therefore, continuous supplementation of *Centella* asiatica could reduce or minimise the detrimental consequences from daily chronic stress exposure in human. Thus, the neuroprotectivity potential of *Centella asiatica* extract shows a promising way as an alternative option to treat chronic stress. The dosage of 800 mg/kg of aqueous *Centella asiatica* extract demonstrated as the optimum dosage for neuroprotective properties in ameliorating the effects of chronic stress.

ACKNOWLEDGEMENTS

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