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Full Length Research Paper

C*entella asiatica* modulates neuron cell survival by altering caspase-9 pathway

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Natural products have been reported to exert positive impact on neurodegenerative diseases which arise as a result of neuronal loss associated with oxidative stress. Therefore, this study was conducted to evaluate the neuroprotective potential of ethanolic extract of *Centella asiatica* (CA) compared to that of tocotrienol rich fraction (TRF) using human neuroblastoma, SH-SY5Y cells. Cytotoxicity and neuroprotective effects of CA and TRF were measured by using 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) against BSO-induced neuron cell death. The rate of apoptotic cell death was measured via FITC Annexin V apoptosis flow cytometer. Presence of live and apoptotic cells were further confirmed with calcein-AM and propidium iodide fluorescence staining. Caspase-8 and -9 were measured to determine the mechanism of activation of the apoptotic pathway. Results showed that CA extract was toxic to neuron cell culture at ≥100 µg/ml. TRF (1 to 50 µg/ml), as positive control and CA (1 to 50 µg/ml) conferred significant protection against BSO-induced cell death. Post-treatment with CA also significantly reduced caspase-9 activity against BSO-induced cell death. In conclusion, this study shows that low concentration of ethanolic extract of CA is able to protect neuron cells from oxidative stress probably by inhibiting the activation of caspase-9 pathway but it can exert neurocytotoxic effect at high concentrations.

Key words: *Centella asiatica* L., antioxidant, neuroprotection, apoptosis, Caspase.

INTRODUCTION

Neurodegeneration has been attributed as one of the causes for the decline in cognitive function in Alzheimer's disease, amyotrophic lateral sclerosis (ALS), and Parkinson's disease. The neuronal loss has been associated with increased oxidative stress (Emerit et al., 2004). Oxidative stress occurs due to imbalance between production and detoxification of reactive oxygen species (ROS). The brain is more susceptible because of its high oxygen consumption, relatively low antioxidant capacity compared to other organs and high contents of lipid and ferum which are the main target of free radicals (Halliwell and Gutteridge, 1999; Crichton et al., 2007). Increased oxidative stress will induce lipid peroxidation, DNA damage, protein damage and induction of apoptosis which will result in cell death (Halliwell and Gutteridge, 1999).

Several findings have indicated that natural products especially herbs are able to exert neuroprotective effects against oxidative stress induced neuron cell death. These include vitamin E (Osakada et al., 2004; Khanna et al., 2005; Musalmah et al., 2006), Ginseng extracts (Rausch et al., 2006; Kim et al., 2008), *Ginkgo biloba* extracts (Watanabe et al., 2001) and green tea extract (Kakuda, 2002). Therefore, this study was conducted to elucidate the potential of *Centella asiatica* L. (CA), family Umbelliferae - traditionally believed to improve memory - to protect neuron from oxidative stress (Jana et al., 2010). CA is a creeping vine-like plant native to India and Southeast Asia (Talbott and Hughes, 2007). It is usually

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consumed as salad or drank as tonic for good health and youthfulness (Mato et al., 2009).

It is traditionally used to promote wound healing, improve circulation, as neuroprotective, relieves hypertension and especially improves memory (Incandela et al., 2001; Chauhan, 2007; Liu et al., 2008; GV, 2010; Heleagrahara and Ponnusamy, 2010). Phytochemical study shows that it contains β-carotene, β-sitosterol, campesterol, camphor, caempferol, stigmasterol, pentacyclic triterpenoid saponins (centelloids), flavonoids, but most of its activity has been attributed to the presence of asiatic acid, madecassic acid, asiaticoside and madecassoside, and several other terpenoids (Inamdar et al., 1996; Balch et al., 2000; Subban et al., 2008; James and Dubery, 2009; Cao et al., 2010; Mustafa et al., 2010).

Several studies on CA have shown that, it posseses antitumor activity (Babu et al., 1995; Yoshida et al., 2005), antiulcer effect (Cheng et al., 2004), neuroprotective potential (Heleagrahara and Ponnusamy, 2010), memory improvement (Kumar and Gupta, 2002) and wound healing properties (Shukla et al., 1999; Liu et al., 2008).

Studies have also shown that CA extracts significantly improved antioxidant status in rat model (Jayashree et al., 2003; Subathra et al., 2005).

Recently, CA extracts was reported to improve physical performance and health-related quality of life in healthy elderly volunteers (Mato et al., 2009). The present study was designed to investigate the neuroprotective potential of CA by using human neuroblastoma, SH-SY5Y cell line.

MATERIALS AND METHODS

Plant extract

C. asiatica (CA) was purchased from Malaysia Agricultural Research and Development Institute (MARDI). Voucher specimen (SK 533/03) was given by the curator of MARDI. The sample was washed, oven dried at 50°C and 11% humidity, grinded and extracted using ethanol solvent. Extraction by using soxhlet apparatus (Eyela, Japan) was done until the ethanol solvent became colourless. Excess solvent was evaporated using the rotary evaporator (Eyela, Japan). The remaining ethanol free substances were freeze dried by using FreeZone 4.5 L (Labconco, USA) and stored at -20°C. Prior to use, the sample was diluted with 100% ethanol.

Tocotrienol rich fraction

Tocotrienol Rich Fraction (TRF) was purchased from Golden Hope, Malaysia. This fraction contains 70% tocotrienol.

SH-SY5Y neuroblastoma cell culture

Human neuroblastoma SH-SY5Y was a gift from Dr. Coral Sanfeliu, Institute d'Investigacions Biomèdiques de Barcelona, Spain. The cells were grown in media mixture of EMEM pH 7.2 (Flowlab, Australia) and Ham's F-12 pH 7.2 (Sigma, USA) supplemented with 200 mM non-essential amino acid (NEAA; HyClone), 10 mg/ml gentamicin (PAA, Austria) and 10% Foetal Bovine Serum (FBS) (PAA, Austria). Cells were harvested when it reached 80% confluent and plated at a density of 1.5×10^4 cells per ml on 96-well plates. Cultures were maintained in 5% CO_2 incubator at 37°C. Medium was changed on the second and fifth day after plating. Addition of 10 µM retinoic acid (Sigma, USA) to the culture changed SH-SY5Y cells to neuron-like cells characteristics which were confirmed microscopically. Experiments were carried out on the seventh day.

Cytotoxicity studies

Plated neuron cells were exposed to various concentrations of TRF (positive control) and CA extracts for 24 h. Cell viability was then determined using the MTS assay.

Neuroprotection studies

Cells at 2×10^4 were plated in 96-wells plate and divided into four groups: 1) control (untreated), 2) incubated with BSO at IC_{50} concentration, 3) pre-treated and 4) post-treated with CA (1 to 50 µg/ml). In pre-treatment group, cells were incubated in various concentrations of CA for 24 h before the addition of BSO and then incubated for a further 72 h in humidified incubator 5% CO_2, 37°C. For post-treatment group, cells were exposed to BSO for 72 h followed by incubation with CA for 24 h. The untreated group formed the control. Cell viability was then analysed using the MTS assay. Experiment was done in triplicates and repeated three times. The same procedures were repeated using TRF.

Analysis of cell viability

Percentage of viable cells in control, BSO-induced and treated groups were determined by CellTiter 96^{TM} Aqueous Non-Radioactive Cell Proliferation Assay (MTS; Promega, USA).The MTS assay employs 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethophenyl) 2-(4-sulfophenyl)-2H-tetrazolium (MTS) and electron coupling agent phenazine methosulphate (PMS). MTS is converted into a medium soluble formazan product by dehydrogenase enzymes found in metabolically active cells. MTS, 20 µl solution was added to experiment culture to terminate the exposure of the tested agent. After 2 h incubation, absorbance was measured at 490 nm using microtiter plate reader (VeraMax, Molecular Devices, USA). The quantity of formazan products is proportional to the number of viable cells in the culture.

Apoptosis studies

FITC Annexin V apoptosis detection kit (BD Pharmingen, USA) was used to determine the rate of apoptosis in cultured neuron cell. Annexin V is a 35 to 36 kDa Ca^{2+} dependant phospholipid-binding protein that binds to phosphatidylserine (PS) which is exposed in apoptotic cells. Annexin V is conjugated to FITC, a fluorochromes, which serve as sensitive probe for flowcytometric analysis of cells that undergo apoptosis. Propidium iodide only stain damaged cells due to its inability to penetrate membrane cells. 2×10^5 cells were plated in 6 well plate and were divided to four groups; untreated groups (negative control), BSO-treated group (positive control), pre-treatment group and post-treatment group. After the treatment, intact cells and detached cells were collected together and washed with cold PBS twice. The cells were resuspended in binding buffer

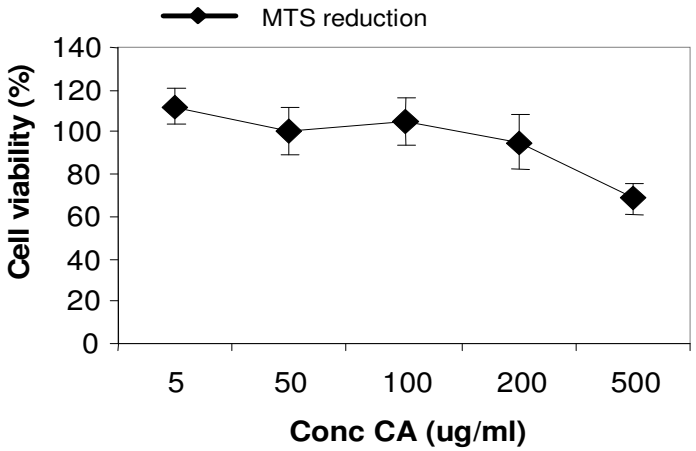

Figure 1. Cytotoxicity of CA on neuroblastoma SH-SY5Y cells by MTS Cell Proliferation assay. Percentage of viable cells is proportional to MTS reduction. Cells were incubated with CA at 5 to 500 µg/ml of concentrations for 24 h at 37°C. CA extract is toxic to neuron cell culture at concentration ≥100 ug/ml. Data is presented as means ± SD, n = 9. * is significant compared to control ($p < 0.05$).

at concentration of 1 X 10^6 cells per ml. Then, 100 µl of the solution were transferred to 5 ml culture tubes and added with 5 µl of FITC Annexin V and 5 µl of Propidium Iodide. The cells were incubated for 15 min at room temperature in the dark. After that, 400 µl of binding buffer were added to the tubes and analyzed by using FACScan flow cytometer (BD Biosciences, USA) within 1 h. A total of 10,000 cells were analyzed in three independent experiments with each experiment run in duplicate.

Fluorescent staining of live and apoptotic cells

Calcein-AM and propidium iodide staining were used to observe live and apoptotic cells via fluorescent microscope. Calcein-AM is highly lypophilic and cell membrane permeable, so it only stains viable cells. Whereas propidium iodide is a nuclei staining dye, so it cannot pass through viable cells and only stains the dead cells where their membranes already damaged. 1.5 X 10^4 cells were plated in chamber slides and the cells were pre- and post-treated with CA or TRF. After treatment, 30 µg/ml calcein-AM and 7.5 µg/ml propidium iodide were added to cultured neuron cells on chamber slides and incubated for 30 min. Then, cultures were washed with PBS, fixed with fresh 2% paraformaldehyde and incubated for 15 min. The cultures were washed again with PBS and cover slip was mounted and examined with fluorescence microscope.

Caspase-8, and -9 activities

Caspase-8 and -9 Colorimetric Activity Assay kit (Milipore, USA) is used to determine caspase-8 and -9 activities. The assays were based on the hydrolysis of Ac-IETD-*p*NA by caspase-8 and of Ac-LEHD-*p*NA by caspase-9, which released *p*-nitroaniline (*p*NA). The free *p*NA will be quantified using Microtiter plate reader (VeraMax, Molecular Devices, USA) at 405 nm.

Neuron cells plated in 96-wells plate were divided into three groups: 1) control (untreated), 2) incubated with BSO at IC_{50} concentration and 3) post-treated with CA (1 µg/ml) where the cells were exposed to BSO for 72 h followed by incubation with CA for 24 h. After treatment, cells were incubated with chilled lysis buffer for 10 min in ice. The cells were then centrifuged at 10, 000 g for 5 min to collect the supernatant. Protein concentration for each supernatant was determined by using BioRad Protein Assay kit (BioRad, Canada). The supernatant (10 µl) was added to reaction buffer which contained 10 µl of Ac-IETD-*p*NA or Ac-LEHD-*p*NA and incubated for 1 h at 37°C. The reaction was measured at 405 nm using Microtiter plate reader (VeraMax, Molecular Devices, USA). The experiments were performed in triplicates and repeated three times. Value from background reading was subtracted from the sample values and the caspase activity was calculated by using the following formula:

Caspase activity = [pNA] µM / amount protein (µg) / 60 min

Statistical analysis

Results are given as mean ± SD. Statistical analysis was performed by using One Way ANOVA at the significance level $p < 0.05$ (SPSS 13 software).

RESULTS

Cytotoxicity studies

The incubation of neurons with ethanolic extract of CA at various concentrations for 24 h, resulted in concentration-dependent reduction in cell viability (Figure 1). CA was found to be toxic to cells at ≥ 100 µg/ml concentration.

Neuroprotection studies

Neuroprotective effects of CA against BSO-induced cell death were evaluated by determining cell viability via the MTS assay and apoptotic cell death via flow cytometry.

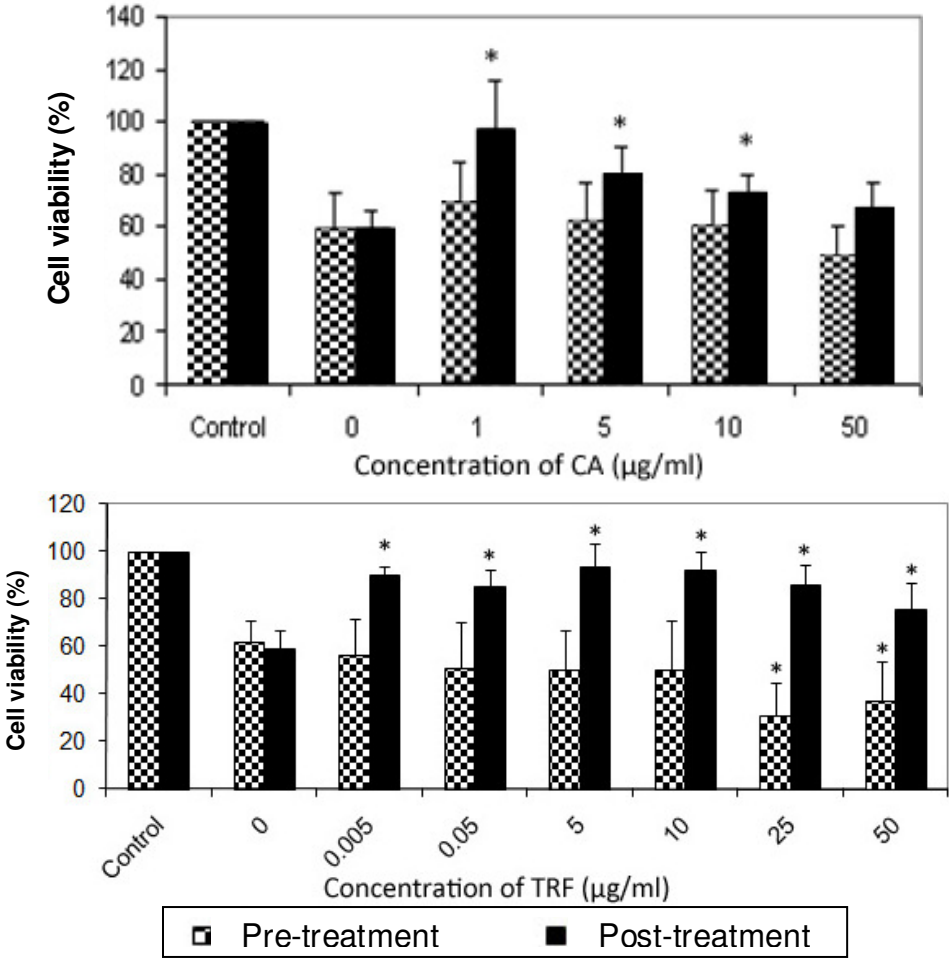

Figure 2. Protective effects of CA and TRF (positive control) against BSO-induced cell death in neuroblastoma SH-SY5Y culture. Cells were pre-treated or post-treated with CA or TRF at 1 to 50 µg/ml concentrations for 24 h before or after exposure to BSO at 37°C. Cell viability was determined by using the MTS Cell Proliferation assay. Cells post-treated with CA or TRF showed higher neuroprotection capability compared to pre-treated groups. Data is presented as means ± SD, $n = 9$.* denotes $p < 0.05$ compared to IC_{50} BSO concentration.

Neuron cultures were pre- or post-treated with TRF (positive control) or CA for 24 h, followed or preceded by BSO for 72 h. From the result, post-treatment with CA showed a significant increase in the number of viable cells compared to the cells exposed only to BSO. Similarly, post-treatment with TRF protected neurons and the neuroprotective effects were observed even at ng/ml concentrations. This neuroprotective effect was concentration-dependent (Figure 2).

Percentage of cells which undergo apoptosis was determined by flow cytometry. Cells with V-/P- were considered viable cells, while cells with V+/P- were taken as a measure of early apoptosis and cells with V+/P+ were considered as necrosis/late apoptosis-like cell death (Figure 3A). Neuron cell cultures were pre- or post-treated with TRF (positive control) or CA for 24 h, before or after exposure to BSO for 72 h. Results showed that post-treatment of either TRF or CA significantly reduced the number of apoptotic cells induced by BSO ($p < 0.05$) (Figure 3B). These results paralleled the results obtained by viability studies using MTS assay. The morphological features of live and dead cells were observed via calcein-Am and PI staining (Figure 4). Live cells were stained by calcein-Am (green) which can be observed abundantly in control cells (Figure 4a), whereas dead cells stained by PI can be observed in BSO-induced cells (Figure 4b). Both pre-treated (Figure 4c) and post-treated (Figure 4d) cells showed that CA at lower concentration effectively protects the cell from BSO induced oxidative stress where the cells were still intact and only a few cells were damaged and stained red.

Figure 5 shows that BSO significantly induced the activities of caspase-8 and -9 which may lead to the activation of the apoptotic pathway. Neuron cell treated

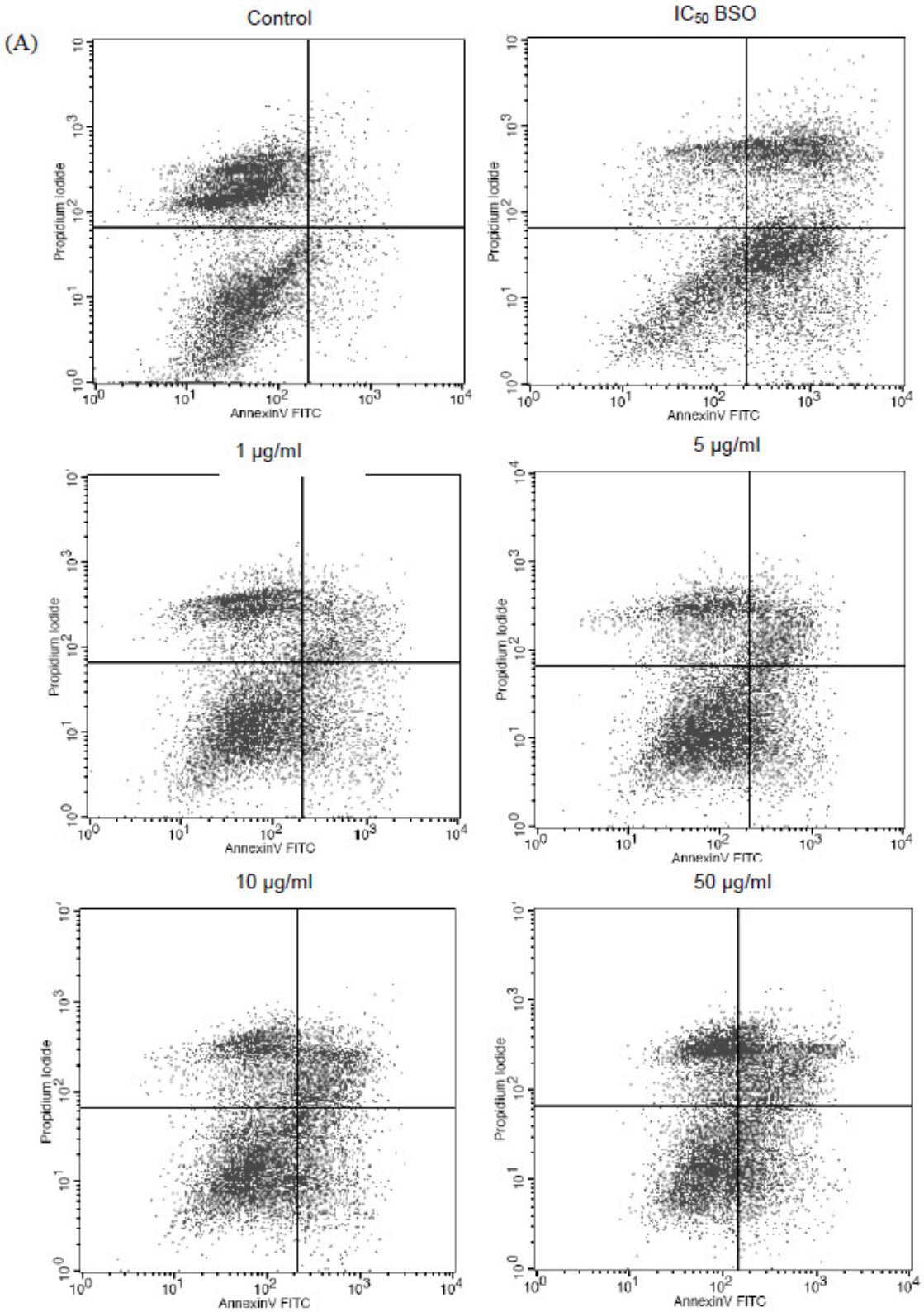

Figure 3a. Cell apoptosis detected by flow cytometry in neuroblastoma SH-SY5Y culture treated with CA or TRF (positive control). Cells were pre-treated or post-treated with CA or TRF at 1 to 50 µg/ml concentrations for 24 h before or after exposure to BSO at 37°C. The percentage of apoptosis was evaluated using FITC Annexin V apoptosis detection kit and it was read by using FACScan flow cytometer. (A) Double parameter dot plot of FITC-Annexin V fluorescence (x-axis) versus PI fluorescence (y-axis) for examination of apoptotic cells post-treated with CA.

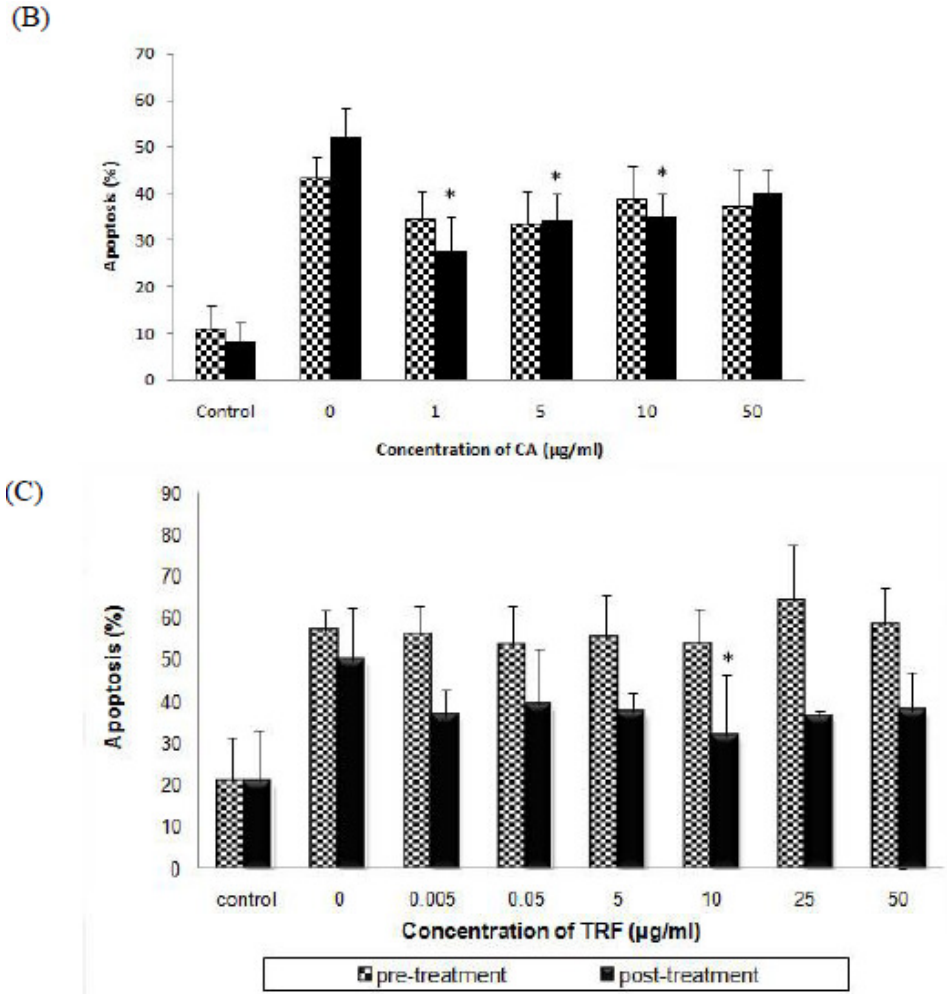

Figure 3B and C. Bar diagrams to show the amounts of apoptotic cells for pre- and post-treatment of CA extract. (C) Bar diagrams to show the amounts of apoptotic cells for pre- and post-treatment of TRF. Cells post-treated with CA also showed higher neuroprotection capability compared to pre-treated groups equal to the data shown by MTS assay in neuroprotection studies. Data is presented as means ± SD, n = 6.* denotes $p < 0.05$ compared to IC50 BSO concentration.

with 1 µg/ml of CA extract after exposure to BSO showed that, CA treatment significantly reduced caspase-9 activity compared to those induced with BSO ($p < 0.05$) but there was no significant changes in the activity of caspase-8.

DISCUSSION

Oxidative stress in central nervous system has been recognized for its role in modulating critical cellular functions which eventually leads to mitochondrial dysfunction, overproduction of reactive oxygen species (ROS) and apoptosis. It is a final common pathogenic mechanism in aging and neurodegenerative diseases (Emerit et al., 2003; Jana et al., 2010). Currently, there are increased interests on medicinal plants with high antioxidant activity that might offer a useful alternative choice to treat neurodegenerative diseases (Kim et al., 2002; Christen, 2004; Heleagrahara and Ponnusamy, 2010). Several studies have shown that ethanol extract of *C. asiatica* (CA) contains high antioxidant activity and was found to be as potent as α-tocopherol (Hamid et al., 2002; Zainol et al., 2003). The phenolic compounds of CA have been demonstrated to be the major contributors to its antioxidative activities (Zainol et al., 2003; Mustafa et al., 2010).

In this study, we explored the cytotoxicity and neuroprotective effects of ethanolic extract of CA by determining cell viability, apoptosis and fluorescence staining. We have demonstrated previously in our laboratory that tocotrienol is toxic to neurons at

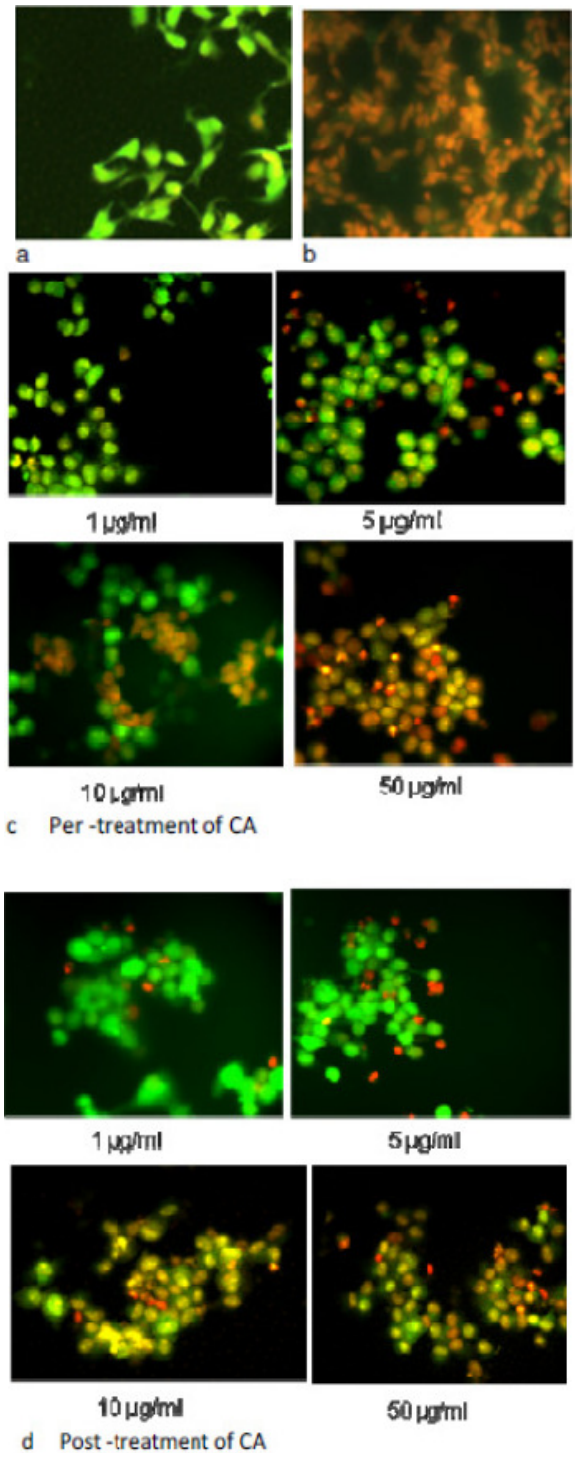

Figure 4. Morphology of live and dead neuron cells stained by calcein-AM and propidium iodide (PI) fluorescence staining. Cells were divided into four groups: (a) control, (b) IC_{50} BSO treated (c) pre-treated and (d) post-treated with CA at 1 to 50 µg/ml concentrations for 24 h before or after exposure to BSO at 37°C. Live cells were stained by calcein-AM (green) and dead cells were stained by Propidium Iodide (red) when observed under fluorescence microscope. Micrographs are shown at 400 X magnification. Data shows that cells exert neuroprotective effects at low concentrations.

concentration >100 µM (Musalmah et al., 2006). The present study demonstrated that TRF was also toxic to neuron culture at concentrations greater than 100 µg/ml. The present data also confirmed the neuroprotective effects of vitamin E as reported earlier (Kamat et al., 1997; Packer et al., 2001; Osakada et al., 2004; Sen et al., 2006; Musalmah et al., 2006). Post-treatment with CA extract showed that, at a concentration range of 1 to 50 µg/ml, CA is capable of protecting neurons from BSO-induced cell death, as determined by viability studies using the MTS assay, apoptotic studies using flow cytometer and further confirmed by the use of calcein-AM and propidium iodide (PI) staining.

Several studies had also shown neuroprotective effects of CA which includes *in vivo* study in which supplementation of CA was observed to increase the endogenous antioxidant enzymes in rat brain while simultaneously enhancing the cognitive function (Kumar and Gupta, 2002; Subathra et al., 2005). The aqueous extract of CA was also reported to markedly ameliorated 3-NPA induced oxidative stress response in brain mitochondria under *in vitro* exposure (Shinomol and Muralidhara, 2008). The neuroprotective effects of CA may be brought about by its action as a scavenger of free radicals. CA was reported to reduce lipid peroxidation by free radicals as well as increase endogenous antioxidant enzyme activities in rats (Hussin et al., 2007) and mice (Jayashree et al., 2003). In addition, the present data suggest that CA may also act by inhibiting the activity of caspase-9, one of the enzymes involved in the activation of the apoptotic pathway.

There are few mechanisms that might contribute to the inhibitory effect of CA on caspase-9 activity. CA might work via inhibiting proapoptotic proteins like p53, Bax and Bad or inducing production of anti-apoptotic family members such as Bcl-2 and Bcl-xL (Wang, 2001). It is also possible that it mimics the anti-apoptotic family members, thus reducing heterodimerization of BCL family members and inhibit release of cytochrome c which results in reduction of apaf-1 and procaspase-9 formation to activate the caspase-9 activity (Pettmann and Henderson, 1998; Hengartner, 2000; Wang, 2001). The observation that CA leaf extract can elicit neuronal dendritic growth (Rao et al., 2007) may point to the possibility of its involvement in stimulating neurogenesis. CA was reported to improve neuronal morphology as well as promote higher brain function in young mice (Rao et al., 2005). Thus, these had been suggestions and recommendations for the use of CA to treat patients with mild cognitive impairment (MCI) (Tiwari et al., 2008) as well as to prevent cognitive decline during aging (Omar et al., 2009).

In conclusion, this study shows that low concentration of ethanolic extract of CA is able to protect neuron cells from oxidative stress probably by inhibiting the activation of caspase-9 pathway but at high concentrations it can exert neurocytotoxic effect.

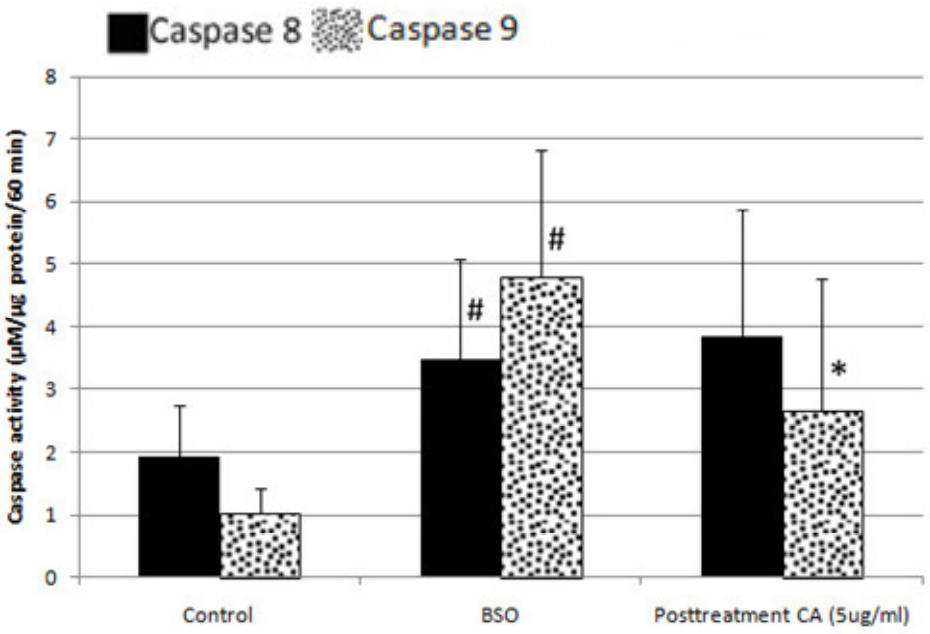

Figure 5. Caspase-8 and -9 activities determined by using commercial kit Caspase-8 and -9 colorimetric activity assay kit. Neuron cells were post-treated with CA 1 µg/ml for 24 h after induced with BSO for 72 h at 37°C. BSO significantly induced the activities of caspase-8 and -9 which may lead to the activation of the apoptotic pathway. Post-treatment with CA significantly reduced caspase-9 activity but not caspase-8 compared to cells exposed to BSO only. Data is presented as means ± SD, *n* = 9. # denotes $p < 0.05$ compared to control, * denotes $p < 0.05$ compared to IC_{50} BSO concentration.

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