

The Effects of *Centella asiatica* Extract (CAE) on Methamphetamine-Induced Neurotoxicity via Human Neuroblastoma Cell Line

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Methamphetamine (METH) was reported to caused neurotoxicity and cell death, *in vitro*. *Centella asiatica* or 'pegaga' is a native tropical herb with antioxidant and neuroprotective activities. Although the effects of *Centella asiatica* against oxidative stress and neuronal cell death have been reported in previous studies, however, the potential effects of *Centella asiatica* against psychostimulant methamphetamine (METH) are limited. Therefore, this study was aimed to evaluate the effects of *Centella asiatica* extract (CAE) against METH on *all-trans* retinoic acid, RA-differentiated human neuroblastoma, SH-SY5Y cells. The RA-differentiated SH-SY5Y cells were used to resemble dopaminergic neuronal-like cells. Cell viability was quantitatively assessed by 3-(4,5-dimethylthiazol-2-yl)-2 tetrazolium bromide, MTS assay. CAE at varying concentrations from 1pg/mL to 1mg/mL significantly decreased the viability of the undifferentiated SH-SY5Y cells in a concentration-dependent manner. At 1mg/mL of CAE, significantly increased the viability of differentiated SH-SY5Y cells. Meanwhile, CAE at 100µg/mL and 1mg/mL significantly reversed the METH-induced neuronal cell death. The results revealed that promising treatment of CAE on METH-induced neurotoxicity is mediated by its high content of asiaticoside, asiatic acid, madecassoside and madecassic acid. Taken together, this study may suggest CAE as a potential therapeutic treatment for METH-induced neurotoxicity, *in vitro*.

Keywords: *Centella asiatica*; neuroprotection; methamphetamine; neurotoxicity

I. INTRODUCTION

Oxidative stress is referred to as an imbalance production between reactive oxygen species (ROS) and antioxidant which eventually result in cellular damage (McDonnell-Dowling & Kelly, 2017). Oxidative stress-related drug abuse leads to neurotoxicity and damage to central nervous system (CNS). Methamphetamine (METH) is an amphetamine-type stimulant (ATS) psychoactive drug with devastating neurotoxic effects on CNS (Gailbraith, 2015). Neuronal-cell death induced by METH have been revealed through *in vitro* and *in vivo* study (Genc *et. al.*, 2003; Nara *et. al.*, 2010; Ramkissoon & Wells, 2015).

Pharmacologically, its cationic lipophilic properties make it easily cross the blood-brain barrier (BBB), thus enhances the release of dopamine (DA) from synaptic vesicles and nerve terminals of the CNS. Excessive release of DA is then auto-oxidised into superoxide radicals (O₂⁻), hydroxyl radicals (OH) and hydrogen peroxide (H₂O₂). These releases of radicals lead to the generation of ROS. Excessive ROS eventually cause neuronal cell damage, thus inducing cell death either via apoptosis or necrosis pathway (Wu *et al.*, 2007).

It is well known that the antioxidant defence system acts as free radical scavengers that prevent and minimise

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cellular damage. The use of antioxidant to attenuate the METH-induced neurotoxicity via targeting oxidative stress have been attempted. Unfortunately, till today limited natural-derived medicines have been synthesised to ameliorate neurotoxicity induced by METH. Thus, safe and cost-effective natural-derived medicines with fewer side effects are being proposed as a therapeutic agent to attenuate oxidative stress and neuronal cell death by METH.

Centella asiatica (L.) Urban (Umbelliferae) is a native medicinal herb of tropical Southern Asian countries such as Malaysia, Thailand and Indonesia have been revealed with its potential anti-tumor (Park *et al.*, 2005), neuroprotection (Ramanathan *et al.*, 2007; Omar *et al.*, 2011), antioxidant (Veerandra Kumar & Gupta, 2002), anti-hyperlipidemic (Kumari *et al.*, 2016) and wound healing (Sripanidkulchai *et al.*, 2013) anti-inflammatory and anti-bacterial activities (Shen *et al.*, 2019). The plant is rich in bioactive constituents mainly triterpenes such as asiatic acid, asiaticoside, madecassoside and madecassic acid which are major contributors to its pharmacological and antioxidant properties (Zainol *et al.*, 2003). Asiatic acid significantly attenuated the apoptotic SH-SY5Y cell death and ROS generation following glutamate induction (Xu *et al.*, 2012). Asiatic acid also revealed its significant protective effects against cytotoxicity induced by cholesterol on differentiated neuroblastoma SH-SY5Y cells (Ternchoocheep *et al.*, 2017). A study was done by Wang *et al.* (2019) further reported the protective role of asiatic acid and madecassic acid against metoprolol tartrate-induced enzymatic antioxidant deficit, *in vivo* male Wistar rat model.

Besides that, asiaticoside has also shown significantly attenuated the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP-induced neurotoxicity via maintaining redox balance and upregulating Bcl-2/Bax (Xu *et al.*, 2012).

The human neuroblastoma SH-SY5Y cell line was used as the most commonly used and established *in vitro* model for cytotoxicity and neurotoxicity study. Thus, this present study was conducted to evaluate the effects of CAE treatment against METH-induced neurotoxicity, via *in vitro* SH-SY5Y cells.

II. MATERIALS AND METHODS

A. Materials

Leaves of *Centella asiatica* were collected and obtained from Herbagus Trading, Pulau Pinang, Malaysia. The leaves were identified and deposited in the Atta-ur-Rahman Institute for Natural Product Discovery UiTM Puncak Alam, Selangor, Malaysia, Voucher Specimen (no. CA-K017). The leaves were washed, oven-dried at 40°C and finely ground and stored in a desiccator before use. Approximately 10kg of leaves powder was extracted using standard extraction protocol and was performed at the extraction facility (Institute of Bioproducts Development, Universiti Teknologi Malaysia) in 95% of denatured-ethanol for 8 h at 60°C. The crude yield extract was freeze-dried and ground into a dried-powder extract and was designated as *Centella asiatica* extract (CAE) (Figure 1).



Figure 1. The CAE powder

Methamphetamine (METH) was obtained from Toronto Supply, Canada. Approval for the use of METH was obtained from the Ministry of Health Malaysia (KKM-55/BPF/213/005/12).

B. The Human Neuroblastoma, SH-SY5Y Cells

The human neuroblastoma SH-SY5Y cell (ATCC® CRL 2266™) (cat no: 94030304) was purchased from Public Health England Culture Collection (Salisbury, UK) and maintained in 1:1 sterile filtered mixture of Minimum Essential Medium Eagle (MEM) and Nutrient Mixture Ham's F12 supplemented with 1% gentamicin (Sigma, USA),

1% non-essential amino acid (Sigma, USA) and 1% L-glutamine (Sigma, USA) and 10% heat-inactivated fetal bovine serum (FBS) (Sigma, USA). Cells were grown in a humidified incubator maintained at 37°C with 5% CO₂ until 80-90% confluent.

The SH-SY5Y cells were harvested once reaching 70-80% confluency. The old medium was discarded from the flask. A 2mL of 1x phosphate-buffered solution (PBS) was added to wash over the cell monolayer. To detach the cell monolayer, 1mL of trypsin-EDTA was added and incubated at room temperature for 1 min. To neutralise the cell pellet, a suspension containing detached cells was collected and transferred into 15mL of Falcon tube then centrifuged at 240rpm for 5 min. The supernatant was carefully aspirated and the cell pellet was gently suspended with media. Cell counting was performed using the Cellometer™ Auto T4 (Nexcelom, Bioscience). A 20µL of harvested cells was aliquoted into a cell counting chamber. Then, the chamber was inserted into the instrument. The concentration, viability and size were determined automatically.

C. 3-(4,5-dimethylthiazol-2-yl)-2 tetrazolium bromide, MTS Assay

The cell viability was assessed by adding 20µL of CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, USA). Cell viability was counted by measuring the production of coloured formazan where the percentage of cell viability is directly proportional to the formazan product. Absorbance reading was taken after 4 hours at 490nm by using a microplate reader (Glomax Integrated Multidetector System, Promega, USA).

D. Cytotoxicity Assay

Undifferentiated SH-SY5Y cells were plated and cultured into the 96-well plates at a concentration of 2x10⁴ cells per well. The cells were incubated at 37°C with 5% CO₂ for 24 hours. Sample dilutions (METH and CAE) were freshly prepared and stored overnight at 4°C. After 24 hours, media on each treatment wells were gently aspirated. A 100µL of 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL, 100ng/mL, 1µg/mL, 10µg/mL, 100µg/mL and 1mg/mL of samples (METH and CAE) were added to each wells. The plates were incubated again for another 1 and 24 hours. Following 24 hours, cell viability was assessed by MTS assay.

E. Neurotoxicity Assay

Undifferentiated SH-SY5Y cells were cultured into the 96-well plates at concentration of 2x10⁴ cells per well. Cells were incubated at 37°C with 5% CO₂ for 24 hours. After 24 hours, media was gently aspirated. For differentiation into neuronal-like cells, *all-trans* retinoic acid (RA) (Sigma) with a final concentration of 10µM was added to each wells containing undifferentiated SH-SY5Y cells (Forster *et al.*, 2016). The plates were wrapped with aluminium foil and incubated again for the next 5 days. The medium was changed every 2 days and the morphology of neuronal-like cells was observed daily. After 5 days, cells were treated with various concentrations of CAE and METH (1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL, 100ng/mL, 1µg/mL, 10µg/mL, 100µg/mL and 1mg/mL) and incubated at 37°C with 5% CO₂ for 24 hours. Following 24 hours of incubation, cell viability was assessed by MTS assay.

F. Neuroprotection Assay

Differentiated SH-SY5Y cells were pre-exposed with 1mg/mL of METH for 24 hours to induce oxidative stress and cytotoxicity. Following 24 hours, cells were post-treated with 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL, 100ng/mL, 1µg/mL, 10µg/mL, 100µg/mL and 1mg/mL of CAE and incubated at 37°C with 5% CO₂ for 24 hours. Cell viability was further assessed by MTS assay.

G. Statistical Analysis

Each experiment was carried out at least in triplicate. The results obtained were analysed by using one way analysis of variance (ANOVA) followed by Tukey's post hoc test using GraphPad® Prism Version 5 (GraphPad Software Inc, USA). The data were expressed as mean±SEM, standard error of mean. When the P-value is less than 0.05, significance was considered exist.

III. RESULTS

A. Cytotoxicity of METH on Undifferentiated SH-SY5Y Cells

Cytotoxicity assay was performed to analyse the effects of METH on undifferentiated SH-SY5Y cell survival. An hour incubation of 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL,

10ng/mL, 100ng/mL, 1µg/mL, 10µg/mL, 100µg/mL and 1mg/mL of METH did not significantly changed the cell viability with 82.08±1.17%, 88.58±2.09%, 87.98±1.97%, 84.16±5.61%, 95.36±1.67%, 94.09±5.20%, 86.46±7.88%, 83.71±0.88%, 83.48±3.89% and 90.28±12.9% respectively compared to control (untreated cells) (Figure 2A). Meanwhile, 24 hours incubation of 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL, 100ng/mL, 1µg/mL, 10µg/mL and 100µg/mL of METH did not significantly changed the cell viability with 98.51±1.48%, 93.08±1.26%, 95.96±5.18%, 90.10±12.26%, 95.79±1.22%, 93.98±5.70%, 93.18±5.29%, 96.90±4.77% and 86.57±5.55% compared to control (Figure 2). There was highly significant decreased in cell viability at 1mg/mL (more than 50%) compared to control (Figure 2A).

B. Cytotoxicity of CAE on Undifferentiated SH-SY5Y Cells

For cytotoxicity of CAE on undifferentiated SH-SY5Y cells, the percentage of cell viability was proportional to MTS assay reduction (Figure 2B). CAE at 1pg/mL did not significantly changed the cell viability compared to control. In contrast, CAE at 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL, 100ng/mL, 1µg/mL, 10µg/mL and 100µg/mL significantly decreased the cell viability with 87.18±1.67%, 88.57±3.39%, 86.34±2.34%, 85.48±0.78%, 83.76±2.98%, 85.62±2.14%, 86.85±1.27% and 87.31±0.38% respectively compared to control. There was highly significant decreased in cell viability at 1mg/mL of CAE with 12.35±2.91% compared to control (Figure 2B).

C. Differentiation of SH-SY5Y Cells into Neuronal-like Cells

Undifferentiated SH-SY5Y cells were added with the final concentration of 10µM of RA into neuronal-like cells (Figure 3A and 3B).

D. Neurotoxicity of METH on Differentiated SH-SY5Y Cells

Neurotoxicity test was performed to determine the neurotoxicity of METH on differentiated SH-SY5Y neuron-like cells, in this study (CAE and METH). There was no significant change in the viability of the differentiated SH-SY5Y cells at 1pg/mL following 24 hours incubation. METH-induced neurotoxicity by decreasing the cell survival and viability in a

dose-dependent manner. METH at 100pg/mL, 1ng/mL, 10ng/mL, 100ng/mL and 10µg/mL significantly decreased in cell viability compared to control. Meanwhile, there were highly significant decreases in cell viability at 1µg/mL, 100µg/mL and 1mg/mL of CAE as compared to control (Figure 4A). METH at 1mg/mL was applied for the subsequent study.

E. Neurotoxicity of CAE on Differentiated SH-SY5Y Cells

Differentiated SH-SY5Y cells were treated with CAE for 24-hour incubation. Results showed that there was no significant change in viability of cells at concentration of 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL, 100ng/mL, 1µg/mL, 10µg/mL, 100µg/mL of CAE compared to control (Figure 4B). Meanwhile, there was highly significant increase in cell viability at 1mg/mL of CAE with 143.31±15.25% compared to control (Figure 4B).

F. Effects of CAE on METH-induced Neurotoxicity and Cell Death

To determine the effects of CAE post-treatment against METH-induced neurotoxicity, differentiated SH-SY5Y cells were pre-exposed with IC₅₀ of METH (1mg/mL) to achieve 50% cell death. Then, cells were post-treated with 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL, 100ng/mL, 1µg/mL, 10µg/mL, 100µg/mL and 1mg/mL of CAE for 24 hours. Results revealed that post-treatment of CAE reverses the toxicity of METH by significantly increased the cell viability through a dose-dependent manner compared to the cells exposed only to METH (Figure 4C). At concentrations of 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL, 100ng/mL, 1µg/mL, 10µg/mL of CAE, there was no significant change in the cell viability compared to IC₅₀ of METH (Figure 4C). Meanwhile, post-treatment of 100µg/mL and 1mg/mL of CAE significantly increased the cell viability with 78.26±2.93% and 93.11±8.05% respectively compared to IC₅₀ of METH. Further, results showed that CAE protects SH-SY5Y cells from METH-induced cell death.

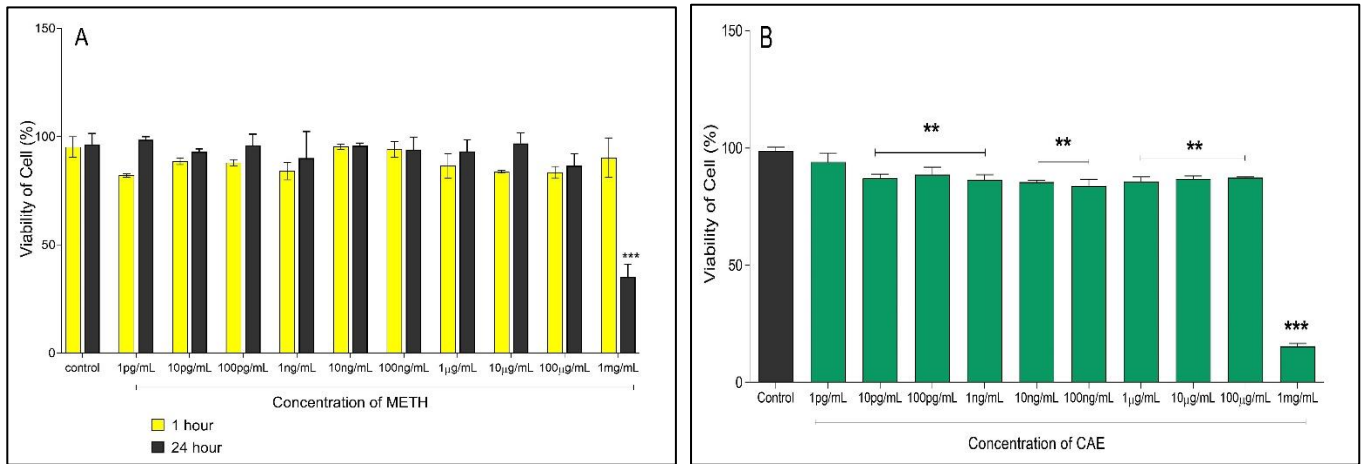


Figure 2. (A) The cytotoxicity of METH on undifferentiated SH-SY5Y following 1 and 24 hour incubation. (B) The cytotoxicity of CAE on undifferentiated SH-SY5Y cells following 24 hours incubation. Statistical analysis was done using one-way ANOVA followed by Tukey’s multiple comparison. Control denoted as 100% cell viability. Data expressed as the mean±SEM. *** P<0.001, **P< 0.01, *P<0.05 statistically significant as compared to control.

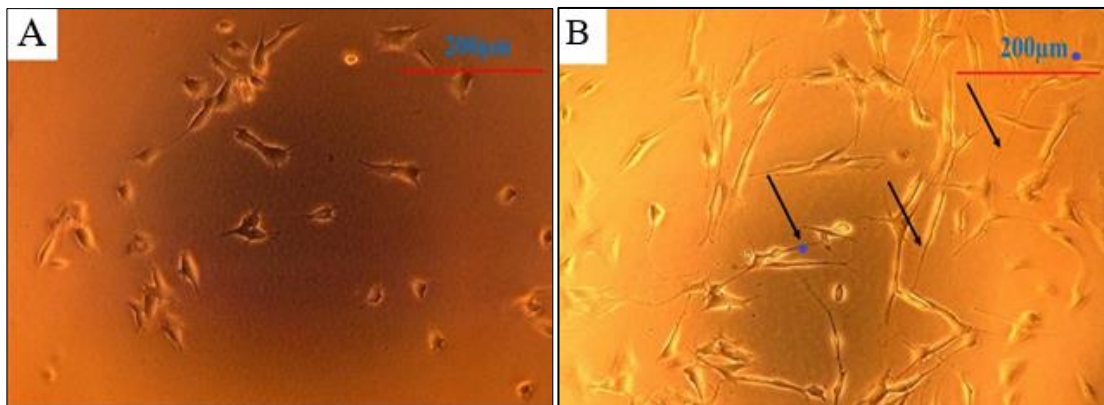


Figure 3. Morphology of undifferentiated and differentiated SH-SY5Y cells as observed under 20x magnification using a light-inverted microscope (Leica, Germany). (A) Undifferentiated SH-SY5Y cells with flat-phenotype. (B) After 5 days differentiation with 10µM *all-trans* RA, cell was transformed with elongated neurite (black arrow).

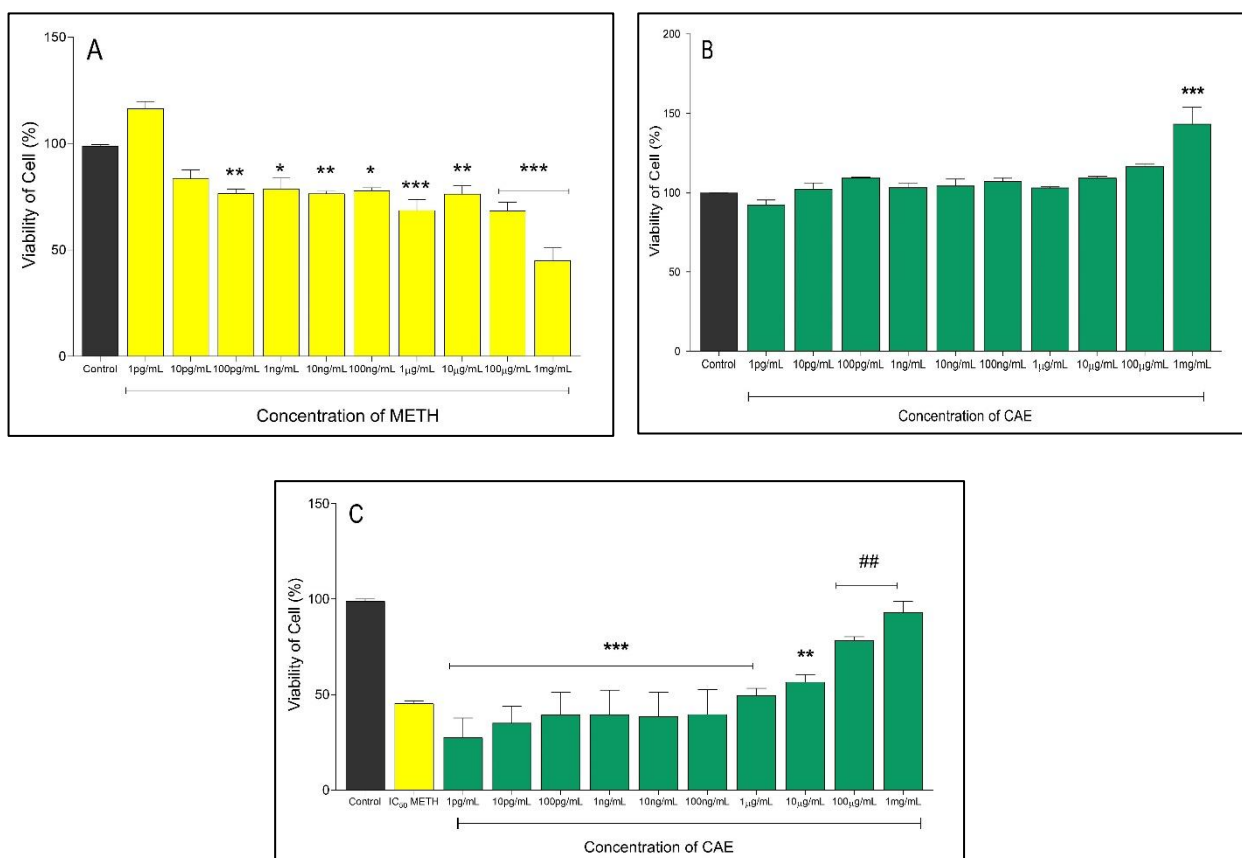


Figure 4 (A) Neurotoxicity of METH on differentiated SH-SY5Y cells following 24 hours incubation. (B) The neurotoxicity of CAE on differentiated SH-SY5Y cells following 24 hours incubation. (C) The effects of CAE on METH-treated differentiated SH-SY5Y cells. Cells were pre-treated with IC₅₀ of METH (1mg/mL) for 24 hours before post-treatment CAE for the next 24 hours. Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparison. Control denoted as 100% cell viability. Data expressed as the mean±SEM. *** P<0.001, **P<0.01, *P<0.05 statistically significant as compared to control s. Data expressed as the mean±SEM. *** P<0.001, **P<0.01, *P<0.05 statistically significant as compared to control while ###P<0.001, ##P<0.01, #P<0.05 statistically significant as compared to METH IC₅₀.

IV. DISCUSSIONS

METH has been reported to cause neurological dysfunction and neurotoxicity in the CNS of humans and rodents model (Davidson *et al.*, 2001; Bowyer & Hani, 2014). METH induces neurotoxicity through several mechanisms including mitochondrial dysfunction, oxidative stress, neuroinflammation and apoptosis (Yang *et al.*, 2018). To date, there is interest in plant-derived medicine as an alternative to treat various neurodegenerative diseases and neuronal-cell death related to METH-induced neurotoxicity.

In this study, we attempted to elucidate the cytotoxicity, neurotoxicity and effects of CAE treatment by determining cell viability via MTS assay using *in vitro* SH-SY5Y experimental model. Firstly, we conducted a cytotoxicity assay to determine

the effects of CAE and METH on undifferentiated SH-SY5Y cells. It is crucial to evaluate the cytotoxicity of METH and CAE at the early phase of the preclinical study to ensure its safety and efficacy prior to clinical study. Suwanjang *et al.* (2010) reported, METH caused a reduction in the cell viability of SH-SY5Y cells through dose and time-dependent manners. Similarly, in this study, we have demonstrated that there was no significant change in the cell viability following short exposure (1 h incubation) of METH on the SH-SY5Y cells. Meanwhile, a significant inhibitory effect was observed at high concentration of METH ($\geq 1\text{mg/mL}$) following longer (24 h) exposure of METH. In this study, METH doses $>1\text{mM}$ induced cell death by time and dose-dependent in agreement with Wu *et al.* (2007) which claimed that METH $<3\text{mM}$ caused

apoptotic cell death in the undifferentiated SH-SY5Y cells. Apart from that, the vulnerability of human cancer cells towards a low dose of METH is probably due to the upregulation of pro-survival protein Bcl-2 (El-Ayadi & Zigmond, 2011). This study shows that CAE (1mg/mL) has strong inhibitory effects on the viability of the undifferentiated SH-SY5Y cells. According to Omar *et al.* (2011), CAE \geq 100 μ g/mL is found to be toxic to the cells. This cytotoxicity is due to asiatic acid found in CAE which induced apoptosis and cell death (Park *et al.*, 2005). Sakonsinsiri *et al.* (2018) also reported antitumor and cytotoxicity of CAE attributed by its high alkaloidal content.

Ethical concern, relatively high cost and maintenance are limitations of primary mammalian cells for toxicity and neuroscience study (Kovalevich & Langford, 2013). Addition of a differentiation-inducing agent such as *all-trans* retinoic acid (RA), the SH-SY5Y cells were differentiated into dopaminergic neuron-like cells (Pahlman *et al.*, 1984). Thus, transformed neuronal-like cell lines can be used to overcome these limitations. Morphological changes of the cells into long and elongated-branch neurites that connect to surrounding cells were observed in RA-differentiated cells as compared to undifferentiated cells are in line with Shipley *et al.* (2017). In this study, we performed both cytotoxicity and neurotoxicity assays using differentiated and differentiated SH-SY5Y cells in order to evaluate the vulnerability of these cells towards CAE and METH. Cecchi *et al.* (2008) reported that undifferentiated and differentiated SH-SY5Y cells display different vulnerability to toxic β -amyloid.

Generally, neurotoxicity is defined as any physical damage to neuronal cells. In this study, METH conferred a significant reduction on cell viability of the differentiated SH-SY5Y through a dose-dependent manner. The result proves that METH gave oxidative stress-environment to neuron-like cells. Nara *et al.* (2010) similarly indicates that METH >7mM significantly caused neuronal cell death on the differentiated SH-SY5Y cells. According to Badisa *et al.* (2019), METH-induced neurotoxicity through generation of ROS. ROS causes inhibition of mitochondrial protein complexes which disturbs the normal function of mitochondria (Shin *et al.*, 2018). This action induced mitochondrial Ca²⁺ dysregulation which leads to cell death (Andres *et al.*, 2015). Interestingly, our data demonstrate a wide range of concentrations from pictogram

(pg/mL) to milligram (mg/mL) of METH. Thus, implying that METH at 1pg/mL significantly possessed neurotoxicity effects on differentiated SH-SY5Y cells. In this study, it was observed that CAE at the highest concentration (1mg/mL) showed highly significant maximal cell viability which are in line with Hafiz *et al.* (2020).

As in Figure 4C, CAE reverses the toxicity of METH by induce the cell viability with a significant increase in cell viability at 100 μ g/mL and 1mg/mL as compared to METH IC₅₀. This effective treatment further showed that protecting the SH-SY5Y cells upon oxidative stress by CAE has also been demonstrated by Omar *et al.* (2011) that CAE at the concentration ranging from 1 μ g/mL to 50 μ g/mL is capable to protect neuron cells from oxidative stress and apoptotic cell death induced by buthionine sulfoximine (BSO). Neuroprotective effects of CAE are probably due to a high content of antioxidant activities (Zainol *et al.*, 2003). Bioactive triterpenes such as asiatic acid and medacassic acid exert protective effects against oxidative cellular damage in dopaminergic neurons *in vitro*. Antioxidant properties of CAE may also mediated by increases in superoxide dismutase (SOD) and catalase activity. Besides that, CAE also acts as a potent scavenger of free radicals. Hussin *et al.* (2007) documented that *Centella asiatica* able to increase endogenous antioxidant enzymes and reduce lipid peroxidation by free radicals. Bioactive constituents of CAE also may activate cell- specific signalling pathways such as activation of pro-survival and anti-apoptotic mechanism thereby attenuating neuronal-cell death. In addition, CAE and its bioactive constituents affects CNS possibly through the anti-inflammatory activity by inhibiting NF-Kb activation and the PI3K/AKT and ERK1/2 signalling pathway (Mairuae *et al.*, 2019).

In future, further study needs to be done in order to comprehensively identify the mechanism which involves in the treatment of CAE on METH-induced neurotoxicity.

V. CONCLUSION

This *in vitro* preliminary study is suggested CAE as therapeutic natural-derived protective agent to attenuate the neuronal cell death induced by psycho-stimulant drug, METH.

VI. ACKNOWLEDGEMENT

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