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Ethanol Extract of *Centella asiatica* Improved Methamphetamine-Induced Neurotoxicity on Mouse Model via Stimulating Superoxide Dismutase II and microRNA-34A Expression

(Ekstrak Etanol *Centella asiatica* Menambahbaik Keneurotoksikan Teraruh Metamfetamin pada Model Tikus melalui Superoksida Dismutase II dan Ekspresi Mikro)

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ABSTRACT

Neurotoxicity induced by a psychostimulant drug, methamphetamine (METH) is associated with devastating and persistent neurotoxicity effects on the central nervous system (CNS). *Centella asiatica* (CA) is known as an antioxidant and neuroprotective agent. However, there is a limited study on natural-derived therapeutic to attenuate neurotoxicity induced by METH. We aimed to investigate the effects of METH and ethanol extract CA (CAE) on motor performance of animal model and the expression of manganese superoxide dismutase II (SOD2) and microRNA-34a (miR-34a) in the brain tissue. Male Sprague-Dawley rats were administered with METH (50 mg/kg per body weight) twice per day for 4 days, CAE (300 mg/kg & 500 mg/kg per body weight for 21 days and combination of METH and CAE for 21 day(s). Weight of rat was measured and motor performance was evaluated using vertical pole and narrow beam tests. Expression of SOD2 and miR-34a were measured using Quantitative Real-time Polymerase Chain Reaction (RT-qPCR). Group III (300 mg/kg CAE); $p<0.001$, Group IV (500 mg/kg CAE); $p<0.001$, Group V (METH+300 mg/kg CAE); $p<0.01$ and Group VI (METH+500 mg/kg CAE); $p<0.01$ significantly improved latency in the vertical pole test compared to METH group. Meanwhile, Group III (300 mg/kg CAE); $p<0.001$ and Group IV (500 mg/kg CAE); $p<0.001$ significantly decreased latency in the narrow beam test compared to METH. Post-treatment of CAE on METH-treated rats, Group V (METH+300 mg/kg CAE) and Group VI (METH+500 mg/kg CAE) non-significantly upregulated the SOD2 expression by 3.78±1.03 and 4.05±0.19 folds compared to METH, respectively. Post-treatment of CAE on METH-treated rats, Group V (METH+300 mg/kg CAE) and Group VI (METH+500 mg/kg CAE) non-significantly upregulated the miR-34a expression by (7.02±3.73) and (6.75±1.94) folds compared to METH, respectively. CAE could be suggested as a promising natural-derived therapeutic for METH-induced neurotoxicity to ameliorating motor performance and triggering SOD2 and miR-34a expression.

Keywords: *Centella asiatica*; methamphetamine; microRNA-34a; superoxide dismutase II

ABSTRAK

Keneurotoksikan yang disebabkan oleh dadah psikostimulan, metafetamin (METH) dikaitkan dengan kesan keneurotoksikan yang teruk dan berterusan pada sistem saraf pusat. *Centella asiatiaca* (CA) terkenal sebagai agen antioksidan dan neurolindung. Walau bagaimanapun, terdapat kajian yang terhad mengenai bahan teraputik semula jadi untuk melemahkan keneurotoksikan yang disebabkan oleh METH. Kami berhasrat mengkaji kesan METH dan ekstrak etanol CA (CAE) pada prestasi motor model haiwan dan ekspresi manganese superoksida dismutase II (SOD2) dan mikroRNA-34a (miR-34a) pada tisu otak. Tikus *Sprague-Dawley* jantan diberikan METH (50 mg/kg setiap berat badan) dua kali sehari selama 4 hari, CAE (300 mg/kg dan 500 mg/kg setiap berat badan) selama 21 hari dan gabungan METH dan CAE selama 21 hari. Berat tikus diukur dan prestasi motor dinilai menggunakan ujian kutub

menegak dan rasuk sempit. Ekspresi SOD2 dan miR-34a diukur menggunakan *Real-time Polymerase Chain Reaction* (RT-qPCR). Kumpulan III (300 mg/kg CAE); $p<0.001$, kumpulan V (METH+300 mg/kg CAE); $p<0.01$ dan kumpulan VI (METH+500 mg/kg CAE); $p<0.01$, meningkatkan latensi secara signifikan dalam ujian kutub menegak berbanding kumpulan METH. Manakala, kumpulan III (300 mg/kg CAE); $p<0.001$ menurunkan latensi secara signifikan dalam ujian rasuk sempit berbanding METH. Pasca rawatan CAE pada tikus yang dirawat dengan METH, kumpulan V (METH+300 mg/kg CAE) dan kumpulan IV (METH+500 mg/kg CAE) secara tidak signifikan meningkatkan ekspresi SOD2 berbanding METH iaitu masing-masing pada 3.78±1.03 dan 4.05±0.19 ganda. Pasca rawatan CAE pada tikus yang dirawat METH, kumpulan V (METH+300 mg/kg CAE) dan kumpulan VI (METH+500 mg/kg CAE) secara tidak signifikan masing-masing meningkatkan ekspresi miR-34a (7.02±3.73 dan 6.75±1.94 ganda) berbanding METH. CAE boleh dicadangkan sebagai terapeutik semula jadi yang menjanjikan untuk keneurotoksikan yang disebabkan oleh METH untuk memperbaiki prestasi motor dan mencetuskan ekspresi SOD2 dan miR-34a.

Kata kunci: *Centella asiatica*; metamfetamin; mikroRNA-34a; superoksida dismutase II

Introduction

National Anti-Drug Agency stated in 2018, the prevalence of drug abuse among Malaysians are in increasing trend. In 2016, approximately, there have been 10,107 cases of methamphetamine (METH) seizure. METH is a colourless, odourless and potent stimulant drug which is categorized under Schedule II stimulant (Moszczynska & Callan 2017). METH-induced neurotoxicity is associated with behavioural and physiological effects as well as addiction (Brecht & Herbeck 2013; Huang et al. 2017; Volkow 2013). METH induces neurotoxicity via several mechanisms such as oxidative stress (Ramkissoon & Wells 2015), mitochondrial dysfunction (Yang et al. 2018) and neuroinflammation (Shaerzadeh et al. 2018). METH also induces degeneration of the dopaminergic system in the brain. The persistent exposure of METH further causes Parkinson's disease (PD)-like symptoms or Parkinsonism (Krasnova et al. 2011; Volkow et al. 2001). Inhibition of oxidative stress by antioxidant defence mechanisms have been previously explored to attenuate METH neurotoxicity (Moshiri et al. 2020; Thanh et al. 2016). However, there are still limited and non-specific antioxidants and treatments are available.

Centella asiatica (CA) or commonly known as *pegaga*, *Indian pennywort*, *Gotu kola* or *biak-biak* is a native herb belongs to Apiaceae family (Jahan et al. 2012). This small, herbaceous plant is widely distributed in the Southeast Asia countries such as Malaysia, Thailand, and Indonesia (Alfarra & Omar 2013). Previously, CA has been extensively studied as antioxidant and free-radical scavengers (Orhan 2012). Besides that, CA was found to be effective in protecting neurodegenerative and reducing oxidative stress performance (Sampath & Janardham 2013). Water extract of CA also reported to improving cognitive deficit of amyloid beta (Aß)-exposed Tg2576 mice models (Gray et al. 2017). In addition, CA possesses neuroprotective effects against 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced neurotoxicity in aged male Sprague-Dawley rats (Haleagrahara & Ponnusamy 2010). It has been proved that, administration of aqueous extract of CA (CAE) decreased lipid hydroperoxides (LPO) and protein-carbonyl-content (PCC) and significantly increased total antioxidant (TA) and antioxidant enzyme levels (Haleagrahara & Ponnusamy 2010). Active compounds found in CA are responsible in its neuroprotective activities. Asiaticoside, is reported to significantly upregulated the bax and bcl-2 expression (gene family that promotes apoptosis) in MPTP-induced mice compared to control group (Sampath & Janardhanam 2013). In addition, asiaticoside also found to attenuate the neurotoxicity induced by hydrogen peroxide (H_2O_2) in SH-SY5Y cells by reducing the reactive oxygen species (ROS), nitrogen oxide (NO) and inducible nitric oxide synthase (iNOS) (Ling et al. 2017).

First-line defence mechanism against ROS begins in mitochondria. Manganese superoxide dismutase II (SOD2) is a major endogenous antioxidant enzyme and oxidative stress biomarker (Flynn & Melovn 2013). Expression of SOD2 was observed on neuronal protection against oxidative stress (Jia et al. 2011) and improving brain function (Parabucki et al. 2012). Thus, activation and expression of SOD2 may be involved in neuroprotective action of CA.

MicroRNA (miRNA) is small, endogenous short-stranded non-coding ribonucleic acids (RNA) approximately 22-nucleotides which is post-

transcriptionally regulates gene expression via imperfect base pairing the 3'-untranslated region (UTR) of its target messenger RNA (mRNA) (Wang et al. 2014). miRNA modulates protein expression by inhibiting mRNA translation and promoting mRNA degradation. Besides that, miRNAs also play important role in the central nervous system (CNS) development and function, potential diagnostic and prognosis biomarkers. According to Tal and Tanguay (2012), miRNAs as an important biomarker of CNS and novel neurotoxicity targets. Studies have found that miR-34a is involved in PD-associated neurotoxins such as rotenone, paraquat, 6-hydroxydopamine (6-OHDA) and METH (Mao et al. 2015). Horst, Titze-De-Almeida and Titze-De-Almeida (2017) reported that downregulation of miR-34a also could attenuate rotenone-induced cell injury. In addition, Alural, Genc and Haggarty (2016) further supported the neuroprotection of lithium on paraquat-induced neurotoxicity on human neuroblastoma (SH-SY5Y) cells via downregulation of miR-34a and activation of antioxidant protein expression regulator nuclear factor 2-related factor 2 (NRF2).

To date, cost-effective, safe and easily available natural-derived therapeutics yet develop for METH-induced neurotoxicity and motor deficit. Thus, this present study was aimed to investigate the effects of CA extract (CAE) administration on METH-induced animal model using narrow beam test and vertical pole test. In addition, this study was aimed to measure the expressions of SOD2 and miR-34a on the brain of METH-induced neurotoxicity in animal model following post-treatment of CAE.

Materials and Methods

SAMPLE PREPARATION

Fresh leaves of CA were obtained from the Herbagus Trading, Pulau Pinang, Malaysia. The leaves were identified and deposited in the Atta-ur-Rahman Institute for Natural Product Discovery (AuRins), Universiti Teknologi MARA (UiTM), Puncak Alam, Selangor, Malaysia, voucher specimen (No. CA-K017). The leaves then were washed and oven-dried at 40 °C. The dried leaves were finely ground and stored in a desiccator before use. Approximately, a total of 10 kg of leaves powder was further extracted at the extraction facility in the Institute of Bioproducts Development, Universiti Teknologi Malaysia (UTM). Extraction was done in 95% of denatured ethanol for 8 h at 60 °C. Crude yield extract was freeze-dried into dried-powder extract and designated as CA extract (CAE). METH was obtained from the Toronto Research Chemicals (Toronto, Canada). Approval for use of METH was obtained from the Ministry of Health (MOH) Malaysia, (KKM-55/BPF/213/005/12, dated: 17 February 2016).

EXPERIMENTAL ANIMALS

Males Sprague-Dawley rats weighing, 250-260 g were used in this study. Rats were obtained from the Laboratory of Animal Facility and Management (LAFAM), Faculty of Pharmacy, UiTM Puncak Alam Campus, Selangor. Rats were properly acclimatized and housed in a polycarbonate cage with controlled temperature (22-24 °C) with 12 h dark-light cycle room throughout study. Rats were given standard commercial dry food pellet and water, *ad libitum*. Experiment or study on the animals were carried out between 0800 h and 1800 h. Animal procedures and protocols were approved by the Ethics Committee of Animal Research Ethics from the Universiti Teknologi MARA, UiTM (Reference No: 600-FF (PS.17/2/1) (Care 202/2017); dated: 3 July 2017). Animal procedures were conducted with minimal animal suffering according to the Organization for Economic Cooperation and Development (OECD) Guideline 420.

ANIMAL TREATMENT

Total of 36 male Sprague-Dawley rats were used in this study and were randomly divided and assigned into six groups consisting of six animals each group namely; Group I (control); rats were orally administered with 1 mL of 0.9% saline for 25 days. Group II (METH-treated); rats were subcutaneously injected with 50 mg/kg per body weight of METH, twice per day (0900 h and 1700 h) for 4 days and were orally given 1 mL of 0.9% normal saline for 21 days. Group III; rats were orally administered with 300 mg/kg per body weight of CAE for 21 days and were orally given 1 mL of 0.9% normal saline for 4 days. Group IV; rats were orally administered with 500 mg/kg per body weight of CAE for 21 days and were orally given 1 mL of 0.9% of normal saline for 4 days. Group V; rats were subcutaneously injected with 50 mg/kg per body weight of METH, twice a day for 4 days and were orally administered with 300 mg/kg per body weight of CAE for 21 days. Group VI; rats were subcutaneously (sc) injected with 50 mg/kg per body weight of METH, twice a day for 4 days and were orally administered with 500 mg/kg per body weight of CAE for 21 days. The METH and CAE samples were freshly prepared on each day of the experiment. The rats were

performed motor performance evaluation on day 26, 27 and 28 and were sacrificed under light anesthesia on day 29. Doses of METH were selected based on work by Ricaurte, Schuster and Seiden (1980). Meanwhile doses of 300 mg/kg and 500 mg/kg of CAE were selected based on the works by Amjad and Umesalma (2015) and Haleagrahara and Ponnusamy (2010), respectively.

EVALUATION OF BODY WEIGHT

To evaluate the METH and CAE-related effects on the growth, body weight was measured and throughout the study. Body weight of rats was compared between each treatment groups.

EVALUATION OF MOTOR PERFORMANCE

Vertical pole test was performed to assess motor performance and motor symptoms in neurodegenerative disease model. This test also is used to measure motor coordination and bradykinesia. Vertical pole test was performed with a slight modification from previous study by Bae et al. (2014). Rats were placed head-up on top of a wooden pole, 100 cm long with 1 cm in diameter. Rats were oriented-downward along the wooden pole. The pole was held in horizontal position and was gradually lifted to vertical position. Animals with deficits in balance and motor performance will fall off easily from the vertical pole. Rats were trained for three trials each session for three days. Total time taken for rats to stay on pole was recorded. Total time 30 s was set as maximum limit. We also performed narrow beam test to further assess the motor performance of rats with a slight modification from previous study by Luong et al. (2001). The narrow beam test is important to evaluate skills of animals to maintain motor balance and walk pattern in narrow beam surface. The narrow beam with a smooth surface of 100 cm in length with flat surface of 6 mm width resting 50 cm above the floor. Rats were placed at starting point of beam at height of 100 cm from the supporting flat surface. Rats were trained for three trials each session for three days. The beam was equipped with escape box at end of the beam. Total time of 120 s was set as maximum limit. Time taken for each rat to across 80 cm beam or reach escape box (escape latency) was recorded. Error was recorded as any failed attempt to reach the escape box, loss of balance and fall from the beam before 120 s of maximum total time.

RNA EXTRACTION AND cDNA SYNTHESIS

RNAs were isolated from brain tissue of rats using Qiazol® lysis reagent (Cat No: 79306) (Qiagen, USA) and miRNeasy mini kit (Cat No: 217084) (Qiagen, USA) according to manufacturer's instructions. RNase-free water and RNase-free reaction tubes were used to avoid RNase activity. Total RNA's yield, quality and integrity were quantified at the ratio of 260 nm and 280 nm (A260/A280) by using NanoDrop ND-1000 spectrophotometer (Thermo-Fisher Scientific, USA). Total RNA (containing miRNAs) was reverse-transcribed into complementary DNA (cDNA) using miScript II RT kit (Cat No: 218161) (Qiagen, USA) according to manufacturer's instructions.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (RT-qPCR)

The RT-qPCR was performed using miScript SYBR Green PCR kit (Cat No: 218073) (Qiagen, USA) and miScript Primer Assay (Qiagen, USA) according to manufacturer's instruction. For mRNA, β-actin (Cat No. 330001 PPR5758A) and miRNA, small nuclear U6RNA (Cat No. MS0003374) were used as reference genes. The PCR program was first set at 95 °C for 15 min for initial denaturation followed with denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s and was repeated for 40 cycles. Each run was performed at least in triplicate with negative reverse transcription (RT) or non-template control (NTC). Relative quantification ΔΔCq (2-ΔΔCq) by Livak and Schmittgen (2001) was applied to measure and quantify the gene expression. The mRNA and miRNA Cq values were normalized to reference genes.

STATISTICAL ANALYSIS

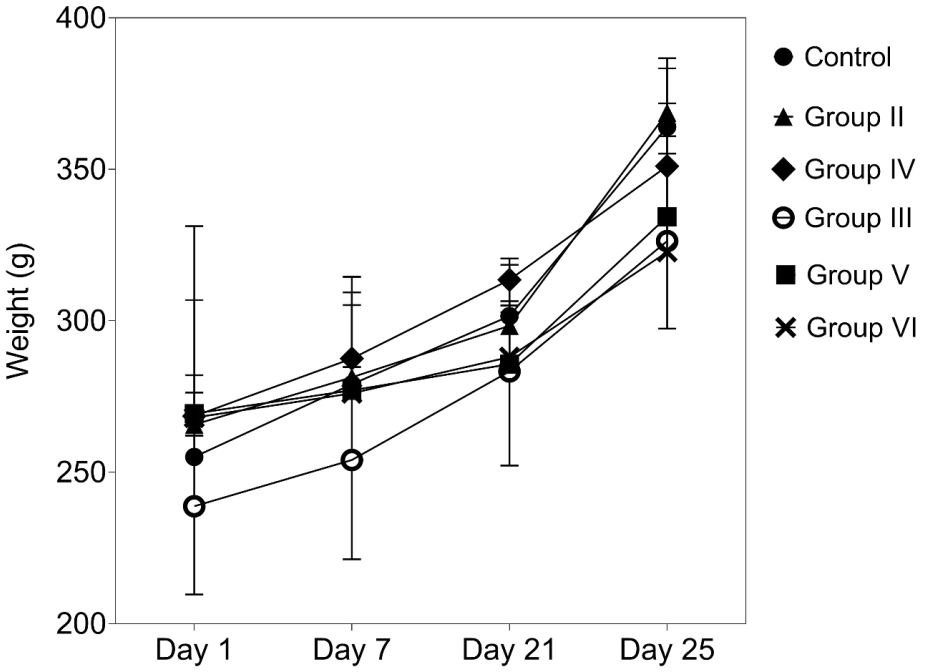
Statistical differences between groups were compared by analysis of variance (ANOVA) followed by Tukey's post hoc test. Analyses were performed by using GraphPad Prism version 8 for Windows (GraphPad Software, San Diego, California, USA). $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

EVALUATION OF BODY WEIGHT

In animal studies, high doses of METH result in damage of dopaminergic neurons which is associated with cognitive and motor deficit. Alteration in the CNS has been observed in the motor performance and coordination of animal model through various mechanisms including oxidative stress which could disrupt the physiological balance of CNS (Chen et al. 2020). In this study, 50 mg/kg per body weight, twice a day for 4 days of METH

was used to induce CNS neurotoxicity and neuronal cell damage in rats as reported by Ricaurte, Schuster and Seiden (1980). The dose intervals exhibited METH-induced neurotoxicity in the dopaminergic region with deficit in behavioural and motor coordination probably due to METH pharmacological effects. There was no significant change in the average body weight of rats from each treatment group (Figure 1). There was no mortality observed following METH and CAE administration. As observed, there was no significant changes in the body weight of rats following exposure to METH and post-treatment of CAE.

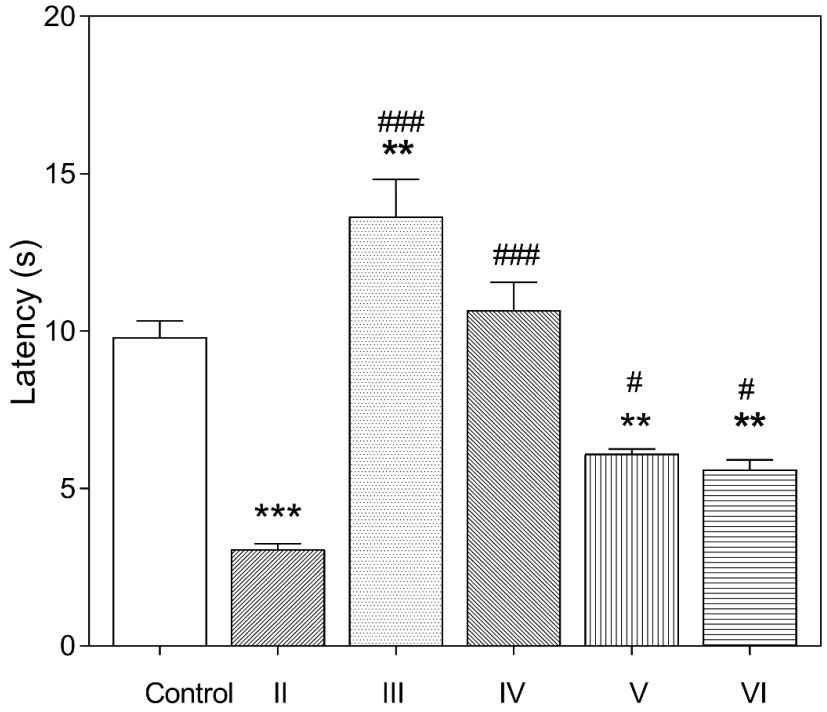


Data are expressed as mean ± standard deviation (SD) (n=6). Control devoted as group without any treatment

FIGURE 1. Changes in body weight of rats in response to CAE and METH treatments for 25 days

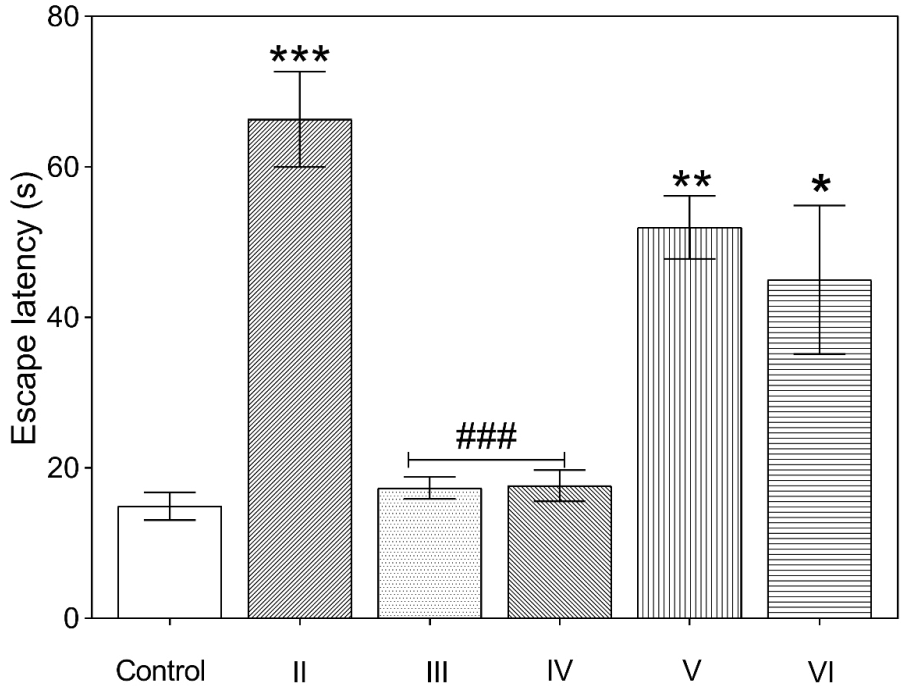
MOTOR PERFORMANCE OF RATS EVALUATED ON VERTICAL POLE TEST AND NARROW BEAM TEST

In the vertical pole test, as observed, there were significant decreases in motor performance among Group II (METH only), Group V (METH + 300 mg/kg CAE) and Group VI (METH + 500 mg/kg CAE) as compared to control (Figure 2). Meanwhile, there was significant increase in latency of rats in Group III (300 mg/kg CAE) as compared to control. Rats in Group II (METH only) fell off in less than 5 s (3.4±1.64 s) with the least grip strength compared to control. Rats in Group III (300 mg/kg CAE), IV (500 mg/kg CAE), V (METH + 300 mg/kg CAE) and VI (METH + 500 mg/kg CAE) significantly increased in latency compared to Group II (METH only). These results indicated that post-treatment of CAE was able to improve motor performance of rats following METH exposure. In the narrow beam test, rats in Group II (METH only) significantly increased their time taken to across beam (escaped latency) compared to control (Figure 3). Meanwhile, Group III (300 mg/kg CAE) and Group IV (500 mg/kg CAE) significantly decrease their time to across beam compared to Group II (METH only). For the total number of errors counted, significant increases of error were observed in Group II (METH only) compared to control (Figure 4). Meanwhile, Group III (300 mg/kg CAE), IV (500 mg/kg CAE), V (METH + 300 mg/kg CAE) and VI (METH+500 mg/kg CAE) significantly decreased in the number of errors compared to METH.



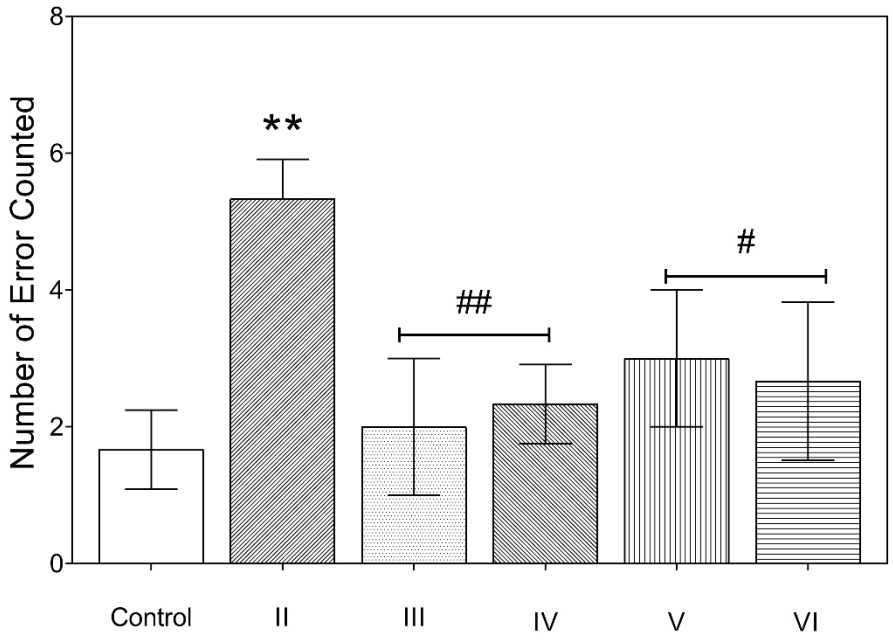
Data are expressed as mean ± standard deviation (SD) (n=6). Control devoted as group without any treatment, Group II (METH), Group III (300 mg/kg CAE), Group IV (500 mg/kg CAE), Group V (METH+300 mg/kg CAE) and Group VI (METH+500 mg/kg CAE). ***p<0.001, **<0.01, *p<0.05 statistically significant compared to control. ### p<0.001, ##p<0.01, #p<0.05 statistically significant compared to Group II (METH)

FIGURE 2. Effect of METH and CAE evaluated through pole test



Data are expressed as mean ± standard deviation (SD) (n=6). Control devoted as group without any treatment, Group II (METH), Group III (300 mg/kg CAE), Group IV (500 mg/kg CAE), Group V (METH+300 mg/kg CAE) and Group VI (METH+500 mg/kg CAE). ***p<0.001, **<0.01, *p<0.05 statistically significant compared to control. ### p<0.001, ##p<0.01, #p<0.05 statistically significant compared to Group II (METH)

FIGURE 3. Average time taken (s) by rats to across beam in the narrow beam test



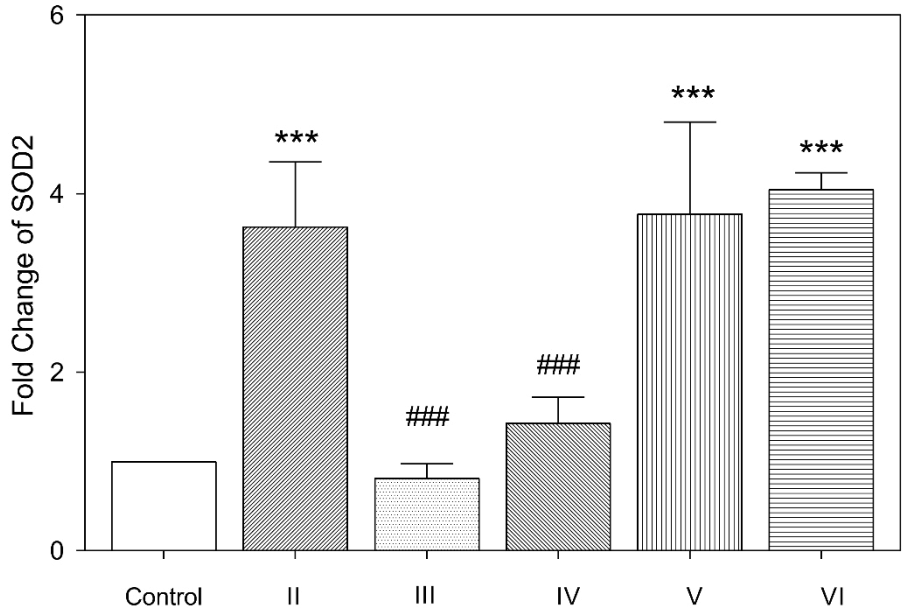
Data are expressed as mean ± standard deviation (SD) (n=6). Control devoted as group without any treatment, Group II (METH), Group III (300 mg/kg CAE), Group IV (500 mg/kg CAE), Group V (METH+300 mg/kg CAE) and Group VI (METH+500 mg/kg CAE). ***p<0.001, **<0.01, *p<0.05 statistically significant compared to control. ### p<0.001, ##p<0.01, #p<0.05 statistically significant compared to Group II (METH)

FIGURE 4. Total number of errors counted in the narrow beam test

Motor performance and coordination of rats can be measured and evaluated through various methods such as rotarod, vertical pole test and narrow beam test. In this present study, vertical pole and narrow beam tests were conducted. Both methods provide a simple and easy method for evaluation of motor performance and coordination in rodent models (Zarruk et al. 2011). As observed in the vertical pole test, there was a significant increase in latency following post-treatment of CAE in Group V (METH + 300 mg/kg CAE) and Group VI (METH + 500 mg/kg CAE) compared to Group II (METH only). In narrow beam test, post-treatment of CAE (Group V; METH + 300 mg/kg CAE and Group VI; METH + 500 mg/kg CAE) significantly reduced the latency to across beam compared to Group II (METH only). Also, we found that post-treatment of CAE in Group V (METH + 300 mg/kg CAE) and Group VI (METH + 500 mg/kg CAE) significantly reduced the error counted on beam (slips and falls) compared to Group II (METH only). This result showed that CAE post-treatment was able to improve motor performance of rats following METH exposure and in line with Bhatnagar et al. (2017) that CAE improved Parkinson's disease (PD)-like symptoms through behaviour recovery in motor performance. CAE doses used here showed that CAE ameliorated motor deficit and performance of rats. The promising effect is also due to the ability of CAE to restore dopamine (DA) and attenuate oxidative stress (Xu et al. 2012). This result further suggests that CAE might offer protection against dopaminergic neurotoxicity induced by the repeated and high dose METH. Animal studies also indicated that flavonoids intake, a bioactive constituent, are capable of reversing cognitive and motor deficit (Ayaz et al. 2019). The effects of flavonoids on cognitive function and performance have been linked to ability of flavonoids on signalling and molecular pathways that are responsible for cognitive performance such as phosphotidylinositol-3 kinase (P13 kinase/Akt) and mammalian target of rapamycin (mTOR) protein kinase (Spencer 2007) and SIRT1/mTOR (Chen et al. 2019). Flavonoid-rich dietary such as water extract CA has shown to improve cognitive performance of mice (Gray et al. 2016).

EXPRESSION OF SUPEROXIDE DISMUTASE II

There were significant increases in SOD2 expression in Group II (METH), Group V (METH + 300 mg/kg CAE) and Group VI (METH + 500 mg/kg CAE) as compared to



Data are expressed as mean ± standard deviation (SD) from triplicate qPCR reaction. Control devoted as group without any treatment, Group II (METH), Group III (300 mg/kg CAE), Group IV (500 mg/kg CAE), Group V (METH+300 mg/kg CAE) and Group VI (METH+500 mg/kg CAE). *** p<0.001, **<0.01, *p<0.05 statistically significant compared to control. ### p<0.001, ## p<0.01, #p<0.05 statistically significant compared to Group II (METH)

FIGURE 5. The expression of SOD2 relative to ß-actin

control (Figure 5). Meanwhile, there was no significant change of SOD2 among Group III (300 mg/kg CAE) and Group IV (500 mg/kg CAE) compared to control. In contrast, there were significant decreases in SOD2 in Group III (300 mg/kg CAE) and Group IV (500 mg/kg CAE) as compared to Group II (METH only).

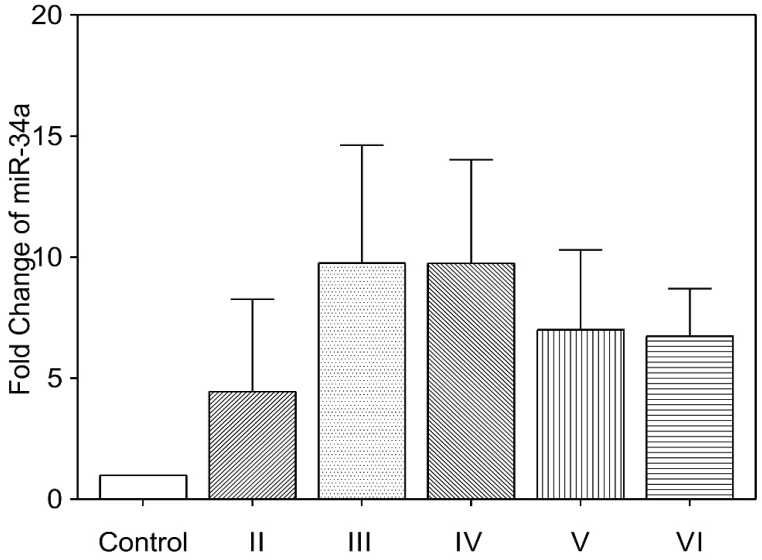
SOD2 plays a crucial role in protection against reactive oxygen species, ROS and superoxide radicals. Similarly, as reported by Thrash et al. (2009), METH induced the upregulation of antioxidant enzymes in response to oxidative stress and ROS. To test our hypothesis, we used RT-qPCR analysis to monitor the changes of SOD2 level following METH exposure and post-treatment of CAE. Notably, β-actin and small-nuclear U6RNA were selected as reference genes for the quantification of SOD2 and miR-34a due to their stability and sensitivity following oxidative stress and brain injury (Balbaa, Abdulmalek & Khalil 2017). Following METH exposure, there was significant increase of SOD2 compared to control. Excessive DA leads to oxidative stress via auto-oxidation of DA in the brain area could be a source of superoxide radicals which increased the SOD2. This upregulation increased the oxygen radicals and antioxidant enzymes following METH exposure. The increase of SOD2 in METH-treated rats in this study is in line with Liu et al. (1993) reported the increased expression of SOD1 and SOD2 in ischemic insults. In another study, higher SOD1 was observed in DA axons of transgenic mice that were injected with METH compared to control wild-type animals (Hirata et al. 1995). In addition, post-treatment of CAE was found to non-significantly increased SOD2 compared to Group II (METH only).

It is believed that the antioxidant effects may have activated the antioxidant response pathway. Triterpenes, major bioactive constituents of CAE such as asiatic acid, asiaticosside, medacassic acid and madecassoside have been shown to mediate the protective effects on dopaminergic neurons through antioxidant activity. The antioxidant constituents such as quercetin and catechins which act as ROS scavenging by inhibiting the generation of free radicals and removing superoxide

radicals (Zainol et al. 2003). In addition, phenolic compounds such as tocopherols and flavonoids are well known as effective hydrogen donating radical scavengers by scavenging lipid alkoxyl and peroxyl radicals (Zainol et al. 2003). However, accumulation of the remaining superoxide radicals due to high dose of METH could influence the SOD2 expression although in the high concentration of CAE. This suggests that ROS plays an important role in the METH neurotoxicity effect. This present study also demonstrated that although CAE could significantly alter SOD2 expression by eliminating superoxide and attenuated oxidative stress (Massad et al. 2009) but the toxicity effect of METH overwhelmed CAE. SOD2 has been reported to be involved in the regulation of superoxide radicals in various age-related disorders and age-senescence activity (Flynn & Melovn 2013). However, numerous studies showed that expression of SOD2 with aging and neurodegenerative diseases are highly tissue-specific and not consistent across studies (Miao & St. Clair 2013).

EXPRESSION OF microRNA-34a

There was significant increase in miR-34a expression between all treatment groups compared to control. Results show that treatment of CAE alone (Group III and IV) increased expression of miR-34a compared to control. Also, there was significant increase expression of miR-34a in Group II (METH) compared to control (Figure 6). Meanwhile, for METH-induced rats, there was no significant increase in miR-34a expression following post-treatment of CAE compared to Group II (METH only).



Data are expressed as mean ± standard deviation (SD) from triplicate qPCR reaction. Control devoted as group without any treatment, Group II (METH), Group III (300mg/kg CAE), Group IV (500mg/kg CAE), Group V (METH+300mg/kg CAE) and Group VI (METH+500mg/kg CAE). ***p<0.001, **<0.01, *p<0.05 statistically significant compared to control. ### p<0.001, ##p<0.01, #p<0.05 statistically significant compared to Group II (METH)

FIGURE 6. The expression of miR-34a relative to small nuclear U6-RNA

miRNA is used as an ideal biomarker due to its sensitivity, specificity, clinical relevance, and efficacy (Krishna et al. 2019). The family of miR-34 including miR-34a has been recognized to play important roles in neuroprotection strategies (Ba et al. 205). Various studies reported that altered function and expression of miRNA has been identified in neurotoxicity, neuroinflammation and neurodegeneration of CNS. Previous study documented the upregulation of miR-34a in a specific brain region in the 3×Tg Alzheimer's disease (AD) mouse model (Li et al. 2011). In addition, miR-34a was upregulated during oxidative stress and anti-apoptosis events of hepatocellular carcinoma (HCC) tissues (Wan et al. 2017) and in the brain of aging mouse (Li et al. 2011). In this

study, we found that the treatment of METH alone (Group II) significantly upregulated the miR-34a. The possible increase in METH treatment may be due to the role of miRNAs in regulating the neuroprotection by modulating genes involved in scavenging ROS in mitochondria (Bai et al. 2011). In contrast, treatment of CAE alone (Group III and IV) was found to highly upregulate the miR34a. The CAE's antioxidant constituents may have a significant role in miRNAs expression in the CNS, as the highest expression of miR-34a were observed following CAE treatment alone (Group III and IV). It has been reported that macronutrients, micronutrients, dietary supplements and bioactive constituents from natural products can modulate the endogenous miRNAs expression (Phuah & Nagoor 2014). Quercetin, has been reported to affect multiple miRNAs expression (such as miR-132 and miR-122) involved in neurodegenerative disease (Smith et al. 2014). Thus, this present study suggests administration of CAE may modulate the miR-34a expression level in the brain of the mouse model. However, the exact mechanism and pathway triggered by CAE's bioactive constituents remain unknown.

Meanwhile, there was no significant upregulation in miR-34a expression following post-treatment of CAE (Group V and VI) compared to METH. The exact mechanisms of CAE's effect on miR-34a expression for healing neurodegenerative rats is still unclear and need to be explored. Many studies reported that post-treatment of CAE against METH may trigger the expression of SOD2 and miR-34a. Thounaojam et al. (2019) previously reported the loss of SOD2 could be a possible mechanism of miR-34a mediating the antioxidant and neuroprotective effects. For the first time, this study evaluated the dynamic change of miR-34a expression in whole brain tissue of male Sprague-Dawley rats. Mechanisms of miR-34a expression could be different between model, mechanism of disease's pathology as well as specificity of brain's region. In addition, animal models with different age, species and strain could probably yield different miR-34a expression levels. Taken together, this present study provides the first description of miR-34a expression in response to CAE on *in vivo* METH-induced neurotoxicity mouse model.

Conclusions

It can be concluded that CAE possess protective effects against METH-induced neurotoxicity on mouse model. Our findings further support that rich-flavonoids dietary could maintain the cognitive and motor performance in METH-induced neurotoxicity. These observations further confirm that CA has potential in the human CNS as an alternative treatment for CNS-related disorders.

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