

Standardized *Centella Asiatica* Increased Brain-Derived Neurotrophic Factor and Decreased Apoptosis of Dopaminergic Neuron in Rotenone-Induced Zebrafish

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Abstract— Rotenone is a pesticide that is widely used to kill insects and nuisance fish in lakes. Its used as Parkinson's Disease (PD) model inducer. The mechanism of toxicity of rotenone is primarily mediated by its potential as mitochondrial complex I inhibition. *Centella asiatica* (CA) is known as neurotonic, but how its potential protection in Parkinsonism is still unclear. In this study, we examined the effect of CA to Brain-derived Neurotrophic Factor (BDNF) as a neuroprotectant and apoptosis as hallmark of PD in rotenone-induced zebrafish (*Danio rerio*). Besides, we also measured the zebrafish motility and dopamine (DA) level in the brain. We used adult zebrafish (8 months). Its exposed to 5 µg/L rotenone and co-incubated with methanolic extract of CA by several concentrations which are 2.5, 5 and 10 µg/mL for 28 days. Motility observed for 5 minutes at 0, 7, 14, 21 and 28 days. Measurement DA by ELISA, BDNF and apoptosis by immunohistochemistry. The results showed that CA significantly ($p < 0.05$) increased motility and dopamine level in all concentration of extract. Interestingly, BDNF expression in 5 and 10 µg/mL groups had no significantly difference to the control group. Concentration 10 µg/mL could protect dopaminergic neuron from rotenone toxicity due to significantly ($p < 0.05$) decreased compare to rotenone group. Together, these data suggest that methanolic extract of CA could protect Parkinsonian syndrome conserved dopaminergic neuron through increasing BDNF as neurotrophic factor.

Keywords—*Centella asiatica*; rotenone; Parkinson's Disease; BDNF; apoptosis; zebrafish.

I. INTRODUCTION

Parkinson's disease (PD) the most common movement disorder and the second most common neurodegenerative disease after Alzheimer's disease, is characterized primarily by the loss of dopaminergic neurons in the substantia nigra pars compacta leading to a dopamine deficit in the striatum¹. The consequent dysregulation of basal ganglia circuitries accounts for the most prominent motor symptoms, including bradykinesia, hypokinesia, rigidity, resting tremor and postural instability. In addition to the typical motor symptoms, various non-motor features may develop, such as autonomic dysfunction, sleep disturbances, depression and cognitive impairment, indicating a more widespread degenerative process.

A pathological hallmark of sporadic PD is the presence of proteinaceous deposits within neuronal perikarya (Lewy bodies) and processes (Lewy neurites), mainly composed of α -synuclein, ubiquitin, neurofilaments and molecular chaperones².

Rotenone is the most potent member of the rotenoids, a family of isoflavonoids extracted from Leguminosae plants and used as an organic pesticide. Being highly lipophilic, it freely crosses the cell membrane, blood-brain barrier and causes neurotoxicity by the inhibition of complex I of the mitochondrial electron transport chain and destroy specific dopaminergic neuron, formation of ubiquitin and α -synuclein-positive nigral inclusions and motor deficit^{3,4,5,6}. Many studies have employed rotenone to generate an experimental animal model of Parkinson's disease (PD) that mimics and elicits PD-like symptoms, such as motor and cognitive decline⁷.

Zebrafish is a promising animal for modelling PD as their dopaminergic nervous system has been well characterized and they have more similar PD gene homology than *C. elegans* and *Drosophila*⁸. Zebrafish have the potential to address some of the more traditional animal model limitation; for example, they have shorter generation time and an enormous amount of offspring, although more studies are require before conclusion can be drawn regarding the viability of this animal to model PD. No studies, to our knowledge, have assessed non-motor symptoms using rotenone zebrafish model of PD. Breaud and colegeus exposed rotenone 2µg/L for 4 weeks to zebrafish found there were no alteration in locomotion, respiration or skin pigmentation, but 10 µg/L was shown to be lethal for adult. So, in this research we used rotenone 5 µg/L for 4 weeks to made parkinsonian symptoms⁹.

Centella asiatica (CA) is a small herbaceous annual plant of the family Apiaceae, and its native to Indonesia, India, Srilanka, northern Australia, Malaysia and other part of Asia. It is used as a medicinal herb in Ayurvedic medicine, traditional African Medicine, and traditional Chinese medicine¹⁰. The primary active constituents of CA are saponins (also called triterpenoids), which include asiaticosides, in which a trisaccharide moiety is linked to the aglycone asiatic acid, madecassoside and madasiatic acid.

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Other components isolated from CA, such as brahmoside and brahminoside, may be responsible for CNS and utero relaxant actions, but are yet to be confirmed by clinical studies¹¹. Our Previous study showed that methanolic extract of CA could increase, BDNF, BCL-2, decreased MAPK-p38, iNOS and apoptosis of neuronal cells of Rattus noevvegicus strain Wistar exposed to lipopolysaccharide (LPS)¹² (Khotimah, et al., 2010) and asiaticoside decreased apoptosis in LPS-induced zebrafish embryo¹³. Xu and collegeus proved that maecososide from CA protected rat induced by MPTP by improved locomotor dysfunction, increased dopamine (DA) in striatum, decreased MDA (malonedialdehyde), while increased GSH, BCL2/Bax ration, and BDNF expression¹⁴. In this research we investigated standardized methanolic extract of CA to rotenone-induced zebrafish especially on the expression of BDNF and BDNF, besides we also measured the DA level, locomotor activity and asiaticoside level as biomarker by ultra-high performance liquid chromatography (UHPLC).

II. METHOD

A. Subject

Adult male and female zebrafish were obtained from commercial suppliers from Tulungagung, East Java, Indonesia. Zebrafish identified at Hydrology Laboratory of Fishery Faculty Brawijaya University. Before treatment zebrafish adapted in semi-static 60 L tank and rear as standard procedure¹⁵. Fish fed three times daily (Tetra Bit and Color Tropical Flakes, Tetra Sales, Blacksburg, Germany), and kept on a 14:10 light–dark cycle. Water temperature was maintained between 28±1 °C. All procedures were approved by the Ethical Committee of Medical Faculty Brawijaya University

B. Collection, Extraction and Asiaticoside measurement from *Centella asiatica*

Centella asiatica was gained from Materia Medica, Batu, Malang, Indonesia. The aerial part (leaves and branch above ground) was washed and dried. Dried powder of CA (100 g) was diluted in 900 ml of 96% methanol (maceration) and evaporate in 67°C. The asiaticoside level in the extract was then measured as one of a biomarker of CA with ultra high-performance liquid chromatography (UHPLC) (Thermoscientific, Accela 1250). Mobile phase 0,1% formic acid in water and 0,1% formic acid in acetonitril, flow rate 250 µL/min, hypersil gold column (50x2.1x1.9µM).

C. Rotenone and CA Treatment

Rotenone (Sigma 8875) concentration based on explorative experiment. Bretau and colleagues⁹ used 2 µg/L rotenone and had no significantly effect on adult zebrafish. We used 2, 5 and 10 µg/L rotenone for 28 days exposure. Finally, we found an appropriate concentration was 5 µg/L. Rotenone concentration 2 µg/L had no effect on zebrafish motility and rotenone 10 µg/L caused fish died after 48 hours (data not shown). CA extract was diluted in aquadest 1 mg/mL as stock, and final concentration were 2.5, 5 and 10 µg/mL after exploration experiment. The medium was added with anti-chlorine, rotenone and CA extract, homogenized before fish introduction. Five fish placed in 25x16x12 cm tank for

each group, fed 3 times daily and change the medium every 48 hours.

D. Motility Observation

The locomotor activity of adult zebrafish was assessed in a 2L tank (LxWxH: 25x16.5x12.5 cm) filled with 2 L system water. As the normal behaviour of fish is to swim back and forth along the length of the tank, simple observation was used to determine the locomotor activity of adult zebrafish. Three vertical lines were drawn on the tank at equal distances, dividing the tank into four zones (the length of each zone was 6.25 cm). Locomotor activity was measured for 5 min by counting the number of lines that adult zebrafish crossed. Therefore, the total distance that the adult zebrafish travelled was in direct proportion to the total number of lines that the fish crossed. The locomotor activity was calculated by the total number of lines that the zebrafish crossed, divided by time, and were expressed in a number of crossed lines/5 min⁹.

E. Dopamine Measurement by ELISA

Zebrafish were euthanized using the standard NIH recommended methods by submersion in ice water (5 parts ice/1 part water, 0–4° C) for 30 seconds following cessation of opercular (i.e., gill) movement. The head part then extracted to get the protein for dopamine ELISA (Fast Track procedure base on LDN). The samples of each group gained from 3 heads of zebrafish.

F. Expression of BDNF and Apoptosis by Immunohistochemistry

After 28 days zebrafish were sacrificed to obtain the brain by decapitation of head in ice water. The head was infiltrated by buffer formalin 10% and immediately immersed in buffer formalin for 24 h preparing for paraffin block. The head was sliced (without decalcification) 0.4 µM thick and prepare for immunohistochemistry. The slide was de-paraffinized and stained based on vendor manual procedure. Primary antibody we used BDNF (SantaCruz) and TUNNEL for apoptosis (ApopTag@Peroxidase). The expression BDNF and apoptosis (brown) observed in the midbrain area of zebrafish brain. Each slide observed in 1000 times magnification for 20 field of view in different area and average the data and each group contained 3 zebrafish head (3 slide)^{16,17}.

G. Statistical Analysis

All the grouped data were statistically evaluated by SPSS/10 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. P values of less than 0.05 were considered to indicate statistical significance. All these results were expressed as mean ± S.D for 5 animals in each group.

III. RESULTS

A. Asiaticoside measurement

Asiaticoside chosen as biomarker and standard active compound in this research. Fig 1 showed the result of UHPLC running and based on standard curve, we found the asiaticoside concentration was 2.94 ppm.

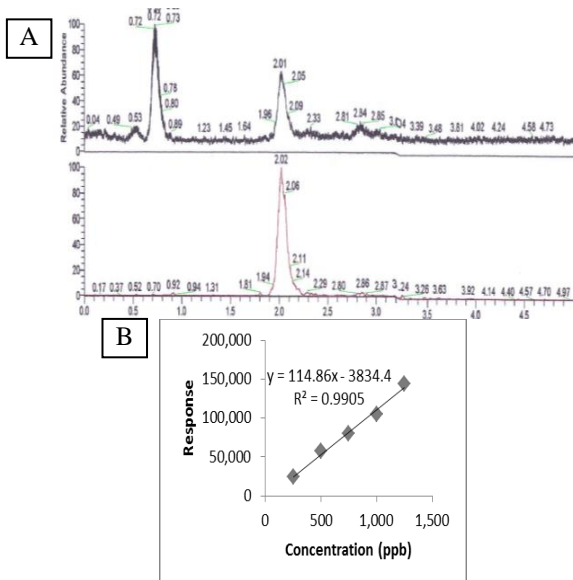


Figure 1. Ultra High Performance Liquid Chromatography (UHPLC). Sample and standard asiaticoside injection showed that retention time is around 2 minutes (left panel), and standart curve for asiaticoside.

B. Motility Assesment

We were figuring out the effect of methanolic extract CA only to the zebrafish motility (Fig.2) to know whether it could affect fish in the same concentration. And we found that CA did not significantly changed the motility. But rotenone caused decreasing locomotor activity starting at day 14th (Fig. 3).

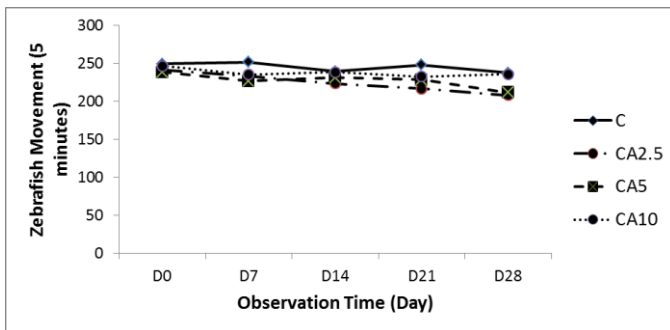


Figure 2. The mean of motility zebrafish measured on day 0, 7, 14, 21 and 28 for each group : Control ©, exposed to CA extract 2.5, 5 and 10 µg/mL. It showed that no significant differences among groups.

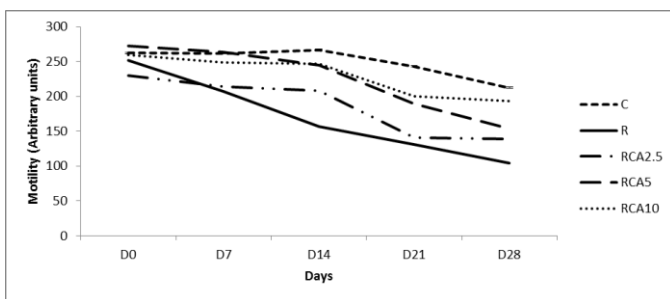


Figure 3. The mean of motility zebrafish measured on day 0, 7, 14, 21 and 28 for each group. Control (C), Rotenone (R), Rotenone and CA 2.5 µg/ml (RCA2.5), Rotenone and CA 5 µg/ml (RCA5), and Rotenone and CA 10 µg/ml (RCA10). The graph showed that the motility tend to decrease with time, but in the control group there are no significant differences amongst time observation. Rotenone decreased motility significantly starting from day 14th and continuing decreased at 21st and 28th days. CA administration gradually increased the zebrafish motility on dose dependent manner.

C. Dopamine Level

Rotenone 5µg/L for 28 days decreased dopamine level in zebrafish brain and methanolic CA extract gradually increased DA by increasing concentration of CA (fig. 4).

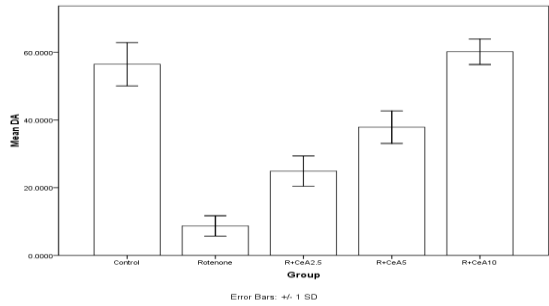


Figure 4. Dopamine level showed significantly decreased by sub-chronic exposure (28 days) of rotenone, and CA gradually increased the dopamine concentration in zebrafish brain by increasing concentration of CA. Interestingly, administration of CA 10 µg/ml had no significant difference with the control group. Different notation shows significantly different from the DA level (P<0.05).

D. BDNF Expression and Apoptosis of Dopaminergic Neuron

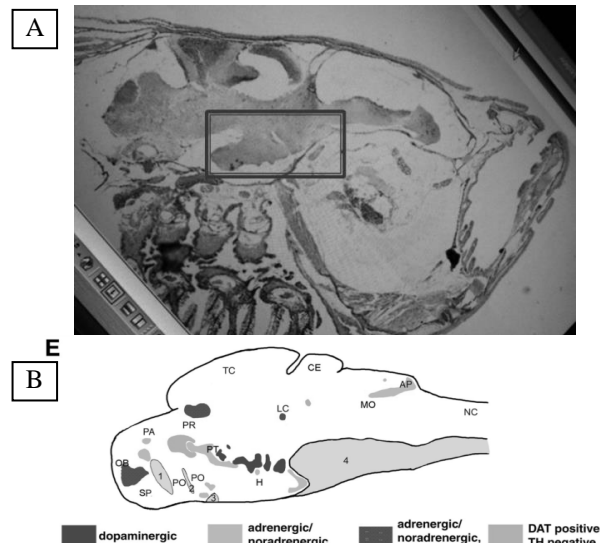


Figure 5. Histological feature of Zebrafish brain by Hematoxylin-Eosin staining of zebrafish brain (A) and Brain mapping of zebrafish¹⁸ (B). We observe Dopaminergic (DA) neuron: area in the box.

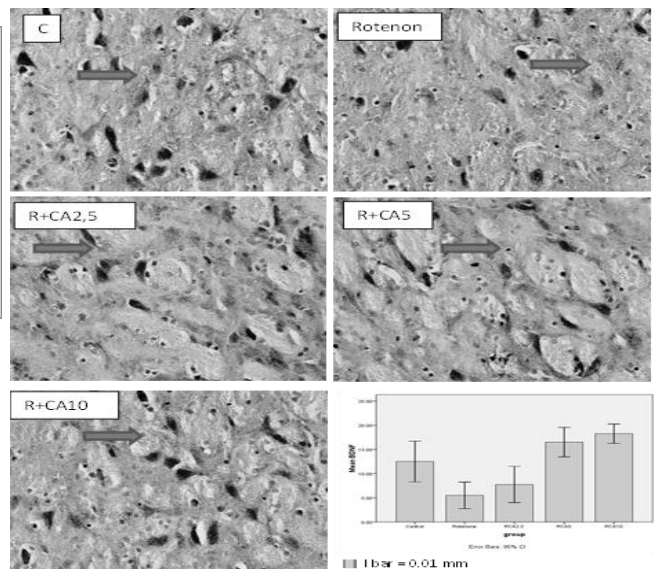


Figure 6. BDNF expression in Dopaminergic Neuron. Rotenone 5 $\mu\text{g}/\text{mL}$ significantly decreased the BDNF expression, and CA extract gradually increased BDNF in dose-dependent manner.

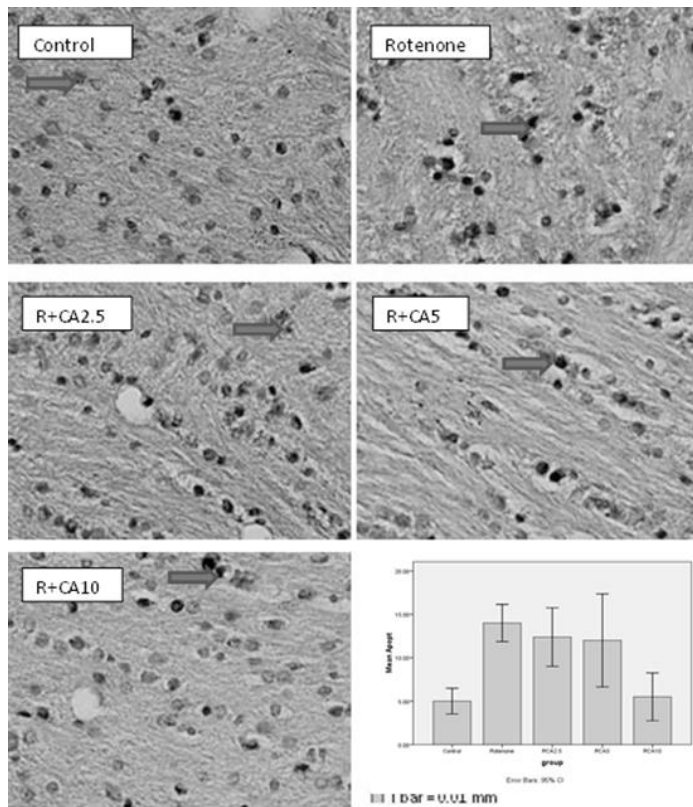


Figure 7. Apoptosis (DNA fragmentation) of Dopaminergic neuron by TUNNEL assay. Rotenone significantly increased apoptosis in dopaminergic neuron, and CA extract 10 $\mu\text{g}/\text{mL}$ significantly decreased apoptosis and had no significant different to control.

IV. DISCUSSION

A. Rotenone-induced Parkinsonian Syndrome

Rotenone is a pesticide derived from the plant roots of leguminosae family. It is highly lipophilic and therefore easily crosses all biological membranes including the blood-brain barrier; hence, it is independent of transporters for entry into cells¹⁹. Rotenone produces the pathologic hallmark features of PD because of its neurotoxic effects. Thus, rotenone exposure in rats provides a valuable model for studying mechanisms of oxidative stress induced dopaminergic damage in PD²⁰. Its lipophilic nature allows systemic administration rendering it less technically demanding than models such as 6-OHDA model, which requires stereotactic injections into the brain. Rotenone inhibits complex I of the mitochondrial electron transport chain leading to reduced ATP production and electron leakage that can form reactive oxygen species such as superoxide, subsequently causing reduced glutathione levels and oxidative stress²¹.

Fig. 3 showed the motility decreased due to rotenone-induced, while CA extract only showed stable motility (fig.2). The decreased motility significantly started from 14 days and continuing decreased until the end of experiment (28 days). This decrease may be due to the decreasing of dopamine (DA) level in the brain (fig. 4). Since its discovery as a prominent chemical neurotransmitter in the vertebrate nervous system,

dopamine (DA) is recognized to have many important physiological functions including the control of movement, cognition, affect, as well as neuroendocrine secretion²². In adult zebrafish DA neurons are conspicuously absent from the ventral midbrain, the ventral forebrain DA neurons ascending to the striatum (where ventral midbrain DA neurons in mammals project) are likely the functional counterpart of the mammalian midbrain DA neurons²³.

Increasing dopaminergic neurotransmission lead to increases locomotor activity and decreasing in dopaminergic neurotransmission lead to decreased locomotor activity²⁴. The decreasing dopamine level due to both synthesis by tyrosine hydroxylase (TH) and degradation of dopamine by free radicals. In this research showed that TH was decreased in rotenone group and increased in CA extract administration groups (data not shown). The decreasing locomotor activity of zebrafish seems due to mitochondrial dysfunction as power house of the cells that caused depleting ATP production, disruption of mitochondrial permeability, increasing Ca^{2+} and overproduction of reactive oxygen species. These conditions can lead to autooxidation of dopamine or its enzyme (TH), therefore decreasing dopamine as neurotransmitter for motility. On the other hand, stress oxidative by mitochondrial dysfunction lead to releasing caspase family protein and apoptosis of dopaminergic neuron in substantia nigra (neuronal loss). There are association between dopaminergic damage and peripheral motor nerve degeneration in an animal model of dopaminergic toxicity. Peripheral motor nerve dysfunction in rats following a chronic exposure to rotenone may serve not only as a relevant experimental model of motor neuropathy but also as a peripheral marker of dopaminergic neuronal damage to the central nervous system²⁵.

B. CA Extract Protect Zebrafish-induced Rotenone through the Increasing BDNF

Secondary metabolites from plant have important role for ecological, agriculture, industrial and medicinal aspects. It has been estimated that over 40% of medicines have their origins in these active natural products²⁶. CA is reported to contain numerous phytoconstituents (terpenes, glycosides, saponins, flavonoids, alkaloids, etc.) as secondary metabolites²⁷. The chemical composition of CA has a very important role in medicinal and nutraceutical applications and it is believed due to its biologically active components of triterpenes saponins²⁸. Major bioactive compounds of this plant contain highly variable triterpenoid saponins, including asiaticoside, asiatic acid, madecassoside, oxyasiaticoside, centelloside, brahmoside, brahminoside, thankunoside, isothankunoside and related saponins²⁹. Asiatic acid, madecassic acid and madecassoside, therefore its used as biomarker for quality assessment of CA³⁰. Methanolic CA extract in this research contain 2.94 ppm of asiaticoside (fig. 1). Many research showed the neuroprotective effect of CA through the antioxidant and anti-inflammatory property of CA³¹. Based on standard curve formula (Fig. 1,B) we found that biomarker concentration of asiaticoside was 2.9 ppm.

Our Previous study³² showed that methanolic extract of CA increased BDNF expression, decreasing of nuclear factor kappa beta (NF κ B), iNOS and apoptosis in LPS-induced neuronal cell of rat. BDNF is abundant in the brain and is important for neuronal growth, survival and differentiation of

neuronal cells in the central nervous system. In the brain, the synthesis of BDNF, as investigated in the hippocampus and cortex, is affected by neuronal activity and has a unique role in synaptic plasticity³³. It was the first neurotrophin described to promote the survival and dopamine uptake of embryonic midbrain dopaminergic neurons in vitro, thus it was considered early on as a crucial protein for Parkinson's disease (PD). BDNF enhances survival of dopaminergic neurons in the substantia nigra, whereas in patients with Parkinson's disease (PD), the expression of BDNF mRNA is decreased³⁴.

In this research, methanolic extract of CA increased locomotor activity by increasing DA level (Fig. 3 and fig. 4), while CA extract alone did not made any significant changing (Fig. 2). It seems that CA could protect from rotenone toxicity through the protection to mitochondria. CA protects rotenone-induced SH-SY5Y cells through the stability of *mitochondrial membrane potential* (MMP) and inhibiting *voltage-dependent anion channel* (VDAC) enzyme^{35,36}. Madecassoside increased inhibition of Monoamine oxidase (MAO)³⁷. MAO A and B are key isoenzymes that degrade biogenic and dietary amines. Both form can oxidize DA³⁸.

In the other hand antioxidant property of CA also have important roles to protect the dopaminergic neuron. Aqueous extract of CA on intracerebrovascular streptozocin-induced memory associated with the sporadic type of AD and pentylentetrazole (PTZ)- induced memory loss in rats showed the increasing neuroprotection through suppression of MDA (malondealdehyde) and increasing antioxidant enzyme catalase, superoxide dismutase (SOD) and glutathione^{39,40} (kumar ; Gupta). Inhibitory activity of the aqueous extract of CA that contained 84% of asiaticoside was tested by the radioenzymatic assay against phospholipase A2 (PLA2), which play a role in neuroinflammation⁴¹, and in this experiment also proved anti-inflammatory property of CA through decreasing inducible Nitric Oxide Synthase (data not shown). Not only mitochondrial protection could protect the cells from oxidative stress, but increasing endogenous antioxidant play important role in the dopaminergic protection showed by decreasing dopaminergic neuron apoptosis (Fig. 7).

Beside the role of mitochondrial protection, CA also protect the cells through the increasing of neurotrophin such as BDNF (Fig. 6) so that apoptosis could suppressed. The leaf extract of *C. asiatica* growing in China was shown to display neuroprotection through enhancing phosphorylation of cyclic AMP response element binding protein (CREB) in neuroblastoma cells in A β (1–42) proteins⁴². In this research based on the path analysis BDNF have bigger contribution to protect the dopaminergic neuron, rather than α -synuclein aggregation and dopamine conservation.

V. CONCLUSION

Our data suggest the methanolic extract of CA protect the dopaminergic neuron from rotenone toxicity through the increasing neurotrophic factor BDNF. Apoptosis or neuronal loss could decreased by BDNF protection and conservation of dopamine neurotransmitter in zebrafish-induced rotenone which administration of methanolic CA extract 10 μ g/mL.

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