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# Decreasing α-synuclein aggregation by methanolic extract of *Centella asiatica* in zebrafish Parkinson's model

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ABSTRACT

**Objective:** To observe the effects of *Centella asiatica* (*C. asiatica*) methanolic extract on α-synuclein aggregation and its expression in rotenone-exposed zebrafish.

**Methods:** Zebrafish (*Danio rerio*) were exposed to 5 μg/L rotenone for 28 days and co-incubated with 2.5, 5.0 and 10.0 μg/mL of *C. asiatica* methanolic extract. The medium was changed every 48 h for maintain the concentration of rotenone and extract. After 28 days zebrafish were sacrificed on the ice block and protein was isolated from zebrafish brain for ELISA of dopamine and Western blotting of α-synuclein. Immunohistochemistry was conducted to observe the α-synuclein expressions from histopathological preparation of zebrafish brain. The head were soaked in 10% formaline for less than 24 h and embedded onto paraffin block, then sliced for immunohistochemistry using anti α-synuclein antibody. We also measured zebrafish motility for 5 min in each week.

**Results:** *C. asiatica* has important bioactive compounds such as asiaticoside that has anti-inflammatory and antioxidant properties. It may inhibit cascade reaction due to oxidative stress induced by rotenone. Decreasing reactive oxygen species proposed probability of radical attack to α-synuclein protein that caused aggregation and increase of its expression. The motility of zebrafish was also maintained in *C. asiatica* groups due to the increasing dopamine level in rotenone-induced zebrafish. High level of reactive oxygen species inactivated enzyme for dopamine synthesis such as tyrosine hydroxylase, and oxidized dopamine itself. Oxidized dopamine increased α-synuclein aggregation. Thus, the dopamine level decreased in rotenone-induced zebrafish, but *C. asiatica* increased dopamine level.

**Conclusions:** *C. asiatica* has a potential to be developed as an anti-Parkinson's disease treatment due to its capability for minimized the sign of Parkinson's such as α-synuclein aggregation and expression, increasing motility and dopamine as well.

## 1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder caused by genetic and environmental factors. PD is also the most prevalent neurodegenerative movement disorder of adults and occurs sporadically in more than 90% of cases. While the primary motor deficits in PD arise from progressive death of dopaminergic substantia nigra neurons, the pathology of PD begins preclinically in lower brainstem nuclei with the appearance of small α-synuclein containing aggregates (Lewy neurites) [1]. α-Synuclein is an abundant presynaptic protein that binds to negatively charged phospholipids [2,3], functions as a soluble *n*-ethylmaleimide-sensitive factor attachment protein receptor complex chaperone 3 and contributes to PD pathogenesis [4,5]. α-Synuclein is a 140 amino acid protein, mainly localized in presynaptic terminals in the brain. α-Synuclein is normally an unstructured and soluble protein [6]. Although the detailed physiological functions of α-synuclein are still not clear, recent studies suggest that it plays a key role in synaptic functions [7]. Clinical diagnosis of PD, in both clinic treatment and research, relies upon characteristic symptoms and cardinal signs. The diagnosis of PD presently depends upon observation of bradykinesia, "lead pipe" rigidity, resting tremor, and subsequent loss of postural reflexes [8]. The

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action of dopamine on the aggregation of unstructured α-synuclein protein may be linked to the pathogenesis of PD. Dopamine and its oxidation derivatives may inhibit α-synuclein aggregation of non-covalent binding [9].

One valuable type of animal model for PD is established by treating animals with PD inducing neurotoxins, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, rotenone, and paraquat [10]. These neurotoxins are thought to inhibit mitochondrial complex I activity leading to oxidative stress, impaired energy metabolism, proteasome dysfunction, and, eventually, dopamine neuronal loss [11]. Rotenone is a pesticide that inhibits mitochondrial complex I activity, thus creating an environment of oxidative stress in the cell [12].

One of the newest model for PD is the zebrafish or *Danio rerio* [13]. According to studies, 70% of protein-coding human genes are related to zebrafish genes, and 84% of the genes known to be associated with human disease have a counterpart in the zebrafish genome. These findings highlight the importance of the zebrafish model in human disease research [14]. Although the molecular mechanisms involved in PD are not fully understood, much progress has been made in identifying some pathogenic pathways, such as inflammation, excitotoxicity, mitochondrial dysfunction, and oxidative stress, that might be involved in ischemic neuronal death [15]. *Centella asiatica* (*C. asiatica*) (local name: pegagan) is a herbaceous plant that might also have medicinal value. It is being used in Ayurvedic and traditional medicine preparations to improve learning and memory [16]. Published data suggest that the plant extract has nootropic effects [17], protects the brain from age-related oxidative damage [18], and promotes nerve growth and neuronal dendritic arborization [19]. Our previous findings showed that *C. asiatica* has anti-inflammatory effects in lipopoly saccharide-treated neuronal cells of rats [20].

In this research we evaluated the expression of α-synuclein and aggregation formation in rotenone-induced zebrafish treated by *C. asiatica* extract. We also analyzed zebrafish motility and dopamine levels in the brain.

## 2. Materials and methods

### 2.1. Subjects

Adult male and female zebrafish were obtained from commercial suppliers from Tulungangung, East Java, Indonesia. Zebrafish were identified in Hydrology Laboratory of Fishery Faculty of Brawijaya University. Before treatment, zebrafish were housed in semi-static 60 L tank and reared under standard procedure [21]. Fish were 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 24 and 26.5 °C. All procedures were approved by the Committee of Medical Faculty of Brawijaya University (No. 253/EC/KEPK/03/2014).

### 2.2. Collection of plant material and extraction

*C. asiatica* was gained from Materia Medica, Batu, Malang, Indonesia. The aerial part (leaves and branches above ground) was washed and dried. Dried powder of *C. asiatica* (100 g) was diluted in 900 mL of 96% methanol (maceration) and evaporated in 67 °C. Active compound composition is very important to know the potential of herbs. One of active compound that used as both standard quality and biomarker for *C. asiatica* is asiaticoside. The asiaticoside level in the extract was then measured as one of biomarker of *C. asiatica* by liquid chromatography-mass spectrometry (Thermo Scientific, Accela).

### 2.3. Rotenone and C. asiatica treatment

The used concentrations of rotenone (Sigma 8875) were based on explorative experiment. We 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 that the appropriate concentration was 5 μg/L. Rotenone concentration at 2 μg/L was found to have no effects on an explorative zebrafish motility and rotenone 10 μg/L caused fish death after 48 h (data not shown). So we used 5 μg/L concentration because it had significant effects and the zebrafish were still alive until 4 weeks. Five fish were placed in 3 tank (25 cm × 16 cm × 12 cm) for each group, fed three times daily and the medium was changed every 48 h. All fish were reared under a photoperiod of 14:10 (dark: light). Methanolic extract of *C. asiatica* was added in various concentrations (2.5, 5.0 and 10.0 μg/mL) in the same time with rotenone for 28 days.

### 2.4. Motility observation

Dysfunction of locomotor activity is clinical syndrome for PD. One of them is bradykinesia (decreasing locomotor activity). The locomotor activity of adult zebrafish was assessed in a 2 L tank (L × W × H: 25.0 cm × 16.5 cm × 12.5 cm) filled with 2 L water system. Normal behavior 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 traveled 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 expressed in the number of crossed lines/5 min (modified [22]).

### 2.5. Dopamine measurement by ELISA

Zebrafish were euthanized using the standard National Institutes of Health recommended methods by submersion in ice water (5 parts ice/1 part water, 0–4 °C) for at least 10 min following cessation of opercular (*i.e.*, gill) movement. The head part was then extracted to get the protein and dopamine level was measured by ELISA method (Fast Track procedure base on LDN's technology). The samples of each group were gained from three heads of zebrafish.

### 2.6. Western blotting

#### 2.6.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

α-Synuclein protein isolated from heads of zebrafish was added by reducing sample buffer and heated at 100 °C for 3 min. Cooled samples were injected into the wells which is 20 μL for each well. The samples (20 μg) were ran in 30 mA and 130 V until reach 0.5 cm from the bottom of the plate. The gel was stained with EtBr and destained for 20 min. After transferring the gel to nitrocellulose, the membrane was rinsed and washed

twice in phosphate buffered saline (PBS) containing 0.05% Tween20 (pH 7.5) for 2 min on shaker at room temperature (RT). The membrane was incubated for 1 h in blocking solution (5% casein in 0.05% Tween20-PBS, pH 7.5) at RT on shaker. The band corresponding to tyrosine hydroxylase was probed by incubating the membrane in monoclonal α-synuclein anti-mouse antibodies (1:1 000 dilution, Santa Cruz Biotechnology, Heidelbery, Germany Inc.) in the same blocking solution on shaker for 1 h at RT. After washing three times (5 min per wash) in washing buffer (0.05% Tween20-/PBS, pH 7.5), a further incubation with secondary antibody, rabbit anti-mouse immunoglobulin G-alkaline phosphatase, conjugated with alkaline phosphatase (1:1 500 dilution, Santa Cruz Biotechnology, Inc.) was carried out for 1 h at RT on shaker. The membrane was washed further three times for 5 min in washing buffer and incubated at RT in detection buffer (100 mmol/L Tris–/HCl, pH 9.5, containing 100 mmol/L NaCl and 50 mmol/L $MgCl_2$). Color detection was carried out by incubating the alkaline phosphatase substrate nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim, Mannheim, Germany) at RT in the same detection buffer. Reaction was stopped manually with water [23].

### 2.6.2. Immunohistochemistry

After 28 days zebrafish were sacrificed to obtain the brain by decapitation of head in ice water. The head was immediately immersed in formalin buffer before preparing for paraffin block. The head was sliced (without decalcification) 0.4 μmol/L thick and prepared for immunohistochemistry. The slide was deparaffinized and stained based on vendor manual procedure. We used α-synuclein (Sigma) as primary antibody. The expression of α-synuclein (brown) was observed in the midbrain area of zebrafish brain. Each slide was observed in 1 000 times magnification for 20 fields of view in different areas and average data and each group contained three zebrafish heads (three slides) [24].

### 2.7. Statistical analysis

All the grouped data were statistically evaluated with SPSS/10 software. Hypothesis testing methods included One-way ANOVA followed by least significant difference test. *P*-values of less than 0.05 were considered to indicate statistical significance. All these results were expressed as mean ± SD for six animals in each group.

## 3. Results

### 3.1. Asiaticoside level in methanolic extract of C. asiatica

To analyze the contents of asiaticoside we used liquid chromatography-mass spectrometry. Based on the standard of curve formula, we found that biomarker concentration of asiaticoside was 2.9 ppm for which the retention time was around

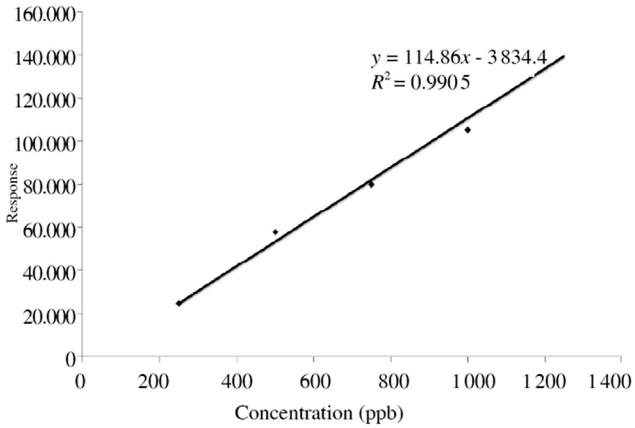


**Figure 2.** The standard curve of asiaticoside.

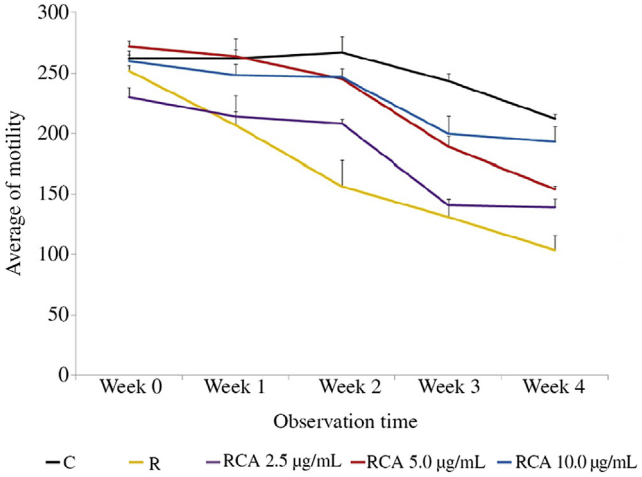


**Figure 3.** Mean of zebrafish motility measured every week for each group. C: Control; R: Rotenone; RCA: Rotenone and *C. asiatica*.

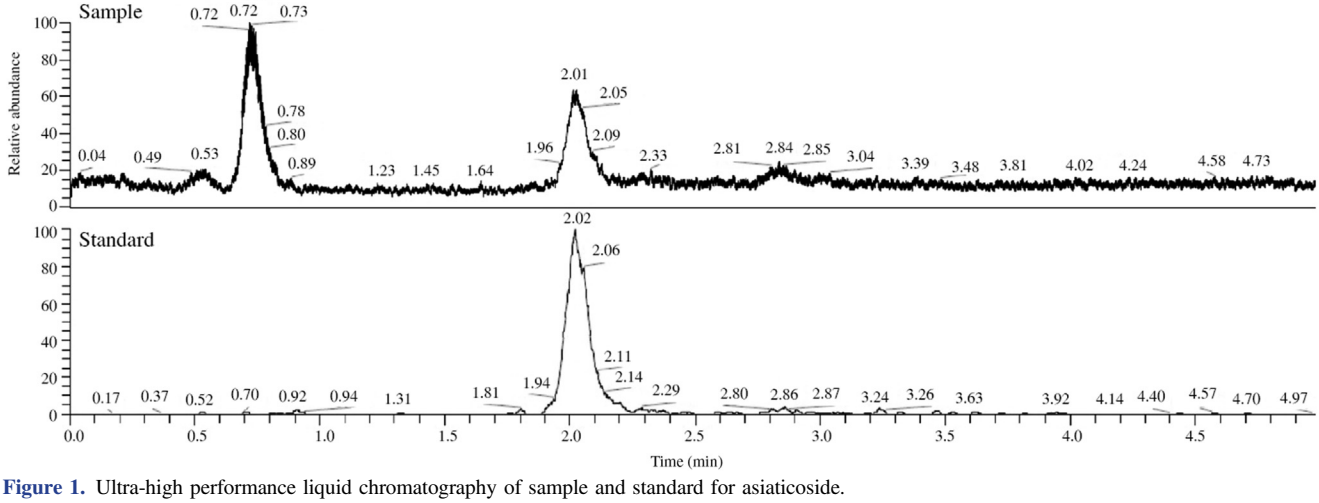


**Figure 1.** Ultra-high performance liquid chromatography of sample and standard for asiaticoside.

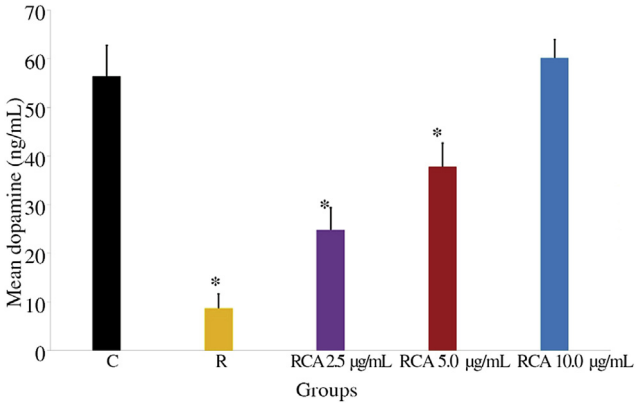


**Figure 4.** Effect of *C. asiatica* on the dopamine level in zebrafish treated with rotenone.
C: Control; R: Rotenone; RCA: Rotenone and *C. asiatica*. *: Significantly different compared with control ($P < 0.05$).

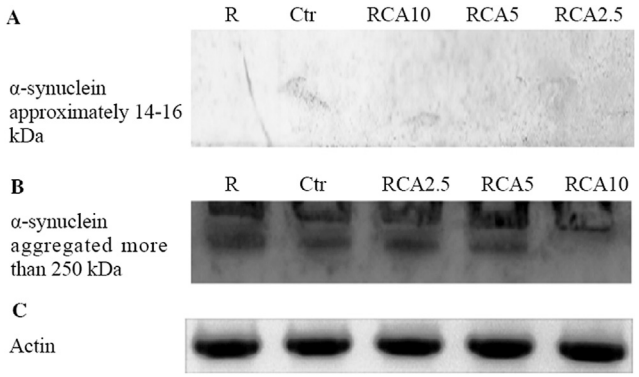


**Figure 5.** Western blot analysis of protein α-synuclein.
A: Western blot analysis of small protein α-synuclein (14–16 kDa). It showed that rotenone-induced zebrafish did not expressed this native form, but control did. And administration of 10.0 μg/mL showed the expression of protein but not 5.0 and 2.5 μg/mL; B: Protein aggregation of α-synuclein (more than 250 kDa). In the rotenone group there were very high expression of α-synuclein in aggregation form. In the control group the aggregation was still found, but the expression was slightly decreased by *C. asiatica* administration and at 10 μg/mL *C. asiatica* the aggregation was not found; C: Actin expression as control. Ctr: Control; R: Rotenone; RCA: Rotenone and *C. asiatica*.

2 min (Figures 1 and 2). Another higher peak was probably madecassoside for which the retention time was about 0.7 min.

### 3.2. Motility assessment

To know the effect of rotenone towards locomotor activity we measured the zebrafish motility for 5 min. The result showed (Figure 3) that rotenone decreased motility significantly starting from 2nd week and continuing decreased at 3rd week and 4th week. *C. asiatica* administration gradually increased the zebrafish motility in a dose dependent manner.

### 3.3. Dopamine level by ELISA method

Locomotor activity is correlated to the dopamine level in the brain. The result showed that rotenone significantly decreased dopamine level ($P < 0.05$) (Figure 4).

Administration of *C. asiatica* extract increased dopamine level in a dose dependent manner, higher concentration higher dopamine level. Interestingly, dopamine level increased significantly start from concentration 2.5 μg/mL ($P = 0.000$) and concentration of 10.0 μg/mL had no significant difference to the control group ($P = 0.644$). It seems that 10.0 μg/mL could protect dopaminergic neuron from rotenone destruction and maintain dopamine levels appropriately.

### 3.4. Western blotting of α-synuclein

Increasing free radical production in rotenone-induced zebrafish lead to aggregation form of α-synuclein and administration of *C. asiatica* could prevent the aggregation of α-synuclein in rotenone-induced zebrafish (Figure 5). To further augment our hypothesis that α-synuclein is indeed involved in rotenone-induced Parkinsonism in zebrafish, we observed α-synuclein expression by immunohistochemistry (Figure 6). In this assay the antibody recognized both native and aggregation form of this protein. Rotenone significantly increased the α-

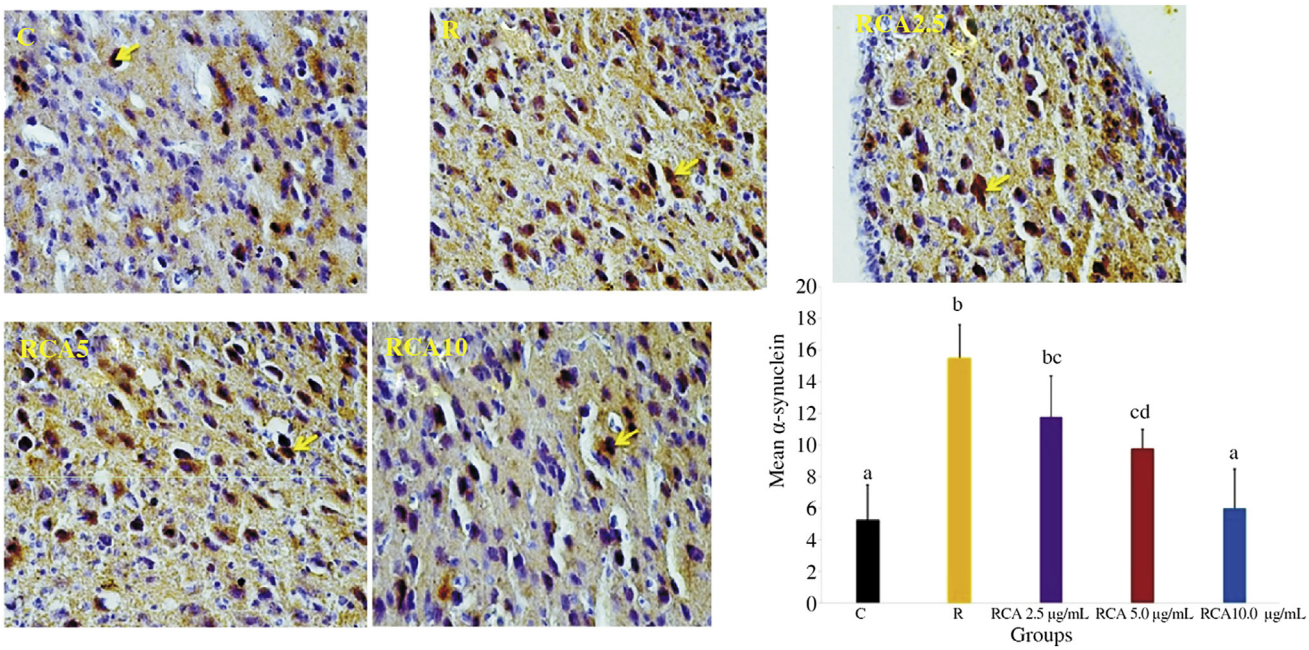


**Figure 6.** Immunoreactivity of α-synuclein.
Diaminobenzidine staining (brownish) showed that positive reactivity of α-synuclein. The graph showed the quantification of α-synuclein expression. Co-incubation of rotenone and *C. asiatica* significantly decreased the expression at 5.0 and 10.0 μg/mL. Different letters show the statistical significantly difference ($P < 0.05$). C: Control; R: Rotenone; RCA: Rotenone and *C. asiatica*. 400×.

synuclein expression (almost 300% increase, $P < 0.05$). Co-incubation of *C. asiatica* extract decreased the expression of protein at concentrations of 5 and 10 μg/mL.

## 4. Discussion

### 4.1. Methanolic extract of C. asiatica

The chemical composition of *C. asiatica* has a very important role in medicinal and nutraceutical applications and it is believed due to its biologically active components of triterpenes saponins [25]. Major bioactive compounds of this plant contain highly variable triterpenoid saponins, including asiaticoside, asiatic acid, madecassoside, oxyasiaticoside, centelloside, brahmoside, brahminoside, thankunoside, isothankuniside and related sapogenins [26]. Asiatic acid, madecassic acid and madecassoside, are used as biomarker for quality assessment of *C. asiatica* [16]. Many researches showed the neuroprotective effect of *C. asiatica* through the antioxidant and anti-inflammatory property of *C. asiatica* [27].

### 4.2. Motility assessment

The zebrafish (*Danio rerio*) has become a widely used model system for the study of development and gene function. Zebrafish are vertebrates and therefore more closely related to humans than other genetic model organisms such as *Drosophila* or *Caenorhabditis elegans* [28]. Many factors suggest that the zebrafish is a powerful tool for the study of human diseases: patterning, path finding and connectivity in the central nervous system have all been deciphered and correlated with the human central nervous system [29], touch and behavioral responses such as movement patterns can be monitored [30].

Rotenone (an inhibitor of mitochondrial reduced form of nicotinamide-adenine dinucleotide dehydrogenase, a naturally occurring toxin and commonly used pesticide) appears to reproduce the neurochemical, neuropathological and behavioral feature of PD in the rat [24]. One of cardinal sign of Parkinson's is the rigidity or decreasing ability of movement. Significant decreasing motility of zebrafish started from week 2 (14 days), which may be caused by decreasing motor nerve conducting velocity (Figure 3). Binienda *et al.* [31] proved an 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 system. Tyrosine hydroxylase, an enzyme that responsible for dopamine synthesis decreases the immunoreactivity in rotenone-induced rat [31]. Increase in dopaminergic neurotransmission leads to increased locomotor activity and decrease in dopaminergic neurotransmission leads to decreased locomotor activity [32]. It seems that the decreasing dopamine level is due to both synthesis of tyrosine hydroxylase and degradation of dopamine by free radicals.

Methanolic extract of *C. asiatica* could protect from decreasing movement ability in rotenone-induced zebrafish. This condition may be caused by antioxidant and anti-inflammatory property of *C. asiatica* [33]. Haleagrahara and Ponnusamy [34] reported that supplementation of *C. asiatica* decreased lipid hydroperoxide, protein carbonyl content, and significantly increased total antioxidant and antioxidant enzyme level in rat induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Asiatic acid, one of the major components in *C. asiatica* could protect rotenone-induced SH-SY5Y cell through the stabilization of mitochondrial membrane potential, decreasing voltage-dependent anion channel both in mRNA and protein level [35]. So, it seems that antioxidant property of *C. asiatica* could protect the zebrafish brain from free radical production by rotenone and decrease auto-oxidation of dopamine neurotransmitter in pre-synapse.

### 4.3. Dopamine level and α-synuclein aggregation

Rotenone is extremely lipophilic, which crosses biological membranes easily and independently of transporters (unlike 1-methyl-4-phenylpyridinium), and it gets into the brain very rapidly [36]. The chronic, uniform, systemic inhibition of complex I caused (over a period of days to weeks) selective degeneration of the substantia nigra dopamine neurons [23,37]. Chronic rotenone exposure leads to mitochondrial respiratory chain inhibition and dopaminergic neuronal loss. At low doses, rotenone alters calcium signaling and can induce oxidative stress, apoptosis and α-synuclein aggregation, which is also a characteristic of early stages of PD [38]. Dopamine metabolism through monoamine oxidase and cathecol-O-methyltransferase contributes to free radical production. These free radicals could interact with α-synuclein to form aggregate of α-synuclein. There is evidence that α-synuclein can interact with the dopamine transporter and tyrosine hydroxylase to regulate the amount of dopamine synthesized and the storage of catecholamines into synaptic vesicles [39]. The evidence of apoptotic in dopaminergic neuron of substantia nigra is decreased dopamine production.

α-Synuclein is a 14 kDa (140 amino acids) presynaptic protein abundant in various regions in the brain but its natural functions are still a matter of speculation [40]. Under physiological conditions, α-synuclein adopts a random conformation, natively unfolded. But if exposed to low pH, high temperature, organic solvents, metal ions, pesticides and so on, it could aggregate in the cell membrane [41] and form ion-permeable pores in the lipid bilayer, resulting in the change of the membrane potential and the subsequent cell dysfunctions [42,43]. The aggregation of α-synuclein in PD always arrests much attention, it has been supposed to be the primary events in the pathogenesis of PD [44].

The major active compounds of *C. asiatica* are known as asiatic acid, madecassic acid (6-hydroxy-asiatic acid), asiaticoside, madecassoside, and madasiatic acid, betulinic acid, thankunic acid, and isothankunic acid [45,46]. Ethanolic extract of *C. asiatica* could inhibit acetylcholinesterase, butyrylcholinesterase and tyrosinase. These enzymes are important in PD since these enzymes play a role in neuromelanin formation in human brain and could be significant in occurrence of dopamine neurotoxicity associated with neurodegeneration linked to PD [47]. The leaf extract of *C. asiatica* growing in China was shown to display neuroprotection through enhancing phosphorylation of cAMP response element binding protein in neuroblastoma cells in Aβ (1–42) proteins found within the amyloid plaques occurring in the brains of Alzheimer's disease patients [48], as we know that cAMP response element binding protein is important for brain-derived neurotrophic factor (BDNF) synthesis. BDNF

has important survival and neuroplasticity [49], and in the present research BDNF level were found to be increased by *C. asiatica* (data not shown).

Inhibitory activity of the aqueous extract of *C. asiatica* that contained 84% of asiaticoside was tested by the radioenzymatic assay against phospholipase A2, which plays a role in neuro-inflammation [50]. Aqueous extract of *C. asiatica* on intra cerebrovascular streptozocin-induced memory associated with the sporadic type of Alzheimer's disease and pentylenetetrazole-induced memory loss in rats showed the increasing neuro-protection through suppression of malondialdehyde and increasing antioxidant enzyme catalase, superoxide dismutase and glutathione [51,52]. Another experiment showed that *C. asiatica* protects rotenone-induced cells through the stabilization of mitochondrial membrane potential and inhibiting voltage-dependent anion channel enzyme [34,35]. It seems that antioxidant and anti-inflammatory property of *C. asiatica* could protect rotenone-induced zebrafish motility through the stabilization of dopamine synthesis, availability and its kinetics. This condition is supported by conserving and protecting α-synuclein to its native form that inhibit progression of dopaminergic neuron degeneration.

Methanolic extract of *C. asiatica* containing asiaticoside could improve the ability of movement in rotenone-induced zebrafish PD model through the stability of dopamine neurotransmitter and the decrease of α-synuclein aggregation at concentrations of 5 and 10 μg/mL.

## Conflict of interest statement

We declare that we have no conflict of interest.

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