

COMPARISON EFFECT BETWEEN PEGAGAN (*Centella asiatica*) EXTRACT AND PRAMIPEXOLE TOWARD LOCOMOTOR ACTIVITIES, α - SYNUCLEIN, AND NRF2 EXPRESSION IN ZEBRAFISH PARKINSON MODEL

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ABSTRACT

Background: Parkinson disease is characterized with deposition of *Lewy Bodies* containing α -synuclein happened due to the effect of chronic neuroinflammation that causes the death of dopaminergic neurons through oxidative stress processes, so it involves the response of Nuclear factor erythroid 2-like 2 (Nrf2). *Centella asiatica* (*C. asiatica*) contains antioxidant effect, inhibits the aggregation of α -synuclein and improves the locomotor on Parkinson-model animals so it needs to compare to the standard medication.

Objective: To compare the *C. asiatica* extract and Pramipexole to the zebrafish Parkinson model by determining the locomotor activity, α -synuclein expression, and Nrf2.

Methods: This study used six groups of zebrafish: negative control, rotenone [5 μ g/L], pramipexole [7 ng/mL], 2, 3 (rotenone + pramipexole [3,5] ng/mL, [7] ng/mL, [14] ng/mL), and *C. asiatica* (rotenone + *C. asiatica* [10] μ g/mL). The observations of locomotor activity of day 0, 14, and 28 were continued to the α -synuclein immunohistochemical examination, and Nrf2 on the midbrain area.

Results: There are significant differences in locomotor activity on day 28 among the *C. asiatica* group with rotenone ($p < 0,05$), while there are no significant differences among the *C. asiatica* group with pramipexole [7] ng/mL and [14] ng/mL ($p > 0,05$). α -synuclein expression of the *C. asiatica* group is the lowest and significantly different from all groups ($p < 0,05$), while Nrf2 had no significant differences ($p > 0,05$).

Conclusion: *C. asiatica* extract [10] μ g/mL is equal to pramipexole [7] ng/mL and [14] ng/mL in improving locomotor activity, but *C. asiatica* extract holds excellence as it decreases α -synuclein expression better than pramipexole, while Nrf2 expression shows no differences.

Keywords: *Centella asiatica*, locomotor activity, α -synuclein, Nrf2, Parkinson disease, zebrafish

Introduction

Parkinson disease is a chronic and progressive aging disease characterized by hypokinesia, resting tremor, rigidity and postural instability along with *Lewy bodies* dominated by α -synuclein protein.^{1,2} This disease can develop at the age between 45 years and 75 years, with prevalence in the world ranging from 160 per 100,000 population and incidence ranging from 20 per 100,000 population with a little bit higher incidence rates in industrial and agrarian countries with more pesticide use.^{2,3} In addition to genetic mutations, pesticide exposure offers 95% of sporadic occurrences of Parkinson's disease.^{1,4,5} Dopamine degradation in the substantia nigra pars compacta resulting from the death of dopaminergic neurons in Parkinson disease causes interference in movement coordination.^{6,7}

Rotenone is a pesticide derived from *Derris spp.*, *Lonchocarpus spp.*, and *Tephrosia spp.*, which can cause Parkinson disease through the hindrance to chain 1 complex

of the mitochondrial respiration and causes oxidative stress, apoptosis that ends in selective cell death in dopaminergic neurons.^{8,9,10,11} Rotenone can also cause *in vivo* accumulation of α -synuclein, beta-amyloid aggregation, tau hyperphosphorylation, loss of dopaminergic neurons and the death of striatal cell neurons and lead to decreased locomotor activity in experimental animals such as rats and zebrafish.^{11,12,13,14,15,16}

Treatment of Parkinson disease may be pharmacological therapy with drugs and non-pharmacological such as stem cells.¹⁶ for Parkinson disease presently intends to control the symptoms occurred by replacing dopamine (*levodopa*) which reduces in number or use agonist dopamine such as pramipexole that works directly on Dopamine D2 receptors.^{17,18} Both classes still have an insufficiency of the high incidence of dyskinesia and *wearing-off* after long-term use or after passing the "honeymoon period". Pramipexole is said to be better than levodopa because of the probability of smaller motor complications, and has neuroprotective effects on mitochondrial ROS inhibition,

increased glutathione (GSH) and BDNF^{20,21,22,23,24} Currently, it is considered a therapy that can reduce the progressiveness of Parkinson disease through the hindrance in α -synuclein aggregation formation which becomes one of the foundations of pathogenesis of Parkinson disease, and Nuclear factor erythroid 2-like 2 (Nrf2) is also considered to be the potential pharmacological target for neuroprotective therapy in Parkinson disease with the aim of enhancing the transcriptional activity of Nrf2 which is a transcription factor inducing the antioxidant gene.²⁵

C.asiatica is herbal plants that have the main content such as *asiatic acid*, *asiaticoside*, *madecassic acid*, and *madecassoside*^{26,27} already known and used as an analgesic, antidepressant, antimicrobial, antiviral, immunomodulator in Southeast Asia and India. *C.asiatica* also holds antioxidant effect and neuro-protectant through the decreased superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase, and it is also able to decrease α -synuclein aggregation and increase BDNF on Parkinson model experimental animal.^{28,29,30,31,32,33} *C.asiatica* extract with a concentration of 10 μ g/L provides an increase in tyrosine hydroxylase (TH) which is a tyrosine catalyst enzyme into L-dihydroxyphenylalanine (L-dopa) which later turns to dopamine, accompanied by improved locomotor activity, as well as decreased α -synuclein expression in zebrafish Parkinson model.^{34,35}

Zebrafish is one of the Parkinson model animals which have the structure and function of organs including a dopaminergic system with genetic homology with mammals reaching 70 - 80%. Besides, zebrafish has other advantages like easy maintenance, affordable, and fast breeding.^{36,37,38,39} This study is an advanced study aimed to compare the effects of the administration of *C.asiatica* with concentration of 10 μ g/mL, which is known to have a positive effect on zebrafish Parkinson model in previous studies, with pramipexole [3,5 ng/mL], [7 ng/mL] and [14 ng/mL] as one of medication for Parkinson disease. The study is established on zebrafish Parkinson model through rotenone exposure, then the locomotor activity, α -synuclein expression, and Nrf2 on the brain were valued.

Methods

This study is a laboratory experimental, randomized pre and post test control group design, using 30 zebrafish aged 8 - 10 months divided into 6 groups i.e group 1 negative control, group 2 positive control given rotenone [5] μ g/L, group 3, 4, and 5 respectively were given rotenone [5] μ g/L plus pramipexole [3,5] ng/mL, [7] ng/mL and [14] ng/mL, and group 6 were given rotenone [5] μ g/mL plus *C. asiatica* extract [10] μ g/mL.

Producing *C. asiatica* Extract

C. asiatica was obtained from Balai Materia Medika Batu. The leaves and stems that are above the ground were washed and then dried using an oven with a temperature of 40°C then are well-blended. As much as 100 g of dried *C. asiatica* was soaked with 96% methanol to 900 ml volume and shaken for \pm 30 minutes and was deposited for a night then filtered using filter paper. The solution of the extraction process is evaporated to ¼ of dry matter. The *C. asiatica* extract is stored in the freezer 4°C in plastic or glass bottles as stocks with a concentration of 10 g/L (10 mg/ml) in distilled water solution.

Producing Pramipexole Solution

Pramipexole (SIGMA A1237) in the form of 10 mg powder was dissolved in 1 ml of distilled water then 20 μ l of the solution was dissolved in 1980 μ l distilled water to be a stock solution. Pramipexole [3.5] ng/ml, [7] ng/ml, and [14] ng/ml were obtained by taking stock solutions as much as 70 μ l, 140 μ l, and 280 μ l.

Producing Rotenone Solution

Rotenone (SIGMA 8875) powder weighed 20 mg was dissolved in 1 ml of DMSO (dimethyl sulfoxide) then diluted to produce 2 x 105 μ g/l stock solutions with rotenone replacement solution from 2 x 107 μ g/l stock as much as 10 μ l, then added distilled water as much as 990 μ l (DMSO level 1%). A concentration of 5 μ g/l was administered by giving rotenone from 2 x 105 μ g/l stock as much as 50 μ l (DMSO concentration of 0.25 ppm).

Administration of Rotenone, Pramipexole, and *C. asiatica* on Zebra Fish

Administration of rotenone, pramipexole, and *C. asiatica* extract is dissolved into an aquarium containing 2 L of fresh water that was previously filtered using *Pure It*[®] according to the respective concentration. Aquarium water replacement, giving of rotenone, pramipexole, and the *C. asiatica* extract was administered every 2 days.

Measuring Locomotor Activity

Locomotor activity was carried out in the aquarium containing 2 L of water measuring 25 x 16.5 x 12.5 cm by giving three vertical lines at the bottom of the aquarium so as to divide it into 4 sections measuring 6.25 cm. Each wall of the aquarium was closed by using white paper to reduce environmental influences. Zebrafish were allowed to move actively, be observed and recorded using a video camera for 5 minutes with previous adaptation for 3 minutes. The movement of the fish was calculated from the video recording by counting and summing the fish move crossing the striped area for 5 minutes.⁴⁰

Sample Preparation of the Zebrafish Brain

After 28 days, the zebrafish was sacrificed by placing on an amount of ice with a little water. After not showing a spontaneous movement, it is decapitated, then the fish head was taken, inserted into a bottle filled with formalin buffer solution and stored at room temperature. Furthermore, the brain tissue was incised and made a slide with paraffin block. Cutting was done sagittally with a thickness of 0.4 μ m starting from the center of the head of the zebrafish towards the lateral.

Calculation of α - synuclein Expression and Nrf2 with Immunohistochemistry

Before the slide was deparaffinized, the slide was preheated at 60°C for 60 minutes in a dry incubator and then added with the following solutions in order: xylol (2x10 minutes), absolute ethanol (2x10 minutes), 90% ethanol (1x5 minutes) ethanol 80% (1x5 min), ethanol 70% (1x5 min), sterile distilled water (aquadest) (3x5 min) followed by retrieval antigen with chamber decloaking. The slides which were prepared for Immunohistochemical examination were dripped with 4% H₂O₂ in methanol, and inserted chamber for 15 minutes and then washed with sterile PBS (*paraformaldehyde buffer solution*) for 3x5 minute. After that, a non-specific protein blocking the process by dripping the background sniper (Biocare

Startrex) is established for 30 minutes at room temperature and then washed with sterile PBS for 3x5 minutes.

The incubation process of primary antibodies was administered by dripping the primary antibody α -synuclein (Santa Cruz) or Nrf2 (Santa Cruz) according to the vendor's manual procedure. Furthermore, the slide was dripped with DAB chromagen (DAB chromogen: DAB buffer = 1:40), incubated for 5 minutes at room temperature, washed with sterile PBS for 3x5 min, and then rinsed with distilled water for 3x5 minutes. After that, the slides were given counterstain with Mayer's Hematoxylen, by dripping Mayer's Hematoxylen: distilled water with comparison 1:20, incubated for five minutes at room temperature, and rinsed with distilled water. The last step of immunohistochemical coloring is the mounting process, with Entellan, then the sides were dried and the observation using microscope was administered.

The calculation of cell total expressing α -synuclein and Nr2 was done by using "hot spot" method aided with binoculars microscope in 10 different fields of view in the midbrain area ($M=1000\times$). Positive cells showed a brown-stained cytoplasm.³³

Processing and Analysis of Data

All data were statistically analyzed using SPSS 20 software using one-way ANOVA and followed by a post hoc test least significant difference (LSD) to compare mean differences between groups of experimental animals. Statistical test results are significant if the value of $p < 0.05$.

Results

Locomotor Activity

The locomotor activity of zebrafish within five minutes in day 0, 14, and 28 in all treatment groups and the analysis result off one-way ANOVA test can be seen in Table 1, the analysis result of post hoc LSD can be seen in Figure 1, and the curve of time series of the entire groups is presented in Figure 2.

Table 1. The comparison of locomotor activity and the analysis of one-way ANOVA test at various times of each treatment group.

Group	Number of Locomotor Activity per 5 minutes (\pm SD)		
	Day 0	Day 14	Day 28
Control (-)	113.67 (± 11.06)	193.00 (± 5.57)	144.67 (± 12.66)
Rotenone	112.00 (± 10.44)	101.00 (± 6.08)	67.00 (± 7.94)
R + Pmx 3,5	119.33 (± 14.01)	83.67 (± 8.39)	142.00 (± 9.85)
R + Pmx 7	86.67 (± 1.15)	96.67 (± 10.26)	100.00 (± 2.00)
R + Pmx 14	92.67 (± 9.02)	97.00 (± 6.08)	101.00 (± 25.71)
R + <i>C. asiatica</i>	109.00 (± 3.61)	97.67 (± 11.02)	97.33 (± 6.66)

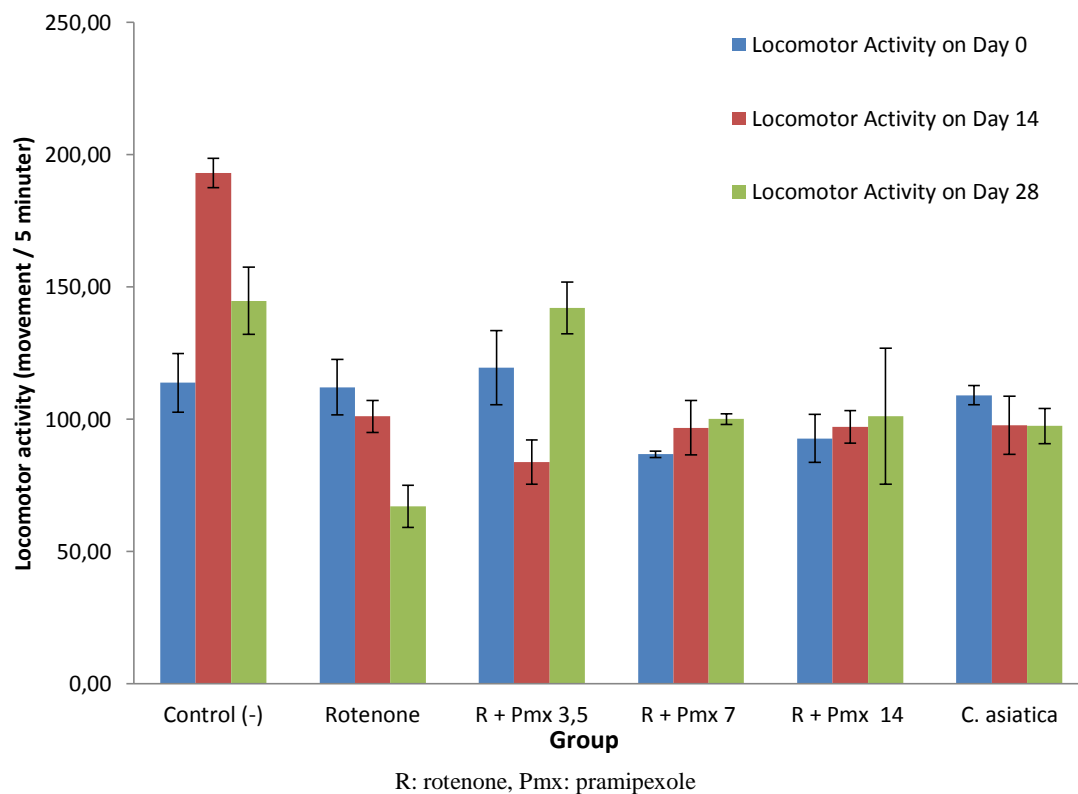


Figure 1. LSD test results analysis of zebrafish locomotor activity on day 0, 14, and 28.

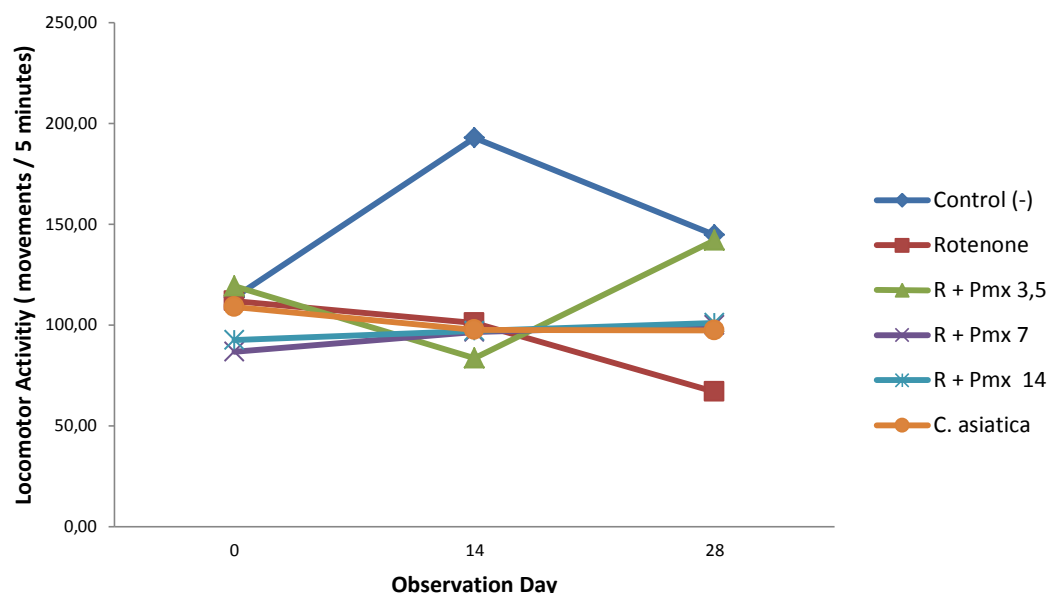


Figure 2. The zebrafish locomotor activity on day 0, 14, and 28.

The result of one-way ANOVA test showed the mean value of the locomotor activity of all treatment groups on day 0, 14, and 28 is significantly different ($p < 0.05$). Later, it was continued with post hoc test LSD, and it was found in the observation day 0 significant difference among control groups, rotenone, pramipexole [3.5] ng/mL, and *C. asiatica* with pramipexole [7] ng/mL and [14] ng/mL. On the rotenone group showed significant progressive degradation on day 14 and 28. Pramipexole [3.5] ng/mL on day 14 showed degradation but it was back increased on day 28. Pramipexole [7] ng/mL and [14] ng/mL and *C. asiatica* was able to maintain the locomotor activity on day 0, 14, and 28.

Immunohistochemical Examination

The observation of α -synuclein and the Nrf2 expression on zebrafish was carried out in the midbrain area in line with Figure 3.

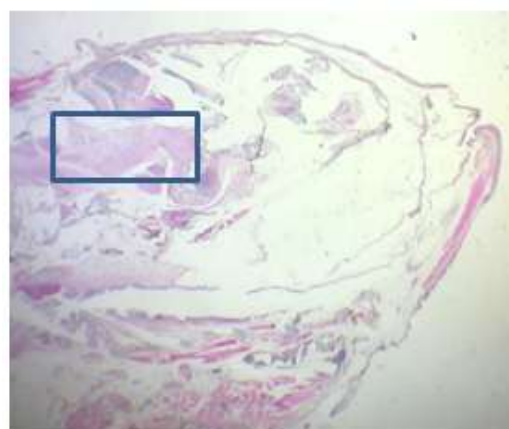


Figure 3. Sagittal Incision on zebrafish head with hematoxylin and eosin coloring. The area inside the blue box is the area in which the immunohistochemical observation was established.

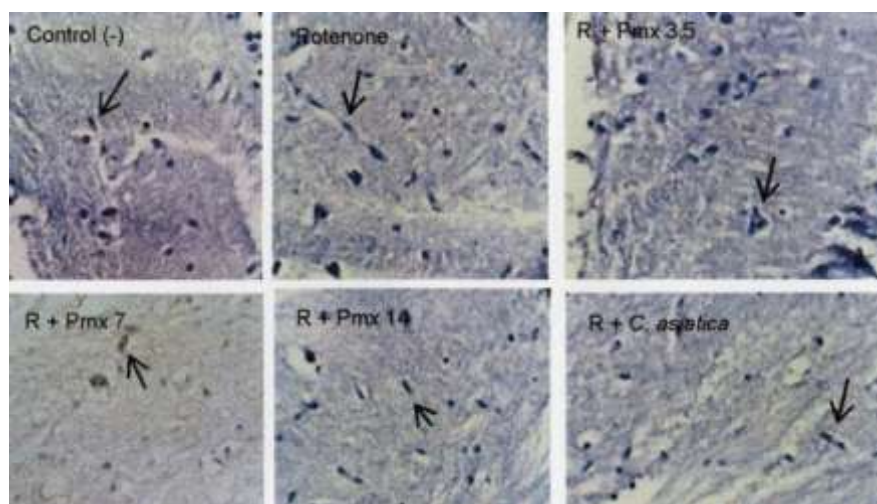


Figure 4. α -synuclein Expression observed with on the midbrain area of each group (M=1000x). Arrow \uparrow represented the cell expressing α -synuclein, having brown-stained cytoplasm. Rot: rotenone, Pmx: pramipexole.

The calculation of α -synuclein expression on each group analyzed by using one-way ANOVA test can be seen in Table 2 and the analysis result of post hoc LSD can be seen in Figure 5.

Table 2. Mean of α -synuclein expression on day 28 and the analysis result of one-way ANOVA of the zebrafish in each group.

Group	α -synuclein expression (\pm SD)		Sign. (p)
Control (-)	33.67	(\pm 1.53)	0.014
Rotenone	66	(\pm 5.57)	
R + Pmx 3,5	53.33	(\pm 11.37)	
R + Pmx 7	53.33	(\pm 9.50)	
R + Pmx 14	49	(\pm 5.57)	
R + <i>C. asiatica</i>	47.33	(\pm 12.06)	

R: rotenone, Pmx: pramipexole

The result of one-way ANOVA test showed the α -synuclein expression among groups were significantly different ($p < 0.05$). It was continued with Post hoc test LSD and it was obtained that the rotenone group showed the highest α -synuclein expression, but there was no significant difference with the pramipexole group with a concentration of 3.5 ng/mL and 7 ng/mL. *C. asiatica* held the lowest value and was not significantly different from the control group.

Nrf2 Expression

The result of the Nrf2 immunohistochemical on each group is presented in Figure 6 with using binoculars microscope on the field of view 10 ($M=1000\times$). The positive cell will have brown-stained cytoplasm.

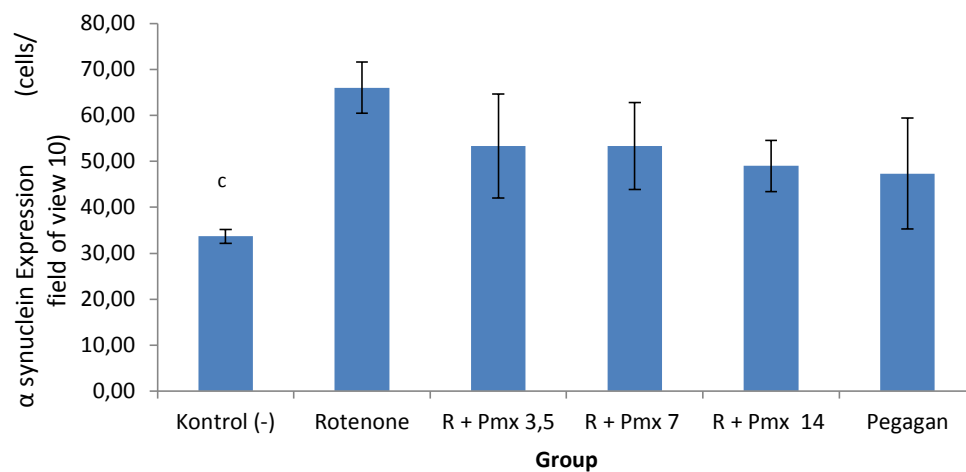


Figure 5. The Mean of the α -synuclein and LSD test. Rot: rotenone, Pmx: pramipexole.

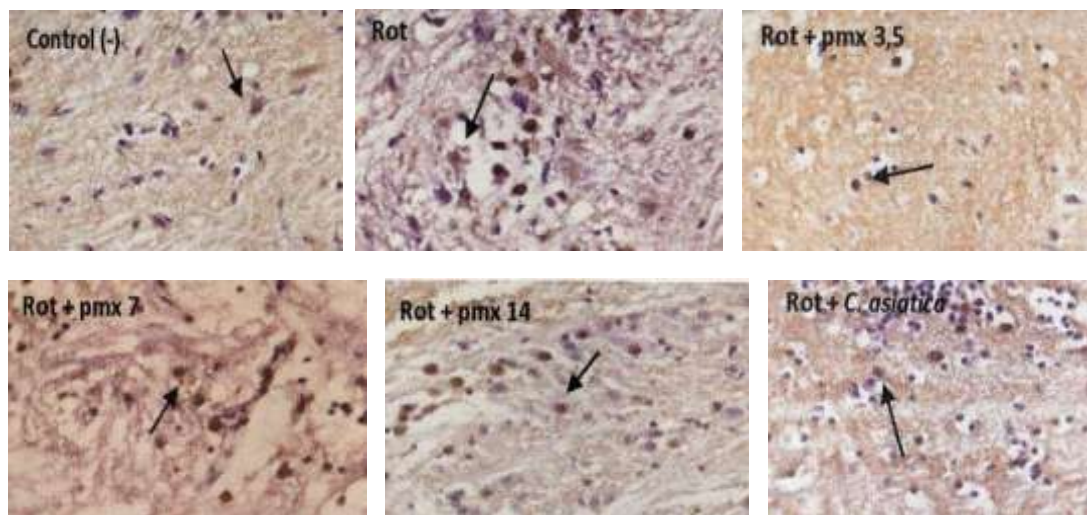


Figure 6. Nrf2 expression seen in the midbrain area of each group ($M=1000\times$). Arrow represented the cell expressing Nrf2, having brown-stained cytoplasm. Rot: rotenone, Pmx: pramipexole.

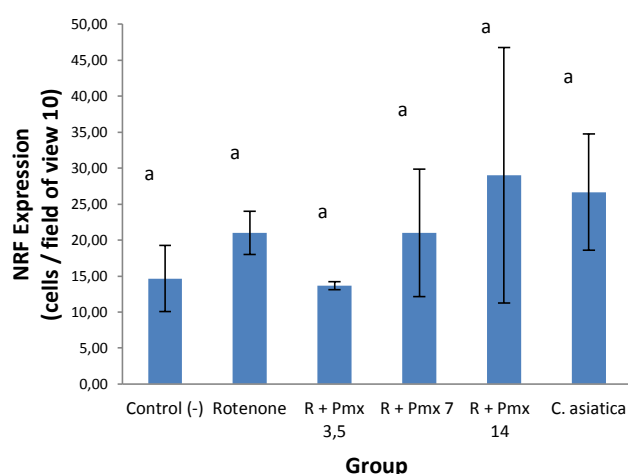
The calculation of Nrf2 expression on each treatment group analyzed by using one-way ANOVA test can be seen in

Table 3 and the analysis result of post hoc LSD can be seen in Figure 7.

Table 3. Mean of Nrf2 expression on day 28 and the analysis result of one-way ANOVA of the zebrafish in each group.

Group	Nrf2 expression (\pm SD)		Sign. (p)
Control (-)	14.67	(\pm 4.62)	0.294
Rotenone	21	(\pm 3.00)	
R + Pmx 3,5	13.67	(\pm 0.58)	
R + Pmx 7	21	(\pm 8.89)	
R + Pmx 14	29	(\pm 17.78)	
R + <i>C. asiatica</i>	26.67	(\pm 8.08)	

R: rotenone, Pmx: pramipexole

**Figure 7.** Graph of Mean of the Nrf2 and LSD test. Rot: rotenone, Pmx: pramipexole.

The result of one-way ANOVA showed that the sig value (p -value) 0.294 is greater than alpha 0.05 ($p < 0.05$), thus it can be concluded that the treatment group had no significant effect on Nrf2.

Discussion

Locomotor Activity

Zebrafish holds a basic motor response of locomotor activity including the speed of swimming, distance, and rotational speed.⁴¹ Zebrafish are sensitive to the administration of neurotoxins including rotenone pesticides that may cause decreased dopaminergic neurons, thyroxine hydroxylase (TH), increased α -synuclein levels, oxidative damage, and increased apoptotic activity.^{14,42} In this study, locomotor activity in the rotenone group decreased progressively until day 28. This result was in line with the previous research where [5] μ g/L rotenone exposure showed decreased motility, whereas [10] μ g/L could cause death in zebrafish,^{40,43} in line with the study conducted by Wrangel et al., 2015, that rotenone-exposed rat showed a general idea of Parkinson disease including decreased motor activity accompanied by loss of dopamine in the nigrostriatal system.⁴⁴ Similarly, a 28-day sub-chronic rotenone administration in zebrafish can lead to decreased dopamine, increased α -synuclein expression and aggregation resulting in the apoptosis of dopaminergic neuron cells leading to the symptoms of parkinsonism throughout decreased locomotor activity.⁴³

This study showed that *C. asiatica* extract is able to maintain the locomotor activity until day 28 better than the administration of rotenone only. This result is in line with the previous study, where the administration of *C. asiatica* [2,5] μ g/L and [10] μ g/mL to zebrafish exposed by rotenone can increase motility.³¹ The administration of the *C. asiatica* active content such as *madecassoside* and *asiaticoside* can prevent the decreased motor activity in rats Parkinson model. Locomotor activity is connected to the level of dopamine, in which the administration of that active content is able to protect dopaminergic neurons on rats Parkinson model exposed with MTPT through the increased glutathione (GSH) which is an antioxidant and the decreased Bcl2/Bax ratio^{45,46} besides, the *C. asiatica* extract is able to decrease apoptosis of dopaminergic neurons in zebrafish Parkinson model exposed with rotenone.³³

Pramipexole [3,5] ng/mL showed the highest improved locomotor activity on day 28 and it did not differ significantly with the control group, but on day 14, it had decreased, while [7] ng/mL and [14] ng/mL are similar to *C. asiatica* capable of maintaining locomotor until day 28.

α -synuclein Expression

α -synuclein protein is the main component of Lewy bodies that becomes one of the pathogenesis of Parkinson disease found in both sporadic and genetic Parkinson disease, which are normally in synapses in unfolded form, but at high concentrations may aggregate.³ A number of pesticides including rotenone can cause α -synuclein aggregation in vitro as well as in neuron cultured cells so rotenone can be one of the factors causing Parkinson's disease.^{47,15}

This study showed all groups exposed to rotenone experienced increased α -synuclein expression if compared to the negative control, with the highest increase was 100% obtained in rotenone group. This is in line with the study conducted by Khotimah, Ali *et al.*, 2015 showing the increased α -synuclein aggregation up to 300% in zebrafish midbrain area exposed with rotenone and then connected to the decreased locomotor activity describing the clinical form of bradykinesia parkinsonism occurring in Parkinson disease.³⁴

α -synuclein expression in *C. asiatica* group showed the lowest result and it did not differ significantly with the control group, thus it can be concluded that the administration of *C. asiatica* extract is able to perform the decreased α -synuclein expression exposed with rotenone. This is in line with the previous study in which the administration of *C. asiatica* extract [5] and [10] μ g/mL is able to decrease α -synuclein aggregation if given with rotenone.³⁴ *C. asiatica* extract is able to inhibit the change of α -synuclein monomer form to oligomers by maintaining α -synuclein in spiral form, and inhibit the oligomers enduring to form aggregated filaments.⁴⁸

Pramipexole group [3.5] ng/mL, [7] ng/mL, and [14] ng/mL showed that the α -synuclein expression did not significantly differ from *C. asiatica*, but pramipexole [14] ng/mL was better compared to the other two concentrations because they were still significantly different from rotenone group.

Excessive α -synuclein aggregation can directly inhibit TH activity resulting in decreased dopamine, it can also inhibit the work of vesicular monoamine transporter 2 (VMAT2)

used in the inclusion of dopamine cytosol into vesicles, so that the accumulation of dopamine in the cytosol can increase oxidative stress that leads it to the death of dopaminergic neurone cell.^{49,50}

Nrf2 Expression

Nrf2 is a transcriptional factor that induces antioxidant genes and detoxification enzymes and has a major role in protecting various organs and tissues including the heart, kidneys, liver, and brain of the body from environmental oxidants such as electrophiles, reactive oxygen species (ROS) and reactive nitrogen species (RNS).^{51,52}

This study showed that there was no significant difference statistically of the Nrf2 expression in all treatment groups. The high expression of Nrf2 in the negative control group may be related to the Nrf2 function as a self-defense mechanism for the cell. Activation and inactivation of Nrf2 are required to maintain the redox cell homeostasis.⁵¹ The natural process of natural oxidation in the fish body can also activate Nrf2.

The nrf2 expression on treatment group with rotenone, pramipexole, and *C. asiatica* may be related to the sub-chronic oxidant exposure causing the continuous expression of Nrf2. This result was different from the study on Hep G2 culture cells induced by tert-butyl hydroperoxide (t-BHP) by modulating Nrf2 signaling through activation of Akt and ERK.⁵³ While Toninelli et al., was able to prove the antioxidant role of dopamine pramipexole agonists through SH-SY5Y neuroblastoma culture cells where pramipexole prevents cell death induced by H₂O₂ and inhibits the formation of mitochondrial ROS.²³

Associated with the phenomenon of locomotory enhancement in pramipexole and *C. asiatica* group, it is required to do further research to know whether there is a high expression of Nrf2 in each group which is proportional to the oxidation process that goes on a cell such as examination of MDA.

Conclusion

Rotenone exposure is able to decrease locomotor activity along with an increase in α -synuclein aggregation of up to 100% in zebrafish midbrain area. *C. asiatica* extract and pramipexole [7] ng/mL and [14] ng/mL were able to improve equivalent locomotor activity, but *C. asiatica* has an advantage because it decreases the lowest α -synuclein expression, whereas Nrf2 expression among *C. asiatica* extracts and pramipexole did not differ in this study.

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