Protection of Pegagan (*Centella asiatica*) Extract Through Hsp60 and Bax Expression on Stunting Model Zebrafish Larvae (*Danio rerio*) By Rotenone Inducted

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**Abstract.** Objective: Rotenone is a pesticide (insecticide and fish killers) that is widely used in water. This study utilised rotenone as a model to initiate the occurrence of stunting on zebrafish larvae. Rotenone acts to inhibit the mitochondrial complex I to form an Adenine Tri Phosphate (ATP), *Centella asiatica*(CA) is commonly used as Ayurveda medication as the triterpene in CA’s ingredient and antioxidant protect cells from oxidative damage. This study aimed to know the effect of CA extract on the stunting model zebrafish larvae induced by rotenone through Hsp60 and Bax expression. Methods: Zebrafish embryos were exposed with rotenone [10 ppb] and CA extracts(concentration 2.5 µg/mL; 5 µg/mL and 10 µg/Ml) from the age of 2 to 72 hpf (hour post fertilization). The body length was measured at the age of 3, 6, and 9 dpf (day post fertilization) using Image Raster software. The measurement of Hsp60 and Bax expression used IHC method on wholemount zebrafish larvae through DAB colouring. Density integrated was observed with Image J software. Results: The results of the study showed, There was an increase in Hsp60 and Bax expression on the zebrafish larvae induced by rotenone. In addition, CA extract with concentration [5 µg/mL] increased the body length at the age of 9 dpf (p<0.05). The observation of Hsp60 and Bax expression showed there was a significant decline on the CA extract groups with concentration [5 µg/mL]. Conclusion: CA extracts with concentration [5 µg/mL] increase the body length of zebrafish larvae and decreased Hsp60 and Bax expression.

1. Introduction

Stunting is a failure to achieve linear growth as a result of suboptimal health condition, the lack of nutrition, and *low-height-for-age* (HAZ) less than-2 standard deviations (-2 SD) based on WHO growth standards¹. The prevalence of stunting worldwide in 2010 was 27% globally, stunting in Indonesia was higher than Southeast Asian countries with around 37.2% in 2013, stunting in Indonesia was higher than Southeast Asian countries with around 37.2% in 2013².

A failure or linear growth retardation in the first year of life is influenced by prenatal factor that one of them is toxin (i.e pesticide) although genetic and environmental factors, for instance, no ventilation in the kitchen and food quality contribute in the occurrence of growth disorders such as stunting. The excessive of toxin and malnutrition affects cell replication, cell migration, apoptosis, cell mature, and the increase of abnormality’s risk of growth and development of embryo and organ³.
Rotenone is a pesticide (non-synthetic) from *Derris elliptica* (Wallich) Benth roots and tefrosia leaves (rotenone 5%) as a poison for fish and insects that can be found in Indonesia. Based on study eksploration Ali et al., (2016) Rotenon concentration 10 ppb induce stunting on zebrafish larvae. Rotenone acts to inhibit oxidative phosphorylation of the electron transport system in the mitochondrial complex I to form an Adenine Tri Phosphate (ATP) 6-7[1], [2], followed by high Reactive Oxygen Species (ROS) and Nitric Oxide (NO) that induce the occurrence of mitochondrial dysfunction and Bim and Bax translocation from cytosol to mitochondria caused the released of cytochrome-c from mitochondria to cytosol and activating caspase-3 to induce apoptosis. Mitochondria stress causes the release of HSP60/HSP10 and the occurrence of apoptosis. This Research using animal, zebrafish as an animal testing for research has several advantages such as rapid growth, transparent embryo, easy to manipulate, low cost, and hundreds embryos, at least around 70% of zebrafish gene is similar to human gene.

2. Method

The research methods were true experimental study with post-test-only control group design. Animal testing: Zebrafish embryos at the age of 2 hpf (hour post fertilization) were from adult zebrafish breeding identified by hydrology laboratory, Fisheries faculty, Brawijaya University, Malang. The study utilised transparent embryos, no moldy, and clear yolk sacks which were divided into five groups including negative control group (normal embryo); the positive control group (exposed with rotenon 10 ppb), treatment group I(rotenon 10 ppb and CA extract concentration 2.5 μg/mL), treatment group II(rotenon 10 ppb and CA extract concentration 5 μg/mL), and treatment group III(rotenon 10 ppb and CA extract concentration 10 μg/mL). The exposure was started from 2 to 7 hpf, followed by the measurement of body length up to the age of 9. The treatment for zebrafish embryo has fulfilled the ethical requirements on animal testing in Health Polytechnic Ministry of Health, Malang. Medium embryonic preparation: Embryonic medium was made from CaCl 0.08 gr; Kcl 0.06 gr; NaCl 2 gr; MgSO4 3.2 gr which will produce 200 ml of embryonic medium. *Centella asiatica* extraction process: *Centella asiatica* was gained from Materia Medika, Batu, Malang, East Java. The aerial part (not include roots and stolon) was used. The extraction process utilised a maceration method with ethanol 96% as a diluter which was conducted at pharmacology laboratory, Faculty of medicine, Brawijaya University. The result of extraction was crude extract within pasta and diluted with normal saline, stored at freezer. Rotenone and *Centella asiatica* treatment: Stunting model zebrafish larva using rotenone concentration 10 ppb. Rotenone is obtained from (Sigma 8875). Centella asiatica extract 10 mg/mL was made as a stock solution using concentration 2.5; 5; 10 μg/mL. All embryos were death before 48 hpf at concentration 20 μg/Ml, hence, it was decided to use concentration 2.5; 5; 10 μg/Ml. The exposure was conducted from 2 to 72 hpf. Medium was changed daily. Body length measurement: Body length of zebrafish larvae was measured at the age of 3, 6, and 9 dpf. Larvae was observed under stereo Olympus SZ61 microscope connected with OptiLab. Image Raster Software was calibrated to measure the body length of larvae or Standard Length (SL) started from tip of the snout to caudal fin or snout-fin. Zebrafish larvae euthanasia: Zebrafish larvae at the age of 9 dpf were performed euthanasia based on NIH protocol. Wholemount zebrafish larvae were placed into micro tube in iced water minimal 5 minutes and ensured that there was no movement. Then, it was rinsed and fixed with paraformaldehyde (PFA) 4% at 4°C overnight, followed by exposing MeOH 3% for 5 minutes and H2O2 3% for 24 hours to remove the color pigments prior to the procedure Immunohistochemistry (IHC) whole mount. Whole mount Immunohistokimia (IHC): After optimal fixation, zebrafish embryo is coloured by IHC method. Embryo is washed with PBS+triton 0.2% (PBSTX) for 3x5 minutes, then, followed by distilled water for 3x5 minutes using 200-300 μl per tube. Then, it is washed with PBSTX 3x5 minutes, incubated with aceton at 4°C for 20 minutes, and re washed with PBSTX 3x5 minutes. It is mixed with 1 mg/ml of collagenase for 15 minutes and re washed again with PBSTX for 3x 5 minutes. Next, it is incubated with acetate acid 10% at 4°C selama 10 seconds and washed with PBSTX for 3x5 minutes. Then, embryo is incubated in blocking reagent (10% normal horse serum (NHS) and 3% bovine serum
albumin (BSA)) at 4°C all night and washed with PBSTX for 2x60 minutes. Next, it is incubated with primary antibody: 1:1500 (antibody anti Hsp60 Ab 210118 (Abcam®), antibody anti-Bax Santa Cruz®) at 4°C all night, re-washed again with PBSTX at room temperature for 4x60 minutes, incubated with secondary antibody 1: 1000 at 4°C all night, and washed with PBSTX at room temperature for 2x60 minutes. Then embryo is incubated in DAB substrat at room temperature for 20 minutes and washed with DW for 3x5 minutes and stored in glycerol 87% to be observed. **Observation and Calculation:** Whole mount zebrafish larvae were observed with Olympus CX21 microscope with magnificent of 40x. The calculation of colour intensity was with integrated density using image J software. **Statistical analysis:** The data analysis of the body length and expression measurement were calculated for mean ± SD and analysed with independent t-test, normality test, and homogeneity, Anova, and LSD, If the requirements for these tests were not fulfilled, it would be continued with Kruskal Wallis and Mann Whitney U test. Significant level in the study was 95%. The analysis utilised SPSS 22.0 software for windows.

3. Result

**The effects of CA extract on the body length**

CA extract with concentration 2.5; 5 and 10 µg/mL has various effects on the body length.

| Treatments | 3 dpf | 6 dpf | 9 dpf |
|------------|-------|-------|-------|
| K: Control |  | | |
| R: Rotenone |  | | |
| RP1: Rotenone with CA concentration 2.5 µg/ml |  | | |
| RP2: Rotenone with CA concentration 5 µg/ml |  | | |
| RP3: Rotenone with CA concentration 10 µg/ml |  | | |

The graph below described 5 lines of the mean of body length growth in control group and four treatment groups. Each group showed the difference in the mean of body length the age of 3, 6 and 9 dpf. Rotenone group was shorter in body length than control, RP1, and RP2 groups. Growth line in RP3 group showed a higher in the mean of body length compared with control group or the other treatment groups.
Figure 1. The graph of mean difference of body length on zebrafish larvae among control, rotenone, and CA group.

There was an increase of body length compared to rotenone group, although the results of Mann Whitney U test showed that the age of 6 dpf had no difference with rotenone group. At the age of 9 dpf [5 µg/mL] had a difference in the body length compared with rotenone group (p-value = 0.024) on RP2.

Table 2. The Results Of Mann Whitney U Test On Body Length Among All Groups

| Group     | p-value | Conclusion | p-value | Conclusion |
|-----------|---------|------------|---------|------------|
| R and RP1 | 0.180   | No different | 0.140   | No different |
| R and RP2 | 0.151   | No different | 0.024   | Different   |
| R and RP3 | 0.685   | No different | 0.968   | No different |

Significant differences if p< 0.05. RP3 group from the beginning had shorter in body length compared with the other groups. This group was also found congenital abnormalities on heart as described in figure 2.

Hsp60 and Bax expression

The colour visualization showed the difference colour among CA extract groups. The difference of Hsp60 expression was described in histogram of figure 6 below:

Figure 3. The histogram of the mean of integrated density among Hsp60 expression groups. The results of statistical analysis test showed a significant difference on integrated density value of rotenone group and RP1 group CA [2.5 µg/mL] and RP2 CA [5 µg/mL] with p-value < 0.05 (p=0.036 and p=0.005). Different notations showed significant differences.

The results of statistical test using LSD of Bax expression on zebrafish larvae at the age of 9 dpf, the measurement of colour density using Image J to Bax expression showed the decrease of expression on CA extract [2.5 and 5 µg/mL] compared with rotenone group as described in the histogram in figure 4 below:
Figure 2. Histogram of the mean integrated density among Hsp60 expression groups. Different notations showed significant differences.

The results of One way Anova test followed by LSD test showed the difference of expression on group [2,5 dan 5 µg/mL], on the contrary, there was no difference between group [10 µg/mL] and rotenone group.

4. Discussion
The lack of nutrition in the first year of life reduces the feet length and total body posture\textsuperscript{34}. Stunting occurs due to prenatal factors through toxin including pesticide\textsuperscript{18,19}. Rotenone triggers oxidative stress acts to inhibit the mitochondrial complex I, decreases the ability of oxidative phosphorylation so the synthesis of Adenine Tri Phosphate (ATP) is delayed. Mitochondria contributes to produce energy to support the need of ATP in body through glycolysis. The failure of this activity decline the level of ATP production by cells and add ROS\textsuperscript{36}. This also influences cell replication, cell migration, apoptosis, cell mature, and increased risk of growth (i.e bone length), and embryo and brain development\textsuperscript{3,20}.

4.1 CA extract protects zebrafish larvae Model of Stunting
Rotenone is a natural pesticide that influences environment and has toxicity effects affecting human biology system as this pesticide triggers oxidative stress\textsuperscript{20}. Rotenone acts to inhibit the mitochondrial complex I, decreases the ability of oxidative phosphorylation so the synthesis of Adenine Tri Phosphate (ATP) is delayed. Mitochondria contributes to produce energy to support the need of ATP in body through glycolysis. The failure of this activity decline the level of ATP production by cells and add ROS\textsuperscript{36}. This also influences cell replication, cell migration, apoptosis, cell mature, and increased risk of growth (i.e bone length), and embryo and brain development\textsuperscript{3,20}.

The ingredient of C. asiatica is terpenoids including triterpene (main and most important component) that consists of asiatic acid, madecassic acid, asiaticosside, and madecassoside. One of active components is antioxidant\textsuperscript{21}. The ingredient of antioxidant are polyphenol, flavonoid, β-caroten, tannin, vitamin C, vitamin B1, B2, E, A, karoten, niasin, volatile, fatty oils, and glycoside. The ingredients of CA are chloride, sulfate, phosphat, zinc, kalsium, magnesium, sodium and potasium\textsuperscript{22}. 100 g of CA contains calcium 171 mg\textsuperscript{23} and zinc 32.51 mg\textsuperscript{24}. The role of calcium and zinc is needed to the growth of body length. The lack of calcium in the early of life influences bone development. In this study, there was no specific identification of CA extract components. However, the same CA were obtained from Materia Medica Batu using metaholic extract and LC-MS measurement (Liquid Chromatography-Mass Spectrometry) (Thermo Scientific, Accela) showed the amount of asiaticosida\textsuperscript{15}.

In addition, CA group with concentration [10 µg/ml] occurred shorter growth since the age of 9 dpf. It may be caused by the influence of the high concentration of CA extract on zebrafish embryo. CA contains triterpene that is asiatic acid, madecassic acid, and asiaticoside, madecassoside\textsuperscript{25}. Higher concentration is given will lead to toxic effects. The induction of asiatic acid 10 mmol/L in the SH-
SY5Y cells causes the toxicity in the cells. The induction of 10 nmol/L decreases the occurrence of cell apoptosis, reduce ROS, and stabilize mitochondriam membrane potential (MMP)26.

4.2 CA extract decreases Hsp60 and Bax expression
Pesticides could disturb endocrine system and immunity system and increase ROS (Reactive Oxygen Species)27. Rotenone inhibits electron transport on the mitochondria that hampers utilization of oxygen on organism respiration system, through death cells and causes the death of organism in high doses of rotenone. This is led to toxicity in organism due to disrupted electron transport28. The inhibition of complex I affects on the stimulation of ROS production29. This is led to the various diseases that influence the long-life, one of them is growth faltung30.

Hsp60 expression decreased in the CA extract groups [2,5 dan 5 µg/mL] in figure 6. It may be caused by the activities in several CA components. The activity of antioxidant of crude CA extract is similar with the antioxidant activity in rosemary and sage31. The antioxidant activity of CA (84%) is compared with grape seeds extract (83%)23. CA high triterpene, In-vitro study, CA leaves contain high antioxidant which works on three lines; superoxide free radical activity (86,4%), inhibiting peroxidation of linoleat acid (98,2%), and scavanging radical activity, DPPH (92,7%)32,33. L-askorbat acid in CA is antioxidant that is soluble in the water, Flavonoid in Centella asiatica are rutin and quercetin, it is important to be applied to diseases caused by free radicals 34,35.

Antioxidant used by aerob organism to protect the cell from oxidative damage by oxidant in oxygen metabolism. The oxidative stress occurs due to unbalance of pro-oxidant with antioxidant producing the cell damage. The main antioxidants such as superoxidase dismutase (SOD), catalase, glutathione peroxidase GSH-Px), glutathione, ascorbic acid andtocopherol are important to protect cells in eliminating free radicals including reactive oxygen species (ROS)36. The Bax expression decreased on CA extract group [2,5 dan 5 µg/mL]in figure 7 because Centella asiatica contains triterponoid. Several research found that isolation active compound (triterpene) in some cancers showed that triterpene acts to its target, Bcl-2 as antiapoptosis37.

The ingredient of Triterpenoid CA is as antioxidant and anti-inflammatory. Asiaticoside contributes in the wound healing by stabilizing the membrane38. CA as cytoprotective effectreduces the level of ROS and protects the potential membrane of mitochondria by increasing the availability of VDAC39.

The increase of Bax and Hsp60 expression on CA extract [10 µg/ml] showed the high ROS as the results of high concentration of CA extract and the availability of congenital abnormalities on the heart of zebrafish larvae.

5. Conclusion
This study showed Centella asiatica extract [5 µg/mL] protects stunting model of zebrafish larvae induced by rotenone, through the decrease of Hsp60 and Bax expression.

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