Centella Asiatica Increased the Body Length Through the Modulation of Antioxidant in Rotenone-Induced Zebrafish Larvae

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Centella asiatica (CA) is herbal medicine that used as traditional medicine including ayurvedic therapy since hundreds years ago. This herb containa of pentacyclic triterpenoids such as asiaticoside, madecassoside, Asiatic acid and brahmoside that proved had anti-oxidant and anti-inflammatory properties. This research aims to know the effect of ethanolic extract of CA extract against the length of rotenone-induced zebrafish larvae through the free radicals mechanism. This research used zebrafish larvae until 6 dpf that consists of 5 groups (controls, rotenone 12.5 ppb on 2 hpf-3 dpf, and group treatment given rotenone 12.5 ppb 2 hpf-3 dpf and 5 µg/mL extract with long exposure to start 2 hpf to 4, 5 and 6 dpf respectively). The body length measured on 3-6 dpf using software Image Raster v 3.0 from optilab v 2.0. Malondialdehyde (MDA), superoxide Dismutase (SOD), catalase were measured by ELISA on 6 dpf. The results showed rotenone can inhibit the growth of length > 2 standard deviation (SD) and CA extract may increased the body in 6 dpf which correction value was 99.6%. CA extract significantly decreased the levels of MDA, and increased the level of SOD and catalase (p=0.000). Ethanol extract of Centella asiatica may increase in length through the modulation of oxidative stress.

Keywords: Centella Asiatica; Catalase; Malondialdehyde; Rotenone; Superoxide Dismutase; Zebrafish.

Rotenone is a natural pesticide extracted from the roots of tropical and sub-tropical plants derived from the leguminosae family and from the genus lonchocarpus in America, and Derris in Asia that can be used for insecticides, pesticides and piscisida. Rotenone as endocrine disrupting chemicals (EDCs) can disrupt hormonal homeostasis and decrease the amount of Adenosine Triphospate (ATP) and increase the production of reactive oxygen species (ROS). ROS interaction with polyunsaturated fatty acid (PUFA) causes lipid peroxidation with one of its products malondialdehyde (MDA). Antioxidants are reductant compounds that function to lower oxidation reactions and bind to free radicals that can inhibit cell damage. Antioxidants are classified into endogenous antioxidants and exogenous antioxidants. Endogenous antioxidants include superoxide dismutase (SOD) and catalase (CAT). SOD is the main antioxidant enzyme that plays a
role in the elimination of oxidative stress. Catalase is an enzyme that plays a role in catalyzing the dismutation of hydrogen peroxide (H2O2) into water and oxygen.

*Centella asiatica* has the main phytonutrients of triterpenoids that act as antioxidants in balancing the oxidants in cells so that oxidative stress can be prevented. In addition *Centella asiatica* contains nutrients such as macronutrients (proteins and carbohydrates) and micronutrients such as vitamins and minerals. *Centella asiatica* significantly reduced levels of malondialdehyde (MDA) and increased antioxidant enzyme levels, i.e., superoxide, catalase, and glutathione peroxidase in diabetic rats.

Previous studies have suggested 12.5 ppb rotenone can induce stunting with 98% confidence degree and administration of 5 µg/mL pre hatching (2-72 hpf) significantly increases insulin growth factor-1 (IGF-1) expression and increases body length. The purpose of this research is to know the effect of ethanolic extract of *Centella asiatica* until the sixth day to rotenone-induced zebrafish larvae through the antioxidant mechanism.

**MATERIALS AND METHODS**

**Animal Treatment**

The mature wild-type zebrafish males and females were identified in the laboratory of Hydrology Faculty of Fisheries and Marine Sciences University of Brawijaya Malang, Indonesia. Zebrafish are kept in semistatic 60 L tank. The water temperature was kept between 26-28°C, pH 6.8-7.5, and a lighting cycle 14:10 (dark: light). Fish were fed three times a day (Tetra Bit Color Tropical Flakes, Tetra Sales, Blacksburg, Germany).

The zebrafish embryo was obtained from the fertilization of male and female zebrafish with a ratio of 2:1. Zebrafish embryos 0-2 hpf (hour post fertilization) with criteria round, transparent, fertile, and not moldy. The number of larvae used was 30 larvae/group. All procedures have been approved by the Ethics Committee, Faculty of Medicine, Universitas Brawijaya (No. 403/EC/KEPK/12/2017). Zebrafish embryos were divided into 5 groups: controls (C), Rotenone (R), Rotenone + CA extract for 4, 5, and 6 days respectively.

**Embryonic Medium**

Embrionic medium was made with concentration of 10x by CaCl 0.25 gr, KCl 0.15 gr, NaCl 5 gr, MgSO4 0.815 gr and 500 ml of aquadest (Modified from Cold Spring Harbor Laboratory Press).

**Extraction of Centella asiatica**

*Centella asiatica* was certified from UPT Materia Medica, Batu, Malang East Java, Indonesia. The extraction was done using maceration method with 98% ethanol solvent.

**Rotenone and Extract Administration**

Rotenone sigma (R8875) with purity of =95% dissolved in DMSO (dimethyl sulfoxide 1%) to obtain a stock solution of 2 x 107 µg/L (ppb). Rotenone was given at 12.5 ppb and *Centella asiatica* extract 5 mg/mL. The extracts were stocked with concentrations of 1 mg/mL. The medium was replaced daily.

**The Body Length Measurement**

The body length measurements of zebrafish larvae were performed at 3-6 dpf. Larvae were observed using the Olympus SZ61 stereometry microscope that was connected to Optilab software version 2.0. The body length was measured from the tip of the nose (tip of the snout) to the base of the caudal fin using the calibrated Immage Raster software version 3.0.15

**Measurements of Malondialdehyde**

The evaluation of MDA, SOD and Catalase levels was performed on day 6. The zebrafish larvae were euthanized by putting the larvae into ice water containing 5 parts of ice and 1 part of water for 40 minutes. The temperature of the ice water was held at a temperature of 0°C monitored using a thermometer.

Zebrafish larvae (n=30 larvae/group) homogenized using 500 µL Ripa Buffer in glass homogenizer. Homogenate was centrifuged at 4°C, 2500 rpm for 20 min. Supernatant was taken for evaluation procedure. Malondialdehyde, SOD, and Catalase were examined in accordance with the ELISA Kit protocol (Bioassay Laboratory Technology, Shanghai, China), Cat.No E0156Ra (MDA), Cat.No E0168Ra (Cat) N. Cat E0869Ra (Catalase).

**Statistical Analysis**

Statistical analysis was performed by IBM ANOVA SPSS v23.0 and continued by LSD post hoc test with 5% confidence level. Normality
testing was performed using the Shapiro-Wilk test and homogeneity testing was performed using Levene test.

**RESULTS AND DISCUSSION**

Based on figure 1, at the 3 dpf the growth chart shows adjacent points in all groups. The result of statistical analysis of length comparison at age 3 dpf got p-value equal to 0.247. So it can be concluded that there was no significant difference between all groups. Figure 2 showed that there was significant difference of the body length at 6 dpf among groups (p-value = 0.000). The rotenone group had the lowest body length compared to the control and treatment groups. This is in accordance with previous studies, where rotenone projections of 2.21 µg/L and 2.75 µg/L for 32 days significantly decreased the length of the body length in rainbow trout. Rotenone can decrease bone ossification in zebrafish larvae, which causes damage. Increased ROS in bone cells causes bone growth disorders. Highly concentration of ROS in the body will induce RANKL (receptor activator of NF-κB ligand) to interact with RANK thus activating the RANKL pathway that may cause imbalance in the formation process and resorption of the bone. In addition, the increasing of ROS causes impairment.

![Fig. 1](image1.png)

**Fig. 1.** The average of body length at 3-6 dpf. Rotenone group showed the average of body length had the shortest compared to others. Linier growth of R+CA 4 days, R+CA 5 days, dan R+CA 6 almost reach the control group.

![Fig. 2](image2.png)

**Fig. 2.** The average of body length at 6 dpf. There were significant different among the groups (p =0.000). The group of R+CA 4 days, R+CA 5 days, and R+CA 6 days have the average of body length more than rotenone group and had no significant different to the control group (p>0.05)
in Insulin Growth Factor-I (IGF-I)\textsuperscript{21}. IGF-I plays a role in mediating growth and development of somatic cells, including the muscles and bones during prenatal and postnatal\textsuperscript{22}.

Rotenone group showed the average of body length had the shottest compared to others. Linear growth of R+CA 4 days, R+CA 5 days, and R+CA 6 days have the average of body length more than rotenone group and had no significant different to the control group (p>0.05).

Figure 3 showed that the rotenone group had higher MDA levels and lower SOD and catalase levels. Another study proved that rotenone 30 mg/kg for 60 days in mice significantly increased levels of MDA and reduced endogenous antioxidants such as GSH\textsuperscript{23}. Rotenone inhibit the mitochondrial complex I which causes a decrease in the amount of Adenosine Triphosphate (ATP) so that the nucleus fails to divide and apoptosis occurs\textsuperscript{24}. Leakage of complex I results in the increasing of free electrons reacting to oxygen molecules resulting in superoxide (O2-) production. Superoxide will be converted to hydrogen peroxide (H2O2) and O2 by SOD as an endogenous antioxidant. Hydrogen peroxide will be convert by catalase to H2O and O2\textsuperscript{26}. Hydrogen peroxide is not reactive\textsuperscript{27}, but if H2O2 reacts with Fe2+ or Cu2+ (Haber-Weiss and Fenton reaction) to form a highly reactive hydroxyl (OH-) radical\textsuperscript{28}. Hydroxyl radicals can attack polyunsaturated fatty acid (PUFA) by lipid peroxidation process to form hydroperoxide.\textsuperscript{4} One of the secondary aldehydes produced by lipid peroxidation is Malondialdehyde (MDA)\textsuperscript{28} which is capable of inactivating many cellular proteins\textsuperscript{26}.

![Fig. 3. The average of MDA, SOD and Catalase. There were significant different among the groups (p =0.000). CA extract significantly decreased the MDA level, and increased the antioxidant (SOD and Catalase) in zebrafish larvae-induced rotenon](image)

**Table 1.** The Average of MDA, SOD, and Catalase at 6 dpf Zebrafish larvae

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Rotenon</th>
<th>R + CA 4 days</th>
<th>R + CA 5 days</th>
<th>R + CA 6 days</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body length 3 dpf (mm) (n=30)</td>
<td>3.40 ± 0.08a</td>
<td>3.36 ± 0.08a</td>
<td>3.40 ± 0.06a</td>
<td>3.39 ± 0.07a</td>
<td>3.39 ± 0.06a</td>
<td>0.247</td>
</tr>
<tr>
<td>Body length 6 dpf (mm) (n=30)</td>
<td>3.76 ± 0.07b</td>
<td>3.59 ± 0.09a</td>
<td>3.73 ± 0.06b</td>
<td>3.76 ± 0.07b</td>
<td>3.74 ± 0.08b</td>
<td>0.000</td>
</tr>
<tr>
<td>MDA (nmol/mL) (n=5)</td>
<td>5.07 ± 1.4a</td>
<td>14.39 ± 2.81c</td>
<td>9.39 ± 2.83b</td>
<td>7.09 ± 1.83ab</td>
<td>7.77 ± 1.83ab</td>
<td>0.000</td>
</tr>
<tr>
<td>SOD (ng/mL) (n=5)</td>
<td>8.80 ± 0.12c</td>
<td>5.05 ± 0.21a</td>
<td>7.07 ± 0.27b</td>
<td>8.7 ± 0.37c</td>
<td>8.54 ± 0.32c</td>
<td>0.000</td>
</tr>
<tr>
<td>Catalase (ng/mL) (n=5)</td>
<td>25.18 ± 1.06c</td>
<td>18.76 ± 0.31a</td>
<td>22.34 ± 1.07b</td>
<td>24.03 ± 1.37c</td>
<td>23.93 ± 1.27c</td>
<td>0.000</td>
</tr>
</tbody>
</table>
There were significant differences among the groups (p = 0.000). CA extract significantly decreased the MDA level, and increased the antioxidant (SOD and Catalase) in zebrafish larvae-induced rotenone.

Research conducted on stunting children increased MDA levels and decreased the amount of antioxidants such as catalase, SOD, and GSH. Stunting is a growth disorder in which the body length corresponds to <-2SD age based on the WHO child growth chart. Stunting children found normal body length at birth, no congenital abnormalities, and have the same body proportions with normal children.

Based on figure 3, 5 µg/mL Centella asiatica extract for 4, 5, and 6 dpf significantly decreased MDA level compared to rotenone group (p<0.05). Administration of Centella asiatica in this study was given from intrauterine to extra uterine. This refers to the occurrence of stunting starting from within the womb and continues until 2 years (first 1000 days of life). Figure 3 also showed that administration of ethanol in extract of Centella asiatica for 4, 5, and 6 days significantly increased the SOD and Catalase levels (p<0.05). Administration of Centella asiatica extract for 4, 5, and 6 days were able to correct the body length of 99.6%. Centella asiatica might increased the body length through the expression of osteoprotegerin (OPG) as receptor for osteoblast and decreasing the expression of RANKL (receptor activator of Nuclear kappa beta ligand) as indicator for osteoclastogenesis. Thus, the binding of OPG and RANKL inhibit of osteoclastogenesis process.

Centella asiatica contains phytonutrients including of triterpenoids, carotenoids, flavonoids, alkaloids, glycosides, and essential oils. The high level of triterpene in Centella asiatica can provide antioxidant protection. Asiaticoside contain in Centella asiatica by UHPLC (ultra high performance liquid chromatography) examination of 2.94 ppm.12 The ethanolic extract of Centella asiatica in this study came from the same simplia as the previous study (Khotimah, et al), so it is assumed to have the same asiaticoside content. In addition, flavonoids contained in Centella asiatica can act as an important antioxidant.

Centella asiatica significantly decreases MDA and increases antioxidant enzymes, such as SOD, glutathione peroxidase, and catalase to protect the body from the ROS reactions. It has potential as a scavenger of superoxide free radicals, hydrogen peroxide, nitric oxide. This condition leads to a decrease in ROS production in the body, thus reducing MDA levels. MDA is a poly-unsaturated fatty acid oxidation product by free radicals hydroxyl and metabolite of cell components production. Another study proved that ethanolic extract of Centella asiatica stabilized free radicals by radical scavenger and show H2O2 scavenging activity. It also increase the activity of SOD, Catalase, and glutathione peroxidase, and glutathione reductase. Centella asiatica increases the expression of the NRF2 gene, which is the key transcription factor regulating the antioxidant response.

Decreasing the MDA levels, elevated SOD and Catalase levels were followed by a significant increase in body length between treatment groups compared to the rotenone group (p-value <0.05). Administration of Centella asiatica showed non-significant body length to control (p-value > 0.05) with correction of body length at 6 dpf of 99.6%.

**CONCLUSION**

It can be concluded that Centella asiatica increased the body length in rotenone-induced zebrafish larvae. Administration of 5 µg/mL ethanol extract of Centella asiatica can decrease free radical activity with decrease MDA, and increase SOD and catalase level in rotenone-induced zebrafish larvae.

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**REFERENCES**


