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Effect of *Centella asiatica* to the Glucose Transporter 4 and Osteocalcin on the Rotenone-Induced Zebrafish Larvae (*Danio rerio*) Stunting Model

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**Abstract.** Stunting is a failure of linear growth during the first two years of life. Rotenone is a pesticide causing stunting. Rotenone as an Endocrine Disrupting Chemical and complex I mitochondrial inhibitor thus causes a decrease of ATP and oxidative stress. *Centella asiatica* is a plant that contains high antioxidants and antiinflammation. The purpose of this study is to evaluate the effect of CA ethanolic extract toward the rotenone-induced zebrafish larvae stunting model toward the expression increasing of GLUT 4 and Osteocalcin. This experimental study used zebrafish larvae as an animal model. Rotenone 12 ppb and CA extract were administered at 2-72 hpf. Observation of body length using optilab Image Raster at 3, 6, and 9 dpf, while the GLUT 4 and osteocalcin observed at 9 dpf by wholemount immunostaining. The results showed that rotenone 12.5 ppb decreased the body length of zebrafish larvae at 6 and 9 dpf significantly and more than 2 SD shorter from control. The expression of GLUT 4 and Osteocalcin in zebrafish larvae in the rotenone-induced group decreased significantly. Administration of 5 μg/mL extract increased expression of GLUT4, while the expression of osteocalcin increased by 2.5 μg/mL and 5 μg/mL. Rotenone 12.5 ppb induced stunting of zebrafish larvae. Ethanolic extract of CA protects the zebrafish larvae from stunting through increased expression of GLUT 4 and Osteocalcin.

**INTRODUCTION**

Stunting is linear growth failure in children under five where the nutritional status based on height by age compared to WHO growth standard, z-score value less than -2 standard deviation (-2SD) [1]. The World Health Organization (WHO) places Indonesia as the third country with the highest stunting prevalence rate in Asia in 2017. The figure reaches 36.4%. However, in 2018 according to Basic Health Research (Riskesdas) data, the figure will continue to decline by 26.6%. The reduction stunting rates in Indonesia is quite good, but still a severe problem because it refers to WHO standards, the maximum limit is 20% or one-fifth of the total number of children under five [2].
Genetic and environmental factors influence the achievement of the child’s height. One of the stunting factors is the environmental factor such as the exposure of toxins or pesticides [3,4]. Pesticides work as Endocrine Disrupting Chemicals (EDCs), which can disturb function hormone homeostasis in the body. Rotenone is one type of pesticide that inhibits mitochondrial complex, which can cause the system transport electron disturbed, reduction total Adenosine Triphosphate (ATP), the increase of ROS (reactive oxygen species) and oxidative stress occurs. Research results show that children who experience stunting showed the enhancement of stress oxidative and decrease antioxidant defense system[5]. Ridlayanti and Wijayanti suggest that Centella asiatica may increase the length of the body and the expression of BDNF (neurotrophin role in the growth and cognitive function) and can reduce the expression of Hsp60 and Bax in stunting zebrafish larvae [6,7].

Glucose transporter 4 (GLUT 4) is a protein that transports glucose from the bloodstream to the muscles in order to help insulin as well as a regulator homeostasis glucose inside the body. Oxidative stress causes the function of GLUT 4 disturbed and causes ATP hampered so that it can interfere with the growth process [8,9]. Osteocalcin is a non-collagen protein as a marker of bone matrix, secreted by osteoblasts in the process of bone formation. The level of osteocalcin in bone related to bone mineral density. Calcium is an essential mineral required in the process of osteogenesis or bone formation and bone growth [10,11]. Osteoblast cells play an essential role in bone growth and work balanced with osteoclasts. If the cell balance osteoblasts lower compared to osteoclasts due to high ROS and inflammation caused, bone growth will be retarded, and stunting occurs [12].

Centella asiatica is a plant that has phytonutrients. The main compounds are triterpenoids which act as an antioxidant and antiinflammation, besides having macro and micronutrient[13]. Wardani et al. proved that the ethanolic extract of Centella asiatica could prevent stunting through increased expression of VEGF and VEGFR-2 in rotenone-induced zebrafish larvae [14]. Zebrafish (Danio rerio) is one of the commonly used experimental animals as a study because it has several advantages such as high fecundity, transparent embryos, and rapid development. Based on literature studies, 70% of proteins that encode genes in zebrafish, as well as 84% of genes known to be related to human disease, have a similarity relationship with zebrafish genes. This finding shows the importance of using the zebrafish model in research on human diseases [15].

### EXPERIMENTAL DETAILS

#### Reagents

The rotenone used in this study was rotenone from Sigma (R8875) with purity $\geq$ 95%, and the dose used was 12.5 ppb. The Centella asiatica used in this research was certified from Materia Medica, Batu, Malang East Java, Indonesia. Extraction using maceration method (ethanol) 98% and divided into three concentrations; 12.5 µg/mL, 2.5 µg/mL, and 5 µg/mL [16]. GLUT 4 antibodies (Ser 488) from Santa Cruz Biotechnology catalog number sc-1755 Lot C2812. Anti-antibody osteocalcin (v-19) from Santa Cruz Biotechnology sc-18319 Lot CO706.

#### Fish and Embryo Treatment

Wild-type zebrafish (Danio rerio) was reared in a freshwater environment that has been filtered in aquariums with a capacity of 60 liters. The temperature maintained 28 ± 1 °C, pH 6.8 – 7.5, with a dark light cycle of 14 dark and ten light. The fish was fed with Artemia twice to three times a day [16].

The embryos used in this study were 0-2 hpf with transparent, clear, and round shape (evaluate under a stereomicroscope; OlympusSZ61). The embryos were divided into five groups, with 20 embryos in each group. Rotenone concentration was 12.5 ppb exposed at 2-72 hpf. Centella asiatica ethanolic extract concentration of 1.25, 2.5, and 5 µg/mL exposed simultaneously with rotenone. The embryonic medium was changed every 24 hours.

The medical research ethics committee of Medical Faculty, University of Brawijaya, stated that the study was declared ethical by number: 154/EC/KEPK/04/2017.

#### Body Length

Body length is measured based on SL (standard length) measured from the mouth snout (snout) to the base of the caudal fin, which is commonly abbreviated as snout-fin in millimeters on days 3, 6, and 9 after fertilization [17]. Zebrafish larvae were observed using a stereometry microscope, and then they were taken using Optilab version 2.0.
and measured using a scale in the Image Raster software. Stunting criteria based on modification from WHO (2010) with the provisions of height/age ≤-2 SD with a 95% confidence coefficient and remain until the 9th days [18].

**GLUT 4 Expression**

Whole-mount immunohistochemical methods observed the expression of the GLUT 4 of zebrafish larvae at the 9 dpf. It observed using Olympus LED microscope Olympus CX-22 in 40-100x magnification and the Panasonic DMC G6 Lumix digital camera then the results are quantified in integrated density using *Image J* 1.46 software.

**Osteocalcin Expression**

The expression of the Osteocalcin zebrafish larvae observed at 9 dpf by whole-mount immunohistochemical methods and evaluated by an LED microscope from the Olympus CX-22 magnification of 40-100x and Panasonic digital camera DMC G6 Lumix. The pictures were quantified by its integrated density using *Image J* 1.46 software.

**Statistical Analysis**

The statistical analysis used in this study was One-Way ANOVA (α=0.05) and continued with the post hoc Least Significance Different (LSD) test with a confidence level of 5%. The normality test used was the One-Sample Kolmogorov-Smirnov Test, and the homogeneity was tested by using the Levene Test.

**RESULTS AND DISCUSSION**

In this study, researchers evaluated the effect of *Centella asiatica* to prevent stunting of zebrafish larvae due to rotenone induction through increased expression of Glut 4 and Osteocalcin.

**Body Length Measurement**

The results of measurements of the average body length and image display of zebrafish larvae aged 3, 6, and 9-day post fertilization (dpf) between the control and the rotenone group are shown in Table 1 as follow :

<table>
<thead>
<tr>
<th>Age (dpf)</th>
<th>3</th>
<th>6</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
<td>Control</td>
<td>Rotenonee</td>
<td>Control</td>
</tr>
<tr>
<td><strong>Mean ± SD</strong></td>
<td>3.37 ± 0.08</td>
<td>3.34 ± 0.10</td>
<td>3.62 ± 0.11</td>
</tr>
<tr>
<td><strong>Ratio</strong></td>
<td>1:5</td>
<td>1:5</td>
<td>1:5</td>
</tr>
<tr>
<td><strong>p-value</strong></td>
<td>0.113</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>Conclusion</strong></td>
<td>Not significant</td>
<td>Significantly different</td>
<td>Significantly different</td>
</tr>
</tbody>
</table>

**TABLE 1. Body Length of Zebrafish Larvae, Head, and Body Length Ratio between the Control and the Rotenonee Group [12.5 ppb] at 3, 6, and 9 dpf**

Picture (zoom in 2.5x)
The results showed that at the age of 3-day post fertilization (dpf) zebrafish larvae (analogous to newborns), there was no significant difference in body length (p-value 0.113) between control and rotenone group. At the age of 6 dpf (analogous to a 2-year-old child) a difference in body length of zebrafish larvae > 2SD was obtained, and it was significantly different (p-value 0.000) from the control group. At the age of 9 dpf (analogous to an 8-year-old child), a difference in body length > 2SD was obtained, and there was a significant difference (p-value 0.000) with the control group. All groups at ages 3, 6, and 9 dpf have the same body length ratio of 1:5. So it can be concluded that stunting due to to 12.5 ppb rotenone induction occurs from the age of 6 to 9 dpf.

Growth in body length between the control and rotenone group in ages 3, 6, and 9 dpf are displayed in Fig. 1 as follows.

FIGURE 1. The body length of rotenone group was shorter than the control group. The difference in body length is significant at ages 6 and 9 dpf.

The results showed at 3 dpf there were no significant differences in the average body length of zebrafish larvae between the control group and rotenone. Whereas at ages 6 and 9 dpf showed significant differences in average body length between the control and rotenone group. This is supported by the theory of stunting, which states that the baby's body length at birth is regular without being accompanied by congenital abnormalities, and shortening arises at the age of 2 years [19]. Research conducted by Melo et al. proved that in zebrafish, rotenone is embryotoxic, which can influence the development of zebrafish embryos, one of which is growth retardation [20].

**Glut 4 Expression**

GLUT 4 is expressed in all zebrafish muscle tissue indicated in brown. In Fig. 2A that is a control group shows a darker brown density by the value of integrated density is higher than Fig. 2B, which is rotenone group [12.5 ppb]. This shows a decrease GLUT 4 expression of zebrafish larvae age 9 dpf in the rotenone group. The results showed a significance value of 0.000 (p-value<0.05), which means that there are significant differences in the expression of GLUT 4 in zebrafish larvae age 9 dpf between the control and rotenone group.

The results showed that the expression of GLUT 4 zebrafish larvae at 9 dpf in the rotenone group was lower than in the control group. The induction of rotenone in zebrafish causes decreased GLUT 4 expression in the rotenone group. Rotenone works by inhibiting the mitochondrial complex I and causes oxidative stress, which is characterized by an increase in ROS [21]. The increase of ROS causes the PI-3K pathway to lead to GLUT 4 translocation so that the role of GLUT 4 as a protein transporter cannot be carried out. As a result, the supply of glucose as an energy source for making ATP is reduced, which can affect cell growth and life [22].
**Osteocalcin Expression**

Osteocalcin is expressed in all zebrafish bone tissue indicated in brown. In Fig. 3A that is the control group shows a darker brown density by the value of integrated density is higher than Fig. 3B, which is rotenone group [12.5 ppb]. This shows a decrease osteocalcin expression of zebrafish larvae at 9 dpf in rotenone group. The results showed a significance value of 0.001 (p-value<0.05), which means that there are significant differences in the expression of osteocalcin in zebrafish larvae age 9 dpf between the control and rotenone group.

**FIGURE 3.** The expression of osteocalcin (A) Control zebrafish larvae and (B) rotenonee exposure.

The results showed that the expression of Osteocalcin zebrafish larvae aged 9 dpf in the rotenonee group was lower than in the control group. Decreased Osteocalcin expression in the rotenonee group can occur due to the induction of rotenonee in zebrafish, which causes oxidative stress, thus increased ROS. The occurrence of oxidative stress can inhibit bone growth by inhibiting osteoblastogenesis and increasing osteoclastogenesis through the increasing of RANKL expression [23]. This causes an imbalance between bone formation and resorption so that growth is inhibited [24].
Effects of *Centella asiatica* Etanol Extract on Zebrafish Larvae Stunting due to Rotenone-Induced Body Length of Zebrafish Larvae Due to Rotenone-Induced Administration of *Centella asiatica* extract in rotenone-induced zebrafish larvae, showed that not significantly different of body length average to the rotenone group at 3 dpf, but significantly different at 6 and 9 dpf by 2.5 and 5 μg/mL, shows in Fig. 4. The 1.25 μg/mL of CA extract may not adequate to protect zebrafish larvae from rotenone destruction in zebrafish larvae.

![Figure 4](image)

**Figure 4.** There are five growth lines in the average body length of zebrafish larvae ages 3, 6, and 9 dpf. The rotenone group [12.5 ppb] has a graph shorter growth than the control group, rotenone+*Centella asiatica* group [1.25 μg/mL], rotenone+*Centella asiatica* group [2.5 μg/mL] and rotenone+*Centella asiatica* [5 μg/mL]. Increased body length the most approaching the control group was the Rotenone+*Centella asiatica* group [5 μg/mL].

The results of this study are supported by previous studies conducted by Ridlayanti and Wijayanti that *Centella asiatica* extract concentrations of 5 μg/mL increased the body length of zebrafish larvae aged 6 and 9 dpf induced by rotenone. Triterpenoids and other active ingredients from *Centella asiatica* contain high antioxidants able to reduce the activity of species oxygen reaction (ROS) in the body's system that increases [25]. *Centella asiatica* also acts as an anti-inflammatory, which can prevent chronic inflammation in children with stunting conditions [26,27] stated in his research one of *Centella asiatica* functions as an anti-inflammatory that is protecting dopaminergic neurons from toxicity caused by rotenone [27]. Therefore, by getting *Centella asiatica* as a supplement or additional nutrition to the child in the womb in addition to completing the nutritional needs for growth, *Centella asiatica* can prevent chronic inflammation and reduce oxidative stress by balancing oxidants in cells so that stunting does not occur [13]. Other studies proved that *Centella asiatica* could prevent stunting in zebrafish larvae by increasing SOD expression and decreasing MDA [28].

**Effect of *Centella asiatica* Etanolic Extract in Rotenone-induced GLUT4 expression**

GLUT 4 expressions of zebrafish larvae induced by rotenone decreased significantly compared to the control group. GLUT4 expression significantly increased (p-value 0.000) with the addition of *Centella asiatica* concentration of 5 μg/mL, shows in Fig. 5.

The effect of ethanolic extract of *Centella asiatica* in enhancing Glut 4 expression is in line with research conducted by Ramachandran & Saravanan [29] that reported that *Centella asiatica* contains large amounts of Asiatic acid. Asiatic acid is an asiaticosida metabolite that can increase insulin levels in plasma, reduce glucose levels, restore carbohydrate metabolic activity, prevent lipid peroxidation, and increase antioxidant activity. These findings indicate that Asiatic acid can increase glucose response by increasing Glut 4 in skeletal muscle through Akt and antioxidant defenses in plasma and increasing glucose homeostasis.
Figure 5. Integrated density Value. Expression GLUT 4 zebrafish larvae age 9 dpf between control groups, rotenonee, and rotenonee+Centella asiatica some concentration. An increase in GLUT 4 expression in the rotenonee+Centella asiatica group [1.25 μg/mL], rotenonee+Centella asiatica group [2.5 μg/mL] and rotenonee+Centella asiatica group [5 μg/mL] compared to the rotenonee group [12.5 ppb]. Nevertheless, the most expression tall and approaching the control group is the rotenonee group [5 μg/mL] (n=5; α=5%).

Effect of Centella asiatica Etanolic Extract in Rotenonee-induced Osteocalcin expression

Osteocalcin expression of zebrafish larvae induced by rotenone decreased significantly compared to the control group. Osteocalcin expression significantly increased (p-value 0.043) with the addition of Centella asiatica concentration of 2.5 μg/mL and significantly increased (p-value 0.000) with the addition of Centella asiatica concentration 5 μg/mL, shows in Fig. 6.

Figure 6. Comparison of integrated density Osteocalcin values of zebrafish larvae age 9 dpf between control, rotenonee and rotenonee+Centella asiatica concentrations.

Osteocalcin expression increased in the rotenonee+Centella asiatica group [1.25 μg/mL], rotenonee+Centella asiatica [2.5 μg/mL] and rotenonee+Centella asiatica [5μg/mL] compared to the rotenonee group [12.5 ppb]. However, the highest expression and approaching the control group [5μg/mL] (n=5; α=5%).
Bone growth and development requires adequate nutrition. Nutritional deficiencies associated with stunting include a lack of energy, protein, and zinc. Lack of or impaired metabolism of vitamin D, magnesium, phosphorus, or calcium can result in the softening of the bones called rickets. Meanwhile, other micronutrients such as vitamins, minerals, and essential elements are needed for organ function, nutrition, and tissue integrity during growth and play a role in absorption and metabolic processes [30]. Inadequate intake and or poor absorption of bone-forming minerals, especially calcium (Ca) and Zinc (Zn), can also cause linear growth retardation [31].

Centella asiatica has several valuable nutritional contents, including a high calcium content. Calcium is essential to use in the process of bone formation. Adequate and adequate calcium absorption in childhood through adolescence is a determinant in achieving bone mineral status in later adulthood [13].

**SUMMARY**

Centella Asiatica ethanolic extract can protect stunting through the increasing of GLUT 4 and osteocalcin expression in rotenone-induced zebrafish in 5 μg/mL significantly.

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**REFERENCES**


