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(RESEARCH ARTICLE)



The effect of *Centella asiatica* to the vascular endothelial growth factor and vascular endothelial growth factor receptor-2 on the rotenone-induced zebrafish larvae (*Danio rerio*) stunting model

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Abstract

Stunting is a condition of obstructed body length growth in the first two year of life. Rotenone as a pesticide specimen which trigger stunting condition, inhibits the mitochondrial complex I causing an increased level of ROS that affects angiogenesis and vasculogenesis process that involve VEGF with VEGFR-2. *Centella asiatica* serves as antioxidant which inhibits ROS production and revitalize blood vessels. The purpose of this research is to discover the effect of *Centella asiatica* ethanolic extract to the enhancement of body length through the expression of the VEGF and VEGFR-2 on the rotenone-induced zebrafish larvae stunting model. The result in this study proved that 12.5 ppb rotenone could induce stunting growth in 6 dpf (days post fertilization) zebrafish larvae (*Danio rerio*). The administration of *Centella asiatica* ethanolic extract improved the body length, along with the increasing of VEGF [2.5 µg/ml] and VEGFR-2 [5 µg/ml]. The correlation test showed a positive and strong relevance the levels of *Centella asiatica* and the expression of VEGF and VEGFR-2. It can be concluded that *Centella asiatica* ethanolic extract could inhibit stunting and improved the body length through increasing the expression of VEGF and VEGFR-2 in stunting model zebrafish larvae.

Keywords: VEGF; VEGFR-2; Stunting; Zebrafish; *Centella asiatica*

1. Introduction

Stunting is a condition in the first 2 years of life where the length- for- age index compared to the WHO standard– MGRS (Multicenter Growth Reference Study), has less than -2 SD z-score on the average growth chart [1–3]. Indonesia is ranked 5th in number of children with stunting in the world, increasing from 35.6% in 2010 to 3.2% in 2013 compared to other ASEAN country [1]. Stunting threatens global health as it inhibits children's physical and mental development. It is related with delayed motoric and intelligence skills, productivity and higher risk of degenerative diseases [4-5].

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Stunting is known affected by internal and external factors such as genetic and environment. One of the environmental factor that causing stunting is pesticide exposure [6].

Rotenone is a pesticide that inhibits the mitochondrial complex I, causing a failure in electron transport of ATP's production and conduce an abnormal level of ROS (Reactive Oxygen Species). The elevation of ROS activate the caspase 3, DNA fragmentation and detached of cytochrome-c which effect of apoptosis [7-8]. Furthermore, it also affects the failure on vasculogenesis and angiogenesis process which play an important role in making the embryo vascular tissue. One of the growth factor which in charge of that is the VEGF (Vascular Endothelial Growth Factor) that will attached with its receptor called the VEGFR-2 (Vascular Endothelial Growth Factor Receptor - 2). The VEGFR-2 has a bigger role than the VEGFR-1 because it is an early sign of endothelium and hematopoietic precursor cells that show autophosphorylation and tyrosine kinase activity [9-10]. The obstruction of mitochondrial complex I by the pesticide affects as a disruption of body's homeostasis that causing a growth disorder such as stunting. Antioxidant is required to preserve life and cell signaling [11].

Centella asiatica contains antioxidant such as triterpenoid, madecassoside and asiaticoside to inhibit the ROS production and revitalize the blood vessels [12–16]. The zebrafish (*Danio rerio*) is chosen as the testing animal because of its 75% genetically similar to human [17], can be externally fertilized to make it easier to provide treatment, can be easily observed because of its transparent body, has a short incubation and growth time and cost cheaper in maintenance [18–21]. It took 9 days of observation, adjusting with number of stunting in 2 years old children. The 4- 10 days old of zebrafish is equal to a 2- 3 years old children (4). The purpose of this study is to prove the effect of *Centella asiatica*'s ethanolic extract to the body length through the VEGF and VEGFR-2 on the rotenone-induced zebrafish larvae.

2. Material and methods

This research used 2 hpf (hour post fertilization) zebrafish embryos from adult zebrafish fertilization, which has been identified on Hydrology Laboratory in Faculty of Fisheries and Marine Science of Universitas Brawijaya. The embryos had inclusion criteria: not moldy, clear and not opaque, has a yolk sac, translucent and hatching on 72 hpf. Rotenone (sigma, R8875) concentration 12.5 ppb exposed from 2 – 72 hpf (3 days). *Centella asiatica* concentration were [1.25 µg/ml]; [2.5 µg/ml] and [5 µg/ml] that made from 10 mg/ml stock. It co-incubate with rotenone for 3 days. The embryonic medium have the composition 0.08 gr of CaCl₂; 0.06 gr of KCl, 2 gr of NaCl and 3.2 gr of MgSO₄ diluted in 200 ml aqueduct (10x concentration) and were replaced every 24 hour. There were 5 groups of embryos in this study: the control group, the [12.5 ppb] rotenone-induced, the rotenone [12.5 ppb] and *Centella asiatica* induction of [1.25 µg/ml]; [2.5 µg/ml] and [5 µg/ml].

The observation of growth was performed by measuring the length of zebrafish larvae on 3, 6 and 9 dpf used image raster V.3 software from Optilab. It was measured from its snout to fin. The treatment has already qualified for ethical clearance in Faculty of Medicine from Universitas Brawijaya (154/EC/KEPK/04/2017). The evaluation of VEGF and VEGFR-2 expression on 9 dpf using the whole mount immunohistochemistry (IHC). The larvae were placed on an iced water micro tube for 5 minutes to sacrificed the larvae. Then, it was soaked on 4% PFA solution at 4°C and 3% H₂O₂ to clear up the pigment. The zebrafish on 9 dpf stained by the DAB; primary antibody VEGF and VEGFR-2 (Santa Cruz Biotechnology) to be observed under the Olympus CX-21 microscope (40 x) connected to DMC-G6 Lumix Panasonic camera. The emerging brown color was quantified using image J software via integrated density in pixel units. The data of VEGF and VEGFR-2 expression were analyzed by one-way Anova test and Pearson's correlation by SPSS V 22.0 for Windows.

3. Results and discussion

The results showed, zebrafish larvae at 3 dpf have no significantly difference (p. 0.05) and has < -2 SD difference on body length average. At 6 and 9 dpf the rotenone induced stunted growth (p < 0.05) and the difference > 2 SD of body length. Administration of *Centella asiatica* [1.25 µg/ml] and [2.5 µg/ml] and [5 µg/ml] increased the body length in dose-dependent manner. But, all groups at 3, 6 and 9 dpf have the same body length and head length ratio with ratio of 1:5. The data shown in Table 1.

Rotenone is a toxic pesticide that inhibits the mitochondrial complex I. It caused the increasing of ROS production and reduction of ATP which is needed in metabolic system [23-24]. The toxic effect of rotenone as pesticide, insecticide and piscicide is more visible in fish [25]. Rotenone obstructs the replication, migration and cell oxygenation which effect of apoptosis and caused abnormality in bone growth, organ maturity and fetal development [25–27]. The rotenone's embryotoxicity effect on the zebrafish larvae observed on tail form abnormality, loss of balance, growth delayed,

cardiomegaly, lack of pigmentation and yolk absorption disorder [28]. The retracted of body length on zebrafish larvae caused by a certain pesticide called malathion [29]. Rotenone is proven induced stunting on zebrafish larvae through the reduction of BDNF and evaluation of BAX and HSp60 [30-31].

Table 1 The comparison of length average of zebrafish larvae on 3, 6 and 9 dpf in control group, the [12.5 ppb] rotenone induced and the rotenone *Centella asiatica* groups

Age (dpf)	Group	Mean Length(mm) ±SD	Mean Head length (mm)	Ratio length and head length
3	Control	3.37± 0.08	0.63	1:5
	Rotenone	3.34±0.1	0.61	1:5
	RCA1.25	3.34 ± 0.09	0.61	1:5
	RCA2.5	3.35 ± 0.08	0.63	1:5
	RCA5	3.35± 0.07	0.72	1:5
6	Control	3.62±0.11	0.77	1:5
	Rotenone	3.39±0.08	0.71	1:5
	RCA1.25	3.41 ± 0.09	0.7	1:5
	RCA2.5	3.44 ± 0.09	0.7	1:5
	RCA5	3.49 ± 0.09	0.73	1:5
9	Control	3.82±0.11	0.81	1:5
	Rotenone	3.6±0.11	0.8	1:5
	RCA1.25	3.62±0.09	0.8	1:5
	RCA2.5	3.65±0.12	0.78	1:5
	RCA5	3.71±0.11	0.69	1:5

Rotenone-Rotenone 12.5 ppb, RCA1.25 -Rotenone+*Centella asiatica* [1.25 µg/ml], RCA2.5-Rotenone+*Centella asiatica* [2.5 µg/ml], RCA5- Rotenone + *Centella asiatica* [5 µg/ml]

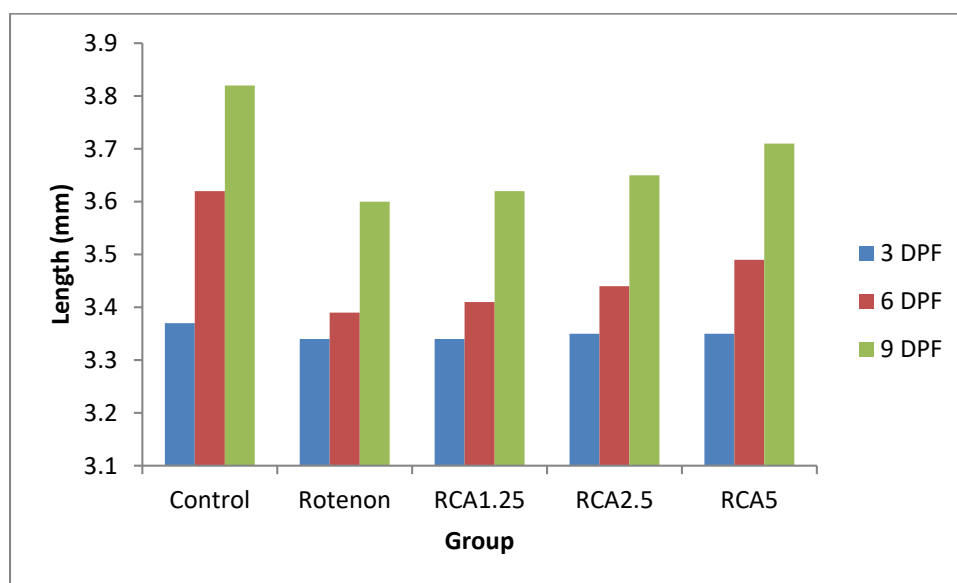


Figure 1 The diagram of body length zebrafish larvae on 3, 6 and 9 dpf

Administration of *Centella asiatica* increased body length of rotenone-induced zebrafish larvae. Concentration of *Centella asiatica* extract have significant effect to the body length of zebrafish larvae. In 5 µg/ml, *Centella asiatica* have

the highest effect on the correction of body length, but still could not reach normal growth. It might need the longer exposure or higher concentration to gain the normal growth.

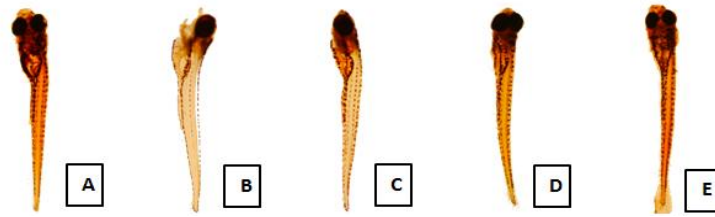


Figure 2 VEGF expression marked by the emerging of brown color of all group at 9 dpf zebrafish larvae. A- Control, B- Rotenone, C-Rotenone+*Centella asiatica* [1.25 µg/ml], D- [2.5 µg/ml] and E- [5 µg/ml]

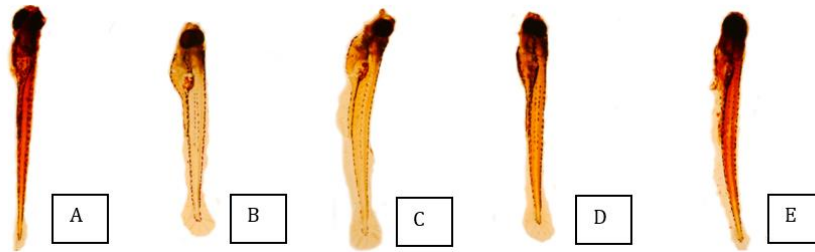


Figure 3 VEGFR-2 expression marked by the emerging brown color. A-Control, B- Rotenone, C- Rotenone *Centella asiatica* [1.25 µg/ml], D- [2.5 µg/ml] and E- [5 µg/ml]

Figure 2 and figure 3 showed that the control group has the thickest brown color (the highest VEGF and VEGFR-2 expression) while the rotenone has the thinnest. The VEGF and VEGFR-2 expression were increased by the administration of ethanolic extract of *Centella asiatica*.

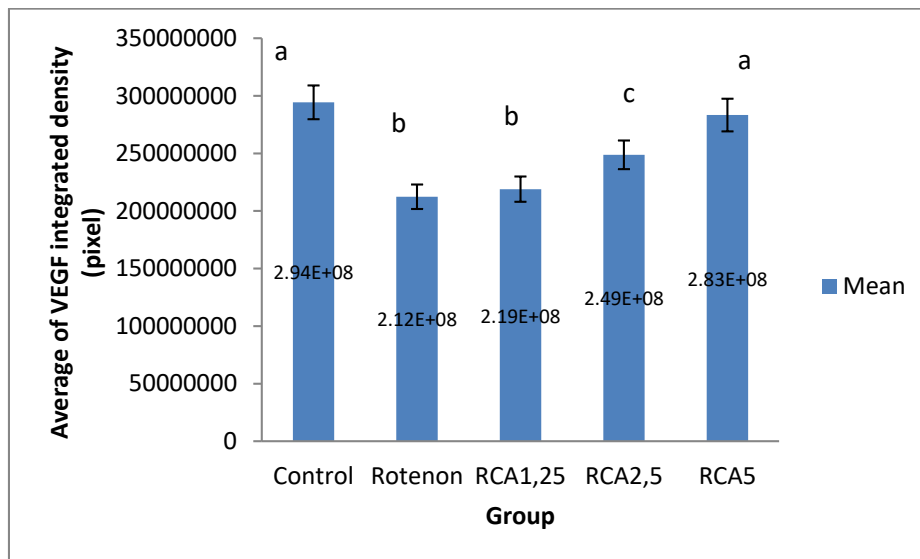


Figure 4 The Comparison on average of VEGF expression integrated density at 9 dpf zebrafish larvae

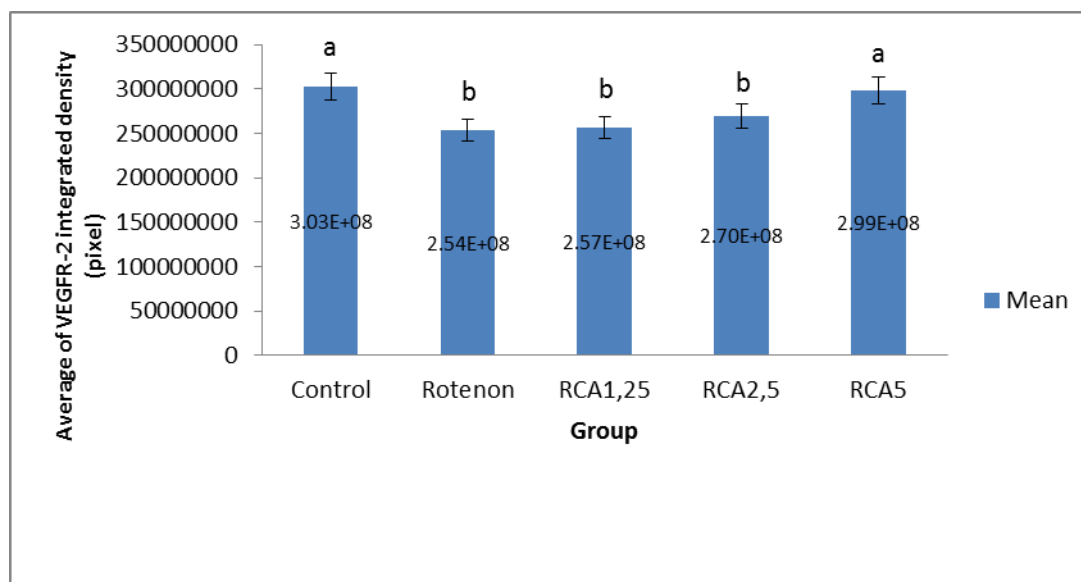


Figure 5 The Comparison on average of VEGFR-2 expression integrated density at 9 dpf zebrafish larvae

Figure 4 and 5 showed the quantification of VEGF and VEGFR-2. Rotenone significantly decreased the expression both of VEGF and VEGFR-2 in 9 dpf zebrafish larvae. The *Centella asiatica* ethanolic extract addition caused a significant increased at concentration of 2.5 $\mu\text{g}/\text{ml}$ and 5 $\mu\text{g}/\text{ml}$. Pearson correlation showed a positive and strong correlation between *Centella asiatica* concentration to VEGF, VEGFR-2 and the body length as well. And in the other side, the VEGF and VEGFR-2 also have strong correlation to the body length of zebrafish larvae. The higher VEGF and VEGFR-2 expression, the longer body length it has.

In normal state, the increased ROS caused hypoxia that stimulate expression of VEGF. The HIF 1- α acts as a transcription factor which binds to specific promoter of VEGF to produce more VEGF [9, 32]. Hypoxia stimulates the VEGF by increase mRNA's transcription and stability that induce natural feedback and blood vessels formation to maintain sufficient oxygen supply [33-34]. The excess ROS caused by oxidative stress leads to cells damage as a result of VEGF gene mutation by a complex signal pathway [33].

Chronic hypoxia caused decreased in VEGF expression, obstruction of tissue vascularization and endothelial dysfunction [35]. Endothelial dysfunction caused disruption in angiogenesis controlled by VEGF interaction with VEGFR-2. This binding stimulates proliferation and migration in growth process [36-37].

VEGFR-2 is an initial marker of a damage endothelial cells in angiogenesis process. During the activation, the Mitogen-activated protein kinase (MAPK) served in endothelial cells proliferation along with the VEGFR-2 [10, 32, 38-39]. Excess ROS affect in endothelial damage that caused reduction of VEGF and VEGFR-2 [40].

In human, growth process can be maximized if the treatment was done in age of 0- 2 years (1000 first day of life) when the rapid growth happen [41]. Based on that statement, the *Centella asiatica* were given since 2- 3 hpf or equal to 2 years in human age.

Nutrition takes an important role in child growth and development. It could cause obstruction such as stunting if it's not fulfilled [42]. *Centella asiatica* has macronutrient, vitamin and mineral [15]. Vitamin C is consider as a chytochrome-c and caspase-3 rotenone induced inhibitor [7]. Active compound such as *Centella asiatica*'s triterpenoid could prevent oxidative stress and clean up free radicals on rotenone-induced zebrafish [43]. Clinical studies shows that *Centella asiatica* is a wild plants that known as the most powerful healing herbs mainly in revitalize blood vessels [44].

Madecassoside found in *Centella asiatica* could reduce damage on endothelial cell caused by oxidative stress by escalate the cell survival and glutathione levels, reduce dehydrogenase and malondialdehyde levels induces by H_2O_2 . H_2O_2 caused apoptosis, prevent caspase-3 activation and mitochondrial membrane's protection. In normal state, HIF-1 α is positively responded by increasing the VEGF and VEGFR-2 expression. It makes the angiogenesis running normally that impacted to the escalation of body length [13]. The administration of *Centella asiatica* has proven in the enhancement of rotenone induced zebrafish body length through the elevation of BDNF, BAX, Hsp60 and SOD also decrease MDA [30-31, 45].

4. Conclusion

In this study proved that [12.5 ppb] rotenone could induce stunting growth in zebrafish larvae. The administration of the ethanol extract of *Centella asiatica* could protect the zebrafish stunting larvae through the increasing of VEGF and VEGFR-2 expression. This research can be improved by examining other factors that influence the occurrence of stunting.

Compliance with ethical standards

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Disclosure of conflict of interest

All authors declare that there is no any conflict of interest regarding the publication of this article.

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