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(RESEARCH ARTICLE)

The effect of *Centella asiatica* ethanolic extract on expression of glucose transporter 1 and osteocalcin on stunting larvae of Zebrafish (*Danio rerio*)

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Abstract

Stunting is known as length for age below minus two standard deviations (<-2 SD) of the WHO Child Growth Standard nutritional status. Several causes are identified, including exposure to pesticides (rotenone), which works by inhibiting mitochondrial complex I as an ATP-producing site and generate oxidative stress that affects glucose delivery and bone elongation involving GLUT 1 and osteocalcin. *Centella asiatica* is a high antioxidant herbal plant with anti-inflammatory properties. This study aimed to investigate the effect of *Centella asiatica* ethanolic extract on the rotenone-induced stunting zebrafish (*Danio rerio*) larvae model through GLUT 1 and osteocalcin expressions. This study found that 12.5 ppb of rotenone can induce about 6 dpf (day post-fertilization) zebrafish larvae into stunting. The effect of *Centella asiatica* ethanolic extract on increasing body length in stunting zebrafish larvae works by increasing the expression of GLUT 1 and osteocalcin expressions. The correlation test showed a positive and strong relationship between this ethanolic extract level with GLUT 1 and Osteocalcin expression. Thus, the *Centella asiatica* ethanolic extract effectively promotes the body length by elevating the expression of GLUT 1 and osteocalcin in stunting zebrafish larvae.

Keywords: *Centella asiatica*; Rotenone; Glucose Transporter 1; Osteocalcin; Stunting; Zebrafish

1. Introduction

Stunting is a condition where the child's height is too low based on the length for age or height for age, where the height is below minus two standard deviations (<-2 SD) from WHO Child Growth Standard nutritional status [1]. According to the Decree of the Minister of Health of Indonesia, Number 1995/MENKES/SK/XI I/2010 concerning Anthropometric Standards for Assessment of Child Nutritional Status, there are 2 types of stunting, stunted (short) where the length <-2 SD and severely stunted (very short) where the length <-3 SD. The incidence in Indonesia is the highest compared to

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other countries in Southeast Asia, around 37.2% in 2013, which has increased compared to 2010 and 2007. About 8 million Indonesian children (1 out of 3 children) experience stunting [2].

Genetic and environmental factors significantly affect the growth and development of children. Environmental factors have two phases, prenatal and postnatal. The prenatal phase is influenced by maternal nutrition, stress, embryonic anoxia, immunity, toxins or chemicals, and infections experienced by the mother during pregnancy. The postnatal phase is caused by biological factors, the family's physical environment, psychosocial and customs that will affect growth and development during the first two years [3–7]. Toxins and chronic malnutrition affect the occurrence of apoptosis, cell proliferation, cell maturity, growth abnormalities, embryonic development [8].

Rotenone is a plant-based pesticide derived from tuba root *(Derris elliptica)* (rotenone 0.3-12%) and tephrosia leaf (5% rotenone) [9], which is used for fishing in North America and agriculture as a fruit and vegetable insecticide [10]. Rotenone is used as an insecticide, pesticide, and piscicide [11]. Humans can be exposed to rotenone through agricultural residues and consuming fish exposed to rotenone [12]. The mechanism of action of rotenone is to inhibit mitochondrial complex I so that ATP production decreases [13,14], followed by high ROS causing the release of cytochrome c and the activation of caspase 3, which causes apoptosis [15]. ATP is the result of processing glucose into pyruvate, as a glucose transporter from outside into cells is GLUT 1 [16]. Failure of ATP formation affects muscle cells, bone (Osteocalcin as a product of osteoblasts) cells nerves that require high energy, which will cause growth disorders such as stunting if not fulfilled [17].

Reducing ROS is by using *Centella asiatica* antioxidants containing terpenoid [18]. This plant's active ingredients include triterpenoids, asiaticoside, madecassoside, and Asiatic acid [19,20]. Besides, important roles were revealed as anti-inflammatory, antioxidant, antiulcer, wound healing factor [21], anti-anxiety [22], and antidiabetic [23].

Zebrafish has been widely used as a research model for system pathology in humans [24]. The advantages of zebrafish are rapid development, transparent embryo, and the genome is easy to manipulate, laying eggs with hundreds of embryos, low maintenance [25], homologous to human genes 70% [26].

This research was the first to create a stunning model in zebrafish larvae induced with rotenone with a concentration of 12.5 ppb and the second to determine the effect of giving *Centella asiatica* extract to the zebrafish stunting model induced by rotenone through the expression of GLUT 1 and osteocalcin.

2. Material and methods

This research was a true experimental research design with a post-test control group design approach used zebrafish embryos aged 2 hpf (hour post-fertilization) from the mating of selected adult zebrafish (transparent, not moldy, and not white). This study was divided into 5 groups: Control group (given embryonic medium); Rotenone group (given induction rotenone concentration of 12.5 ppb); Treatment group I (RP1) rotenone (12.5 pp) + *Centella asiatica* extract concentration 1.25 µg/ml; Treatment group II (RP2) rotenone (12.5 pp) + *Centella asiatica* extract concentration of 2.5 µg/ml; Treatment group III (RP3) rotenone (12.5 pp) + *Centella asiatica* extract concentration of 5 µg/ml. Exposure started from 2 to 72 hpf, followed by body length development up to 9 dpf, then terminated.

Centella asiatica was obtained from UPT Materia Medika – Batu, Malang using the irrigated part (the part above the ground) without stolons and roots [27]. Extraction was using the maceration method with 96% ethanol solvent [27]. The extraction results are in the form of a paste and diluted with normal saline, stored at 0 °C, carried out at the Pharmacology Laboratory of Brawijaya University.

The body length of zebrafish larvae was carried out at 3, 6, 9 dpf using an Image Raster calibrated on a stereo microscope connected to an Optilab viewer microscope. Body length was measured from the tip of the nose to the snout-fin [28].

Wholemount zebrafish larvae were observed using an Olympus CX21 microscope with 40× magnification, while the images were taken using a Panasonic DMC-G6 Lumix camera. Calculation of color intensity with Integrated Density using Image J.F

The body length and expression measurements were calculated for the mean and analyzed using SPSS version 22 with the Independent t-test statistical test followed by ANOVA and LSD since the data were normally distributed and homogeneous.

3. Results and discussion

The addition of *Centella asiatica* extract with 3 different concentrations gave different effects on zebrafish body length, as shown in Table 1.

Table 1 Overview of zebrafish larval body length

Age (dpf)	3					6					9				
Group	C	R	RP1	RP2	RP3	C	R	RP1	RP2	RP3	C	R	RP1	RP2	RP3
Picture															
Height(mm)	3,37	3,34	3,34	3,35	3,35	3,62	3,39	3,41	3,44	3,49	3,82	3,6	3,62	3,65	3,71
Mean ±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
SD	0,08	0,10	0,09	0,08	0,07	0,11	0,08	0,09	0,09	0,12	0,11	0,11	0,09	0,12	0,11

Note: C= control, R= rotenone, RP1= Rotenon + *Centella asiatica* extract concentration 1.25 µg/ml, RP2= Rotenon + *Centella asiatica* extract concentration 2.5 µg/ml, RP3= Rotenon + *Centella asiatica* extract concentration 5 µg/ml.

The difference in body length in Table 1 can be illustrated by a graph of all groups' average body length growth.

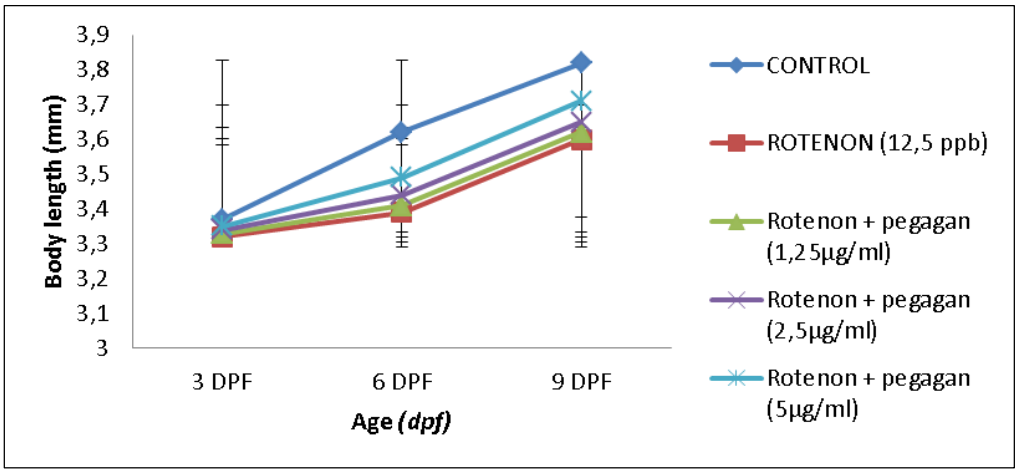

Figure 1 The growth of the average body length of zebrafish larvae in all groups

The rotenone group had the lowest growth line compared to the control group, and the rotenone + *Centella asiatica* group had 3 different concentrations (Figure 1).

An exploratory study conducted from September 2016 to February 2017 found that a rotenone concentration of 12.5 ppb caused stunting. The occurrence of differences in body length between the control group and the rotenone group in zebrafish larvae aged 6 dpf continued until the age of 9 dpf when the age of 6 dpf was analogous to 2 years old, and 9 dpf was analogous to 8 years [29].

Stunting is a condition experienced by children; they were born normally without any congenital abnormalities, but at 2 years, they experience a short body length due to symptoms of chronic undernutrition experienced[3]. Stunting is different from cretinism; the stunting condition has a body length under the normal with proportional body and height[30]; meanwhile, cretinism has a disproportionate body and height, less than normal [31]. Age 3 dpf in zebrafish larvae in the control and rotenone groups showed nobody length difference. The ratio of head length to body length was proportional, which was a prerequisite for stunting conditions that must be distinguished from cretinism.

The difference in the size of the control group and the rotenone group at the age of 6 dpf and 9 dpf showed a significant difference of >-2 SD according to the stunting condition at the age of 2 years affect the reproductive period [32].

Factors that cause stunting are multifactorial, environmental factors, and genetic factors are the main influences on growth in the first 2 years of life [33]. Environmental factors that cause stunting are the presence of toxins, such as exposure to pesticides [4]. Rotenone is a natural pesticide that can affect the body's system and trigger the formation of oxidative stress [34]. Rotenone inhibits the mitochondrial Complex I as a site of ATP synthesis, resulting in decreased ATP production in cells and increased ROS [35]. An increase in ROS affects cell proliferation, apoptosis, cell migration, cell maturation and interferes with the growth process of bone elongation and growth and development during pregnancy [8]. The body's metabolic processes require ATP [36]. The apoptosis caused by rotenone is characterized by DNA fragmentation, the release of cytochrome C, and the presence of caspase activity 3 [37].

The prenatal period experiencing chronic malnutrition and inflammation can lead to IUGR (Intrauterine Growth Retardation) and LBW (Low Birth Weight). In addition, the presence of inflammation in newborns also affects the first thousand days of life, so it is also related to stunting growth [32]. Fatty is also caused by nutritional deficiencies, including protein, energy, and Zn, affecting bone growth and development [38].

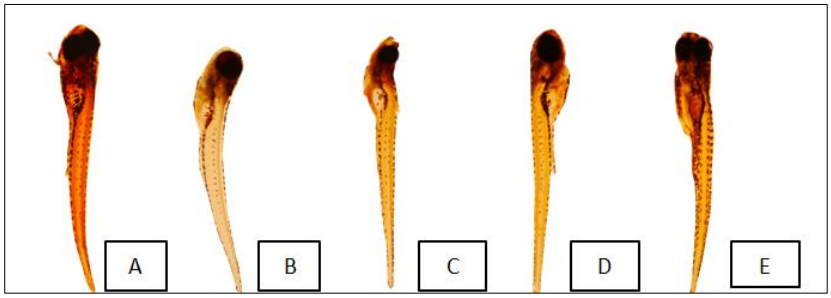

Figure 2 Expression of GLUT-1 in zebrafish larvae at the age of 9 dpf induced by rotenone and *Centella asiatica* at 3 different concentrations. Figure (A) depiction of IHC control group without rotenone induction; (B) Rotenone-induced group; (C) Rotenone + *Centella asiatica* induced group with a concentration of 1.25 µg/ml; (D) The rotenone + *Centella asiatica* group with a concentration of 2.5 µg/ml; (E) Rotenone + *Centella asiatica* group with a concentration of 5 µg/ml

GLUT-1 expression in zebrafish larvae was stained with IHC with DAB staining so that a yellowish-brown color appeared, which would be observed on an Olympus microscope with a magnification of 40-100×.

The darkest brown color was the control group, and the faintest color was the rotenone group. After treatment with *Centella asiatica*, the rotenone-induced group with a 5 µg/ml concentration had almost the same color density as the control group.

Glucose Transporter 1 (GLUT-1) is any glucose transporter that transports glucose from outside the membrane into cells found in the brain, placenta, muscle, and fat [39]. The brain, retina, and placenta are the organs most abundant in GLUT-1 [40], but fat, liver, and muscle are the least [41]. GLUT-1 expression in zebrafish can be seen at 6 hpf (hour post-fertilization) [42], whereas in humans, it begins to be expressed in the preimplantation period [43]. Glucose metabolism is an essential requirement as an energy source for cell proliferation, which if not smooth, cell apoptosis can occur [44].

The presence of metabolism in the body is characterized by glucose carried by glucose transporters (GLUT) across the plasma membrane from high concentrations to low concentrations. In mitochondria, Hexokinase (HK) phosphorylates glucose to glucose-6-phosphate (G6P) via the glycolytic pathway, generating NADH, ATP, and pyruvate, or the pentose phosphate (PPP) pathway. More ATP will be produced in the presence of sufficient oxygen [45]. The presence of metabolism in the body is characterized by glucose carried by glucose transporters (GLUT) across the plasma membrane from high concentrations to low concentrations. In mitochondria, Hexokinase (HK) phosphorylates glucose to glucose-6-phosphate (G6P) via the glycolytic pathway, generating NADH, ATP, and pyruvate, or the pentose phosphate (PPP) pathway. More ATP will be produced in the presence of sufficient oxygen [16]. Rotenone is a pesticide whose function is to inhibit mitochondrial complex I so that ATP is not formed and increases oxidative stress [46]. Rotenone interferes with the endocrine system and the body's defense system and increases ROS [47]. Electron transport in mitochondria is disrupted so that oxygen utilization is hindered, which has a role as a respiratory organism resulting in cell death/apoptosis and then the organism's death if high doses of rotenone occur [48]. ROS's hypoxic state activates AMP-activated protein kinase (AMPK) [49] and Tools for energy metabolism and cellular adaptation to ROS

[50]. The condition of the lack of inhibition in mitochondrial complex I will reduce ROS so that cells will grow. The inhibition in the mitochondrial Complex I can cause various diseases that affect growth disorders [51].

The study results in figure 2 show differences in GLUT-1 expression as seen from the integrated density value between the control group and the rotenone group, where the rotenone group was lower than the control group. This condition was caused by rotenone induction in zebrafish larvae. It is in line with the theory that ROS causes apoptosis, then cells do not proliferate so that growth is inhibited and stunting occurs.

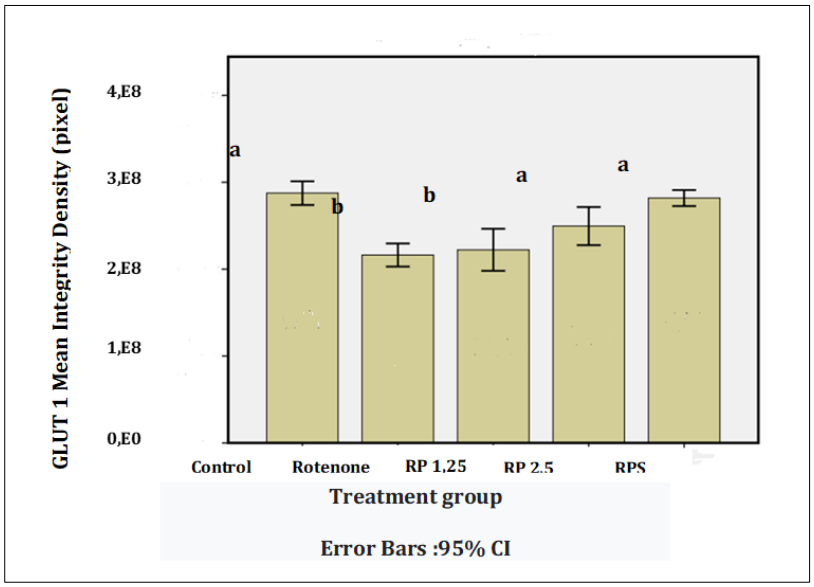

Figure 3 Differences in Integrated Density Expression of GLUT-1 aged 9 dpf zebrafish larvae between the control and treatment groups. Different letters show significant differences

The highest expression was in the control group, while the lowest was in the rotenone-induced group. The increase in Glut-1 expression close to the control group was the RP3 group in the rotenone + *Centella asiatica* group with a 5 µg/ml concentration.

The relationship between increasing the concentration of *Centella asiatica* and the expression of GLUT 1 through the Pearson correlation statistical test showed p= 0.873. It means a very strong correlation between the higher the concentration of *Centella asiatica* affected the increasing the expression of GLUT-1. The increase in GLUT 1 expression strongly correlated with the increase in body length p= 0.739.

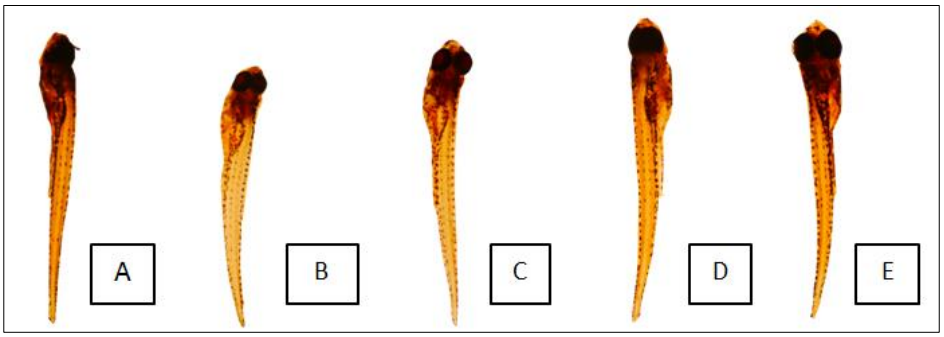

Figure 4 Osteocalcin expression at 9 dpf age of zebrafish larvae induced by rotenone and *Centella asiatica* at 3 different concentrations. Figure (A) control group without rotenone induction; (B) Control group with rotenone; (C) The rotenone + 1.25 µg/ml *Centella asiatica*; (D) The rotenone + 2.5 µg/ml *Centella asiatica*; (E) Rotenone + 5 µg/ml *Centella asiatica*

The color density shown in each group that looks the most concentrated was the control group, then the color density increased in the treatment group along with the increase in *Centella asiatica* concentration. The difference in color quantity in all treatment groups is illustrated in figure 4.

Osteocalcin is a protein found in the bone as a product of osteoblasts [51]. Osteocalcin in zebrafish begins to be expressed at 48 hpf [52]. The 2-day-old embryo begins to be expressed in humans during pregnancy, which originates from the maternal circulation [53].

ROS (Reactive oxygen species) contain oxygen molecules that, when in a state of overproduction in the absence of a balance between antioxidants and pro-oxidants, will cause oxidative stress that causes inflammation [54]. ROS plays an important role in osteoporosis by inhibiting osteoblast [55]. The emergence of oxidative stress can be influenced by insulin/IGF-1, target rapamycin, sirtuins, and AMPK (AMP-activated protein kinase-dependent pathways). The factors mentioned above have the same target, namely stem transcription factor O (FoXo), which has 4 members, where FoXo's role is to maintain osteoblasts [56]. FoxOs are a defense against overproduction of ROS that causes bone disease [57]. Osteoblasts and osteoclasts carry out bone remodeling, and if there is no balance, abnormal bone resorption will occur [58]. ROS produce H_2O_2 which can affect osteoclast differentiation, thereby increasing the number of osteoclasts and bone resorption occurs. These conditions stimulate the expression of RANKL and TNF-α, which can damage cells through the activation of ERK and NF-κβ [59]. Increased osteoclastogenesis stimulates increased O_2 anion resulting in concurrent bone destruction [60].

The study results showed an increase in body length between the rotenone group and the rotenone + *Centella asiatica* extract group starting at a concentration of 2.5 µg/ml at 9 dpf. A significant increase in body length occurred at a five µg/ml, aged 6 dpf. From the analysis, it can be concluded that *Centella asiatica* extract can increase the body length of zebrafish larvae induced by rotenone.

Centella asiatica has a cytoprotective effect by suppressing ROS by protecting the mitochondrial membrane through an increase in VDAC [61]. Another study stated that this plant protects rotenone by protecting the mitochondrial membrane potential, reducing apoptosis of dopaminergic neurons [27]. It also reduces oxidative stress by suppressing fat peroxidation due to an increase in the H_2O_2 defense system in mice [62]. Wijayanti et al. (2016) proven that *Centella asiatica* extract can reduce the expression of Bax and HSP60 in rotenone-induced stunting. Its triterpenoids and active ingredients reduce ROS and prevent oxidative stress due to rotenone exposure [63]. Vitamin C in *C. asiatica* can inhibit the production of cytochrome-c and caspase 3 [12]. *Centella asiatica* potentially prevents chronic inflammation in stunting [18].

This study revealed that the addition of *Centella asiatica* extract could increase the expression of GLUT 1, which was significantly different at a concentration of 2.5 µg/ml *Centella asiatica*. The most significant increase in GLUT 1 expression near the control was *Centella asiatica* at a 5 µg/ml. Based on the Pearson correlation analysis test in this study between the concentration of *Centella asiatica* extract and an increase in GLUT 1 expression, there was a strong positive correlation (0.873), which means the increasing concentration of *Centella asiatica* was followed by an increase in GLUT 1 expression. Then the correlation test between increased GLUT 1 expression and increased body length also had a strong correlation (0.739), which means an increase in GLUT 1 expression followed by an increase in body length in zebrafish larvae.

Centella asiatica is rich in antioxidants in the leaves, stems, roots and can counteract ROS [64]. Its bioactive works as antioxidants, including triterpenes, which have a pharmacological effect on wound healing [65]. The triterpene content inhibits enzymes involved in glucose metabolism, prevents insulin resistance development, and normalizes plasma glucose and insulin levels [66]. The presence of other constituents of gotu kola asiatic acid in large amounts can normalize and enhance the glucose response through signal transduction of PI3K and AKT as a defense of glucose homeostasis. [67].

The bioactive content of *Centella asiatica* affects GLUT 1, which is a glucose transporter for cell metabolism throughout the body, especially for body length growth.

The highest expression data was in the control group, and the rotenone group was the group with the lowest expression. The addition of *Centella asiatica* with increasing concentration affects the increase in osteocalcin expression. The rotenone + *Centella asiatica* concentration 5 µg/ml experienced an increase in osteocalcin expression close to the control group.

The increase in the concentration of *Centella asiatica* could increase osteocalcin expression and had a strong relationship after the Pearson correlation test with the results p= 0.671. It means that the increase in the *Centella asiatica* concentration was followed by an increase in the osteocalcin expression. Meanwhile, the increase in osteocalcin expression also had a strong relationship with the result p= 0.727, which means an increase in osteocalcin expression could increase the body length of zebrafish larvae.

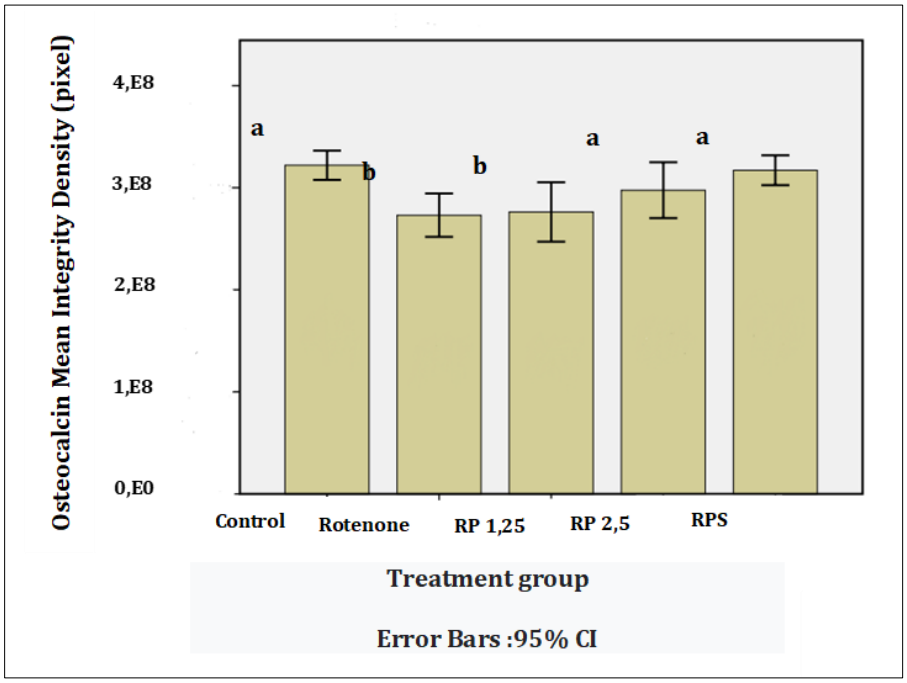

Figure 5 The Integrated Density Expression of Osteocalcin aged 9 dpf zebrafish larvae in all groups. Different letters show significant differences

Based on the analysis results of the One Way Anova test, the addition of *Centella asiatica* extracts together with rotenone in zebrafish larvae from the age of 2 hpf (hour post-fertilization) to 3 dpf (day post-fertilization) showed that the concentration of 2.5 µg/ml started to give a significant difference to increased osteocalcin expression. Then, the Pearson correlation analysis test was carried out between the *Centella asiatica* extract and the increase in osteocalcin expression. The result was a strong positive correlation (0.671), which means that the higher the concentration of *Centella asiatica* was followed by the increase in osteocalcin expression. An increase in osteocalcin expression affects body length, as proven by the correlation test between the two parameters, which has a strong positive correlation (0.727) which means that an increase in osteocalcin expression is followed by an increase in body length in zebrafish larvae.

Osteocalcin, a protein in bone, is very influential on increasing body length, a product of osteoblasts [51]. Adequate micronutrient and macronutrient nutrition greatly affects bone growth and development, which if not met will cause growth disorders such as stunting due to lack of energy, Zn and protein, then rickets due to vitamin D deficiency, abnormal bone growth due to deficiency of Cu, Zn, and Vitamin C [68].

Stunting among infants is caused by chronic inflammation in the womb and persists for the first 2 years of life [32]. *Centella asiatica* contains terpenoids (asiatic acid, madecassic acid, madecassoside, asiaticosside) which are active ingredients that contain high antioxidants [69]. The *Centella asiatica e*xtraction carried out by Jamil et al. (2007) explained that it contains sulfate, phosphate, calcium, potassium, iron, chloride, magnesium, and sodium. Another study explains that 100 grams of *Centella asiatica* consist of 171 mg calcium and 32.51 mg zinc [70]. The calcium and zinc content is essential for bone development, which affects the increase in body length.

The data above shows a strong correlation between the expression of GLUT 1 and osteocalcin. It means that the osteocalcin expression followed an increase in GLUT-1 expression. The addition of *Centella asiatica* extract to both expressions was stronger in GLUT-1 than osteocalcin.

Through the Pearson correlation test, the results obtained that the correlation between GLUT-1 expression and osteocalcin has a strong relationship (0.735), which means an increase in GLUT-1 expression followed by an increase in osteocalcin expression.

The element of bone formation is the role of osteoblasts where the energy source is glucose, where the formation of osteoblasts requires the largest glucose absorption in the body [71]. Osteoblasts are endocrine cells that produce osteocalcin, which supports glucose homeostasis [72]. Osteocalcin is influenced by the process of energy expenditure, glucose homeostasis, and male fertility [53]. The insulin-independent glucose transports from the outside into the cells via the facilitating glucose transporter GLUT-1, whose expression precedes Runx2 during skeletogenesis. Increasing the number of RUNX2 and differentiation of osteoblasts requires glucose by inhibiting AMPK activity, increasing osteocalcin expression. The transcription factor RUNX2 is a major determinant of osteoblast differentiation [73].

Table 2 The increase in GLUT 1 and osteocalcin expression with a Pearson correlation test

		GLUT-1	**Osteocalsin**
GLUT-1	Pearson Correlation	1	0.735
	Sig. (2-tailed)		0.002
	N	25	25
Osteocalsin	Pearson Correlation	0.735	1
	Sig. (2-tailed)	0.000	
	N	25	25

Thus, it is in line with the previous research that the increase in GLUT 1 expression is greater than the increase in osteocalcin expression since GLUT 1 is a glucose transporter for cell metabolism, especially for osteocalcin formation.

4. Conclusion

In this study, it was shown that the rotenone treatment group and the *Centella asiatica* extract at a concentration of 1.25 µg/ml had not been able to give a significant effect on increasing body length. Adding *Centella asiatica* extract at 2.5 µg/ml can give a significant difference effect at 9 dpf. Meanwhile, *Centella asiatica* extracts at a 5 µg/ml concentration at 6 dpf significantly increased body length. This is in line with research conducted by Ridlayanti et al. (2016) and Wijayanti et al. (2016), giving *Centella asiatica* extract at 5 µg/ml can provide a significant difference in age of 9 dpf in rotenone-induced zebrafish larvae.

Compliance with ethical standards

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Disclosure of conflict of interest

We warrant that the article is the Authors' original work and ensure no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is not under review at any other publication.

Statement of ethical approval

The treatment on zebrafish embryos have fulfilled the ethical requirements for test organisms at the University of Brawijaya, Malang, numbered 154/ EC/KEPK/04/2017.

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