Bioscientist : Jurnal Ilmiah Biologi
E-ISSN 2654-4571; P-ISSN 2338-5006
Volume 12, Issue 1, June 2024; Page, 633-642
Email: bioscientist@undikma.ac.id

Colocasia esculenta L. EXTRACT INCREASE GROWTH AND MOTILITY OF ROTENONE INDUCED ZEBRAFISH (*Danio rerio*) LARVAE STUNTING MODEL

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Submit: 24-01-2024; Revised: 04-04-2024; Accepted: 16-04-2024; Published: 30-06-2024

ABSTRACT: This study aims to determine the effect of *Colocasia esculenta* L on the growth and motility of zebrafish (*Danio rerio*) larvae after induction of rotenone as a stunting model. The Zebrafish have divided 3 groups i.e Normal (embryonic medium without rotenone induction), Negative Control (embryonic medium with rotenone induction), and Treatment Groups (embryonic medium with rotenone induction and *Colocasia esculenta* extracts, dose 30-60-90 ppm). The stunting model is designed by induction of 12.5 ppb rotenone to Zebrafish larvae. The growth of the larvae was observed by using a microscope to measure the body and head length. Evaluation of motility was carried out by recording the movement of the fish for 1 minute, and the pace was analyzed by using Video Tracker. The evaluations were observed at 3, 6, 9 dpf (day post-fertilization). Colocasia Esculenta Extract (CEE) groups have higher body lengths compared to the Negative Control Group. The lengths were 125.4 µm for CEE30, 119.4 µm for CEE60, and 121.4 µm CEE90 while the length for the Negative Control Group was 118.3 µm at 9 dpf. However, there were no different values for the ratio of head length to body length of all groups. The ratio was 1:6 at 3 dpf, and 1:4 at 6 and 9 dpf. Moreover, the motility was higher in CEE groups compared to the Negative Control at 3, 6, 9 dpf. The body length and motility of Zebrafish larvae higher compare to the Negative Control Group showing the potential of *Colocasia esculenta* extract on growth and enhancement of motoric activity.

Keywords: *Colocasia esculenta*, *Danio rerio*, Motility, Rotenone, Stunting.

How to Cite: Furqon, M., Kumala, S., Farida, Y., Zaidan, S., & Sumiyati, Y. (2024). *Colocasia esculenta* L. Extract Increase Growth and Motility of Rotenone Induced Zebrafish (*Danio rerio*) Larvae Stunting Model. *Bioscientist : Jurnal Ilmiah Biologi*, *12*(1), 633-642. https://doi.org/10.33394/bioscientist.v12i1.10670

INTRODUCTION

The prevalence of stunting in Indonesia was 30.8% based on The Baseline Health Research that conducted by the National Institute of Health Research and Development, Ministry of Health, Republic of Indonesia. It was a very high number that requires attention from the government and communities to address this problem (Dasman, 2019). Stunting causes many negative consequences, such as decreased development of cognitive, memory, and language function leading to an increment of morbidity and mortality. The conditions will impact not only individuals, but also nations (WHO, 2021).

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Stunting leads to diminished motor skills because it hinders the development of muscle maturity, resulting in decreased muscle capability. Insufficient long-term nutrient intake, particularly protein, fat, and energy, impedes the proper formation and maturation of muscle tissue (Supriatin et al., 2020). The long-term impacts that may occur are inhibition of cognitive and psychomotor development also the higher risk to affected by degenerative diseases. Those describe the low quality of human resources (Dasman, 2019).

Colocasia esculenta Schott belongs to the Araceae family which is popular as Satoimo or Taro. The leaves and tubers of this plant are used as food in several countries such as Japan, China, India, and the Philippines and others (Ubalua et al., 2015). There are about 13 amino acids contained in *Colocasia esculenta*, 7 of which are essential amino acids (Elfita, 2014; Darmayanti et al., 2017). In addition, hyaluronic acid as part of the natural formation of collagen in the body was also identified (Eliantosi & Darius, 2015).

Zebrafish (*Danio rerio*) have many advantages including having a transparent embryonic wall and extra-uterine development so that it be chosen for a growth and development study (Teame et al., 2019; Liu et al., 2023). Due to its resemblance to humans in linear growth and nervous system, zebrafish can be used as a stunting experimental animal model. Previous research showed the induction of 12.5 ppb rotenone during prenatal as a stunting model (Laizé et al., 2014; Zahara et al., 2018). Rotenone is a type of natural insecticide that has a broad spectrum. The toxic effects of rotenone arise through the mechanism of inhibiting complex I of the respiratory chain in mitochondria which in turn causes oxidative stress (Gupta et al., 2017), and reduces the amount of ATP produced. Low concentrations of ATP in the body can inhibit the formation of bone and osteoblasts (Orriss et al., 2017). The objective of this was to determine the effect of *Colocasia esculenta* extract on body length, a ratio of head length to body length, and the motility of zebrafish larvae induced by rotenone.

METHODS

The study was conducted as a true experiment in a laboratory, utilizing either a randomized control group post-test-only design or a random control group post-test design, with sample assignment to each group performed randomly.

Materials

Colocasia esculenta tubers are cultivated by CV. Herbagold in Purwakarta, West Java, and then determined at the National Research and Innovation Agency with letter number B-3171/II.6.2/DI.05.07/9/2022. The characteristics of the taro plant are having a fibrous, wild, and short root system. The tubers weight is more than 4 kg. It has cylindrical or round in shape and brown in color. The leaves are heart-shaped, 20-50 cm in length, with stalks up to 1 m, and have various colors for the midrib. Flowering consists of stalks, sheaths, and cobs. Separate male and female flowers, male flowers are at the top of the female, and barren flowers are at their peak. Buni fruit type flowers, many seeds with an oval shape and 2 mm long (Bago, 2020).

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Extraction

Colocasia esculenta simplicia is dried in an oven at a temperature of 35-45ºC and then coarsely ground using a 60-mesh mill. The extraction using water solvent at a temperature of 40-50ºC. The liquid extract was filtered and then concentrated by evaporation at 45-50ºC. The viscous extract was placed in the oven at 35-45ºC, then finely ground with a grinding machine and sieved using an 80-100 mesh.

Zebrafish Farming

The type of embryo used is transparent, not moldy, round in shape, and aged 0-2 hpf (hour post-fertilization). The embryo is obtained from the fertilization of male and female parents. The zebrafish used as a wild type has been identified at the Laboratory of Fish Cultivation, Faculty of Fisheries and Marine Sciences, Universitas Brawijaya.

Preparation of Embryonic Medium

A 200 ml of embryonic medium was prepared by placing a paper on a digital scale and tared. Weigh all the ingredients ($CaCl_2$ 0.08 g, NaCl 2 g, KCl 0.06 g, $MgSO_4$ 3.2 g) and place them into a test tube, adding 200 ml of filtered water, then shake gently to homogenize. The stock portion is placed in a tightly closed bottle and stored at 2-8 ºC in the refrigerator. When used, the embryonic medium is added to filtered water at a ratio of 1:9 (Avdesh et al., 2012).

Measurement of Body Length and Head Length

Body length (BL) is the standard length that is measured from the tip of the snout to the last vertebrate/caudal tail fin, it's known as snout-fin, in micrometer (µm) unit. Measurement of body length was carried out by using a scaled lens in millimeters (mm) on days 3, 6, and 9 dpf (day post-fertilization). Head length (HL) was measured from the snout to the operculum, then compared with body length, from the snout to the caudal fin.

Motility Determination

Determination of motility at the age of 3 dpf is conducted by using a touch-evoked test. A circle on paper with a diameter of 5, 10, 15, and 20 mm was placed under the well. The zebrafish were in the middle of the smallest circle and the tail is stimulated by using a touch-evoked test. The distance that zebrafish swim away from the stimulus passes the circle and the pace of its movement was observed (Basnet et al., 2017). At 6 and 9 dpf, motility was assessed by observing the distance of spontaneous swimming on a watery petri dish. A camera is used to record the motility for a minute and the pace was analyzed by using video tracker analysis software.

RESULTS AND DISCUSSION

The following are the results of research regarding the effect of *Colocasia esculenta* extract on body length, the ratio of head length to body length, and rotenone-induced motility of zebrafish larvae.

Exploration Studies

The studies were carried out for 9 doses of *Colocasia esculenta* extract i.e. 25, 30, 40, 50, 60, 70, 80, 90, and 100 ppm. This range of doses also determines

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the toxicity value of the extract to zebrafish larvae which would be observed in the two-activity test, survival, and hatching rate.

Survival Rate Study

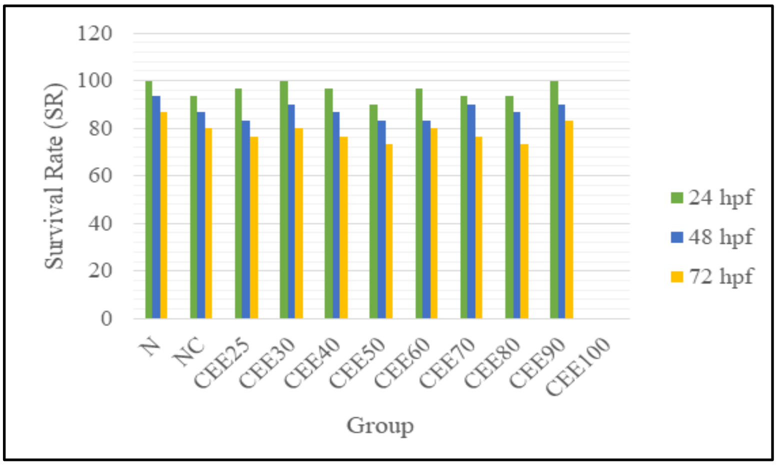

Figure 1. Survival Rate (SR) of Zebrafish Larvae.

The data showed (Figure 1) that all groups except CEE100 have SR values of >80% at 24 hpf which describes the good quality of the embryos. The survival rate of some groups was >80% at 72 hpf, which shows no teratogenic effects of the exposure.

Hatching Rate Study

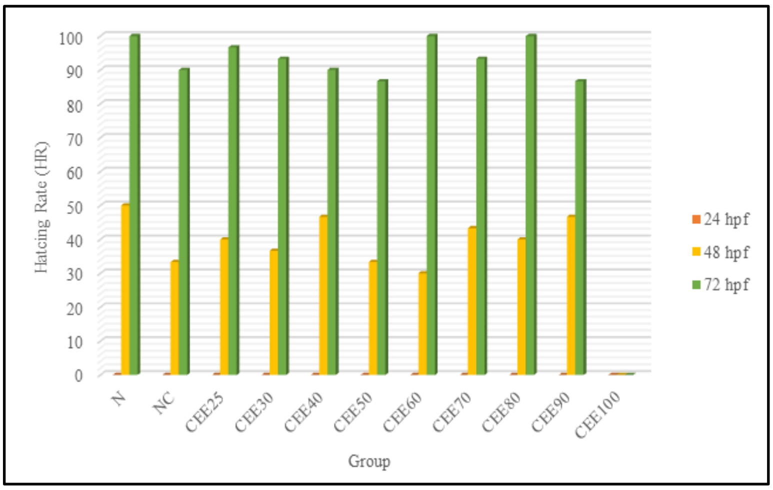

Figure 2. Hatching Rate (HR) of Zebrafish Larvae.

The data showed (Figure 2) that no embryos had hatched in all groups at the age of 24 hpf. The embryos start to hatch at the age of 48 hpf where the hatching rate is increasing at the age of 72 hpf. CEE100 showed no hatching rate nor survival rate, considering the toxic concentration. Based on the data on explorations studies, further determination for body length, ratio body-head length, and motility was applied for CEE30, CEE60, and CEE90.

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Body Length Analysis

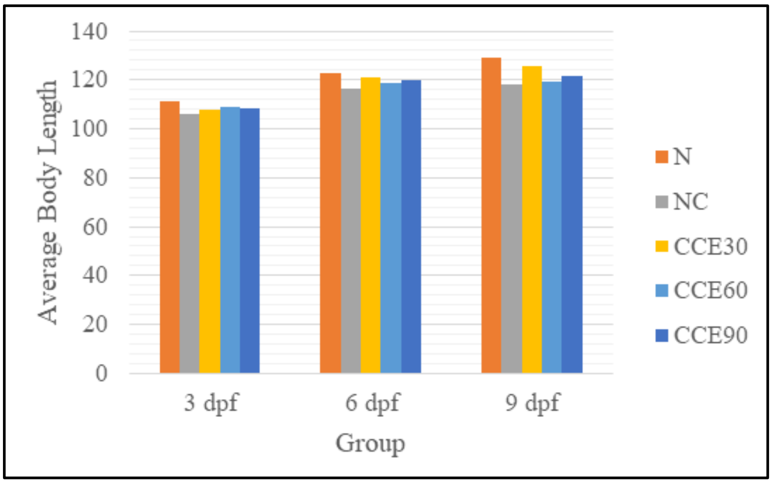

Figure 3. Average Body Length of Zebrafish Ages 3, 6, and 9 dpf.

The data showed (Figure 3) that at the age of 3 dpf there was a significant difference between the Normal and negative control groups, but not significantly different in the CEE30, CEE60, and CEE90 groups. The average body length of zebrafish larvae aged 6 and 9 dpf indicates that there was a significant difference between the Normal, Negative Control, and *Colocasia esculenta* extract groups. Statistical analysis showed no significant difference between the concentrations of CEE60 and CEE90. These data showed that the body length of zebrafish larvae grows normally and consistently from 3 dpf to 9 dpf, and the best concentration for improvement of the body length is at the concentration of CEE30.

The average head-to-body length ratio between Normal (N), Negative Control (NC), and *Colocasia esculenta* extract treatments aged 6 and 9 had the same ratio, 1:4, whereas at age 3 dpf the ratio is 1:6 (Table 2). At the age of 3 dpf, there was still a yolk sac between the head and body of the zebrafish larvae. The equal ratio between groups showed the same and normal growth proportions. The data showed that the administration of *Colocasia esculenta* to zebrafish larvae of the stunting model can affect the growth of body length but does not affect the growth ratio of head length to body length.

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Table 1. Zebrafish Average Body Length.

Age (dpf)	**3**					**6**					**9**				
Group	**N**	**NC**	**CEE30**	**CEE60**	**CEE 90**	**N**	**NC**	**CEE30**	**CEE60**	**CEE90**	**N**	**NC**	**CEE30**	**CEE60**	**CEE 90**
Figure															
BL (µm)	111	106	107.6	108.8	108.6	123	116.2	120.8	118.6	120	129.2	118.3	125.4	119.4	121.4
SD	0.894	0.894	0.8	0.748	0.8	0.632	0.748	0.98	0.49	0.748	0.748	0.748	0.49	0.8	1.02

The average of body length at the age of 3 dpf is different, and none is shorter than -2SD compared to the control group. ANOVA tests showed a significant difference between groups ($p < 0.05$). For the extract treatment group, the highest average body length was shown in CEE60, can be seen in table 1. There was an increase in the average body length in all treatment groups and there was a significant difference between all treatment groups at the ages of 6 and 9 dpf. CEE30 is considered as the optimum dose to increase the body length in zebrafish larvae after rotenone induction.

Table 2. The Ratio of Head Length to Body Length of Zebrafish.

Age (dpf)	**3**					**6**					**9**				
Group	**N**	**NC**	**CEE30**	**CEE60**	**CEE 90**	**N**	**NC**	**CEE30**	**CEE60**	**CEE90**	**N**	**NC**	**CEE30**	**CEE60**	**CEE 90**
Average HL (µm)	19.0	15.9	17.9	18.6	18.3	35.0	29.8	34.4	32.8	31.8	32.0	27.8	30.4	28.8	29.8
Average of HL(µm)	111	106	107.6	108.8	108.6	123	116.2	120.8	118.6	120	129.2	118.3	125.4	119.4	121.4
Rasio BL:HB	1:6	1:6	1:6	1:6	1:6	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4

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Motility Test

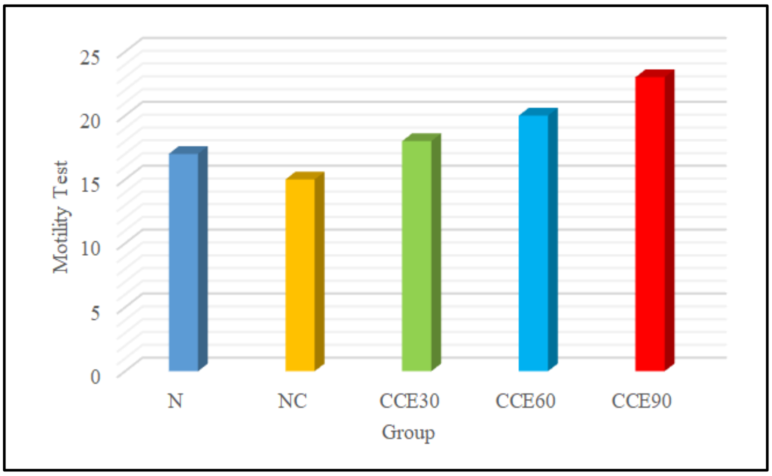

Figure 4. Motility Test of Zebrafish at the Age of 3 dpf.

Motility at 3 dpf has a different distribution between groups. Administration of Rotenone and 90 ppm of *Colocasia esculenta* extract (CEE90) had the highest motility compared to the other groups, can be seen in Figure 4.

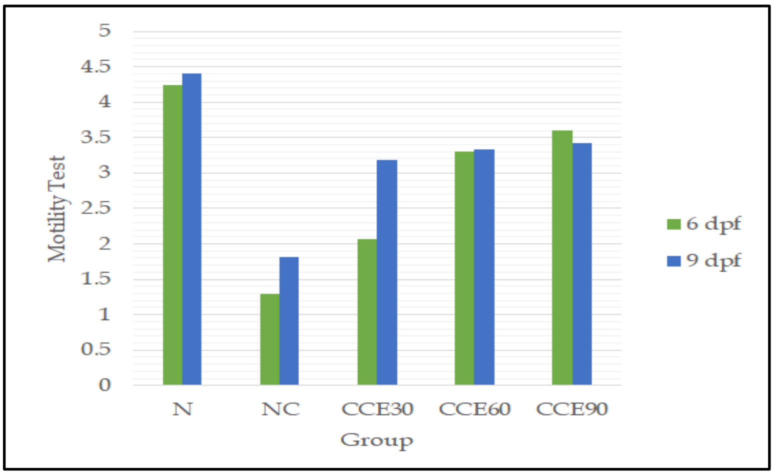

Figure 5. Motility Test of Zebrafish at the Age of 6 and 9 dpf.

The motility of zebrafish larvae at ages 6 and 9 dpf had the same pattern as 3 dpf whereas the highest is in the Normal group and the lowest is in the Negative Control group, can be seen in Figure 5. CEE90 had the highest motility compared to other doses. The average motility was increased according to the lifetime. One Way ANOVA with the Post Hoc LSD follow-up test showed the motility at 6 dpf had a significant difference between the Normal and Negative Control group ($p < 0.05$).

Stunting is a condition when a z-score of height to age is below -2SD based on World Health Organization (WHO) standard (WHO, 2017). Therefore, body length is the main indicator in this study. To observe the growth of the body represented by comparing the body length between the Normal and Negative

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Control groups. The data showed that the shortest body length was in the Negative Control group. Zebrafish larvae are at the age of 3, 6, and 9 dpf equal to new-borns, 2 and 8 years old in human (Primihastuti et al., 2022). The results of this study are in accordance wherewith stunting, congenital abnormalities in new-born are often unidentified and will appear at 2 years old.

Rotenone induction with a concentration of 12.5 ppb for 3 days showed no acute toxic effects. This survival of the embryo is more than 80% at the age of 3 dpf. It represented that stunting occurs as a result of a chronic and multifactorial process. The ratio of head length to body length remained 1:6 at 3 dpf, and 1:4 at 6 and 9 dpf. Some stunting children not showing different body proportions with the normal (Zahara et al., 2018). Stunting and cretinism are 2 different things where in cretinism the short stature accompanied by change in body proportions compared to normal (Syed, 2015).

Exposure of rotenone 12.5 ppb during embryonic period induced the stunting model (Zahara et al., 2018; Khotimah et al., 2018). Rotenone inhibits complex I mitochondrial resulting in a decrease of ATP and an increase in free electrons that can bind with oxygen and form Reactive Oxygen Species (ROS). ROS reacts with polyunsaturated fatty acids (PUFA) cause lipid peroxidation which causes an increase in the amount of Malondialdehyde (MDA) (Gupta et al., 2017). In addition, rotenone decreases the number of endogenous antioxidants such as catalase, GSH, and SOD (Indah & Safnowandi, 2020; Zahara et al., 2018). MDA can react with various macromolecules such as proteins and nucleic acids and induce apoptosis of progenitor nerve cells resulting in developmental disorders. Apoptosis also occurs in dopaminergic neurons so dopamine, which functions in motor function, decreases and reduces motility.

Nutrition such as amino acids are micronutrients needed by the body, especially during pregnancy. Amino acids play a role in DNA synthesis which is very important in the initial process of growth and development. In addition, amino acids prevent oxidative stress and scavenging ROS and prevent lipid peroxidation. Amino acids can reduce the activity of NADPH oxidase which plays a role in producing ROS and increases GSH an endogenous antioxidant. It can be understood that decreasing the number of ROS and increasing antioxidant activity can prevent oxidative stress. The amino acid contains in *Colocasia esculenta* extract has the potential effect as an antioxidant and enhances the growth and motility of the zebrafish.

CONCLUSION

Colocasia esculenta extract enhances the growth and motility of rotenone-induced zebrafish and thus has a potential effect as anti-stunting.

SUGGESTION

More investigation is necessary regarding the developmental progress of zebrafish larva models exhibiting stunting, extending beyond the age of 9 days post-fertilization (dpf) up to adulthood. Further studies are warranted to analyze biomolecular markers like IGF-1, MDA, and dopamine to validate the impact of administering Japanese taro extract.

ACKNOWLEDGMENT

We'd like to thank the Ministry of Education, Culture, Research, and Technology for Matching Fund Scheme with contract number: 243/E1/KS/06.02/2022.

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