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Protection of *Centella asiatica* Extract Through BDNF Expression on Stunting Model Zebrafish Larvae (*Danio rerio*) by Rotenone Induced

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***Abstract*—Objectives: Stunting in WHO Child Growth Standards is based on the length-for-age indexes with less than -2 SD of z score. Rotenone is a pesticide model that acts as an mitochondrial complex I inhibitor and Endocrine Disrupting Chemicals (EDCs). *Brain Derived Neurotrophic Factor* (BDNF) is one of growth factors mostly found in central nervous system and slightly in peripheral nerves. One of natural herb, *Centella asiatica*, has antioxidant effects and grows in tropical areas in Indonesia. The aim of this research was to know the effects of *Centella asiatica* extracts on *zebrafish larvae (Danio rerio)* by Rotenone induced, through *Brain Derived Neurotropic Factor* (BDNF) expression. Methods: Zebrafish embryos were divided into five groups including control (C), rotenone group (R), and *Centella asiatica* group (Ca1;Ca2;Ca3) with concentration of the each 2.5, 5, and 10 □g/mL. The entire groups had been started since 2 until 72 hpf (half post fertilization) and followed its development up to the age of larvae 9 dpf (days post fertilization). It was followed by measuring the body length at the age of 3, 6 and 9 dpf using Image Raster software. After termination zebrafish larvae, the BDNF expression measurement was measure by using an Immunohistochemistry (IHC) wholemount method with diamino benzidine (DAB). Brown colour density values was quantified by using Image J software. Results : There was a significant difference in the mean of body length which was shown by Ca2 group (5 □g/ml) compared with rotenone group (p < 0,05). The BDNF expression increased in all three *Centella asiatica* groups compared with rotenone group. However, only *Centella asiatica group* (5 □g/ml) showed the most significant effect on the increase of BDNF expression (p <0.05). Conclusion: Protection of *Centella asiatica* extract increases the body length and BDNF expression on the stunting model zebrafish larvae induced by rotenone.**

***Keywords*—Centella asiatica, rotenone, zebrafish, stunting, body length, BDNF**

I. INTRODUCTION

Stunting in WHO Child Growth Standards is based on the length-for-age or height-for-age indexes with the z-score less than -2 SD. The stunting prevalence categories are low (<20%), medium (20-29%), high (20-29%), and very high (>40%). The prevalence of stunting in children under five in Indonesia is still relatively high. Based on the complete analysis of national assessment in 2010, approximately 37% of Indonesian children experienced stunting. The Indonesia basic health research (2013) reported that the national prevalence of stunting increased from 35.6% (2010) to 37.2% (2013). The increase of the prevalence showed that one in three children in Indonesia experiences impaired growth due to stunting growth[1]. Factors affecting child development consist of genetic and environmental factors. Environmental factors during prenatal period are maternal nutrition during pregnancy, toxic or chemical substances, radiation, stress, anoxia embrio, immunity, infection, etc[2]. In the Kolsteren chart[1], toxin or chemical substances became a determining factor for the occurrence of stunting. One type of toxin or chemical substances is pesticide[3]. Pesticide, categorized as endocrine disrupting chemicals (EDCs), is a chemical substance which interferes synthesis, secretion, transport, metabolism, binding and elimination of various hormones in the body including thyroid hormone[4]. Rotenone is a natural compound obtained from roots and stems of plants and can be used as an ingredient in a broad spectrum insecticide, piscicide, and pesticides[5]. Rotenone acts as an inhibitor of mitochondrial complex I and a source of Reactive Oxygen Species (ROS)[3]. The effects on the body are reducing the availability of Adenosine Tri Phospate (ATP), oxidative damage, and cell death[6].

II. DESIGN, MATERIALS, AND METHODS

This research design was true experimental study with post-test-only control group design approach. Animal testing. Z*ebrafish* embryos at age of 2 hpf was obtained from adult zebrafish breeding. The embryos must be transparent, not moldy and white. Death embryos and unfertilized could not be used. Embryos were divided into 5 groups including negative control group (normal), positive control (induced by rotenone 10 ppb/ exploration study report), treatment group I (Ca1) (rotenone 10 ppb+Ca extract 2,5 µg/mL), treatment group II (Ca2) (rotenone 10 ppb+Ca extract 5 µg/mL), and treatment group III (Ca3) (rotenone 10 ppb+Ca extract 10 µg/mL). The exposure was started from 2 until 72 hpf and measured the development of body height up to the age of 9 hpf and then it was terminated. The treatment on the zebrafish embryos had fulfilled the ethical requirements towards animal testing in Health Polytechnic, Ministry of Health Malang. Embryonic medium preparation. Embryonic medium was made from CaCl 0,08 gr; Kcl 0,06 gr; NaCl 2 gr; $MgSO_4$ 3,2 gr which will produce 200 ml of embryonic medium medium[7].*Centella asiatica* extraction process. *Centella asiatica* was gained from Materia Medika, Batu, Malang, East Java. The aerial part (not include roots and stolon) was used[8]. Extraction utilised a maceration method with ethanol 96% as a diluter[8][9]. The results of extraction was crude extract within pasta and diluted with normal saline, stored at 00C. The process of extraction was conducted at pharmacology laboratory, Faculty of medicine, Brawijaya University. Rotenone concentration and Centella asiatica extract. Rotenone (Sigma 8875) concentration used in the study was 10 ppb (based on explorative experiment). Explorative study utilised concentration of 0,625; 1,25; 2,5; 5; 10; 20; 40; 80 ppb. The results of the first exploration study were congenital abnormalities (concentration 20 ppb), low survival rate (concentration 40 ppb), and death (concentration 80 ppb) less than 72 hpf. The second exploration with various concentrations (0,625; 1,25; 2,5 and 5 ppb) has not shown a reduction in body length towards stunting growth. Centella asiatica extract 10 mg/mL as a stock, using concentration 2,5; 5; 10 µg/mL[8]. Embryo medium is a mixture of embryonic medium, rotenone, and CA extract which is relevant with the concentration of research groups. The exposure was conducted from 2 to 72 hpf. Medium was changed daily. Body length measurement. Body length of Zebrafish larvae were measured at the age of 3, 6 dan 9 dpf using calibrated Image Raster. Larvae were observed with a stereo microscope connected with OptiLab viewer. The body length of larvae or Standard Length (SL) were measured from snout to caudal fin[9]. Zebrafish larvae euthanasia. Zebrafish larvae at the age of 9 dpf were performed euthanasia based on NIH protocol. Wholemount zebrafish larvae were placed into micro tube in iced water minimal 5 minutes and ensured that there was no movement. Then, it was rinsed and fixed with parafolmaldehide (PFA) 4% at 40C overnight, followed by exposing MeOH 3% for 5 minutes and H2O2 3% for 24 hours to remove the color pigments prior to the procedure Immunohistochemistry (IHC) whole mount. Wholemount Imunohistochemistry (IHC). Larvae at the age of 9 dpf was in methanol, then rinsed with PBSTx (3x5'). Placed larva in the distilated water then washed with PBSTx (3x5'). Acetone was given for 20 minutes, stored at 40C and rewashed with PBS (3x5'). Larvae were given collagenase enzyme 1 mg/ml and rewashed with PBS (3x5'). Larvae were placed into acetate acid 10% (40C;10"), then washed 3 times with PBSTX. NHS 10% and BSA 3% (room temperature; 3 hours) were given to the larvae. Primary antibody (1/10) 15 □l/ NHS 15 □l/ PBS 1 ml were needed, then washed 4 times with PBSTX (room temperature; 1 hour). After that, larvae were given secondary antibody around 1 □l/ NHS 15 □l/ PBS 1 ml, then washed twice with PBSTX (room temperature; 1 hour). ABC reagent was given including reagent A 20 □l/ PBS 1 ml, then it was followed with reagent B 20 □l, after that rinsed with PBSTX (3x5') and stored at room temperature. Larvae were given DAB colouring for 5 minutes in the room temperature, then washed with PBSTX (3x5'), stored at room temperature. Larvae can be stored in the 87% gliserol (survive up to 6 months). Wholemount zebrafish larvae at the age of 9 dpf were placed on the slide, then they were observed under inverted microscope connected with digital camera (Panasonic DMC G6 Lumix) to get the pictures. The results of brown colour density were quantified using image J software within integrated density value. Statistical analysis. The data of body length was analysed using independent t test, Kruskal Wallis test, and Mann Whitney. The difference of BDNF expression among treatment groups used One Way Annova. Significant level in the study was 95%. The analysis utilised SPSS 22.0 software for windows.

III. RESULTS

The effects of *Centella asiatica* extract on the body length There was a difference in the body length of CA group compare to rotenone group with the mean of body length of CA was higher than rotenone group and closed to control.

TABLE I. THE BODY LENGTH OF ZEBRAFISH LARVAE INDUCED BY ROTENONE AND CA EXTRACT.

Hari ke-	3 dpf					6 dpf					9 dpf				
Kelompok	K	R	RP1	RP2	RP3	K	R	RP1	RP2	RP3	K	R	RP1	RP2	RP3
Gambar															
PB (mm) Means ± SEM	3,24 ±0,02	3,20 ±0,02	3,15 ±0,02	3,19 ±0,02	2,83 ±0,03	3,63 ±0,02	3,49 ±0,03	3,53 ±0,04	3,54 ±0,02	3,41 ±0,06	3,79 ±0,02	3,67 ±0,02	3,70 ±0,03	3,74 ±0,02	3,65 ±0,05

Notes :
K : Control
R : Rotenone
RP_1/Ca1 : Rotenone with Ca 2,5 µg/ml
RP_2/Ca2 : Rotenone with Ca 5 µg/ml
RP_3/Ca3 : Rotenone with Ca 10 µg/ml

Based on the graph below, there were growth lines of body length among 5 groups at the age of 3, 6, and 9 dpf. Rotenone group had lower in the body length than control group. On the other hand, the body length of Ca1 and Ca2 groups was higher than rotenone group and closed to control group.

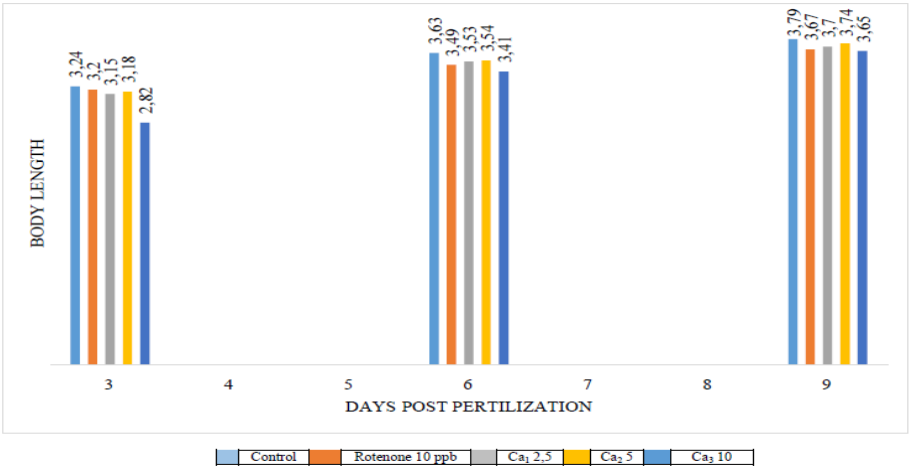

Fig. 1. The graph of the mean of zebrafish larvae body length among control, rotenone, and CA group.

Rotenone group showed lower in the mean of body length than control group. The additional of body length occurred on the Ca1 and Ca2 compared with Rotenone group

TABLE II. THE RESULTS OF KRUSKALL WALLIS TEST AND MANN WITHNEY U TEST ON THE BODY LENGTH AMONG GROUPS.

Tested groups	t test *p-value*		Conclusion
	Day 6th	Day 9th	
Rotenone with Ca1; Ca2; Ca3	0,233	0,191	(*) Significant P< 0,05

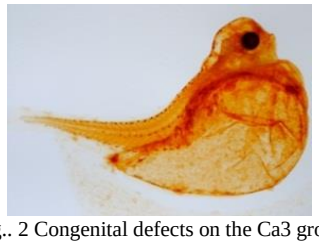

Fig.. 2 Congenital defects on the Ca3 group

On the day 6th, there was no difference in the body length between rotenone group and Ca1, Ca2, and Ca3 groups. In addition, on the day 9th, the body length of rotenone group and Ca2 group showed a significant difference. Therefore, from the results of analysis table. 2, it can be concluded that Ca2 group with concentration 5 μg/ml showed that there was a significant effect on the addition of body length in the rotenone group. Ca3 group (10 μg/ml) has showed the shortest in the mean of body length compared with the other groups since at the age of 3 dpf. Congenital defects were also found in this group.

BDNF Expression

From several analysis below, it can be concluded that there was an increase in the BDNF expression among three groups including Ca1 group (concentration 2,5 μg/ml), Ca2 group (concentration 5 μg/ml), and Ca3 group (concentration 10 μg/ml) compared with rotenone group, however, only Ca2 group (concentration 5 μg/ml) showed the relevance between the addition of body length and BDNF expression. It showed that there was a significant effect of Ca concentration 5 μg/ml on the addition of body length of stunting model zebrafish larvae induced by rotenone. The results of brown colour density measurement on the CA group described in the table III.

TABLE III. THE RESULTS OF BROWN COLOUR DENSITY MEASUREMENT ON THE ZEBRAFISH LARVAE AT THE AGE OF 9 DPF USING IHC-WHOLEMOUNT METHOD

Groups	*Integrated density*	SD	SEM	*p-value*	Conclusion
K	288075417	$3{,}96 \times 10^7$	$1{,}77 \times 10^7$	0,005	Difference
R	231511717	$2{,}33 \times 10^7$	$1{,}04 \times 10^7$	0,005	No Difference
Ca1	262129267	$1{,}67 \times 10^7$	$7{,}48 \times 10^6$	0,098	No Difference
Ca2	290757179	$2{,}79 \times 10^7$	$1{,}25 \times 10^7$	0,003	Diferrenece
Ca3	232426827	$2{,}56 \times 10^7$	$1{,}28 \times 10^7$	0,961	No Difference

Table.III showed that there was a difference in whole groups with *p-value* < 0,05. It means that *integrated density* value on the Ca1, Ca2, and Ca3 was higher than rotenone group, but only Ca2 group closed to value of control. Integrated density value on the Ca3 group had no difference with rotenone group. This shows that Ca2 group has the most significant effects on the stunting model zebrafish larvae induced

IV. DISCUSSION

Protection effects of *Centella asiatica* extract on the stunting model zebrafish induced by rotenone through BDNF expression The results of the study showed the increase of body length mean on CA group compared with rotenone group and the mean value of body length of zebrafish larvae on CA group closed to control group (table and figure 1). At the age of 3 dpf, the body length between rotenone group and CA group is not much different, however, several samples were obtained congenital abnormalities on the Ca3 group (figure 2). Based on some analysis results above, it can be concluded that CA increase the body length of zebrafish larvae exposed by rotenone.*Centella asiatica* is a natural herb that has the effects of antioxidant[10]. Some ingredients of antioxidant of CA are polifenol, flavonoid, □□ caroten, tannin, vitamin C[11]. Moreover, antioxidant capacity in CA is associated with phenolic and flavonoid in CA[12]. Kumar (2009) reported that CA against colchicine that induce cognitive disorders through oxidative damage[13]. Additionally, Khotimah et al (2015) found that CA extract was used as anti-inflammatory and antioxidant on the dopaminergic neuron from toxic influences of rotenone on zebrafish[6][14].

As described in the previous sections, rotenone increases oxidative stress[15]. Oxidative stress is dangerous because it can activate chemicals such as Reactive Oxygen Species (ROS) which is a free radical. Free radicals have unpaired electrons that are highly reactive and unstable. Free radicals have various chemical structures such as hydroxyl, superoxide, nitrite oxide, and radical peroxil lipid[16]. To reach stability, free radicals damage nearest molecules to gain electrons, this condition is dangerous for the structure and function of the molecule. The reactivity of free radicals can harm all macromolecule cellular including proteins, carbohydrates, lipids, and nucleic acids, including DNA because it can be a precursor genotoxicity. When the presence of free radicals is excess while the antioxidant is low, oxidative stress condition will develop which may lead to chronic diseases and dangerous permanent[17].

The increase of body length on the Ca1 and Ca2 groups compared with rotenone group may be caused by the antioxidant function of Ca. If antioxidant in the body increases, the possibility of oxidative stress as the results of rotenone induction would decline and metabolic process in the body would continue[10]. The rotenone induction on zebrafish elevates oxidative stress and cause the harm in mitochondria function[18][19]. The exposure of Centella asiatica declines oxidative stress. Centella asiatica also acts in mitochondria by repairing voltage-dependent anion channel (VDAC) and cleaning free radicals[20]. Tewari et al., (2016) found that Centella asiatica has protection effects on N2a cells by against ischaemia reperfusion (IR) injury and reduce ROS. Moreover, Centella asiatica prevents the increase of intracellular calcium and dan the depletion of mitochondria potential membrane due to oxygen glocose deprivation (OGD)[20]. Centella asiatica also has sitoprotection effects by stabilising human VDAC-1 and protect mitochondrial permeability transition pore (mPTP) from oxidative stress[11]. Based on table 2, three groups showed no differences in the mean of body length at the age of 9th compared with rotenone group (p value= 0,191), on the other hand, on the Ca2 group (5 □g/ml) had a significant result with p < 0,05 which means that there was a significant difference of body length between Ca2 group and rotenone group (p value= 0,024). Therefore, it can be concluded that CA group with concentration 5 □g/ml had a significant effect on the addition of body length on the day of 9th. In addition, this study found that

Ca1 and Ca3 groups showed the effect of the addition body length was not significant compared with Ca2 group. Significant effect of Centella asiatica concentration 5□□g/ml started at the day 9th as Ca had low bioavaibility. Another finding in this study was the availability of congenital abnormalities of the sample in figure 2. This sample was in Ca3 (10 □g/ml) group. In addition, the mean of body length was less than normal average (2,83 mm) on the day 9th due to toxicity effect of CA or unknown genetic factor. This study did not measure specific causes of mutated gene. In other research there was no toxicity on experimental animals exposed by Centella asiatica such as death and no clinical symptoms of poisoning at dose levels up to 2000 mg/kgBW[7]. Another study reported that there was no poisoning effect on mice with dose 3-7 g/kgBW. Ca plants juiced which was consumed by experimental animals around 2 ml had no toxic effects[21]. The side effects of this plant were gastrointestinal disorders and nausea, redness on the use of topical, jaundice with the elevation of liver enzyme with unknown dose consumed for 20-60 days[11].

This study found that there was an increase in BDNF expression on stunting model zebrafish larvae induced by rotenone. BDNF expression in the all Ca groups groups increased compared with rotenone (table 3). However, the most significant shown in the Ca2 group with concentration 5 □g/ml. The data proved that Centella asiatica is rich of antioxidant that reduce oxidative stress due to rotenone induction so transcription process and translation of mRNA BDNF continued and BDNF expression could increase[22]. BDNF regulates TGF□□ and BMP-2 contributes in the bone development, so the bones grow normally[23][24][25]. The increase of BDNF expression was relevant with data of body length mean that increased on the day 6th and 9th compared with rotenone group. Nevertheless, based on One Way Annova test, the significant effect of Ca on the BDNF expression was shown in the Ca2 group on the day 9th. Another finding, Ca3 group (10 µg/ml) had anomaly abnormalities, hence, an increase of BDNF expression was not significant compared with rotenone group.

Therefore, it can be concluded that *Brain Derived Neurotropic Factor* (BDNF) contributes in patomechanism the occurrence of stunting model zebrafish larvae induced by rotenone with the decline of BDNF expression induced by rotenone. Moreover, there are protective effect of *Centella asiatica* extract with elevates BDNF expression on stunting model zebrafish larvae induced by rotenone.

V. CONCLUSION

Centella asiatica extract [5 µg/mL] has a protection in the elevation of body length zebrafish larvae (*Danio rerio*) at the age of 9 dpf through BDNF expression.

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