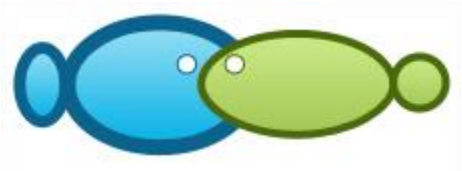

Centella asiatica alleviates neurotoxicity and development of lead-exposed zebrafish larvae

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Abstract. Lead exposure causes excessive reactive oxygen species (ROS). It can cause opened mitochondrial permeability transition pore (PTP), followed by the translocation of proapoptotic molecules from mitochondria to cytosol, which are the causes of apoptosis. If this happens to the neuronal cells, it will disrupt the neurotransmitter system including its receptors, like the dopaminergic system characterized by decreased expression of tyrosine hydroxylase (TH), disruption in dopamine synthesis and its receptors, which can decrease locomotor activity and affect ossification as well. Increased ROS can reduce the expression of SIRT1 and also increase apoptosis. *Centella asiatica* can prevent the disruption of embryonic development in stage proliferation and differentiation. The aim of this research was to evaluate the level of dopamine, TH and the expression of its receptors (DRD1 and DRD2), ossification, sirtuin-1 and apoptosis. This experiment used lead-induced zebrafish (*Danio rerio*) larvae. The expression of dopaminergenic genes was analyzed through RNA analysis, and locomotor activity was observed using JPG Video converter software and ImageJ V 1.50. Apoptosis was measured using a fluorescent microscope, while the ossification of bone analysis uses alizarin red staining and was analyzed by ImageJ V 1.50. The results showed that *C. asiatica* increases the expression of TH and SIRT1, the level of dopamine, and dopamine receptor D1 expression. *C. asiatica* can also decrease the expression of dopamine receptor D2 and apoptosis, increase the locomotor activity and ossification in lead-exposed zebrafish larvae. It can be concluded that *C. asiatica* can prevent developmental disorders in lead-exposed zebrafish larvae.
Key Words: bone ossification, dopamine receptor, locomotor, SIRT-1, tyrosine hydroxylase.

Introduction. Pollution is a worldwide problem with a wide impact. Heavy metal compounds are often a source of pollution arising from human influence and interference, due to anthropogenic activities (Richetti et al 2011). A heavy metal harmful to the environment is lead. Lead is a chemical element known to have neurotoxic effects, especially in humans and in animals (Wani et al 2015).

One of the causes of lead toxicity is oxidative stress. Lead ions are associated with increased reactive oxygen species (ROS). Moreover, lead can reduce antioxidant defenses, including antioxidant enzymes and non-enzymatic antioxidants (Dobrakowski et al 2016). The effects of low lead toxicity during pregnancy are abortion, pregnancy hypertension, infertility, premature membrane rupture, premature and pre-eclampsia labor in humans (Assi et al 2016). During childhood, damage occurs to the development of the central nervous system. The brain injuries due to exposure to lead early in life include loss of intelligence, lack of attention, and behavioral disorder (WHO 2010).

Centella asiatica is a plant that is widely used for treatment as an anticancer agent (Wu et al 2017), antiinflamatory agent (Ju Ho et al 2018), wound healing agent (Azis et al

2017), antibacterial and antioxidant agent (Kesornbuakao et al 2018), etc. Among other nutrients, *C. asiatica* has triterpenoids, which act as antioxidants, preventing oxidative stress. In addition, *C. asiatica* also contains quercetin, and it is able to chelate heavy metals (Chandrika & Prasad Kumara 2015; Flora et al 2012). Some studies have proven that *C. asiatica* has a nootropic effect, being able to protect the human brain from damage caused by adulthood age, induces cell growth and acts as an anti-inflammatory antioxidant (Hashim 2011; Khotimah et al 2010). Lead toxicity has been demonstrated in several animal experiments, including zebrafish (*Danio rerio*). Lead affects nerve morphogenesis and dendrites branching in zebrafish (Zhang et al 2011). Zebrafish is one of the experimental animals that are widely used for toxicological research because it has similarities with humans in gene homology and spinal arrangement (Chakraborty et al 2009), so it is also widely used for research in neurodegenerative diseases (Orhan 2012).

The aim of this study was to evaluate the protection mechanism ensured by *Centella asiatica* to zebrafish larvae by analyzing the dopamine system (DA level, expression of TH, receptor DRD1 and DRD2), expression of SIRT1, apoptosis and ossification.

Material and Method

Collection and maintenance of zebrafish eggs. Zebrafish maintenance was carried out in 60 L aquariums. Freshwater with a temperature of 26-28.5ºC, and a pH of 6.8-7.5 was used (Avdesh et al 2012). Tetramine flakes are administered as feed *ad libitum*, 3 times a day (Khotimah et al 2015). The light:dark cycle was 14:10 hours (Avdesh et al 2012). The eggs obtained from the reproduction of the fish are cleaned by changing the medium several time until the debris of food and feces were eliminated, and examined under a microscope. The eggs were fertilized, clear and rounded. The selected eggs were placed on a well plate. 180 eggs were placed in 6 wells (30 eggs per well) and treated differently, according to each group. This procedure was conducted before the embryo was 2 hpf (hours post fertilization). The treated eggs were stored in an incubator at 28ºC and the embryonic medium was changed daily.

Research design. The experimental study using zebrafish embryos used 5 treatment groups, by following their development from 2 hpf to 6 dpf (days post fertilization). The treatments were: G1 - control group without exposure to lead or *C. asiatica*; G2 - group exposed to lead with a concentration of 2.5 ppm; G3 - group exposed to lead (2.5 ppm) and *C. asiatica* (1.25 µg mL^{-1}); G4 - group exposed to lead (2.5 ppm) and *C. asiatica* (2.5 µg mL^{-1}),; G5 - group exposed to lead (2.5 ppm) and *C. asiatica* (5 µg mL^{-1}). The research was conducted at the Pharmacology Laboratory of the Faculty of Medicine, Universitas Brawijaya, Malang, from March to June 2019.

Lead acetate trihydrate. Lead acetate trihydrate - $Pb(CH_3COO)\ 2.3H_2O$ - in the form of white powder (MERCK, produced in Indonesia) was dissolved in distilled water (AquaDest) to obtain a stock concentration of 3793.3 ppm. The stock was diluted to 50 ppm and 10 ppm. For obtaining a 2.5 ppm stock, 1.25 mg mL^{-1} lead solution is retrieved from a stock of 10 ppm, and dissolved in the embryonic medium (5 mL for each well).

C. asiatica extraction. *C. asiatica* was obtained from UPT Materia Medica Batu, Malang, East Java, Indonesia. *C. asiatica* extract was obtained from the leaves and stems. They were cut and dried at 40ºC using an oven. Dry simplicia was blended and sifted. The fine powder of *C. asiatica* was macerated with 98% ethanol for 24 hours 3 times and then filtered. The obtained filtrate was concentrated with a rotary evaporator until thick extracts were obtained and evaporated to remove the residual ethanol. Based on preliminary studies, *C. asiatica* extracts were administered in the following concentrations: 1.25 µg mL^{-1}, 2.5 µg mL^{-1}, 5 µg mL^{-1}.

Embryonic medium. The embryonic medium contained 0.25 g CaCl, 0.15 g KCl, 5 g NaCl, 0.815 g MgSO4, and 500 mL AquaDest. When used, it was further diluted in AquaDest, to obtain a 10% concentration.

Expression of tyrosine hydroxylase, SIRT1, and dopamine receptors D1 and D2. For gene expression analysis, embryos were collected immediately after the 72 hours. Isolation of RNA was conducted using RNA isolation kit (Geneaid No. Cat RT-100 produced in Taiwan). RNA was purified using the manufacturer protocol prior to cDNA synthesis to purify the samples and remove genomic DNA. The synthesis of cDNA (TOYOBO FSQ-301 produced in Japan) was conducted using the manufacturer protocol. The specific zebrafish primers were designed for TH: F"GAC GGA AGA TGA TCG GAG ACA"; R"CCG CCA TGT TCC GAT TTCT". The remaining zebrafish primers were designed by us for an annealing temperature of approximately 55.6–56.2°C. The primer sequences for SIRT 1 are: F"CAA GGA AAT CTA CCC CGG ACA GT", R "CAG TGT GTC GAT ATT CTG CGT GT". The remaining zebrafish primers were designed by us for an annealing temperature of ~60°C. The primer sequences for DRD1 are: F "TGG TTC CTT TCT GCA ACC CA", R"AGT GAT GAG TTC GCC CAA CC". The primer sequences for DRD2 are: F" TCC ACA AAA TCA GGA AAA GCG T", R"CAG CCA ATG TAA ACC GGC AA". The remaining zebrafish primers were designed by us for an annealing temperature ~60°C. β-actin zebrafish primers: F "ATG GAT GAG GAA ATC GCT GCC", R " CTC CCT GAT GTC TGG GTC GTC". The thermal cycling parameters for real-time PCR were as follows: pre denaturation at 95ºC for 3 minutes; denaturation at 95ºC for 3 minutes; 62ºC for annealing for 30 s; elongation at 72ºC for 90 s; post elongation at 72ºC for 3 minutes; and 4ºC for cooling.

Dopamine. Zebrafish embryos were exposed to 2.5 ppm lead for 72 hours and were incubated with 1.25, 2.5 and 5 μg mL^{-1} of ethanolic extract of *C. asiatica*. After 6 days, zebrafish larvae were euthanized and protein isolation was carried out for dopamine ELISA examination. Dopamine examination was carried out using the ELISA KIT Elabscience No. Cat E-EI-0046 (produced in China).

Apoptotic measurement. Apoptosis in zebrafish larvae (after hatching at 3 dpf) was examined for the head, body and the caudal fin. The zebrafish embryos were incubated with propylthiouracil (PTU) in a concentration of 0.0003% for 3 days, to remove pigmentation. The larvae were incubated in acridine orange (5 μg mL^{-1}) for 60 minutes, washed with the embryonic medium 3 times and anesthetized with tricaine 0.016%. Images of zebrafish larvae were captured with a FSX 100 fluorescent microscope with green filter and analyzed using ImageJ V1.50 software to determine the color density and total fluorescent signal.

Locomotor activity. Locomotor activity in zebrafish was evaluated by the ability of zebrafish larvae to swim past a specified line pattern. Locomotor activity was measured at 4-6 dpf using a recording device and measured using the JPG video converter software and ImageJ V1.50. The calculation of locomotor activity was assessed for 1 minute.

Bone ossification. Examination of bone ossification in zebrafish larvae was carried out at 6 dpf using alizarin red staining (Art. 6279 Alizarinrot S). Zebrafish larvae are fixed with 96% alcohol for 12 hours, soaked with distilled water for 24 hours, then soaked with 1% KOH with 3% hydrogen peroxide for 15 minutes. The larvae are rinsed with distilled water for 10 minutes, then soaked with alizarin red (1 mg mL^{-1}) for 3 days, then washed with AquaDest 5 times. After staining, they were photographed with a stereomicroscope and the integrated density was measured using ImageJ V1.50.

Statistical analysis. Data were analyzed statistically using one-way ANOVA test, followed by LSD post hoc test (5% confidence level) and Kruskall-Wallis test. Statistical test results were considered statistically significant if $P<0.05$.

Results and Discussion

Expression of tyrosine hydroxylase. Lead exposure in zebrafish larvae causes a decrease in the expression of TH. In groups G3, G4 and G5 there was an increase in TH

expression in larvae aged 6 dpf, with an increase in the concentration ethanolic extract of *C. asiatica*. The lowest expression of TH was found in G2. The administration of *C. asiatica* can increase the expression of TH (Figure 1).

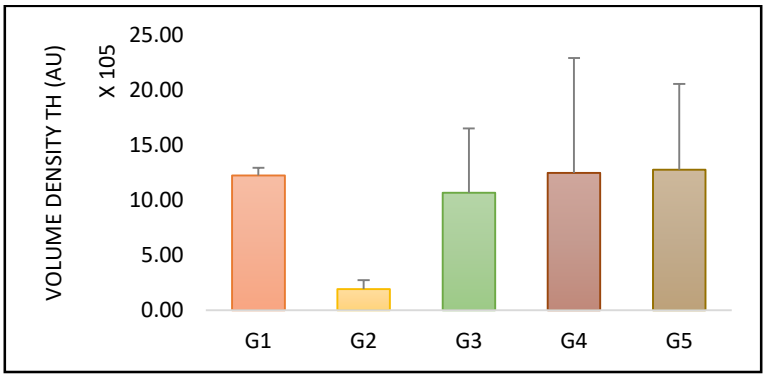


Figure 1. *Centella asiatica* treatment increase the expression of TH in lead-induced zebrafish larvae. G1 - control group without exposure to lead or *C. asiatica*; G2 - group exposed to lead with a concentration of 2.5 ppm; G3 - group exposed to lead (2.5 ppm) and *C. asiatica* (1.25 µg mL^{-1}); G4 - group exposed to lead (2.5 ppm) and *C. asiatica* (2.5 µg mL^{-1}); G5 - group exposed to lead (2.5 ppm) and *C. asiatica* (5 µg mL^{-1}).

Dopamine levels. There was a decrease in dopamine levels in zebrafish larvae exposed to lead, but *C. asiatica* administration can increase dopamine levels. A decrease in the expression and activity of TH will reduce dopamine synthesis. The normality testing of dopamine levels showed a P-value of 0.428, the data is normally distributed. Homogeneity testing of dopamine levels showed a P-value of 0.448, so the data have a homogeneous variety. Based on the one-way Anova test, the P-value was 0.031 (P<0.05) (Figure 2).

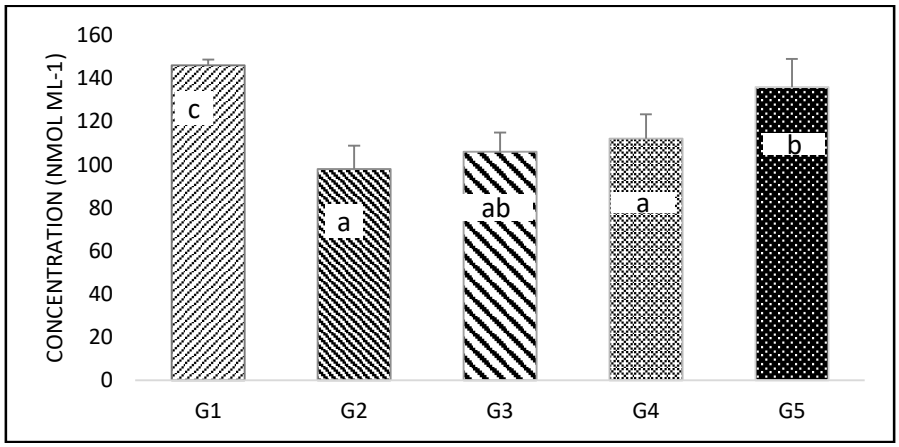


Figure 2. The dopamine levels were significantly increased by the administration of *C. asiatica* in lead-induced zebrafish larvae (G5). Different letters suggest significant differences. G1 - control group without exposure to lead or *C. asiatica*; G2 - group exposed to lead with a concentration of 2.5 ppm; G3 - group exposed to lead (2.5 ppm) and *C. asiatica* (1.25 µg mL^{-1}); G4 - group exposed to lead (2.5 ppm) and *C. asiatica* (2.5 µg mL^{-1}); G5 - group exposed to lead (2.5 ppm) and *C. asiatica* (5 µg mL^{-1}).

Expression of SIRT 1. Lead decreased the expression of SIRT1, but administering *C. asiatica* can increase its expression. The results of the post-hoc LSD test show that the expression of the SIRT1 lead group did not have a significant difference compared to that of G1, G3 and G5, but differed significantly from that of G4. The expression in G3 group differed significantly from those of the control group. The expression of SIRT1 in groups

G4 and G5 is significantly different compared to that of G2 and not significantly different from that of the G1 (Figure 3).

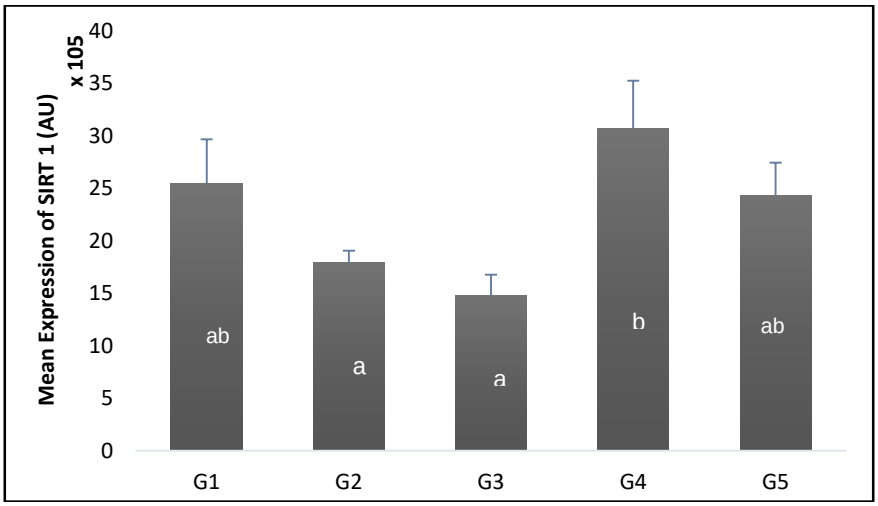


Figure 3. Comparison of the mean expression of SIRT 1 in all groups. Different letters suggest significant differences. G1 - control group without exposure to lead or *Centella asiatica*; G2 - group exposed to lead with a concentration of 2.5 ppm; G3 - group exposed to lead (2.5 ppm) and *C. asiatica* (1.25 µg mL^{-1}); G4 - group exposed to lead (2.5 ppm) and *C. asiatica* (2.5 µg mL^{-1}); G5 - group exposed to lead (2.5 ppm) and *C. asiatica* (5 µg mL^{-1}).

Expression of dopamine receptor D1 (DRD1). According to the results of the study, lead caused downregulation in the expression of DRD1, indicating a decrease in the number of DRD1. Lead caused decreased DRD1 expression, but administering *C. asiatica* can increase the expression of DRD1. The G4 and G5 treatments produced an increase of DRD1 expression, but just the expression in G5 treatment was significantly different than that of the lead group (Figure 4).

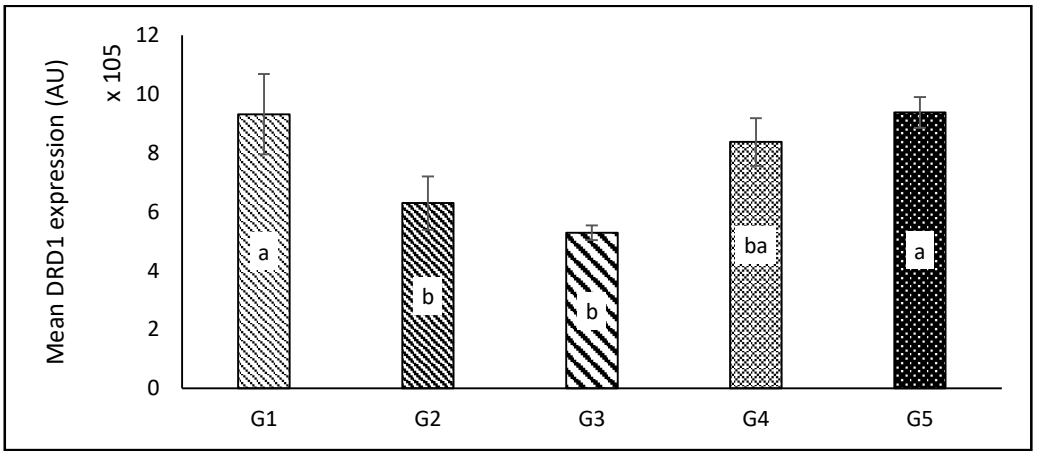


Figure 4. Mean expression of DRD1 in all groups. Different letters suggest significant differences. G1 - control group without exposure to lead or *Centella asiatica*; G2 - group exposed to lead with a concentration of 2.5 ppm; G3 - group exposed to lead (2.5 ppm) and *C. asiatica* (1.25 µg mL^{-1}); G4 - group exposed to lead (2.5 ppm) and *C. asiatica* (2.5 µg mL^{-1}); G5 - group exposed to lead (2.5 ppm) and *C. asiatica* (5 µg mL^{-1}).

Expression of dopamine receptor D2 (DRD2). The results of the study showed that lead caused upregulation in the expression of DRD2, indicating an increase in the total dopamine receptor D2. However, *C. asiatica* extract can decrease the expression of DRD2.

The G3 and G5 treatments showed a decrease of DRD2 expression, while the expression in the G5 treatment was significantly different than that in the lead group (Figure 5).

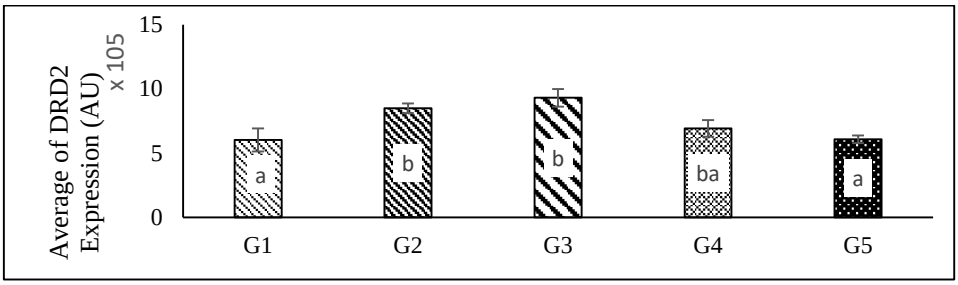


Figure 5. Mean expression of DRD2 in all groups. Different letters suggest significant differences. G1 - control group without exposure to lead or *Centella asiatica*; G2 - group exposed to lead with a concentration of 2.5 ppm; G3 - group exposed to lead (2.5 ppm) and *C. asiatica* (1.25 µg mL^{-1}); G4 - group exposed to lead (2.5 ppm) and *C. asiatica* (2.5 µg mL^{-1}); G5 - group exposed to lead (2.5 ppm) and *C. asiatica* (5 µg mL^{-1}).

Apoptosis. Lead can increase apoptosis in the body and caudal fin of zebrafish larvae. Based on the post-hoc LSD test on apoptosis, the body region showed that G2 experienced an increase in apoptosis that was significantly different from that of G1. The decrease in apoptosis in groups G3, G4 and G5 was not significantly different compared to that of G1. It can be concluded that the administration of ethanolic *C. asiatica* extract in groups G3, G4 and G5 can significantly reduce apoptosis in the head and the body (the body limit is anus). Apoptosis in the caudal peduncle and fin (from the anus to the tip of the fin) showed no significant difference (Figures 6 and 7).

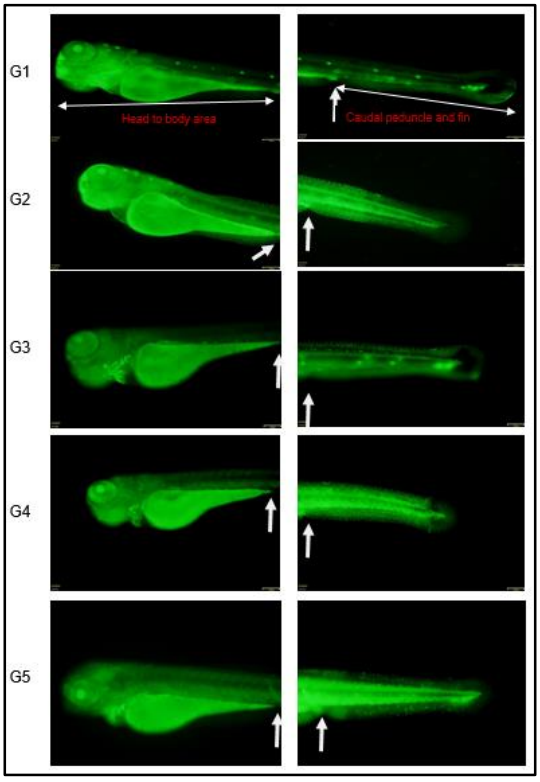


Figure 6. The representative of acridine orange staining for each group of zebrafish (*Danio rerio*) larvae. G1 - control group without exposure to lead or *Centella asiatica*; G2 - group exposed to lead with a concentration of 2.5 ppm; G3 - group exposed to lead (2.5 ppm) and *C. asiatica* (1.25 µg mL^{-1}); G4 - group exposed to lead (2.5 ppm) and *C. asiatica* (2.5 µg mL^{-1}); G5 - group exposed to lead (2.5 ppm) and *C. asiatica* (5 µg mL^{-1}).

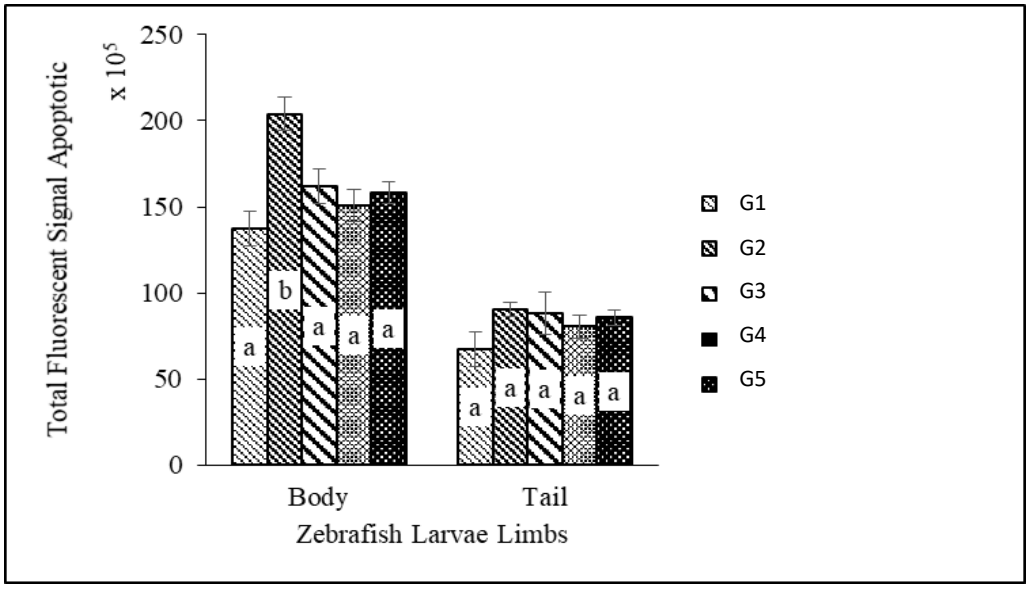


Figure 7. Mean apoptosis values in all groups of zebrafish (*Danio rerio*) larvae. Different letters show significant differences; dpf - days post fertilization. G1 - control group without exposure to lead or *Centella asiatica*; G2 - group exposed to lead with a concentration of 2.5 ppm; G3 - group exposed to lead (2.5 ppm) and *C. asiatica* (1.25 µg mL^{-1}); G4 - group exposed to lead (2.5 ppm) and *C. asiatica* (2.5 µg mL^{-1}); G5 - group exposed to lead (2.5 ppm) and *C. asiatica* (5 µg mL^{-1}).

Locomotor activity. Lead toxicity significantly reduced locomotor activity in zebrafish. In the groups administered *C. asiatica* extract, there was an increase in the average distance and different pattern of locomotor activity compared to the lead group (Figures 8 and 9). Interestingly, 5 µg mL^{-1} of *C. asiatica* administration has no significant effects when compared to the control group in all days of observation.

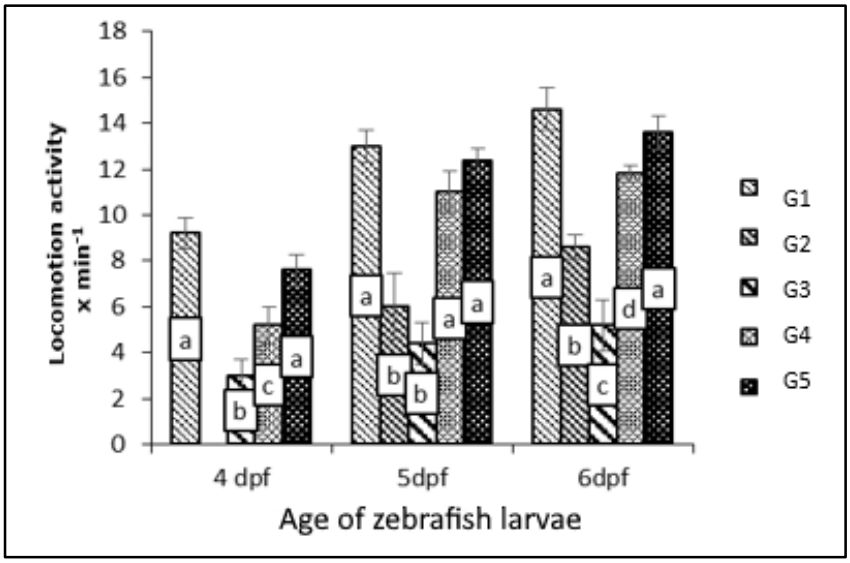


Figure 8. Mean locomotor activity in zebrafish larvae (*Danio rerio*) aged 4, 5 and 6 dpf. Different letters show significant differences; dpf - days post fertilization. G1 - control group without exposure to lead or *Centella asiatica*; G2 - group exposed to lead with a concentration of 2.5 ppm; G3 - group exposed to lead (2.5 ppm) and *C. asiatica* (1.25 µg mL^{-1}); G4 - group exposed to lead (2.5 ppm) and *C. asiatica* (2.5 µg mL^{-1}); G5 - group exposed to lead (2.5 ppm) and *C. asiatica* (5 µg mL^{-1}).

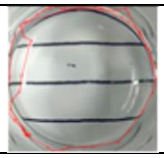
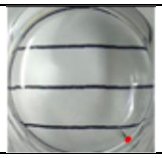
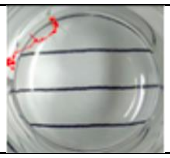
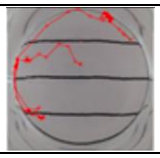
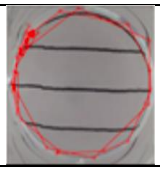
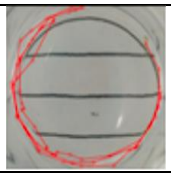
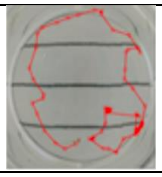
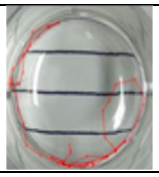
Day (dpf)	Note	G1	G2	G3	G4	G5	P value
4	Locomotor pattern						
	Mean ± SD (cm)	8±2.4	0±0.0	3.2±2.9	6.6±4.6	7.2±4.3	0.007
5	Locomotor pattern						
	Mean ± SD (cm)	15±1.6	8.8±0.8	10±2.7	11±2.0	12.4±1.1	0.00
6	Locomotor pattern						
	Mean ± SD (cm)	14.6± 2.	9.2±0.5	9.8±1.3	11.4±0.9	12.6±2.4	0.02

Figure 9. The pattern of locomotor activity in zebrafish larvae (*Danio rerio*) aged 4, 5 and 6 dpf (days post fertilization). G1 - control group without exposure to lead or *Centella asiatica*; G2 - group exposed to lead with a concentration of 2.5 ppm; G3 - group exposed to lead (2.5 ppm) and *C. asiatica* (1.25 µg mL^{-1}); G4 - group exposed to lead (2.5 ppm) and *C. asiatica* (2.5 µg mL^{-1}); G5 - group exposed to lead (2.5 ppm) and *C. asiatica* (5 µg mL^{-1}).

Ossification. The results of the study showed that lead exposure can decrease bone ossification. According to the post-hoc LSD test, the expression of bone ossification in G2 had a significant difference compared with those of G1, G3, G4 and G5. The ossifications in G3, G4 and G5 were not significantly different from G1. It can be said that lead exposure coupled with the administration of ethanolic *C. asiatica* extract in various doses can increase the expression of bone ossification, without a significant difference from that of the control group (Figure 10).

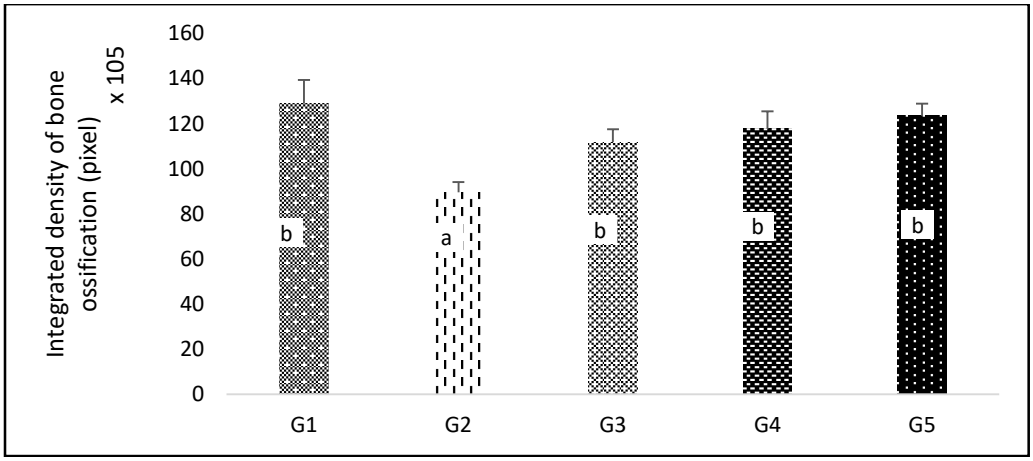


Figure 10. Comparison of mean bone ossification in all groups. G1 - control group without exposure to lead or *Centella asiatica*; G2 - group exposed to lead with a concentration of 2.5 ppm; G3 - group exposed to lead (2.5 ppm) and *C. asiatica* (1.25 µg mL^{-1}); G4 - group exposed to lead (2.5 ppm) and *C. asiatica* (2.5 µg mL^{-1}); G5 - group exposed to lead (2.5 ppm) and *C. asiatica* (5 µg mL^{-1}). Different letters show significant differences.

Lead can replace calcium as a second messenger, mimicking calcium entering cells through calcium channels. It binds to calmodulin and interferes with intracellular calcium homeostasis. As a result of impaired calcium homeostasis, mitochondrial Ca^{2+} increases mitochondrial electron transport and generation of ROS (Sanders et al 2009). In addition, Pb exposure also inhibits complex mitochondrial respiratory enzyme activity, namely complex I and III, and significantly decreases MTT reduction (Mitochondria Transport Chain), which alters mitochondrial function (Ma et al 2017; Venkareddy 2015). Decreased mitochondrial function resulted in increased production of ROS and its inability to deal with excess cytosolic calcium due to the mitochondrial calcium disorder. This condition causes excess calcium in the mitochondria. The impact of the presence of ROS and the production of calcium in the mitochondria will lead to the transition of permeability transition pore (PTP), which is followed by the translocation of the proapoptotic molecule from the mitochondria to the cytosol (apoptosis) (Gandhi & Abramov 2012). Proapoptotic proteins such as Bax can increase apoptosis; p53 protein accumulates during the apoptotic process; p53 can promote neuronal apoptosis by increasing the transcription of Bax; p53 induction leads to the release of cytochrome c. The release of cytochrome c activates caspase-3. Caspase-3 activation induces apoptosis (Mousa et al 2018). A result of that is the blockage of SIRT1 transcription activity, resulting in a decrease in SIRT1. Oxidative stress can reduce the level of NAD+ and thus inhibit the activity of SIRT1 (Salminen et al 2013). ROS caused by lead exposure can be involved in bone resorption by a direct contribution of osteoclast superoxide to bone degradation, so oxidative stress increases osteoclast differentiation and function and inhibits bone protein production such as osteocalcin, collagen, and osteopontin (Gargouri et al 2016; Tarasco et al 2019).

If the increasing ROS occurs in the central nervous system, it will disrupt the neurotransmitter system including its receptors, such as the dopaminergic system characterized by disruption of the TH enzyme. TH is an enzyme that plays a role in the biosynthesis of dopamine (Jadhav & Ramesh 1997). Dopamine is a neurotransmitter that binds to its receptors and produces the electrical potential of presynaptic neuron cells. The dopamine receptor in the post synapse will spread the signal, sending it through synaptic neurons and stimulating motor functions (Best et al 2009). In addition, decreased dopamine is also caused by degeneration of the nigrostriatal system (substantia nigra pars compacta). In this system, dopamine plays an important role in movement, control of motor functions and in learning new motor skills (Ayano 2016). Lead selectively reduces dopamine binds and dopamine transporter receptors in the nucleus accumbens, suggesting that lead exposure can predispose to associated neurodegenerative diseases with dopaminergic dysfunction (DA).

C. asiatica acts as a SIRT1 stimulator (Cheng et al 2014). Recent studies report that one of the components of *C. asiatica*, quercetin, can suppress cell apoptosis by regulating the expression of SIRT1 (Feng et al 2019). Quercetin is one of the SIRT1 indirect activators that can activate the activity/expression of NAMPT and AMP-activated kinase (AMPK). AMPK can also activate NAMPT so that it increases the level of NAD+ (Chung et al 2010). Endogenous antioxidants function as a large system that maintains the redox balance in the body. When ROS levels rise and threaten the homeostatic processes, endogenous antioxidants are activated. SIRT1 is expressed when several factors are activated such as Nrf 2. Endogenous antioxidants can work together with exogenous antioxidants from food to reduce ROS levels. Although everything works together, perhaps antioxidant proteins that have enzymatic activity, such as superoxide dismutase, catalase, and glutathione peroxidase, are the first line of defense against oxidative stress (Fregoso Aguilar et al 2016).

C. asiatica can work by inhibiting proapoptosis proteins, such as p53, Bax and Bad or encouraging the production of members of the anti-apoptosis family, such as Bcl-2 and Bcl-xL. *C. asiatica* is also thought to mimic members of the anti-apoptosis family, thereby reducing the heterodimerization of the BCL family members and inhibiting cytochrome c release, resulting in a reduction of the formation of apaf-1 and procaspase-9 to activate caspase-9 activity (Omar et al 2011). Asiatic acid from *C. asiatica*, when administered in high concentrations, can reduce the protective ability and even show toxic effects, because,

in high doses, asiatic acid can induce apoptosis through caspase 9 and 3 activation and can increase intracellular calcium (Xu et al 2012).

The results of this study also prove that the administration of *C. asiatica* can significantly increase locomotor activity in zebrafish larvae exposed to lead. This is supported by previous research, in which it was demonstrated that *C. asiatica* can increase dopaminergic neurons through increased neurotrophin (Khotimah et al 2015). In line with these results, Widodo (2016) proves that *C. asiatica* extract improves locomotor activity in adult zebrafish exposed to rotenone. *C. asiatica* contains phytonutrients like triterpenoids, carotenoids, flavonoids, glucosides, and essential oils. Triterpene contained in *C. asiatica* leaves (asiatic acid, madecassic, asiaticoside, and madecassoside) acts as an anti-oxidant (Chandrika & Prasad Kumara 2015; Rahman et al 2013; Hashim 2011). *C. asiatica* also contains many active ingredients such as polyphenols, flavonoids, carotenoids, tannins, and vitamin C (Rahman et al 2013). The use of *C. asiatica* as an antioxidant is expected to reduce ROS due to lead exposure so that it can increase locomotor activity in zebrafish larvae exposed to lead. Quercetin, which is also present in *C. asiatica*, shows a significant stimulating effect on osteoblast proliferation and mineralization in mice, which encourages the process of bone formation (Karis et al 2019). The ortho-phenolic group of quercetin located in the quercetin B ring is also known as the chelating agent, which can form coordination bonds with lead. Quercetin markedly reduced the level of ROS and reduced the GSH/GSSG ratio, suppressed an increase in deoxyguanosine 8-hydroxy level, along with the restoration of Cu/Zn-SOD, CAT and GPx activity in rat kidneys exposed to lead (Flora et al 2012). *C. asiatica* has antioxidant properties in addition to being an anti-inflammatory agent and contains micronutrients including calcium as a mineral responsible for bone formation (Octaviana et al 2019).

Conclusions. C. *asiatica* can prevent developmental disorders in lead-induced zebrafish larvae through the increased expression of Th, dopamine level, expression of SIRT1, DRD1, Locomotor activity, bone ossification and decrease expression of DRD2, apoptotic zebrafish larvae. The future direction is to evaluate the active compounds from *C. asiatica* that have strong indication to protect against lead intoxication. Further, the compounds could be developed as supplements or drugs for heavy metal detoxification, especially for lead intoxication.

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