Effect of Estradiol on Gamete Development and Behavior in Zebrafish Model

Abstract— Xenobiotic substance released in the environment is a concern among the public at large. The example of this xenobiotic release into the environment is xenoestrogens. Xenoestrogens have the capability to bind to the estrogen receptors in the body even at low affinity. Food, pesticides and contraceptive pills are known sources of xenoestrogens. In this study, acute toxicity test was conducted to evaluate toxicity of synthetic estrogen such as estradiol to the embryo-larvae of zebrafish model. Morphological changes in the embryo-larvae of zebrafish were also observed. The parameters that were evaluated in acute toxicity study were half lethal concentration (LC50) and few apical endpoints such as coagulation of embryos, development of pericardial edema, and eyes size. Toxicity effect of the compound was evaluated in term of behavior activity of the larvae. Results showed that certain concentration of estradiol caused toxic effects to the embryo-larvae of the zebrafish (p<0.05). The examples of toxic effects that were observed from this test were development of pericardial edema and small eyes in high concentration groups of estradiol used (1700, 850, and 425 mg/L), p<0.05. In terms of behavioral activity, the larvae were greatly affected by the estradiol in which the high group concentrations of 850 and 425 mg/L resulted in the inactivity of the larvae compared with the negative control group, p<0.05. In conclusion, estradiol has effects on mortality rate as indicated by the half lethal concentration (LC50), morphological changes and behavior activity of the embryo-larvae of zebrafish.

Keywords — Xenobiotic, estradiol, zebrafish, toxicity, development, behaviour.

1 INTRODUCTION

Xenobiotic substance such as estrogen has been synthesized either naturally in the body or synthetically from the environment. Most synthetic estrogen that has been exposed to human are mainly through contraceptive pills, certain foods especially soy product such as soy bean and chickpea as well as from industrial wastes [1]. The estrogen-derived food is known as phytoestrogen. Estradiol which is a natural estrogen steroid also contains medical importance especially for preventing osteoporosis and other health related problem in menopausal women. Women at menopause age are no longer capable to synthesize estrogen naturally, hence estrogen supplements have frequently been used. Hormone replacement therapy (HRT) that uses synthetic estrogen can also be administered to reduce discomfort especially for premenopausal or postmenopausal women [2].

Estradiol has certainly benefited women at certain ages in the population but the use of this hormone has also resulted in many problems. Natural and synthetic estrogen either from foods, contraceptive pills or industrial wastes can be introduced to the environment via sewage treatment or river [3]. This xenobiotic hormone will later lead to exposure to certain types of animals such as fish or other vertebrae that live around the river habitat. Natural estrogen is easily metabolized compared to the synthetic estrogen [4]. Synthetic estrogen of which the animal is exposed to will bind to estrogen receptors in the pituitary gland to prevent release of follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH are important for the ovulation process and for the maturation of the egg. Additionally, there are also controversial issues regarding the side effects of synthetic estrogen in hormone replacement therapy [5]. Development of cancers such as breast, prostate and cervix have been reported as the side effects of hormone replacement therapy [6]. Other than that, some ecotoxicology studies have also reported the presence of xenobiotics in estrogen forms which can disrupt aquatic animal behavior [7]. The examples of behavior interruption caused by these xenobiotics are predator avoidance, reproductive, and social behaviors.

One of the current model to test xenobiotic substance is zebrafish. Zebrafish could be used in various studies such as toxicological, genetic,
ecological and environmental monitoring and pharmacology studies [8]. This model is a versatile model since it can be used as a toxicological test for each stage of life as represented by the zebrafish egg, embryo, larvae, juvenile and adult stages [9]. This thus make the model suitable for acute and chronic toxicological studies. Furthermore, the zebrafish offered many advantages in toxicity testing such as being low in cost, robust, high fecundity and less time consuming compared to other models. In addition, estradiol has been extensively studied recently using zebrafish model to observe gene regulation, RNA sequencing, behavior and fertility [10,11,12].

The objectives of the research project are to determine the lethal concentration 50% (LC₅₀) and No Observed Effect Concentration (NOEC) of estradiol in zebrafish embryo-larvae. The project also aims to observe the effects of estradiol toward the development and behavior activity of embryo-larvae of the zebrafish using light/dark conditions as intervention.

2 MATERIALS AND METHODS

2.1 Husbandry of Zebrafish

Wildtype Zebrafishes were placed in special room under Central Research & Animal Facility (CREAM) surveillance. Zebrafishes aged between 6 until 24 months were used for egg production since eggs that would be optimal for research shall be produced in this period. Females and males zebrafishes were kept together in the tank and sufficient space was provided to ensure the fishes were comfortable. Temperature of the tank was maintained at 26 ± 1°C and fishes were kept under 14/10h dark/light condition. Permanent and constant filtering of flow-through condition of water was ensured to guarantee ammonia, nitrite and nitrate were kept below the permissible limit (0–5, 0.025–1 and 0–140 mg/L, respectively). Artificial diets (TetraMin™flakes; Tetra, Melle, Germany) were fed to the fishes twice daily.

2.2 Egg Production

Adult male and female zebrafish were distinguished first before spawning process was conducted. Ratio 2 female to 1 male (2:1) was placed in breeding tank a day before spawning process. The maximum number of zebrafish that allowed in breeding tank was 10 fishes using 2:1 ratio. A total of 3 breeding tanks were prepared to ensure the sufficient eggs would be obtained in the morning. The fishes later were left in dark condition overnight. Automatic lamp was set to light on automatically in the morning. The spawning process usually take place around 30 to 60 minutes after the light was on. After spawning process successfully, transparent eggs were identified at the bottom of the breeding tank. The eggs later were collected and placed in E3 solution. Unfertilized eggs were removed after observing the eggs under inverted microscope.

2.3 Ethical Consideration

The use of embryo-larvae of zebrafish before 6 days post fertilization (dpf) does not required permission from animal ethic committee. The approval from Institutional Animal Care and Use Committee (IACUC) are only required if work with live fish six days post fertilization and above. Approval from the committee was not necessary for breeding purposes since the zebrafish was not exposed to any chemical substances [16].

2.4 Preparation of Working Solution (E3 solution) for Zebrafish Embryo

50X E3 stock solutions was prepared by dissolving 14.6g NaCl (5.0mM), 0.65g KCl (0.17mM), 2.20g CaCl₂ (0.33mM) and 4.05g MgSO₄ (0.33mM) in 1 litre of distilled water. The pH of the stock was adjusted to 7.0 with NaHCO₃. The dilution of E3 solution (1X) was prepared by pipetting 200uL of the E3 stock solution (50X) together with 10uL of methylene blue. Methylene blue functions as fungal preventive agent for the embryo. Autoclave was done before every use of media.

2.5 Stock Solution Preparation

Pure synthetic estradiol powder (E2) previously purchased from Sigma Aldrich was used. Stock solution was prepared by weighing 0.1g of estradiol powder on digital balance and diluted it with 1 mL of Dimethyl sulfoxide (DMSO) to make final stock concentration of 100 g/L. The stock solution was stored in 4°C wrapped with dark paper until use.

2.6 Fish Embryo Toxicity Testing (FET Test)

Using a 96-well plate, zebrafish embryos were placed into three control group and exposed to six different nominal test concentrations of the estradiol. 20 embryos for each experimental concentration were treated to the six different concentration of estradiol respectively (1700 mg/L, 850 mg/L, 425 mg/L, 212.5 mg/L, 106.25 mg/L and 53.125 mg/L) and 20 embryos for negative control group (0 mg/L) and 20 embryos

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for control solvent (0.1% DMSO) as well as 20 embryos for positive control (3, 4-DCA) [15]. The entire embryo was placed in 96 well plates where one well contained one embryo. All of the embryos-larvae were observed at 24, 48, 72 and 96 hours post fertilization (hpf) exposure. Images were captured using digital camera connected to the inverted microscope for all embryos for 72 and 96 hpf only. All dead embryos were recorded during observation. Mortality rate was observed for each hpf until the end of project (96 hpf). The assessments of morphology defects in embryos for each concentration were observed under inverted microscope and compare them with negative control group. The acute lethality and abnormalities of embryo were observed by few basic parameters such as coagulation of embryo, irregularities in somite formation, non-detachment of the tail and lack of heartbeat [16].

2.7 Behavioral of Larvae

Besides development of the embryo, behavioral of the larvae was observed as overall behavioral characteristics of larvae locomotion can enable the differentiation of characteristics between negative control and treatment group. White light routine setting was used to study behavioral activity of larvae [17]. Total of 15 minutes was set up from the setting whereby 5 minutes dark followed by 5 minutes white light and end with 5 minutes dark condition will be set. The behavioral test was conducted after finishing toxicity and developmental study. The effects of the four highest concentrations (850, 425, 212.5 mg/L) after 1700 mg/L was chosen for investigation. Comparisons between these concentrations were made with the negative control group by observing the distance travelled by the larvae over time. Behavior of the larvae was recorded using Daniovision observation chamber instrument. Behavioral of the larvae was observed at 120 hpf.

2.8 Data Recording and Data Analysis

Half lethal concentration (LC50) for estradiol of the embryo was determined by dose response relationship and graph was plotted using GraphPad software. The statistical analysis was done using analytical software SPSS version 18 to find significant value using One Way ANOVA test. Value of \( p < 0.05 \) was considered as significant.

3 RESULTS

3.1 Mortality Rate

In the study, zebrafish embryos were treated to six concentrations of estradiol excluding negative, positive and solvent controls. The negative and solvent control used E3 solvent and DMSO respectively. Mortality rate for these control was less than 10% while positive control uses 3, 4 DCA and mortality rate was at 100%. From the findings, negative and solvent controls showed mortality rates of less than 10%. Meanwhile positive control showed 100% mortality rate by coagulation of the embryo as early as 24 hours post fertilization (hpf).

Mortality rate was observed and recorded as the death of the zebrafish embryo-larvae from the formation of fertilized eggs (2-4 hpf) until 96 hpf when exposed to the certain concentrations of the estradiol in this project (1700, 850, 425, 212.5, 106.25 and 53.125 mg/L) (Figure 1). All forms of lethality characteristics as guided by OECD 236 such as coagulation of the embryo and lack of heartbeat were regarded as death of zebrafish embryo-larvae. The dose response relationship graph to find lethal concentration that caused 50% mortality in embryo-larvae of zebrafish and also no observable effect concentration (NOEC) was plotted (Figure 2). LC50 was automatically calculated from the graphpad software and the data was presented as mean ± standard error (Supplementary Data A).

![Figure 1: Mortality rate of the embryo-larvae of the zebrafish at different post hour fertilization. Data was represented as means ± SE (n = 60). From post hoc Tukey test, the mortality rate indicates a 197 significant rate higher than negative control group (*p<0.05) except for 212.5, 106.25 and 53.125 mg/L. From the graph, the highest concentration showed 100% mortality while the lowest concentration showed the lowest mortality.

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Figure 2: Dose response relationship of mortality percentage of embryo-larvae of the zebrafish in the 6 different concentration of estradiol at 96 hours post fertilization. Data was represented as means ± SE (n = 60). From the graph, the 
LC50 was obtained at Log (2.99) which equivalent to 976.2 ± 3.702 mg/L.

3.2 Developmental Study (Morphology)

Developmental study was conducted using similar methods applied in the toxicity study. The differences between toxicity study and developmental study emphasized on the final outcome whereby the toxicity study was used to observe mortality rate and developmental study was used to observe the effect of estradiol towards development of zebrafish embryo-larvae. However, in this finding, there were only two parameters of developmental study determined, which were formation of heart edema and size of the eyes. All of the measurement of the parameters was presented as mean and standard error (SE) for each treatments group and negative control group. From the findings, it showed that as the concentration of estradiol was increased, more pericardial edema incidence and small eye size formation were observed and recorded. The other developmental defects after estradiol was introduced in this study were development of heart edema, coagulation of embryo and bending shape of the larvae (Figure 5).

Figure 3: The pericardial area of the zebrafish larvae at post hour fertilization. The values represent 224 the mean ± SE for every treatment including control group whereby n=100 for post hour fertilization. 225 From post hoc Tukey test, the pericardial area indicates a significant bigger than negative control 226 group (*p<0.05) except for 106.25 and 53.125 mg/L.

Figure 4: The eye size of the zebrafish larvae at post hours fertilization. The values represent the 231 mean ± SE for every treatment including control group whereby n=100. From post hoc Tukey test, the 232 eye size indicates a significant smaller size than the negative control group (*p<0.05) except for 212.5 mg/L.
<table>
<thead>
<tr>
<th>No.</th>
<th>Abnormal Development</th>
<th>Normal Development</th>
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</thead>
<tbody>
<tr>
<td>1.</td>
<td>Development of pericardial edema around pericardia area in 1700mg/L at 72 hpf. Pericardial edema was indicated by swollen of pericardia area.</td>
<td>Normal development of pericardial area of control group at 72 hpf. Pericardial area was flat and no swollen detected.</td>
</tr>
<tr>
<td>2.</td>
<td>Bending shaped of the larvae due to toxicity of estradiol at 72 hpf.</td>
<td>Straight shape of the larvae in control group at 72 hpf.</td>
</tr>
<tr>
<td>3.</td>
<td>Coagulation of embryo at 1700mg/L of estradiol at 24 hpf.</td>
<td>Normal development of embryo in control group at 24 hpf.</td>
</tr>
<tr>
<td>4.</td>
<td>Small eyes size development of the larvae due to toxicity of estradiol at 72 hpf.</td>
<td>Normal eyes size development of the larvae in control group at 72 hpf.</td>
</tr>
</tbody>
</table>

**Figure 5:** Normal and abnormal development of zebrafish embryo-larvae at 72 and 96-hour fertilization showing morphology changes (pericardial edema, small eyes size, bending shape and coagulation of embryo) in highest concentration group and normal morphology development in negative control group.
3.3 Behavior Study
Behavioral study was conducted to observe whether presence of the estradiol can affect the activity and behavioral of the zebrafish larvae. Three concentration of estradiol (850, 425 and 212.5 mg/L) that was believed to have effects on activities of larvae was chosen. The highest concentration (1700 mg/L) was not able to be used since most of the embryo died at this concentration. Meanwhile, for the two lowest concentration (106.25 and 53.125 mg/L), it showed no significant result compared to negative control as described in Figure 1 (Mortality rate of zebrafish embryo-larvae). The activity of the larvae was measured as distance travelled of the larvae per time. Two types of results were presented for this behavioral study. First was the overall behavior activity of the larvae per time using light and dark condition and second was the activity of the larvae based on light and dark condition that act as an intervention.

All of the measurement on the activities was presented as mean and standard error (SE) for each treatments group and negative control group. From the overall behavior activity of the larvae per time using light and dark condition (Figure 6), it significantly showed that the activities or movement of larvae in concentration 850 and 425 mg/L were reduced compared to negative controls. Meanwhile for the activity of the larvae based on light and dark condition that act as an intervention (Figure 7). The activity of larvae in concentration 850 and 425 mg/L was reduced significantly compared to the negative control. In addition, in the alternate dark-lightdark condition, the larvae in the concentration 850 and 425 mg/L did not follow the trends as the larvae in control group which might suggest that the larvae in treatment group cannot differentiate light and dark condition when compared to negative control.

![Figure 6](image)

**Figure 6:** Overall behavior activity of zebrafish larvae

![Figure 7](image)

**Figure 7:** Behavior activity of the larvae based on alternate white and dark condition

4 DISCUSSION

From Figure 1, the concentration of estradiol at 1700 mg/L caused total mortality to the embryo of the zebrafish. However, there were delays in terms of mortality rate at the highest concentration whereby the total death at 1700 mg/L can only be recorded at 96 hpf. This finding was quite different from the results of positive control where the total death was able to be recorded as early as 24 hpf. Along with this, most of the death of the larvae at 1700 mg/L of estradiol was indicated by the lack of heartbeat or no heartbeat in larvae in 96 hpf. This finding was similar to a previous study about estradiol conducted by Kishida et al (2001) in relation to mortality rate [15]. However, the concentration of estradiol used that caused 100% mortality of embryo-larvae by previous study was slightly lowered than the concentration used in this project [15]. From the results, the death of...
embryo and larvae decreased significantly as the concentration of the estradiol was decreased. In short, the results showed that there was a significant difference between mortality rate of the embryo and larvae at different concentrations of the estradiol. Lethal effect of estradiol on zebrafish embryo-larvae seemed to be dose dependent. However, lethal effects of estradiol on zebrafish were observed to be non-dose dependent solely. Increasing exposure duration and higher developmental stage can also affect the development of zebrafish embryo-larvae as the uptake of estradiol was increased [14].

For determination of half lethal concentration, LC$_{50}$ for estradiol in embryo of zebrafish was found to be 976.2 mg/L. LC$_{50}$ for estradiol in zebrafish embryo was defined as the death of 50% of the model. However, no observable effect concentration (NOEC) cannot be determined from the graph. The lowest concentration of estradiol, 53.125 mg/L still caused death even only 6.67%. So, it was assumed that NOEC for estradiol must be lowered than 53.125 mg/L.

Estradiol is well known to bind to the fish estrogen receptors (ER) [34]. The binding of xenobiotics such as estradiol with the receptors will influence the transcriptional, translational, and post-translational modifications. Introduction of xenobiotic compounds during the critical developmental period between 2 hpf to 24 hpf may cause deformation and interruptions such as induced mortality, development of pericardial edema, interruption of eye development, and altered body shape [35]. These deformed larvae were known as mutants. Alterations to the central nervous system (CNS) development may occur following introduction of xenobiotic substance such as estradiol.

Heart edema and size of eyes were categorized under sublethal toxicity in zebrafish toxicity study [18]. Even though sublethal toxicity did not necessary to cause death to the model but it can be an indicator of the presence of the toxic substance in the environment. The others sublethal examples were decreased growth, reduced reproduction, behavioral changes and many more [19].

Heart edema was defined as a swelling in the pericardial area of the larvae. In normal circumstances, pericardial area would not be bulging and it is usually flat. The swelling in the pericardial region indicated the presence of toxic substances. This occurrence would usually would affect heartbeat of the larvae [13]. The study conducted by Kishida et al (2001) also showed that treatment with 10 µM estradiol (E2) induced pericardial enlargement and curved tail phenotypes in the larvae [15].

The mechanism involved in development of normal pericardial area was osmolarity [20]. There is existence of water permeability barrier at the surface of the zebrafish embryo [21]. This mechanism ensures regulation of the osmolarity or water balance between the fish and environment was maintained. From this study, it however suggested that water permeability barrier was affected by the presence of estradiol in the larvae’s surroundings. Surface permeability toward water may have been influenced by the estradiol at a critical period in the early development of the zebrafish larvae, resulting in the formation of edema at pericardia area. This may be due to influx of water into the body of the larvae was at higher rates than the efflux processes [22]. In normal conditions, the influx process of water should be balanced with efflux process. This therefore demonstrated that an effect on a permeability barrier of the embryo was a likely contributing factor, also as a sign of estradiol toxicity.

For the second parameter, the eyes of the larvae were measured to observe the toxicity effect of estradiol. It was observed that the higher the toxicity levels the smaller the measurement of the eyes of larvae. The smaller the eyes of the larvae due to toxicity effects were reported by few studies [23; 24]. From the findings, there were correlations between the concentrations of the estradiol with the size of the eyes of larvae. As the concentration of the estradiol was increased, the smaller the eyes of the larvae were detected as recorded at concentrations of 1700, 850 and 425 mg/L.

For the mechanism of normal development of the eyes, the development organs including the eyes were highly dependent on activities of hormones and enzymes such as thyroid hormones [25]. The eyes were one of the earliest organs developed in the zebrafish model as it is developed as early as 24 hours post fertilization (hpf). So, the presence of the toxicant could affect the development of this organ at an early stage. There was a theory regarding the inhibition of normal development of growth including eyes of larvae. The theory was the toxicant inhibited the synthesis of thyroid hormone by inhibition of thyroid peroxidase (TPO) activity [26]. Thyroid hormone was known as regulator of the animal

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growth. So, the alteration of thyroid activity could affect animal growth including the eye.

Zebrafish was increasingly used in developmental toxicology for detailed understanding on their behavior [27]. Behavioral changes can be an indicator of CNS interruption by xenobiotic substances [36]. From Figures 6 and 7, the results showed significance differences between concentrations of estradiol with behavioral activity of the larvae. Behavioral activity of the larvae was measured by distance travelled by the larvae in the well per time. From the study, it clearly showed that the higher concentration showed slow activity (distance travelled per time) of the larvae and the activity of zebrafish larvae started to increase as the concentrations was lowered.

In terms of stimuli given, a white light was used to demonstrate the effects of estradiol in the larvae. White light stimulus also acts as an intervention in the studies. In the presence of white light, as there was no distance travelled, the larvae were recorded as inactive. This finding (Figure 7) was consistent with the findings from another study which showed low motility of zebrafish larvae in white light condition compared to dark condition [28]. From the graph, it showed that the larvae were inactive between minutes 5 until minutes 10 when the white light is on. Meanwhile, in dark conditions, the activity of the larvae showed significant increase in terms of distance travelled. This result was supported by the finding from other study which demonstrated normal zebrafish larvae active during dark condition [29]. The increased activity during dark condition was due to the natural prey-predator behavior of the larvae.

For the first five minutes, the condition was set up to maintain dark conditions. During this period, there was an intermediate activity (distance travelled) of the larvae. Between minutes 5 until 10, the condition was changed to a white light routine. The activity of the larvae was recorded inactive during this period. From minutes 10 until 15 (the end of the test), the settings were changed back to the dark condition and a peak activity of the larvae recorded. This time-dependent of activity result was similarly reported by other previous study [30].

From the observations, two concentrations, 850 and 425 mg/L showed significant results in terms of behavioral activity during white light routine whereby the larvae in these two groups kept maintained active even in the white light condition. This was probably due to the toxicity of estradiol towards the developmental of locomotor sensor in the brain of the larvae. Another theory was due to the deformities of eye of the larvae, input signals from the environmental condition (white light) could not be received, hence the brain was unable to functionally interpret an impulse to turn it into any activity [25]. According to Emran et al (2007), pharmacological substances may block photoreceptor terminals in the zebrafish brain area [29].

The previous study suggested that the decreased activity during white light condition was due to habituation [31]. Habituation refers to the condition of the organism that learns to stop responding to a stimulus which is no longer biologically relevant. Light created an inhibitory effect on activity that was slow to dissipate and dark created a more rapid-acting excitatory effect. An alternating light–dark conditions produced a rebound excitation of activity in the dark. This condition can be made several times (2 or 3) rather than only one alternating in this experiment to obtain more accurate data regarding the habituation concept.

The inactivity of the surviving larvae at the concentration group of 850 mg/L of estradiol may due to a combination of estradiol. Locomotor sensor and muscular activity of the larvae were affected and these may be the cause of slow behavioral activity. Locomotor sensors in the brain were used by the larvae to detect changes in their environment. The brain of the larvae usually developed around 72 hpf [32]. Brain development of the larvae was interrupted by the intervention of the chemical substance such as estradiol while the muscular was only initiated when impulses are received from the locomotor sensors [33].

In summary, the current findings showed that the observed behavioral effects of the zebrafish larvae in 96 microplates were reliable and quantifiable. Data also seemed to indicate sensitivity to dark, light conditions and estradiol. The studies have established a complex and highly reproducible, 3 phases of condition, 1) moderate initial activity in dark condition, 2) decreased activity during white light routine, and 3) increased activity upon return to dark conditions. This study is however only restricted to phenotypes of zebrafish model when xenobiotics such as estradiol is introduced to the model. Recommendations for the future study would include molecular study using quantitative polymerase chain reaction (qPCR) or western

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blotting to observe the specific genes and proteins affected by this xenobiotic.

5 CONCLUSIONS
Estradiol has already been established as the main active ingredients in contraceptive pills along with other materials present in food and in the environment. However, lack of toxicity studies using this substance has caused curiosity regarding safety of the substance. This work illustrated the potential of zebrafish embryo-larvae model for high-throughput screening of chemical substances such as estradiol. Zebrafish embryo-larvae has been chosen as a pre-screening model for toxicity study in this project. This is the first time the LC$_{50}$ of estradiol has been reported using zebrafish embryo-larvae model. Plus, the toxicity effects of the compound to the zebrafish embryo-larvae in term of mortality rate, morphology changes and behavior activity can also be observed in this study. Estradiol showed toxicity effects to the embryo-larvae of zebrafish at concentrations of 1700, 850, and 425 mg/L of estradiol and the value of LC$_{50}$ recorded was 976.2 mg/mL. Zebrafish embryo-larvae can be a promising model for toxicity study of commercial chemical or natural products.

CONFLICT OF INTEREST STATEMENT
The authors declare that they have no conflict of interest.

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