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Acute Toxicity of Keladi Tikus (*Typhonium flagelliforme* (Lodd.) Blume) Ethanol Extract on Zebrafish (Danio rerio) Embryo in vivo

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ABSTRACT

Keladi tikus (*Typhonium flagelliforme* (Lodd.) Blume) is an Indonesian medicinal plant that has various pharmacological properties. Zebrafish (*Danio rerio*) has been proposed as a model that can bridge the gap between cell assays and rodent assays. Evaluation of the toxic effects of natural products using the Zebrafish model can be assessed starting from the blastula stage of embryonic development. This study aims to investigate the potential acute toxicity effect of keladi tikus-ethanol extract (KTEE) using zebrafish embryos. A static non-replacement regime for acute toxicity testing was used. Wild-type zebrafish embryos were exposed to various concentrations of KTEE (50, 100, 200, 400, 800, 1600 µg/mL) starting at 6 hours post-fertilization (hpf) until 96 hpf. The results showed that the survival rate of zebrafish embryos decreased as the concentration of the test extract increased. The LC_{50} values of KTEE were 494.553 µg/mL at 96 hpf and 555.787 µg/mL at 72 hpf. Embryotoxicity effect of KTEE includes hatching delays and decreased heartrate on zebrafish embryos, especially at high concentrations. KTEE also caused abnormalities in embryo morphology, including pericardial edema, jaw and tail deformity.

Key words: Keladi tikus, ethanol extract, zebrafish embryo, acute toxicity

INTRODUCTION

Herbal remedies have been widely utilized in traditional medication to cope with various diseases. The use of medicinal plants as complementary and alternative medicines is believed to be more effective in the management of diseases with a lower risk of toxicity compared to conventional medicines. However, the development of research suggests that some medicinal plants are reported to exhibit toxic effects especially in long-term use (Amadi and Orisakwe, 2018; Navarro *et al*., 2017). Some medicinal plants contain toxic compounds that are suspected to cause damage to the kidneys or liver (Abt, 1995; Amadi and Orisakwe, 2018; B. Yang *et al*., 2018). Therefore, evaluating the toxicological effect of the intended medicinal plant extract is an important aspect to predict the potential toxic effects inflicted.

Keladi tikus (*Typhonium flagelliforme* (Lodd.) Blume) is a medicinal plant found in the countries of Southeast Asia, Sri Lanka and southern India (Nicolson and Sivadasan, 1981). Traditionally, keladi tikus are utilized to treat coughs, traumatic injuries, abscesses and cancer treatments. The tuber of keladi tikus have been used as a health supplement to treat various types of cancer, such as lung cancer, rectum, liver, cervical, breast cancer and leukemia (Mohan *et al*., 2011). In practice, keladi tikus are often consumed as herbal drinks and combined with other herbs or honey (Lai *et al*., 2008). Keladi tikus are reported to have a variety of pharmacological activities, such as immunomodulatory, anti-ulcerogenic, antibacterial, antioxidant and anticancer effects (Bardi *et al*., 2011; Mohan *et al*., 2008; Nurrochmad *et al*., 2015). Several compounds were successfully isolated from keladi

tikus, among them phenylaltridekanoic acid, coniferin, β-sitosterol, and β-daucosterol. Recent research, indicates a pheophorbide compound isolated from keladi tikus was reported to have an antiproliferation activity on several types of cancerous cells in vitro (Lai *et al*., 2010). In addition, dichloromethane extract of the tuber parts of keladi tikus proved to have anti-leukemia activity both *in vitro* and *in vivo* (Mohan *et al*., 2010). Despite the pharmacological effects reported, the safety-related information of keladi tikus is still very limited. Therefore, it is important to have a toxicity study on keladi tikus to ensure its safety profile.

Zebrafish has been a model in toxicological, biomedical research and drug discovery (Ali *et al*., 2011a; Kari *et al*., 2007). The embryonic and larvae form of the zebrafish which is small and transparent allows for the visualization of internal organs during its formation and development. The main organ systems of zebrafish such as the nervous system, cardiovascular, digestive and vision have similarities with mammals both anatomical, physiological, molecular, and genetic levels (Howe *et al*., 2013; Kanungo *et al*., 2014). In addition, zebrafish embryos experience a relatively rapid embryogenesis, making it more efficient in terms of time and cost for research. Therefore, zebrafish is ideal for evaluating *in vivo* toxicity that cannot be done *in vitro*, such as in cells, tissues or sliced organs (d'Amora and Giordani, 2018).

This study aims to evaluate the effects of exposure to keladi tikus ethanol extract on the development of zebrafish embryos and larvae through acute toxicity testing. The toxic effect of keladi tikus ethanol extract was assessed based on the LC_{50} value and its effect on the changes in embryogenic morphology, heart rate, and embryonic hatching rate.

MATERIALS AND METHODS

Plant Material

The tuber parts of keladi tikus (*Typhonium flagelliforme* (Lodd.) Blume) were obtained from plantations in the area of Bogor, West Java. Determination of plant samples were conducted in School of Life Sciences and Technology (SITH) ITB, Bandung, West Java.

Extraction Process

Dry powder of keladi tikus tubers were extracted by maceration with 96% ethanol solvent (Enseval) for 3 days with occasional stirring. Then the extract solution was filtered with filter paper. All macerate was collected and evaporated using rotary evaporator at 40°C so that the extract obtained was thickened.

Zebrafish Maintenance

Healthy adult Zebrafish (*Danio rerio*) from the *wild-type* strain (age 3-4 months) were maintained in the aquaculture system at room temperature with a bright 14/10h light/dark cycle. The fish were fed using TetraMin® Tropical Flakes twice a day in the morning and afternoon. Meanwhile, aquarium water was changed every 3 days by replacing 60% of the aquarium water volume to prevent drastic changes in aquarium environmental conditions.

Zebrafish Embryos Handling

Spawning was done by placing zebrafish male and female with a ratio of 2:1 into a breeding tank that had been mounted with mesh to separate the eggs with adult fish, and left overnight. Egg collection process was done in the morning according to the lighting cycle using a fine sieve. Then the eggs were washed several times with water flowing to remove impurities and debris attached to the eggs. Zebrafish embryos could be observed under a stereomicroscope (Nikon SMZ-1). The fertile embryos ware then transferred into a number of Petri dish containing E3-medium. Dead or unfertile embryos will appear white or opaque in color and was separated after the first few hours of developmental phase (0-3hpf) and periodically afterwards using pipettes to prevent a delay in the development of healthy embryos (Avdesh *et al*., 2012; Harper and Lawrence, 2011). Furthermore, eggs were acclimatized in incubator for 2-3h at 28-29°C before given the treatment.

E3-Medium

E3 for embryonic medium was used as the medium of delivery of the test extract solution during testing. E3-medium was prepared by making a 60X stock solution consisting of 17.2g NaCl, 0.76g KCl, 2.9g $CaCl_2.2H_2O$ and 4.9g $MgSO_4.7H_2O$ was dissolved in 1L aquadest and adjusted to pH 7.2 with NaOH solution. The E3-medium stock solution was sterilized using autoclave and stored at 4°C. The solution was then diluted 1X (working solution) by taking 16.7mL of 60x stock solution and diluted with aquadest to 1L.

Preparation of various concentrations of KTEE

The various concentrations of KTEE test solution was dissolved in E3 medium. To increase

solubility of the extract, 0.02% DMSO (Sigma-34869) was added and continued with sonication for 30min the temperature of 40°C.

Acute Toxicity using Zebrafish Embryos

Acute toxicity testing using zebrafish embryos refers to the protocols from the Organization for Economic Co-operation and Development (OECD) No. 236, 2013, and modified from some literature (Ali *et al*., 2011b; Truong *et al*., 2011; Yang *et al*., 2018). The range-finding concentrations test from KTEE as a preliminary test was conducted logarithmically, i.e. 10, 100, 1000µg/mL. Furthermore, the various concentrations of KTEE were determined by the dividing factor 2, i.e. 50, 100, 200, 400, 800, 1600µg/mL. Where, the highest concentration caused 100% lethality or abnormalities, and the lowest concentration showed no observed effect. In addition to variations in the concentration of the test extract, there was also a negative control group using 0.02% DMSO, a positive control group using 4ppm 3,4-dichloroaniline (Merck-820431), and a plate control group using E3-medium.

The zebrafish embryo at 6 hpf was transferred into the 24-well plate (1 embryo per well) and each group comprises 20 embryos. Then, the variation of the concentration of the test extract solution was added to each well with a volume of 1000µL per well with 3 repetitions of the test. A static non-replacement regime was used. Thus, there was no replacement or refreshment of test solutions during the test. Furthermore, 24-well plates (Nest®) containing embryos and variations of concentration of KTEE were incubated at the temperature of 28°C.

Zebrafish Phenotype Observation

Observations were performed every 24 hpf for 96 hpf (4 days) using stereomicroscope (Nikon SMZ-1) at 20-25X magnification. The observed parameters were coagulation of the embryo, lack of somite formation, lack of heartbeat, and defects in jaws, fins, body shapes, and tail of the larvae zebrafish at 96 hpf. If any of these parameters were observed, then the embryo/larva zebrafish was considered dead. In addition, the hatching rate was also observed in 48 hpf and 72 hpf.

Heartbeat Assessment

Zebrafish larvae at 96hpf were anesthetized with 50µg/mL tricaine (Sigma-E10521) in E3-medium and were observed and recorded using a stereomicroscope (Nikon SMZ-1) equipped with a computer and camera for one minute. At the end of the observation the Zebrafish larvae were euthanized by immersing them in a tricaine solution concentration of 0.3mg/mL.

Data Analysis

Determination of LC_{50} value used probit regression analysis, and statistical significance analysis was obtained using the One-way ANOVA method followed by the post-hoc LSD using SPSS version 25. All data was interpreted as Mean±SD with 3 times repetition.

RESULTS AND DISCUSSION

Lethal concentration of KTEE

Previous studies showed that keladi tikus had the potential to be developed as anti-cancer agents, the safety of the components contained in the plant and its effects on the development of the embryos in vivo could not be explained. Exposure of KTEE at certain concentrations indicates embryotoxicity, which was assessed from the hatching rate, heartrate, and defects in zebrafish embryos. In this study, we demonstrated the effect of exposure to KTEE which caused lethality and malformations on zebrafish embryo test animal models. Percentage of mortality in the embryo and larvae of zebrafish increases with increasing concentration and duration of exposure (Figure 1). The mortality rate increased very clearly at concentrations of 800µg/mL and 100% mortality at concentrations of 1600µg/mL both at 72hpf and 96hpf.

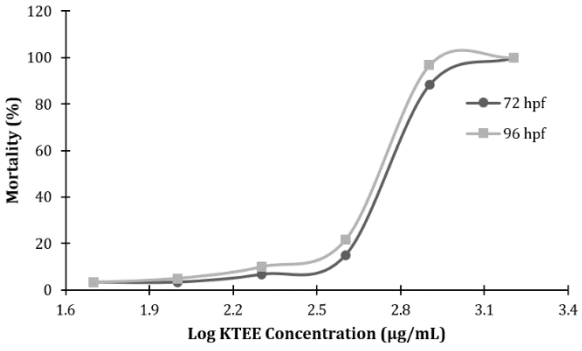

Figure 1. Relationship of concentration and mortality at 72 hpf and 96 hpf

The results of probit regression analysis obtained the LC_{50} values at 72hpf and 96hpf, which were the concentration of the extract that caused 50% of lethality/abnormalities in the embryo and larvae zebrafish (Table I).

The LC_{50} value of KTEE at 96hpf (494,553µg/mL) is smaller than 72hpf (555,787µg/mL). In this study, toxicity of KTEE on zebrafish embryos and larvae increases with duration of exposure at 96hpf. In general, the value of LC_{50} higher indicates lower toxicity level, while the value of LC_{50} lower indicates the sample is more toxic because at a lower concentration can cause lethality or abnormalities of the organism in large quantities (Thiagarajan *et al*., 2019). The LC_{50} value will be the reference to determine concentration of the sample for the next pharmacological activity testing with the wild-type strain zebrafish embryo model.

Tabel I. LC_{50} Value after exposure of KTEE at 72hpf and 96hpf.

The acute toxicity of KTEE	LC_{50} (µg/mL)
72hpf	555.787
96hpf	494.553

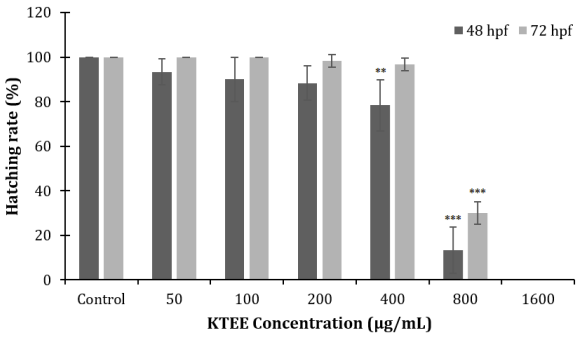

Figure 2. The hatching rate of the zebrafish embryo after being exposed to various concentrations of KTEE at 48 HPF and 72 HPF. Significant different with normal groups *** P<0.001; ** P<0.01 (Mean±SD).

Effect of KTEE on Hatching Rate

Hatching is an important period in zebrafish embryogenesis. Under normal conditions, the zebrafish embryo will begin to hatch at 48hpf and finish at 72hpf (Huang *et al*., 2018). In zebrafish embryo hatching there was a delay at 48hpf with an increase in the KTEE exposure concentration compared to negative controls (Figure 2). However, the zebrafish embryo exposed to KTEE undergoes a 100% hatchery at 72hpf, except at a concentration of 800µg/mL where embryos did not undergo perfect hatching at both 48hpf and 72hpf. The decreased hatchability of KTEE-treated embryos is caused by structural and functional disorders that occur during embryonic development (Sun and Liu, 2017). Meanwhile, at the highest concentration of 1600 µg/mL, embryos coagulated at 48hpf.

Effect of KTEE on the heart rate of zebrafish larvae

The heart rate of the zebrafish larvae that was exposed to KTEE did not exhibit significant differences in concentrations of 50, 100, and 200µg/mL compared to normal groups (193 beats/min). Meanwhile, at concentrations of 400 and 800µg/mL showed a significant decrease in heart rate (P<0.01). Meanwhile, there was no heartbeat at the highest concentrations of 1600 µg/mL that induced mortality in the early development of zebrafish embryos (Figure 3). Heart function is closely related to heart rate and blood circulation (Männer *et al*., 2010; Yalcin *et al*., 2017). In this research, KTEE affected the heart function of the larvae zebrafish in the term of a decrease heart rate, especially at high concentrations. Sun and Liu (2017) reported that the decrease in heart function of zebrafish larvae could be influenced by pericardium abnormalities and distance of sinus venosus (SV)-bulbus arteriosus (BA) heart, thus resulting in an irregular heartbeat.

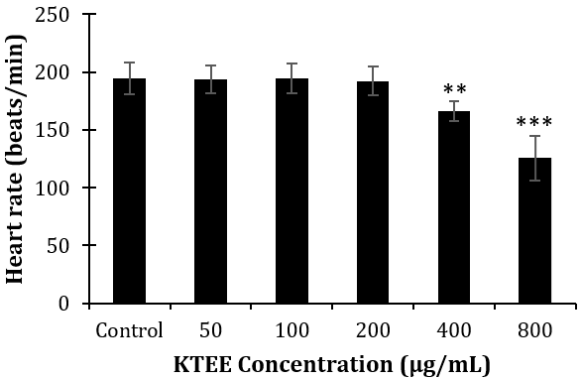

Figure 3. The heart rate of the larvae zebrafish at 96 hpf. Different means with normal groups *** P<0.001; ** P<0.01 (Mean±SD).

KTEE effect on Morphology of zebrafish larvae

Based on the observations of the zebrafish larvae morphology at 96 hpf (Figure 4), there were several malformations that occurred due to the exposure to KTEE including pericardial edema, abnormalities of the jaw and tail. It was suspected that the chemical compound in KTEE affected the development of the embryo and larvae zebrafish. In this study, we found that KTEE exposure at concentrations of 800µg/mL caused pericardial edema on zebrafish larvae.

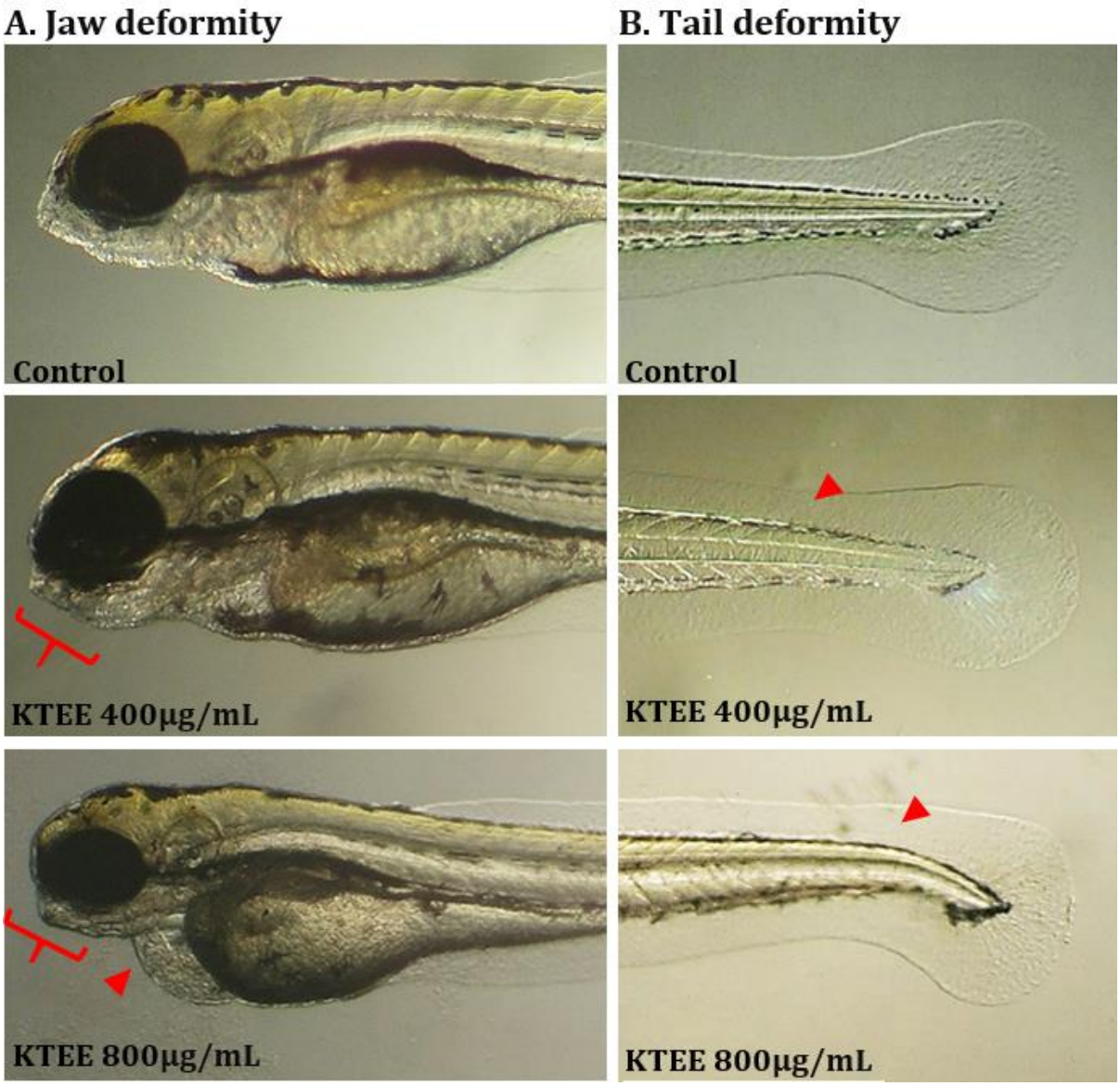

Figure 4. A change in the morphology of zebrafish larvae at 96 hpf. All pictures showed lateral view; anterior left.

T. flagelliforme, one of the plants from the family Araceae, commonly contains calcium oxalate (raphides) which would potentially cause inflammation and edema when consumed (Ceretto and Nacca, 2018; Watson *et al*., 2005). The compound can be absorbed into the zebrafish embryo by crossing the permeability of the chorion and vitelline membranes. Meanwhile, zebrafish larvae beyond 72 hpf are able to absorb the compound through the skin and ingestion from the medium during the treatment (Tye and Masino, 2019; Zhang *et al*., 2015).

In general, edema can be caused by increased hydrostatic pressure caused by heart failure, decreased plasma oncotic pressure in blood vessels in nephrotic syndrome, or hepatic failure (Hanke *et al*., 2013). In this study, it showed that exposure to KTEE during a certain period may be suspected to cause cardiotoxicity or nephrotoxic especially at high concentrations. Meanwhile, the jaw and tail deformation in zebrafish larvae, allegedly occur due to the primary defect of bone formation on zebrafish embryos. Scoliosis studies on the rat model showed that there was a change in

the formation of osteoblasts and chondrogenesis that affected bone formation in the embryonic phase (Liang *et al*., 2018).

Although the several isolated compounds from keladi tikus are known, there has been no follow up studies that shows the direct relationship of each compound (phenylaltridekanoic acid, coniferin, β-sitosterol, and β-daucosterol) in the toxicity inducing effect. Hence, a direct conclusion of each compound being the active isolate that causes the toxicity effect can't be withdrawn. Currently, there are 17 phytochemicals that are proven to show the toxicity effect towards Zebrafish that ranges from the group of alkaloids, terpenoids, cannabis, quinone and its derivatives (Jayasinghe and Jayawardena, 2019).

Keladi tikus shows the toxicity effect on the embryonic developmental growth. *Rumex vesicarius* is another medicinal plants that has been proven to have anti-cancer effect by induced anti-angiogenic activity towards human breast, colon and liver carcinoma cell lines. The choloroform extract of *R. vesicarius* however did not show any significant level of toxicity towards Zebrafish embryos even at high level of concentrations (300 µg/mL) (Farooq *et al*., 2020). *Curcuma longa* is also another type of medicinal plants proven to have anti-cancer activity in lung and prostate cancer (Wu *et al*., 2016; Yallapu *et al*., 2014). It is observed that the exposure of *C. longa* methanol extract towards Zebrafish embryos at variation of concentration causes delayed hatching to no hatching of the egg with increasing of the concentration (0 – 125 µg/mL). It is also observed that exposure towards high concentration of *C longa* causes embryonic deformity in the larvae's trunk area after hatching. However, there has been no observed significant different of heart rate of the embryos (Alafiatayo *et al*., 2019).

CONCLUSION

In this study, it showed that keladi tikus (*Typhonium flagelliforme* (Lodd.) Blume) can cause certain toxic effects on the development of zebrafish embryos and larvae, especially at high concentrations. Embryotoxicity effect of the keladi tikus ethanol extract includes hatching delays, decreased heart rate, and malformations on zebrafish larvae. In addition, further research is needed to identify compounds from *T. flagelliforme* that causes the toxic effects and its molecular mechanisms.

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