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Acute toxicity of arumanis mango leaves (*Mangifera indica* L.) extract against zebrafish (*Danio rerio*) embryos

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ABSTRACT: Arumanis is one of the cultivars of Indonesian mangoes used as a horticultural commodity. Young leaves arumanis can be used for traditional herbal medicine. Pharmacological activity of young leaf arumanis extract are known to be antidiabetic, anticancer, antibacterial, anti-inflammatory, and analgesic effects. However, it is necessary to carry out toxicity testing before young leaf arumanis extract is used in traditional herbal medicine. This study aimed to determine the LC_{50} value of young leaf arumanis extract and identify the hatching time of embryos, heart rate of larvae, swimming movement of larvae, and malformations in both embryos and larvae. Forty five embryos of zebrafish were exposed to several concentrations of young leaf arumanis extract at 24 h post-fertilization until 96 h post-fertilization. Percentage of embryonic death calculated using probit analysis model LC_{50}. Hatching rate, swimming movements, and heart rate were analyzed using the IBM SPSS software version 26. The LC_{50} values of the young leaf arumanis extract were 42.65µg/mL at 96 hpf and also 42.65µg/mL at 72 hpf. The embryotoxic effects at high concentrations of the extract are hatching delay and decreasing heart rate. The extract also caused abnormalities in embryo morphology, including pericardial edema and tail bending.

KEYWORDS: Acute toxicity; arumanis; *Danio rerio*; embryo; zebrafish.

INTRODUCTION

Indonesia is the fifth largest country in the world after India, producing mangoes [1]. In line with this, West Java Province is one of the highest regions to develop and produce mango cultivars. Among the cultivars developed in West Java are Arumanis, Gedong, Dermayu, and Golek [2]. Arumanis (*Mangifera indica* L.) belongs to the genus Mangifera [3]. The mango fruit can be consumed directly. Besides that, several studies disclosed that other parts of mango could be used as traditional herbal medicine [4],[5],[6],[7].

Recently, young arumanis leaves have been consumed by the public as traditional herbal medicine. However, no studies have examined the long-term side effects of arumanis leaves as traditional herbal medicine. Young arumanis leaves contain various secondary metabolites, which play a role in their pharmacological activity. These secondary metabolites include flavonoids, saponins, phenols, polyphenols, tannins, alkaloids, steroids, terpenoids, anthraquinones, and mangiferins [6], [8], [9]. Therefore, it is necessary to conduct a toxicity study to prove the safety and side effects in living organisms.

Toxicity tests aim to determine the toxic effects of a compound that can cause structural, functional, and damage to an organism within a certain time and dose [10], [11]. The toxicity level of this test was measured by LC_{50} or lethal concentration of 50% [12]. Zebrafish embryos have been widely used to study the toxicity of natural ingredients and herbal medicines, often referred to as embryotoxicity. Embryos of zebrafish are transparent, making them easier to observe [13]. Transparency of embryos could allow evaluation effects of compounds on various organs development, such as heart, brain, intestines, pancreas, bones, liver, and kidneys without difficult process [13], [14], [15].

One animal model that has been developed for research is zebrafish. Many studies have focused on the development of molecular genetics, toxicology, and testing methods for new drug discovery [14], [15]. Approximately 60 new drugs have been identified using zebrafish as animal models [16]. Zebrafish is easy to maintain in laboratory, has high level of fecundity which is capable of laying 200-300 eggs a day, as well as a fairly fast stage of organogenesis [14], [17], [18]. Zebrafish also have a fairly close genetic similarity to humans and mammals. The identification of zebrafish and human genomes showed that there is a relationship between the genes involved in causing diseases that occur in humans [19].

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Based on this description, it is important to determine the safety of young leaf arumanis as a traditional herbal medicine. This study was conducted to evaluate toxicity of young leaves arumanis extract in terms of LC_{50} values of young leaves arumanis extract and determine effects on zebrafish embryos and larvae malformations.

▪ MATERIALS AND METHODS

Materials

Plant material used were young leaves arumanis (*Mangifera indica* L.), young leaves taken are reddish, brown, yellow-brown, yellow-green-brown from the tip of the stem, with the tip of the top to the part bordering the old leaf [6] from Kuningan, Indonesia; E3 medium (5.0 mM NaCl; 0,17 mM KCl; 0.33 mM $CaCl_2$; 0.33 mM $MgSO_4$); Zebrafish (*Danio rerio*) wild-type from Depok, Indonesia.

Extraction of young leaves arumanis

Dry powder (200 g) of young leaf arumanis was extracted using the maceration method with 700 mL of methanol for 3 days and followed by stirring [20]. The extract solution was filtered through a filter paper. Then, the macerate was evaporated using a rotary evaporator to obtain a viscous extract.

Preparation of animal model and embryos handling

Three until four months of adult zebrafish (*Danio rerio*) wild-type were maintained in an aquarium system with 14h:10h light: dark. The fish were fed commercial feed twice a day, in the morning and afternoon. The water in the aquarium was changed every two days by replacing 60% of the aquarium water volume [13]. Therefore, the fish were protected from stress by the treatment given in [21]. During the afternoon spawning process, zebrafish were separated into 10 spawning aquariums with a female: male ratio of 1:2. Each spawning aquarium was equipped with fine gauze to avoid cannibalism of the eggs by the mother zebrafish. Embryos were carried out in next day by transferred into petri dishes using a pipette, and then washed in distilled water to remove debris [22]. Zebrafish embryos were selected under a stereomicroscope. Fertilized embryos were marked with a transparent yolk sac and transferred into a petri dish containing E3. The dead embryo was marked with a milky white color [23].

Acute toxicity assays

Acute toxicity test on zebrafish embryos refers to the OECD protocol no 236 of 2013 that concerning the acute toxicity test of zebrafish embryos. Young leaf arumanis extract was put in 24 well plates, and 2 mL of each plate was added. Each well was filled with 1 embryo only. Acute toxicity tests were performed at concentrations of 1000, 750, 500, 250, 50, 30, and 10µg/mL [22]. Five embryos, with four replicates, were used for each concentration. Embryos were incubated at 26± 1 °C. Observations were made every 24–96 hours post fertilization (hpf) after exposure [22].

Heartbeat observation

96 hpf of zebrafish larvae were observed and recorded using a stereomicroscope (Nikon C-FLED 2, Japan) equipped with a personal computer and cameras for one minute.

Swimming movement observation

Observations were performed on 96 hpf zebrafish larvae using a stereomicroscope (Nikon C-FLED 2, Japan) equipped with a personal computer and cameras. Swimming movements of the zebrafish larvae were observed for 20 min.

Embryo morphology observation

Observations were carried out every 24–96 hpf using a stereomicroscope (Nikon C-FLED 2, Japan) equipped with a personal computer and cameras. The observed parameters included defects in the pericardial areas and body shapes.

Data analysis

At the end of exposure, LC_{50} was analyzed and determined using the analyzed probit method for LC_{50}. Other parameters, including hatching rate, heart rate, swimming movements, and malformations, were also observed. The data were analyzed using IBM SPSS version 26 non-parametric Kruskal Wallis and Kolmogorov-Smirnov tests.

▪ RESULTS AND DISCUSSION

The toxicity test of the young leaf arumanis extract on zebrafish embryos was performed according to OECD protocol no. 236 of 2013. Figure 1 illustrate probit value of young leaf arumanis extract at 72 hpf and 96 hpf. The concentrations used were 10, 30, 50, 250, 500, 750, and 1000µg/mL. Every 24 h, apical observations were recorded as indicators of embryo death, coagulation, lack of somite formation, lack of tail from yolk sac, and lack of heart rate [22].

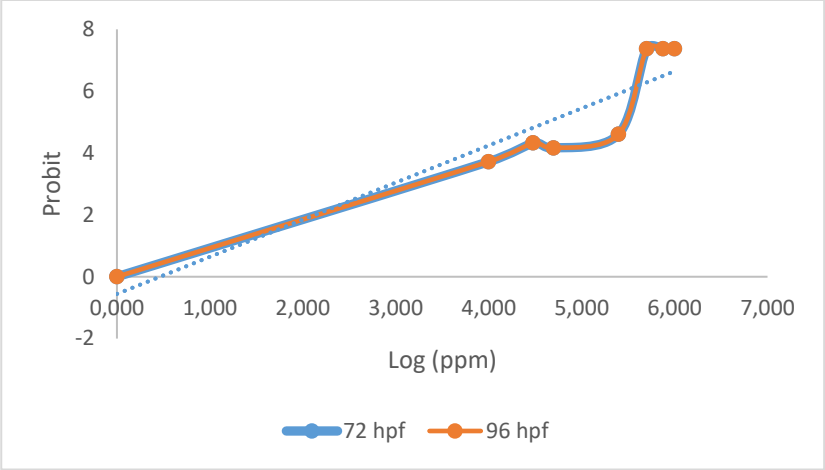

Figure 1. Relationship between mortality of embryo shown by probit value with concentration of young leaf arumanis extract at 72 hpf and 96 hpf. The regression equation obtained is y = 1.2003x – 0.5571, R2 = 0.8686

Exposure to young leaf arumanis extract affects zebrafish embryo mortality The highest mortality occurred in concentrations of 500, 750, and 1000µg/mL (Figure 1). Embryo mortality in each treatment mostly occurred 24 h after exposure to the extract. At that time, embryos entered the segmentation phase where the embryo began to form somites, body axes, pigmentation, and started having heart rate and blood circulation.

Table 1 show the LC_{50} values of the young leaf arumanis extract. Overall, it can be seen that LC_{50} values 42.65µg/mL at 96 hpf and also 42.65µg/mL at 72 hpf. Based on the probit analysis shown in Table 1 the LC_{50} value of the young leaf arumanis extract was 42.65µg/mL. At this concentration, it could be the cause of death in the animal test by 50%. Higher concentrations of the young leaf arumanis extract resulted in higher mortality in zebrafish embryos. High mortality at high concentrations is thought to be due to the presence of secondary metabolites, which can inhibit embryonic development [23]. The extract of arumanis young leaves has been known to have various secondary metabolites, but in this study, no fractionation was carried out, so it is not certain what compounds have a highly toxic impact and cause several deaths in zebrafish embryos.

Table 1. LC_{50} Values of Young Leaves Arumanis Extract on 72 hpf and 96 hpf.

Time	LC_{10}	LC_{50}	LC_{90}
72 hpf	0.02µg/mL	42.65µg/mL	91676.8µg/mL
96 hpf	0.02µg/mL	42.65µg/mL	91676.8µg/mL

Hatching is an important phase in embryogenesis, especially in zebrafish. Under normal conditions, zebrafish embryos can hatch at 48 hpf [13]. The bar chart on Figure 2 describe the hatchability that occurs in in the control and in concentration of arumanis young leaves extract at 72 hpf and 96 hpf.

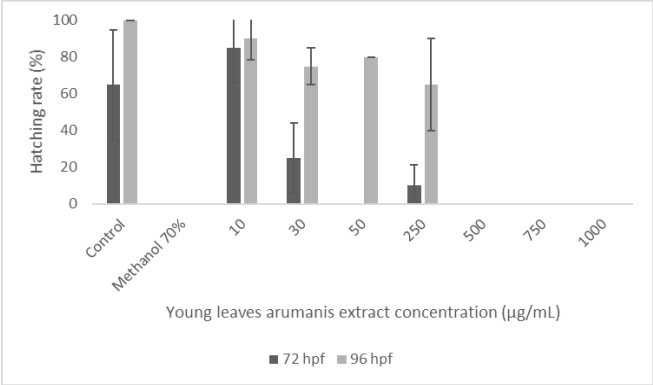

Figure 2. Hatching rates at 72 hpf and 96 hpf in zebrafish embryos after exposure to various concentrations. P1=control, P2=methanol, P3=10 µg/mL, P4=30 µg/mL, P5=50 µg/mL, P6=250 µg/mL, P7=500 µg/mL, P8=750 µg/mL, P9=1000 µg/mL. Significant difference compared to the control group (P<0.05).

Based on Figure 2, it could be seen that the highest hatchability occurred in the control and in concentration of arumanis young leaves extract 10µg/mL, while the zero-hatchability occurred in concentrations of 500µg/mL, 750µg/mL, and 1000µg/mL. Delayed hatching of embryos was thought to be due to the high concentration of arumanis young leaves that were exposed. High concentrations of extracts in natural ingredients and long exposures could result in changes in the chorionic protein profile [24]. This could impact the opening or widening of chorionic pores and damage the chorion. Therefore, there was a decrease in the embryonic survival rate of the chorion.

Figure 3 demonstrated the effect of young leaf arumanis extract on the heart rate of zebrafish larvae. At concentrations of 10µg/mL, 30µg/mL, 50µg/mL, and 250µg/mL, the heart rate (165-174 beats/minute), and there was no significant difference compared to the control group (Kruskal-Wallis test, P<0.05).

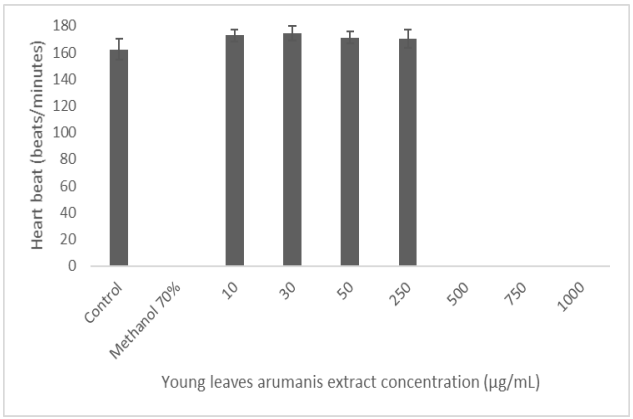

Figure 3. Heart rates at 72 hpf and 96 hpf in zebrafish embryos after exposure to various concentrations. P1=control, P2=methanol, P3=10 µg/mL, P4=30 µg/mL, P5=50 µg/mL, P6=250 µg/mL, P7=500 µg/mL, P8=750 µg/mL, P9=1000 µg/mL. Significant difference compared to the control group (P<0.05).

Heart rate is an important factor in toxicity testing because it can be an important sublethal endpoint to be observed or as an index of toxicity. Figure 3 demonstrated the effect of young leaf arumanis extract on the heart rate of zebrafish larvae. At concentrations of 10µg/mL, 30µg/mL, 50µg/mL, and 250µg/mL, the heart rate (165-174 beats/minute), and there was no significant difference compared to the control group (Kruskal-Wallis test, P<0.05). The average normal heart rate in zebrafish larvae are between 120-180 beats/minute, close to normal heart rate in humans [25], [26], [27].

At concentrations of 500µg/mL (P7), 750µg/mL (P8), and 1000µg/mL (P9), the average heart rate of zebrafish larvae was not observed due to coagulation of all embryos after 24 h being of exposure to young leaf arumanis extract. The development heart of zebrafish larvae is a potential organ target due to the toxicity of young leaf arumanis extract. This was especially true at high concentrations of the extract. Some natural ingredients in the extract can cause heart function disorders, abnormal heart rate, and blood circulation failure [23], [28].

Swimming movement of zebrafish larvae was observed for 20 min in the control group at concentrations of 30µg/mL and 50µg/mL, due to embryo death at other concentrations. At concentration 50µg/mL of young leaves arumanis extract that exposed to embryos zebrafish caused minimal movements in swimming activity of zebrafish larvae. Exposure to 30µg/mL led to increased swimming activity in zebrafish larvae. Exposure to natural ingredients at low doses can cause hyperactivity in larvae, whereas exposure to high doses of natural ingredients affects hypoactivity in larvae [29]. Generally, exposure of zebrafish embryos to high concentrations of natural compounds delays the development of locomotor organs, nervous system, and visual disturbances in zebrafish larvae. Hypoactivation in larvae is also associated with other malformations, such as defects in locomotion and reduced swimming activity [30].

The main toxicological reference that evaluates the teratogenicity of natural substances is malformations. These malformations occur in various forms, and can occur in embryonic or larval stages. The embryo was at 48 hpf, when it entered the pharyngeal phase. The pharyngeal phase is characterized by forming a straight body axis, tail detachment from the yolk sac, and pigmentation [31]. Figure 4. provide information about malformation that was observed. Malformation was founded at concentration of 50µg/mL, with a type of malformation that lacked somite formation.

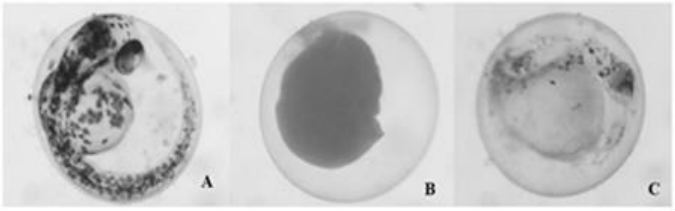

Figure 4. Several conditions in zebrafish embryos and larvae A=normal development (control); B=coagulating embryo (methanol); C=lack of somit formation (50µg/mL concentration). (7x magnification).

Malformation was observed, as shown in Figure 4, with a type of malformation that lacked somite formation at a concentration of 50µg/mL. Somite formation is regulated by Fibroblast Growth Factor (FGF) and retinoic acid, which are determinants of somites [32]. Contaminant compounds that enter the body of the embryo inhibit FGF, which is expressed more in the posterior area. The presence of these barriers has an impact on the disruption of retinoic activity. Therefore, it inhibits the maturation of presomites into somites.

Phenotypic observation of zebrafish embryos was an additional parameter in toxicity tests to evaluate the teratogenic effects of a compound. Figure 5 show some malformations in zebrafish larvae on 50µg/mL concentration of young leaf arumanis extract. In addition to malformations in zebrafish embryos, exposure to young leaf arumanis extract at high concentrations also leads to malformations in larvae. Based on Figure 5, 50µg/mL showed a bending-type tail accompanied by yolk sac edema. During embryogenesis and larval stages, zebrafish absorb food from the yolk. When exposed to high concentrations of natural compounds, the yolk sac becomes larger than usual. It is known as yolk sac edema. The occurrence of yolk sac edema could be influenced by the accumulation of a high concentration of natural compounds in the yolk sac, so it is directly exposed to the embryos. Yolk sac edema causes malnutrition in larvae, reducing larval mobility, and even death in larvae [27], [28].

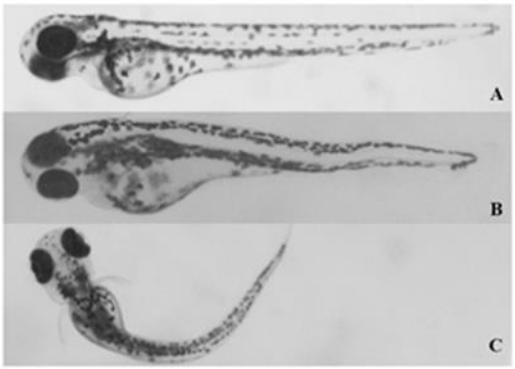

Figure 5. Some malformations in zebrafish larvae. A=normal larvae zebrafish (control); B=tail bending with yolk sac edema (50 µg/mL concentration); C=tail bending (50 µg/mL concentration). (3x magnification)

Several malformations in embryos and larvae are thought to be caused by the influence of high concentrations of secondary metabolites. However, there have been no further studies on the types of secondary metabolites that cause malformation. Although the mechanism of teratogenicity of chemicals in zebrafish still needs further research, xenobiotic-induced teratogens may lead to misregulation of Matrix Metalloproteinases (MMPs) [33]. MMPs are important in the development and impairment of retinoic acid signalling. MMPs are a conserved protein group involved in embryogenesis, specifically in the degradation of the extracellular matrix.

CONCLUSION

Based on this study, the LC_{50} values of the young leaf arumanis extract were 42.65µg/mL at 96 hpf and also 42.65µg/mL at 72 hpf. Embryotoxic effects of young leaf arumanis extract include hatching delay and decreased heart rate, especially at high concentrations. The extract also caused abnormalities in embryo morphology, including pericardial edema and tail bending. Further research is needed to determine the natural compounds from young leaf arumanis extract that cause toxicity and to identify natural compounds from young leaf arumanis extract.

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