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Original Article

Acute Toxicity Tests of Fermented Robusta Green Coffee Using Zebrafish Embryos (*Danio rerio*)

Tedjo Narko[1,2], Marlia Singgih Wibowo[1,*], Sophi Damayanti[1], Indra Wibowo[1]

ABSTRACT

Background: Green coffee beans are coffee beans of coffee fruit that have not yet been roasted. The use of green coffee beans as a weight-loss agent has been widely used worldwide, but nowadays there is a new way to enjoy coffee by adding kombucha culture to it, or what is known as kombucha coffee. The development of this fermented product preparation still requires a more in-depth study, one of which is related to the toxicity of the kombucha green coffee preparation. **Objective:** This research was aimed to determine LC_{50} values for robusta green coffee fermented with kombucha culture using zebrafish (*Danio rerio*) embryos using an *in vivo* method. **Methods:** This study observed the development of 20 zebrafish embryos administered one of five different concentrations of kombucha coffee preparation 24 hours up to 96 hours, with the experiment repeated three times. The percentage of embryo deaths was observed and analyzed using a probit model for LC_{50} concentration and analyzed using IBM SPSS Ver 23. **Results:** An LC_{50} for kombucha green coffee of 1294.29 ppm was obtained. Statistical tests on the concentration of kombucha coffee extracts were not significant differences with p-values > 0.05. **Conclusion:** The LC_{50} of robusta green coffee extract using zebrafish embryos of 1294.29 ppm included in the safe category.

Key words: Green coffee, Kombucha, Toxicity, Zebrafish embryos.

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INTRODUCTION

Coffee plants are included in the genus *Coffea,* family Rubiaceae. The genus *Coffea* has more than 100 species. Of these, there are three species that are cultivated for commercial purposes, namely *Coffea arabica*, *Coffea canephora*, and *Coffea liberica*. In general, only the seeds of coffee plants are used and extracted from as drinks. But in some societies, there are also those who consume the leaves by brewing such as tea leaves.

Indonesia has great opportunities and excellent prospects for developing coffee in terms of both domestic consumption and export markets. The demand for coffee worldwide is quite large and shows an increasing trend. Data from the International Coffee Organization shows that the recent increasing trend in world coffee consumption began in 2010 with an average increase of 2.5% / year. In 2020, it is estimated that world coffee needs will reach 10.3 million tons.[1] Coffee is an agricultural commodity in Indonesia that has further economic value, as it contains a variety of chemical compounds with different characteristics and requires research in its biological activities development.[2] Most of the coffee beans traded globally are produced from the *Coffea arabica* and *Coffea canephora,* which has the popular names arabica and robusta coffee, respectively. The remaining insignificant amount is made from *Coffea liberica,* which is traded under the name of liberica or excelsa coffee.[3]

Green coffee beans are coffee beans that have not yet been roasted. Green coffee contains various chemical compounds such as carbohydrates, proteins, minerals, caffeine, trigonelline, aliphatic acids (carboxylic acids), chlorogenic acids, fats, and derivatives, glycosides, and volatile components. Chlorogenic acid is one of the isolated compounds with antioxidant activity found in a large number of coffee varieties.[4,5] Chlorogenic acid belongs to the hydroxycinnamic acid group,[6] a member of the larger polyphenol group, many members of which have antioxidant activity. The polyphenol content found in robusta coffee is higher than arabica coffee or other plants.[7] Chlorogenic acid in green coffee is considered to have health benefits for heart disease, diabetes, weight loss, and others. Several clinical trials on humans have been conducted to test the effectiveness and safety of chlorogenic acid in green coffee to reduce body weight and body mass in adults.[8,9] While, the results of a clinical study which has been conducted on 30 overweight people showed that the consumption of instant coffee enriched with chlorogenic acid for 12 weeks showed a significant decrease in body mass index and body fat, compared with control.[10]

Kombucha is a traditional fermented tea with a slightly sour and sweet taste and is beneficial for health. This tea is widely consumed by people in all parts of the world. In the past, it was used in China, Russia, and Germany. Kombucha is ready to drink after its pH ranges from 2.5-3.5 with a fermentation time of 10–14 days.[11] Kombucha is defined as a combination of bacteria and yeast or commonly called 'SCOBY' ('symbiotic colony of bacteria and yeast'), where microorganisms create symbiotic relationships in the matrix in the form of fibers

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resembling mycelium.[12] *Acetobacter xylinum* is the primary bacterium found in kombucha culture. The primary species of yeast successfully isolated from kombucha is *Saccharomyces cerevisiae.*[13]

Kombucha has been proven to increase stamina, intensify the work of the small intestine, lose weight, reduce cholesterol, normalize the functions of organs, treat gout, prevent cancer and increase immunity.[14-16] Thus, there has been considerable interest in adding kombucha to coffee. The process of adding kombucha culture to robusta green coffee has been conducted by using a fermentation method where fermentation is defined as a process to generate useful products by utilizing microorganisms as a part of further processing.[17]

Assessing the toxicity of preparation or compound can be done using *in vivo* methods. *In vivo* acute toxicity tests can be carried out using zebrafish embryos (*Danio rerio*).[18] Toxicity tests using species of fish are also an important element of whole effluent toxicity (wet) testing in North America and some European countries.[19,20] Based on these conditions, this research was aimed to determine LC_{50} values for robusta green coffee fermented with kombucha culture by using an *in vivo* method involving zebrafish (*Danio rerio*) embryos.

MATERIALS AND METHODS

Materials

The materials and tools used were robusta green coffee beans from Garut, Indonesia, kombucha culture (Indokombucha), sucrose, water, E3 Medium (5.0 mM NaCl; 0.17 mM KCl; 0.33mM$CaCl_2$; 0.33mM $MgSO_4$) (Merck), dichloroaniline, filter membrane 0.45 µm, as well as Zebrafish (*Danio rerio*) wild-type Bogor.

Research design

This research was experimental design using *in vivo* methods (zebrafish embryos) to determine acute toxicity test in robusta green coffee fermented with the kombucha culture. In this study used 20 zebrafish embryos using five concentrations of kombucha coffee preparations, then the development of these embryos was observed every 24 hours up to 96 hours, and experiment was repeated 3x times. This research received registered ethical approval from Padjajaran University, No. 268/UN6.KEP/EC/2019.

Fermentation of Kombucha Coffee

Robusta green coffee beans were put into boiling water with a ratio of 375 mg of simplicia per 300 mL of water, then 30 g of sucrose was added and stirred until homogeneous, then the mixture was cooled and strained. After that, 30 mL of kombucha vinegar was added and stirred until homogeneous. Then, ± 20 g of kombucha culture with 10^8 CFU/ml (symbiosis between the bacteria *Acetobacter xylinum* with several types of yeast that have been isolated from Kombucha such as *Saccharomyces* sp., *Torulopsis famat*, *Pichia membranae*, and *Candida* sp.[21,22] was inoculated into each jar. The jar containing the inoculant was covered with a sterile porous cloth and placed in an incubator. The test was carried out at room temperature of ± 25 ° C. The incubation time used was 18 days. The procedure was modified from standard kombucha fermentation with tea by previous study.[11]

Phytochemical screening and simplicial characterization

Phytochemical screening was carried out on the simplicia of robusta coffee beans as well as those that have been fermented using kombucha culture, including screening for alkaloids, flavonoids, saponins, steroids/triterpenoids, tannins, and quinines. The screening procedures for each phytochemical was based on Indonesian Herbal Pharmacopoeia without any modification.

Zebrafish maintenance

Healthy adult zebrafish (*Danio rerio*) from wild type strain (3-4 months), were used in the experiment and kept in an aquarium at room temperature with the addition of bright lighting for 14 hours and 10 hours of dark cycles. The fish were fed using Tetramin twice a day in the morning and evening. Meanwhile, the aquarium water was replaced every 3 days by replacing 60% of the aquarium water volume to prevent drastic changes in the aquarium environment.[23]

Toxicity test using zebrafish embryos

Breeding was done by placing male and female zebrafish in a ratio of 2:1 into a breeding tank that has been installed with a net to separate the embryo from adult fish and left overnight. Zebrafish egg collection was carried out in the morning according to the lighting cycle using a fine net. Furthermore, the eggs obtained were washed with running water several times in order to remove any dirt that adhered to the egg. Then the embryos were observed under a stereomicroscope. Healthy or fertile embryos were then transferred to some Petri Dish embryos containing E3 medium. Embryos that died or were infertile appeared white or opaque and the separation was done after the first few hours of the development phase (0–96 hours post-fertilization (hpf)) by using a pipette to prevent a delay in the development of healthy embryos.[23,24] Furthermore, the eggs were acclimatized in an incubator at 28–29°C for 2–3 hours before being given the test treatment.

The test solution of kombucha coffee was made by mixing kombucha coffee with E3 (reconstituted water) medium. E3 medium is a medium which was used as a zebrafish embryo maintenance medium. The composition of E3 medium (per 1 L) contained: 5.0 mM NaCl; 0.17 mM KCl; 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$. After that, five concentrations of test solutions of kombucha coffee were determined as follows: 100 ppm; 200 ppm; 300 ppm; 500 ppm; 1000 ppm, with a ratio of each concentration of 1.8. Positive controls used in the test treatment were in the form of 4 mg / l of Dichloroaniline, while the negative control was in the form of E3 solution. Zebrafish embryos at 6 hpf were transferred into a 24-well plate (1 embryo per well) and every 20 embryos were compared in each group. Then, to the wells were added the various concentrations of the Kombucha coffee extract solution, i.e., 100, 200,300,500 and 1000 ppm per well with 3 repetitions. A static non-surrogate regime was used. Thus, there was no replacement for the test solution refresh during the test. Furthermore, the well plate containing the embryo and variations in the concentration of the kombucha coffee extract solution was incubated at 28 °C. The replacement of the test solution was carried out every 24 hours to 96 hours of observation time. The toxicity test of kombucha coffee was repeated 3 times.[25]

The observations were carried out every 24 hours by observing and recording the number of dead embryos based on these characteristics: coagulated embryo, non-detachment of the tail, lack of somite formation, lack of heartbeat. If one of these characteristics appears in the embryo, the embryo was classified as a dead embryo. The activities of acute toxicity test of kombucha coffee using zebrafish embryos were based on OECD 236 of 2013 are: carrying out egg production, collecting eggs, conducting pre-exposure immediately after fertilizing in a glass container, selecting fertilized eggs with inverted microscope or binoculars and distributing fertilized eggs into containers with 24 wells prepared with each concentration/test control, n = number of eggs needed per concentration/control test (here is 20), as well as hours post-fertilization monitored.[25] The research scheme can be seen in Figure 1.

Statistical analysis

All data are expressed as mean ± SD (Standard Deviation). Statistical analysis was performed using IBM SPSS Ver 23 software (IBM Corporation, North Castle, New York, USA). Statistical comparisons

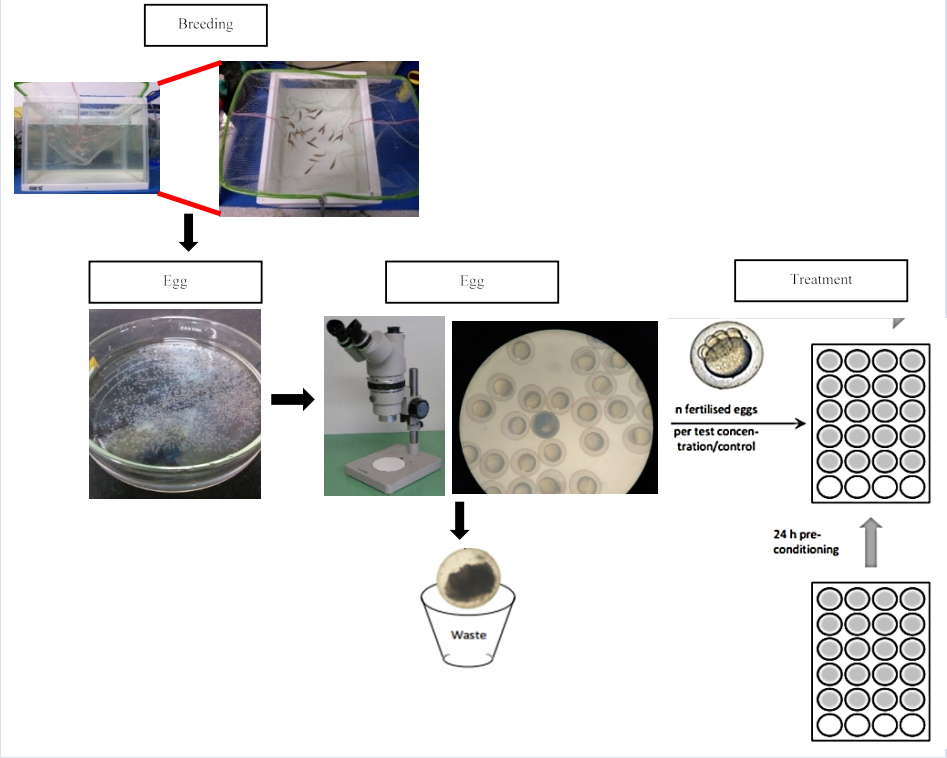

Figure 1: The procedural scheme of toxicity test using zebrafish embryos (OECD, 2013).

were assessed with one-way ANOVA followed by an LSD mutation comparison test with a significance level of 0.05.

RESULTS AND DISCUSSION

Phytochemical screening was carried out to determine the chemical compounds contained in robusta green coffee beans. Phytochemical screening was carried out both on simplicia and kombucha coffee formed from the result of fermentation. The results of the phytochemical screening are listed in Table 1, and the results of simplicia characterization and standardization can be seen in Table 2.

Based on Table 1, it shows that simplicia and kombucha coffee contained alkaloids, flavonoids and tannin compounds. Steroids/Triterpenoids were found in the simplicial as the blue and green color indicator for steroids and the orange, yellow, and golden indicator for terpenoid were formed.[26] There were no saponins and quinone either in simplicia and kombucha coffee as the formed bubbles were unstable for saponin test and no red indicator appeared after NaOH addition for quinone test.[27]

The alkaloid substance in green coffee is caffeine. Caffeine is a non-specific antagonistic for adenosin receptor, which is widely distributed in the cortex.[28] The caffeine is a stimulant that works by inihibiting adenosin receptor, thus blocking the neurotransmitter.[29] The simplicia and the green coffee kombucha extract also had cholorogenic acid flavonoid, a strong antioxidant in the group of hydroxycinnamic acid group that commonly found in coffee and tea.[6] Roasting can reduce the chlorogenic acid; the darker the beans, the least amount of chlorogenic acid will be.[30] Roasting temperature also affect the content; the higher temperature will further decrease the antioxidant activity.[31]

Interestingly, the phytochemical screening of the green robusta coffee bean simplicia and coffee kombucha showed a different result with previous study[32], who reported alkaloid, tannin, saponin, flavonoid, terpenoid content in the robusta coffee beans. These results indicated the secondary metabolite content in coffee beans was affected by type, processing, and geographical origin.[33]

Table 1: The results of the phytochemical screening of simplicia and kombucha coffee.

	Coffee Bean	
Groups	Simplicia	Kombucha Coffee
Alkaloids	+	+
Flavonoids	+	+
Saponins	-	-
Steroids/Triterpenoids	+	+
Tannins	+	+
Quinone	-	-

(+) : identified; (−) : not identified.

Table 2: The results of simplicia characterization.

Parameter	Result (%w/w)
Water-soluble extract content	18.56 ± 0.21
Ethanol soluble extract content	13.42 ± 0.13
Water content*	9.14
Total ash content	2.83 ± 0.08
Acid insoluble ash content	1.04 ± 0.02

(*) % v/w

As seen in Table 2, the simplicia characterizations carried out include the determination of water-soluble extract content, ethanol-soluble extract content, water content, total ash content, and acid insoluble ash content. The water content of robusta coffee used in this study was 9.14%. Moisture content can guarantee safety during the storage process. This water content corresponds to the robusta coffee water range in general, which is 7–13%.[34] In this research, water is used as a solvent because water has a better ability to dissolve bioactive compounds in Robusta coffee than isopropanol. Also, water is able to dissolve other components such as carbohydrates, aroma-forming compounds, and colors.[4] The acute toxicity test of kombucha coffee using zebrafish

resulted in the regression equation in percent (%) of the concentration of kombucha coffee seen in Table 3 and Figure 2.

Based on Table 3, shows that the higher the concentration (ppm) of kombucha coffee given to zebrafish the higher the mortality percentage, which is 32% at 1000 ppm, so that the probit value is 4.53.

Based on Figure 2, the regression equation of the results of an acute toxicity test using zebrafish with the observation time of 96 hours is obtained, and the LC_{50} value for kombucha coffee is 1294.29 ppm. These results are obtained from the equation y = 0.0014X + 3.188. Solving for a probit value for LC_{50} = 5, an x value of 1294.29 ppm can be obtained. This is based on whether there are features: coagulated embryo, Non-detachment of the tail, lack of somite formation, lack of heartbeat. LC_{50} or Lethal Concentration 50 is the concentration that causes death as much as 50% of the test organisms. The OECD categorized the toxicity of pollutants in a zebrafish model as being dangerous (10 ppm $<LC_{50}<$100 ppm), toxic (1 ppm $<LC_{50}<$10 ppm), and very toxic ($LC_{50}<$1 ppm). Based on these categories, kombucha coffee is included in the safe category. The characteristics seen in the zebrafish embryo during 96 hpf observation can be seen in Figure 3.

Based on Figure 3, it can be seen that there are defects in the zebrafish embryo which was given kombucha coffee during 96 hpf observation, indicated by lack of somit formation, pericardial edema and spinal curvature (scoliosis). Therefore, based on this observation, the embryo is classified as a dead embryo.

Zebrafish embryos exposed to dichloroaniline resulted in death at the 24th hour before the hatching process took place. According to Kumar *et al.*, this condition is thought to occur due to interference with cell organelles, as well as mitochondria as a place for aerobic respiration.[35] The process of ATP formation is slow or stopped, resulting in the failure of the active membrane of the sodium pump, intracellular sodium accumulation, and outward diffusion of potassium, which can kill cells. Cells that experience continual swelling can result in lysis of the cell wall, and this can impact all of the cell organelles (necrosis).

Somit formation abnormalities in zebrafish embryos can be caused by the inhibition of Fibroblast Growth Factor (FGF) by genistein, which is expressed more in the posterior region.[36] so that it can make easier for Retinoic Acid (RA) to increase the expression of genes determining the somite pattern, while FGF suppresses RA activity and inhibits presomitic mesoderm maturation into somites. RA is expressed in a rostrocaudal gradient, while FGF is expressed in a caudorostral gradient.[37]

Pericardial edema abnormalities in zebrafish embryos according to Kim et. al can occur due to exposure to genistein which can reduce the frequency of zebrafish heart rates.[38] Genistein is a tyrosine kinase inhibitor that functions to influence the activity of various ion channels, both through the process of phosphorylation and direct bonding. Meanwhile, according to Chen which explains that pericardial edema can occur because it is influenced by many factors. Factors that cause zebrafish embryos to experience stress due to treatment using any means will have an impact on the occurrence of impaired heart function (pericardial edema) and impaired circulation.[39]

Body axis abnormalities that occur in zebrafish embryos due to kombucha coffee namely bent and curly up. Bent body axis abnormalities

Table 3: The results of the calculation of mortality percentage and probit value.

Concentration (ppm)	% Mortality			Mortality	Probit
	1*	2*	3*	Percentage (%)	Value
100	5	5	5	5	3.36
200	10	5	0	5	3.36
300	15	5	0	7	3.52
500	25	5	30	20	4.16
1000	25	10	60	32	4.53

(*) repetition using the same methods

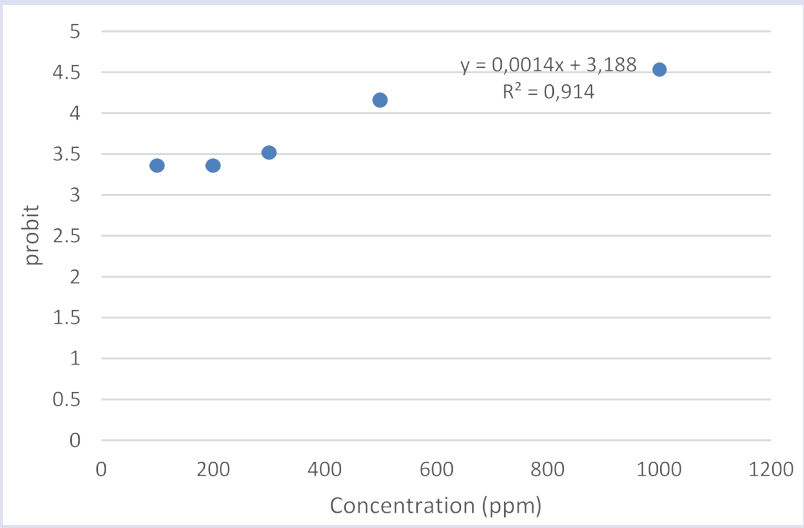

Figure 2: The curve between Probit vs Concentration (ppm) of kombucha coffee in the acute toxicity test towards zebrafish embryos. The regression equation obtained is y = 0.0014X + 3,188; R^2 = 0.914.

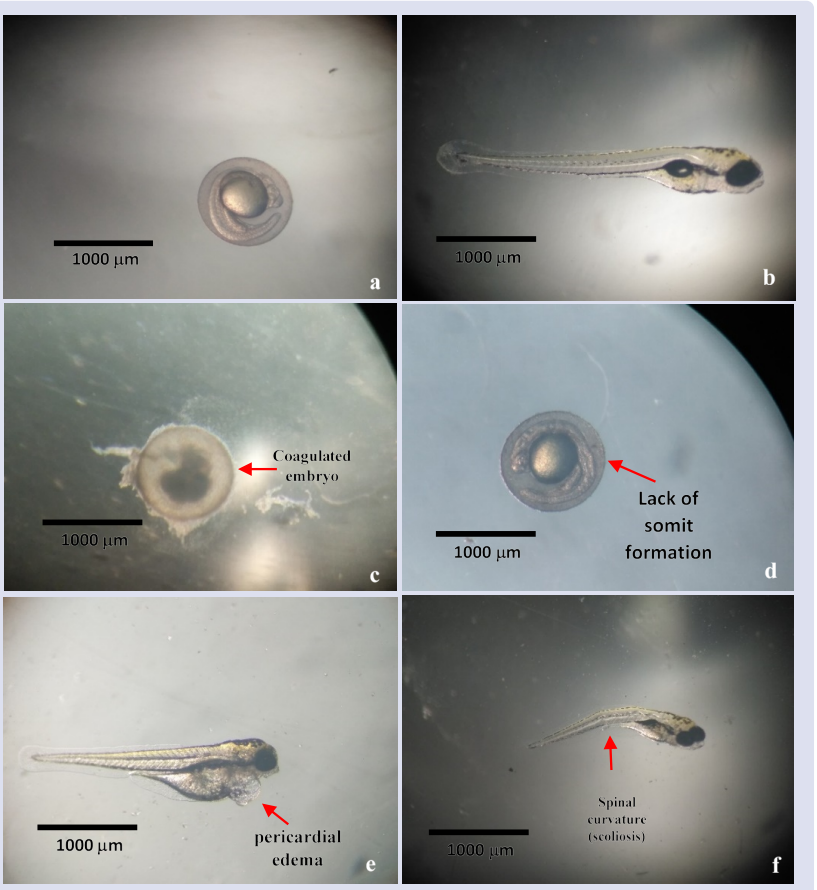

Figure 3: Examples of zebrafish conditions. Healthy: (a) negative control 24 hpf, (b) negative control 96 hpf, (c) Positive control (Dichloroaniline 4 mg/l), Characters appeared in the zebrafish embryo after 96 hpf (observation zebrafish embryo classification deemed dead according to the OECD, 2013), (d) 500 ppm 24 hpf (lack of somit formation), (e) 1000 ppm 96 hpf (pericardial edema), (f) 300 ppm 96 ppm (scoliosis).

(Figure 3f) are shown to the body bent sideways due to damage to the spinal cord, while curly up body axis abnormalities are shown with the body bent up which occurs due to the influence of the Polaris gene and pkd2 (polycystin) and β-catenin protein.[40,41]

Acute toxicity test using zebrafish embryos is intended to obtain safe preparation, where the measured parameter is LC_{50}. The data in Table 3 show a safety level of 95% at concentrations of 100 and 200 ppm, 93% for concentrations of 300 ppm and decreased to 80% at concentrations of 500 ppm and 1000 ppm to 68%. From the results of the toxicity test on kombucha coffee, the LC_{50} value of 1294.29 ppm is obtained. This value, if it is classified using the category of OECD, is included in the safe category. Therefore, based on these conditions, green kombucha coffee is safe for consumption. It is important to develop green coffee kombucha with green coffee ingredients so that a clear toxicity description can be obtained about the types of coffee used as the basic ingredients of fermented coffee drinks.

CONCLUSION

Based on LC_{50} values, 1294.29 ppm, obtained from the test using zebrafish embryos and classified according to the OECD category, kombucha coffee is included in the safe category. This condition is also thought to occur in kombucha coffee with arabica green coffee ingredients.

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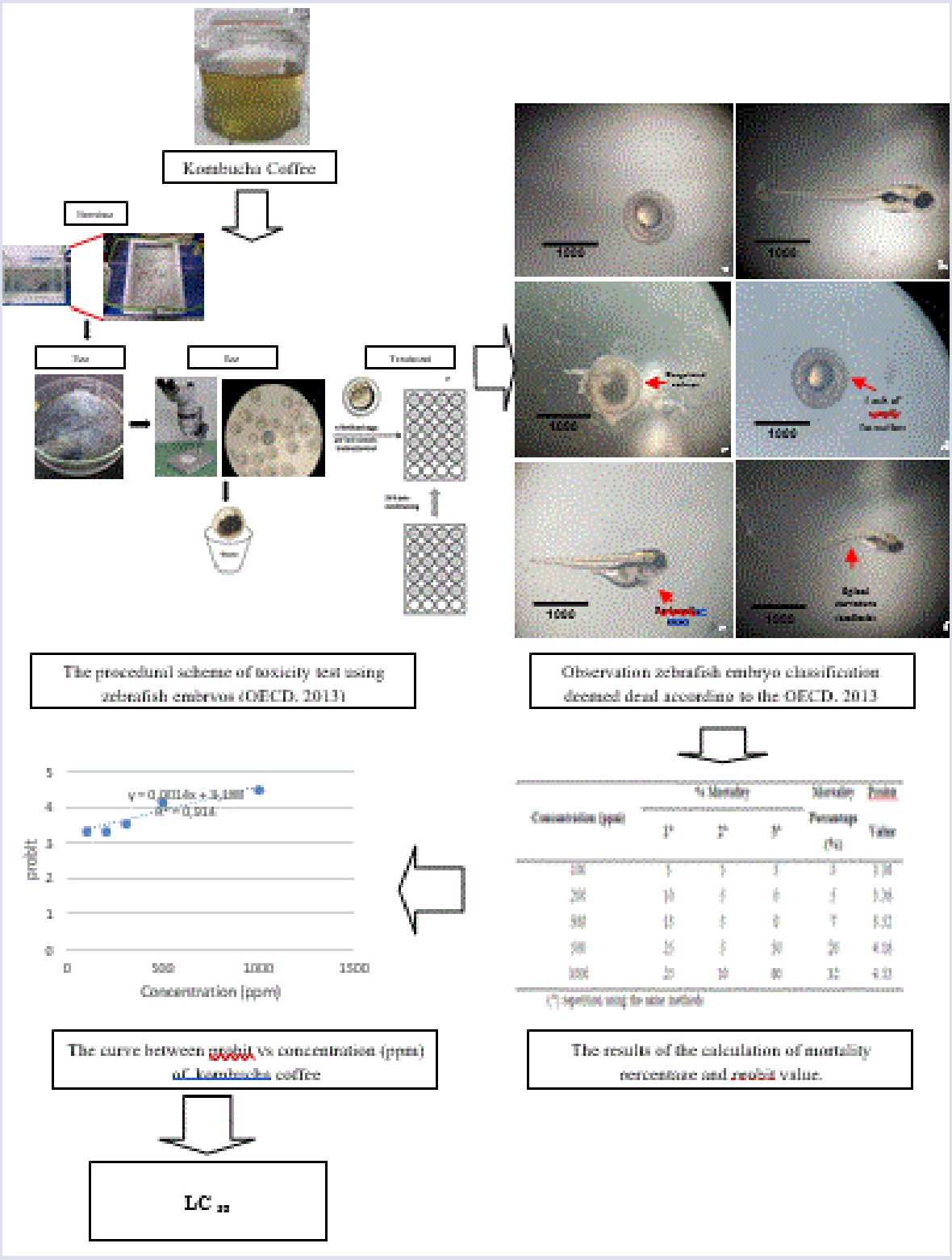
GRAPHICAL ABSTRACT



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