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Farida Hayati, Lutfi Chabib, Rizki Awaluddin, and Tirta Aji Permana

The Effect of *Ipomoea reptans*, Poir Self-Nanoemulsifying Drug Delivery Systems (SNEDDS) on the Development of Zebrafish (*Danio rerio*) Embryos

Farida Hayati[1, a)] Lutfi Chabib[1)], Rizki Awaluddin[1)], Tirta Aji Permana[1)]

[1]*Pharmacy Department, Faculty of Mathematics and Natural Sciences, Islamic University of Indonesia, Yogyakarta, Indonesia*

[a)]Corresponding author: farida.hayati@uii.ac.id

Abstract. This study aimed to determine the effect of Self-Nanoemulsifying Drug Delivery Systems SNEDDS extract of kangkong (*Ipomoea reptans*, Poir) on the development of zebrafish embryos. Toxicity studies on zebrafish embryos each used 6 groups consisting of 3 control groups and 3 treatment groups. The control group consisted of solvent control, normal control, and positive control. The treatment group consisted of a concentration of 100 µg/mL, 50 µg/mL, and 25 µg/mL. Observations were carried out every 24 hours for 96 hours (4 days). Parameters of embryo mortality observed were coagulation, the formation of somites, detachment of tailbud from the yolk, and heart rate. From the experiment, it was concluded that the SNEDDS of kangkong (*Ipomoea reptans*, Poir) leaf extract did not affect the development of zebrafish embryos.

INTRODUCTION

Kangkong is a natural ingredient frequently studied for its safety and activity as an antihyperglycemia [1-5]. One of the methods to formulate nanoemulsions is Self-Nanoemulsifying Drug Delivery Systems (SNEDDS), an isotropic mixture of oil, surfactant, and co-surfactant that can rapidly and easily form nanoemulsions when mixed with water [6]. Self-nano-emulsifying can occur spontaneously since it requires neither additional treatment nor external energy. The obtained nanoemulsions generally have a size of 20-200 nm [7]. The main components of SNEDDS are oil, surfactant, and cosurfactant with oil as the drug carrier phase, surfactant as the oil emulsifier in water, and cosurfactant to support the role of emulsifier surfactant. The benefits of SNEDDS include the ability to accelerate the dissolution time of lipophilic compounds, reduce the first-pass metabolism in the liver, as well as improve absorption [8]. This technique is also relatively affordable and easy to perform [9], making it a high potential for practical application.

Toxicity studies are required to examine the safety of SNEDDS compounds, and such screening can be performed by observing the development of zebrafish embryos [10]. Toxicity studies are important for nano compounds because their increased absorption ability makes the possibility of toxicity also higher. Toxicity screening in zebrafish embryos can assess the safety of nano compounds, particularly during the development of the embryonic phase. This research is important to ensure the safety of the use of kangkong leaves in the development of zebrafish embryos as an initial step before testing teratogenic in mammals. This study aims to test the safety of SNEDDS kangkong extract on the development of zebrafish embryos, as a first step to ensure the utilization of kangkong leaf extract for pregnant mammals.

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MATERIALS AND METHODS

Ethical Clearance

This study has received ethical clearance from the Ethics Committee of Medical and Health Research in the Faculty of Medicine, Universitas Islam Indonesia No. 32/Ka.Kom.Et/70/KE/VIII/2018.

Materials

The materials consisted of kangkong leaves collected from Gantiwarno in Klaten District, distilled water, surfactant (Tween 20), Capriol 90, 96% Ethanol, cosurfactant (PEG 400), 70% Ethanol, surfactant (Tween 80), cosurfactant (Propylene Glycol), and 3,4-DCA (Dichloroaniline). The experimental animal was zebrafish (*Danio rerio*) and their embryos. Zebrafish have been identified at the Indonesian Institute of Sciences (LIPI) Bogor (B-3853/IPH.1./KS.02.03/XI/2017). The inclusion criteria were physically healthy zebrafish aged 4-6 months and fertile embryos aged less than 6 hours after fertilization.

The tools used in this study include a set of glass (Pyrex), rotary evaporator (Heidolph), Particle Size Analyzer (PSA) (HORIBA SZ 100), ultrasonicators (Biologics Model Inc. 300 V / T), analytical scales (Melter Toledo XS205 Dual Range), ovens, cabinet dryers, stereo microscopes (BELL), microwell-plates (IWAKI).

Extraction Process

Kangkong leaf was extracted through maceration using 96% ethanol solvent (1:10) for 6 days. The yield was then evaporated to obtain viscous extract and further concentrated in a water bath [3].

SNEDDS Preparation: Process and Evaluation

SNEDDS Kangkong Leaf Extract

Capriol oil phase was mixed with kangkong leaf extract followed by the addition of Tween 20 as the surfactant and PEG 400 as the cosurfactant. Mixing was done in an ultrasonicator (Biologics Inc. Model 300 V/T) with a 450-rpm stirrer. Evaluation of SNEDDS parameters was performed to assess the particle size, polydispersity index (PDI), and zeta potential using a particle size analyzer (HORIBA SZ 100) [11].

Toxicity Studies on Zebrafish Embryos [12]

Preparation and selection of zebrafish embryos

Adult zebrafish were placed in an aquarium with 7.2 pH and 28°C temperature under a photoperiod cycle of 14-hour daylight and 10-hour dark with daily feeding. Prior to fertilization, male and female zebrafish (2:1) were separately placed in a mating tank for one night. Fertilization was stimulated on the next day at an early photoperiod by putting the male and female zebrafish together. The eggs were then observed and separated in Petri dishes, and fertile embryos without coagulation were selected by observation under a stereomicroscope (BELL).

Observation of zebrafish embryos

Observation of zebrafish embryos was done on 24-well plates. There were 8 plates divided for the negative control (1 plate), positive control (1 plate for 4mg/L 3,4-DCA), and solvent control (1 plate for SNEDDS without the extract) while the other 5 plates were for SNEDDS at concentrations of 428 ppm, 578 ppm, 763 ppm, 1007 ppm, and 1328 ppm. Each plate used 20 embryos with one well for each embryo and remaining wells for the solvent control. All the test solutions without embryos were placed in an incubator at 24 hours prior to addition of embryos to the wells. The Observation was conducted every 24 hours for 96 hours under a microscope. The parameters to observe consisted of embryo coagulation, somite formation, detachment of the tail bud from the yolk, and heart rate.

Data Analysis

After observing zebrafish embryos, then calculating the percentage of zebrafish embryo mortality, the percentage of embryos with coagulation, the percentage of embryos with somit formation, the percentage of embryos detachment a tail-bud from the yolk, and the percentage of embryos that can be observed for their heartbeat.

$$\text{percentage of zebrafish embryo mortality} = \frac{number\ of\ embryo\ death}{total\ number\ of\ embryo}\ x\ 100\% \quad (1)$$

$$\text{percentage of coagulation embryo} = \frac{number\ of\ coagulation\ embryo}{total\ number\ of\ embryo}\ x\ 100\% \quad (2)$$

$$\text{percentage of somit formation} = \frac{number\ of\ somit\ formation}{total\ number\ of\ embryos\ living}\ x\ 100\% \quad (3)$$

$$\text{percentage of somit formation} = \frac{number\ of\ somit\ formation}{total\ number\ of\ embryos\ living}\ x\ 100\% \quad (4)$$

$$\text{percentage of embryos releasing tailbud} = \frac{number\ of\ embryos\ experiencing\ tail\ bud\ release}{total\ number\ of\ embryos\ living}\ x\ 100\% \quad (5)$$

$$\text{percentage of visible heart rate} = \frac{number\ of\ heart\ rate\ visible\ embryos}{total\ number\ of\ embryos\ living}\ x\ 100\% \quad (6)$$

RESULTS AND DISCUSSION

The raw material of this research is kangkong as a natural ingredient that has been empirically used and pre-clinically proven with a number of pharmacological activities. Observation is conducted on zebrafish embryos, and the extract is processed as a SNEDDS preparation in an effort to improve the bioavailability.

TABLE 1. Formulation of Kangkong Leaf Extract SNEDDS [11]

Function	SNEDDS of Kangkong Leaf Extract	
	Material	**Quantity**
Oil Phase	Capryol	0.5 mL
Surfactant	Tween 20	3.5 mL
Cosurfactant	PEG 400	1 mL
Active Ingredient	Kangkong leaf extract	1600 mg

TABLE 2. Results of SNEDDS Evaluation Using Particle Size Analyzer (PSA) (n=3) [11]

SNEDDS of Kangkong leaf extract	Particle size (nm)	PDI (D)	Zeta Potential (mV)
	32.8 ± 0.37	0.377 ± 0.067	-42.53 ± 0.47

The evaluation results show that the SNEDDS preparation has fulfilled the pharmaceutical criteria of a stable nanoparticle preparation. The particle size is 32.8, indicating the fulfillment of the nanoparticle size range (20 – 200 nm). Polydispersity index represents the width and breadth of particle distribution, and in this study, the index has a good range of 0.05 – 0.7 [13]. Therefore, SNEDDS can further be tested on embryos as an effort to examine safety. One of the methods to investigate the toxicity of nano preparation is by observing its effect on zebrafish embryos [10].

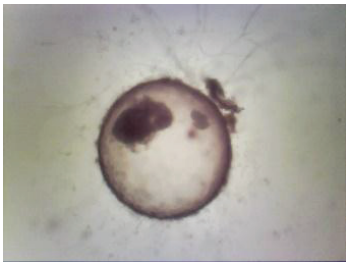

FIGURE 1. Coagulation of an embryo

At the initial stage, zebrafish embryos are selected among 2-hour-old fertilized embryos. The age of fertilized embryos is determined by referring to OECD 236. Coagulated embryos are excluded from the subsequent stage as coagulation indicates that the embryos are infertile and fail to develop.

The exposure of zebrafish embryos is conducted using a 24-well microplate for each embryo group. There are 6 groups of embryos comprising positive control group (4mg/L 3,4-DCA), negative control group (water), solvent control group (SNEDDS without extract), and 3 treatment groups (SNEDDS of kangkong leaf extract) with 100µg, 50µg, and 25µg concentrations. Using a microscope, embryos are observed every 24 hours for 96 hours with four assessment parameters of embryo coagulation, somite formation (formation of the spinal column), detachment of the tailbud from the yolk (detachment of the tail in the embryonic nucleus), and heart rate. The observation results for five embryo groups are presented in Table 3-5.

TABLE 3. Observation results of post-exposure embryonic development from coagulated embryos and embryos with somite formation

Group	% embryos with coagulation				% embryos with somite formation			
	24 hours	48 hours	72 hours	96 hours	24 hours	48 hours	72 hours	96 hours
Control (+)	45	45	45	45	70	100	100	100
Control (-)	10	10	10	10	100	100	100	100
Solvent Control	10	10	10	10	58.8	100	100	100
100 µg/mL	85	85	85	85	100	100	100	100
50 µg/mL	55	55	55	55	100	100	100	100
25 µg/mL	20	20	20	20	100	100	100	100

According to OECD 236, the percentage of embryo coagulation in the negative control and solvent control groups is considered normal at a maximum of 10% or a minimum of 80% incubation or embryonic development in up to 96 hours [12]. This is indicated by 10% coagulation in the negative control and solvent control groups, and more than 30% mortality in the positive control group. The method applied in this study has fulfilled the validation standard from OECD 2013. The embryos used in this research are fertile while coagulated embryos are excluded, and during the 96-hour observation, no coagulation occurs to fertile embryos. This shows that the embryos have good quality, and the administration of the nano compound does not trigger embryo coagulation.

Somite is a spinal column formed at the early stage of zebrafish embryonic development. In a normal condition, somite formation occurs in 10 hours up to 72 hours. In this study, somite is formed in a large majority of the groups in 24 hours, indicating the development of embryos. Somite formation in the solvent control group experiences a problem in 24 hours because the spinal axis that straightens the somite is inhibited by the exposure to SNEDDS through a 35% increase that occurs in 48 hours. Somite formation increases by 40% in the 25µg concentration group and 35% in the solvent control group both in 48 hours. The positive control group (3,4-DCA 4mg/L) inhibits somite formation as 70% somite is formed in 24 hours. This indicates that DCA can inhibit somite development, which subsequently affects the morphology of the zebrafish inbreeding.

TABLE 4. Observation results of tailbud detachment from the yolk and heart rate

Group	% embryos with the detachment of tailbud from the yolk				% embryos with observable heart rate			
	24 hours	48 hours	72 hours	96 hours	24 hours	48 hours	72 hours	96 hours
Control (+)	70	100	100	100	0	55	55	50
Control (-)	100	100	100	100	0	100	100	100
Solvent Control	88,2	100	100	100	0	100	100	100
100 µg/mL	0	100	100	100	0	100	100	100
50 µg/mL	0	100	100	100	0	100	100	100
25 µg/mL	100	100	100	100	0	100	100	100

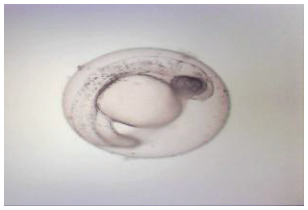

FIGURE 2. Zebrafish embryo from the negative control group at 24 hours

FIGURE 3. Zebrafish embryo from the negative control group at 48 hours

The detachment of the tailbud from the yolk occurs when the tail becomes detached from the embryonic nucleus (yolk). Tailbud detachment normally occurs in the first 24 hours as shown by the negative control group (Table 4). Inhibition of tailbud detachment is shown by the positive control DCA as a continuation of the inhibited somite formation. In the groups of 50 µg/ml and 100 µg/ml SNEDDS, the absorption of SNEDDS into embryos does not inhibit somite formation, but it decelerates the detachment of tailbud from the yolk without causing embryo mortality.

Heart rate is normally observable after 48 hours, and this is proven in this study as the heart rate of embryos in all groups can be observed in 48 hours. This is because the heart rate appears in 48 hours during the pharyngula period of zebrafish development.

TABLE 5. Observation results of the mortality of zebrafish embryos after 96 hours of exposure

Group	Number of dead embryos (%)			
	24	48	72	96
Positive Control	30	40	45	50
Negative Control	0	0	0	0
Solvent Control	0	0	0	0
100 µg	0	0	0	0
50 µg	0	0	0	0
25 µg	0	0	0	0

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TABLE 6. Observation results of embryos with malformation using a stereomicroscope after 96 hours of exposure

Group	Malformation after 96 hours of exposure (%)
Positive control	50% **
Negative control	0%
Solvent control	0%
100 µg	0%
50 µg	5%*
25 µg	5%*

Note: (*) abnormality of the body axis, (**) pericardial edema

FIGURE 4. Abnormality of pericardial edema in zebrafish embryonic development

None of the treatment groups causes mortality to zebrafish embryos. A sign of toxicity is indicated by the malformation of zebrafish embryonic morphology. In DCA administration, malformation appears in the form of pericardial edema, swelling of the heart due to obstruction of the blood circulation. Meanwhile, the administration of kangkong leaf extract SNEDDS can lead to abnormality of the body axis due to embryo weakening during the hatching phase. SNEDDS of kangkong leaf extract does not cause mortality of embryos, but instead, it inhibits the development in the form of body axis abnormality. The mechanism of the effects of kangkong leaf extract SNEDDS on the development of zebrafish embryos has yet to be determined and therefore requires further studies.

CONCLUSION

Administration of SNEDDS of kangkong leaf extract has no effect on the development of zebrafish embryos.

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