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THE TOXICITY OF PEGAGAN (*CENTELLA ASIATICA* (L.) URB) FRACTIONS ON ZEBRAFISH EMBRYO

FARIDA HAYATI[1,*], ARDE TOGA NUGRAHA[1],
WAN SYARIFAH FAIZAH[1], MERINA INDAH SARI[2], LIA NURKHASANAH[3]

[1]Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Universitas Islam Indonesia, Jl. Kaliurang km. 14.5, Yogyakarta 55584, Indonesia
[2]Students of Pharmacist Professional Program, Department of Pharmacy, Universitas Islam Indonesia, Jl. Kaliurang km. 14.5, Yogyakarta 55584, Indonesia
[3]Students of Master of Pharmacy Program, Department of Pharmacy
Universitas Islam Indonesia, Jl. Kaliurang km. 14.5, Yogyakarta 55584, Indonesia
[*]Corresponding Author: farida.hayati@uii.ac.id

Abstract

Pegagan (*Centella asiatica)* is an Indonesian medicinal plant with many advantages. Due to its various pharmacological effects on humans, the development of the plant as a new drug has become prominent. However, there has been no toxicity appraisal towards pegagan in fraction formulation. Thus, this research aims to determine its level of safety on zebrafish embryos. Fractionation was accomplished using the Vacuum Liquid Chromatography (VLC) method. Zebrafish embryos are divided into seven groups for each fraction, 5 groups as a various concentration and 2 groups as a control. The groups were exposed to ethyl acetate fractions in different concentrations (100 ppm; 50 ppm; 25 ppm; 12.5 ppm; 6.25 ppm). The groups were exposed to ethanol fractions in different concentrations (250 ppm; 125 ppm; 62.5 ppm; 31.25 ppm; 15.625 ppm).The positive control group was exposed to (3,4- dichloroaniline), meanwhile the negative group received dilution water. The embryos were observed every 24 hours for 96 hours using microscopes. During the study, somite abnormality, yolk-sac edema, and pericardial edema were found in zebrafish embryos. The LC_{50} value of the ethyl acetate fraction was 26.61 ppm, indicating a moderate level of toxicity. The LC_{50} value of the ethanol fraction was 808.81 ppm, categorized as low toxicity.

Keywords: *Centella asiatica*; Embryo; Fraction toxicity, Zebrafish.

1. Introduction

Pegagan is an Indonesian medicinal plant, reported to have antihyperglycemic effects, anti-inflammatory, neuroprotective, hepatoprotective, and cardioprotective effects [1]. Pegagan (C. *asiatica* (L.) Urb) is among those plants widely utilized as traditional medicine by Indonesian people from generation to generation for the treatment of diarrhea, dysentery, epilepsy, fever, and allergy, as well as for its antibacterial, memory improving, and central nervous stimulating effects [2-4]. Pegagan contains active ingredients, including saponins, triterpenoids, flavonoids, tannins, and steroids [5]. Meanwhile, in the development of natural products, it is necessary to evaluate the safety, efficacy, and quality. One of the safety evaluations processes to carry out is toxicity testing.

Toxicity testing aims to examine the toxic effects of a compound in a short period of time after administrations of a specified concentration or dose, teratogenic effects, mutagenic effects, or others [6, 7]. In the previous study acute toxicity test, the LD50 of the leaf ethanolic extract of *C. asiatica* on the zebrafish model was determined to be 1250 mg/L [8]. A toxicity test is required to strengthen the scientific evidence. The zebrafish embryotoxicity model has advantage due to lower costs, transparency of embryos, short life cycle, high fertility, and genetic data similarity [9, 10]. Toxicity tests are performed using zebrafish embryos since their transparent form facilitates the evaluation of the effects of a compound on some organs, including on the heart, pancreas, and spine, without having to pass a complicated process [11,12]. In addition, at least 70% of human genes have at least one orthologous zebrafish gene similarities [13, 14]. Toxicity tests involving zebrafish embryos are carried out by exposing fertilized embryos to chemical compounds for 96 hours and making observation for 24 hours [15]. This research aims to determine *C. asiatica* fraction (the ethyl acetate fraction and ethanol fraction) level of safety on zebrafish embryos with LC_{50} parameters and the kind of abnormality of embryos.

2. Materials and Methods

2.1. Ethical Clearance

This study has received the ethical clearance released by the Medical and Health Research Ethics Committee of the Faculty of Medicine of Universitas Islam Indonesia (UII) with letter No. 12/Ka.Kom.Et/70/KE/II/2021.

2.2. Materials

Pegagan (C. *asiatica* (L) Urb) was obtained from Kalibawang, Kulonprogo, Yogyakarta and has passed the identification phase at the Plant Systematics Laboratory of the Faculty of Biology of Universitas Gadjah Mada with the letter No. 014963/S/.Tb./II/2021. The test was done with zebrafish and zebrafish embryos. The zebrafish has been identified at LIPI Bogor with the letter No. B-3853/IPH.1./KS/02.03/XI/2017. The inclusion criteria for zebrafish were those aged 4-6 months, having good health, being free from symptoms of microscopically observable infections and diseases, and currently not undergoing a treatment process. Meanwhile, the inclusion criteria for embryos were having recent fertilization and aged 6 hours after fertilization. Other materials included 96% ethanol (Sigma Aldrich, USA), n-hexane (Sigma Aldrich, USA), ethyl acetate

(Sigma Aldrich, USA), and 3,4-dichloroaniline/DCA (Sigma Aldrich, USA), Bouchardat (Mercks, Germany), Chloroform (JT Baker), H_2SO_4 (Mercks, Germany), Viscous HCl (Mercks, Germany), H_2SO_4 (Mercks, Germany), $FeCl_3$ (Mercks, Germany), HCl 2N (Mercks, Germany).

2.3. Extraction and Fractionation Processes

The selected pegagan leaves were dried in an oven at a temperature of 40ºC. The pegagan leaf simplicia was then ground to obtain smooth powder. Then, 150 g of pegagan powder was macerated using 96% ethanol as the solvent at a ratio of 1:10 (simplicia powder : solvent) using a sonication device. The maceration process was done for 3 days with occasional stirring. The result was then filtered using a Buchner funnel, and the solvent in the obtained extract was evaporated using a vacuum rotary evaporator, leaving a viscous extract of pegagan leaves [16]. The viscous extract was then fractionated using VLC filled with 10 g of silica gel 60 GF_{254}, and a mixture of 1 g of ethanol extract and 2 g of silica gel was added. Then, n-hexane, ethyl acetate, and 96% ethanol were added gradually each as much as 200 mL. The fraction obtained from each mobile phase was then collected and dried in a fume hood.

2.4. Identification of the Compounds in Pegagan Leaves Extract

The identification was done in a chamber with a mobile phase ratio of 4:1 (n-hexane : ethyl acetate). Spotting was then performed on a TLC plate for the extracts and fractions of n-hexane, ethyl acetate, and 96% ethanol, and the TLC plate was viewed under UV light at 254 nm and 366 nm. Next, the plate was inserted into a chamber after the mobile phase was saturated, and elution was done as far as the edge with the spots observed under UV light at 254 nm and 366 nm followed by spraying the anisaldehyde reagent.

The identification of the compounds in pegagan leaf extract was performed by testing the secondary metabolites, such as the alkaloids, flavonoids, steroids, terpenoids, tannins, and saponins by referring to previous research methods [17, 18].

2.5. Toxicity test on zebrafish embryos

The identification of the location of the centre of pressure of a projectile body is motivated by the need for calculating aerodynamic moments, stability, and structural analyses. The centre-of-pressure location of bodies composed of conical noses and cylindrical afterbodies is determined as follows [5].

2.5.1. Preparation and selection of zebrafish embryos

Zebrafish were reared in an aquarium at a temperature of 21-25ºC and a pH of 6.0-8.5 with a 14-hour light/10-hour dark cycle and two times a day of feeding. During the fertilization, the male and female zebrafish were placed in one aquarium at a ratio of 2:1 in the afternoon, and the eggs were transferred to a plate in the morning. Zebrafish embryos were selected through observation under a stereo microscope (Zeiss). The embryos used were those aged less than 6 hours after fertilization and not coagulated [15].

2.5.2. Embryo observation

Embryos were observed every 24 hours up to 96 hours under a stereo microscope (Zeiss). Prior to the first 24-hour observation, the embryos in the Microwell plate were kept in the same zygote-cleavage shape to allow uniform growth of each embryo in the Microwell plate during the 96-hour observation in 96% ethanol fraction as well as in ethyl acetate fraction of pegagan leaves. The concentrations of 96% ethanol fraction were 250 ppm, 125 ppm, 62.5 ppm, 31.25 ppm, and 15.625 ppm, while the ethyl acetate fraction had concentrations of 100 ppm, 50 ppm, 25 ppm, 12.5 ppm, and 6.25 ppm. Some parameters were observable, including embryo coagulation, somite formation, tailbud detachment from the yolk, and heartbeats [1, 15].

The zebrafish medium was reverse osmosis water added with ocean salt (0.1%). The fish was living in a glass aquarium completed with an aerator, water filter, and digital thermometer to monitor the water temperature of 26ºC ± 1. The indicators of water quality included conductivity, acidity (pH), temperature, hardness, nitrate, nitrite, and oxygen [19].

2.5.3. Data analysis

The quantitative data consists of the percentage of zebrafish embryo mortality, percentage of coagulated embryos, percentage of embryos with visible heartbeats, and percentage of malformations after 96 hours of exposure. The qualitative data includes the embryo abnormalities [1].

$$\%\ embryo\ mortality = \frac{number\ of\ dead\ embryos}{total\ number\ of\ embryos\ used}\ x\ 100\% \quad (1)$$

$$\%\ embryos\ with\ coagulation = \frac{number\ of\ coagulated\ embryos}{total\ number\ of\ embryos\ used}\ x\ 100\% \quad (2)$$

$$\%\ embryos\ with\ somite\ formation = \frac{number\ of\ embryos\ with\ somite\ formation}{total\ number\ of\ live\ embryos}\ x\ 100\% \quad (3)$$

$$\%\ embryos\ with\ tailbud\ detachment = \frac{number\ of\ embryos\ with\ tailbud\ detachment}{total\ number\ of\ live\ embryos}\ x\ 100\% \quad (4)$$

$$\%\ embryos\ with\ visible\ heartbeats = \frac{number\ of\ embryos\ with\ visible\ heartbeats}{total\ number\ of\ embryos\ used}\ x\ 100\% \quad (5)$$

$$\%\ embryos\ with\ abnormalities = \frac{number\ of\ embryos\ with\ abnormalities}{total\ number\ of\ embryos\ used}\ x\ 100\% \quad (6)$$

3. Result and Discussion

The toxicity test of pegagan leaf extract (C. *asiatica* (L.) Urb) on zebrafish and zebrafish embryos was performed to ensure safety. The research began by extraction and fractionation of pegagan leaves. The obtained viscous extract of pegagan leaves was 70.32 g, and the calculation of the percent yield resulted in 14.064%. According to the Indonesian Herbal Pharmacopoeia, the percent yield of pegagan viscous extract should not be less than 7.3%, indicating that the percentage obtained in this study has met the provision [20].

The fractionation process produced three fractions of n-hexane, ethyl acetate, and 96% ethanol. However, since the n-hexane fraction has nonpolar and volatile properties, only a small fraction can be obtained. In addition, it is suspected that the n-hexane fraction contains only a small amount of asiaticoside, the marker compound of pegagan, which therefore can be excluded from the category of active fraction of pegagan (C. *asiatica* (L.) Urb) [21]. As a result, only the ethyl acetate and 96% ethanol fractions were used as the test samples with the viscous fraction weighing 1.354 g and 0.915 g and having a percent yield of 49.88% and 33.87%, respectively. The results of compound identification for the leaf extract of pegagan (C. *asiatica* (L.) Urb) are shown in Table 1.

Table 1. Compound identification for the leaf extract of pegagan (C. *asiatica* (L.) Urb).

Compound	Reagent	Positive Result from References	Observation Result	Ref.
Alkaloids	*Bouchardat*	Blackish brown precipitation is formed	Blackish brown precipitation was formed (+)	[22]
Steroids	Chloroform and H_2SO_4	A green ring is formed	A green ring was formed (+)	[17]
Flavonoids	Viscous HCl	Red to orange colour is formed	Red to orange colour was formed (+)	[18]
Terpenoids	H_2SO_4	Reddish brown colour is formed	Reddish brown colour was formed (+)	[17]
Tannins	$FeCl_3$	Dark green to blue or black colour is formed	Dark green colour was formed (+)	[22]
Saponins	HCl 2N	More foam is formed after being agitated	More foam was formed (+)	[22]

Note: This test has been in accordance with similar research on the active ingredients and benefits of pegagan, which shows that pegagan leaves contain alkaloids, flavonoids, steroids, terpenoids, tannins, and saponins [5].

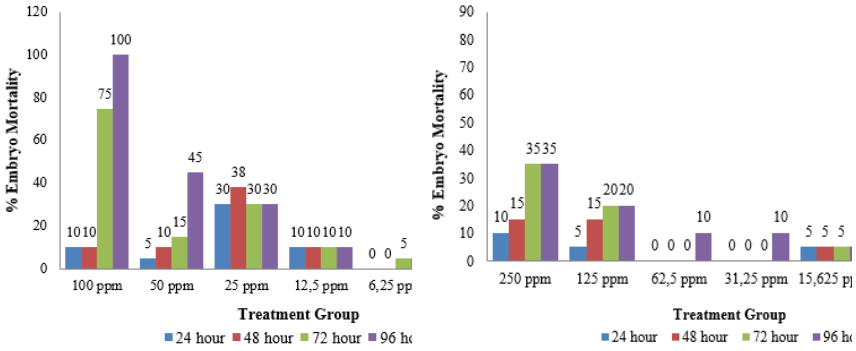
In this study, an identification of pegagan leaf fractions was further done using TLC, and the spray reagents indicated that the pegagan leaves possibly contained asiaticoside. This is evidenced by the Rf value obtained in this study, which was the same as the Rf value of pegagan leaf marker compound of 0.33 [20].

The parameters of toxicity test on zebrafish embryos include the percentage of zebrafish mortality, percentage of embryo coagulation, percentage of embryos with visible heartbeats, and percentage of abnormal embryos. Percentage of zebrafish embryo mortality in both groups of fractions have been shown on Fig. 1.

Coagulation is one of the parameters observed in zebrafish embryos in order to determine toxicity. Coagulated zebrafish embryos are characterized by the existence of white or black lumps when being observed under a microscope.

Based on Table 2 the positive control group (+) showed that, after 96 hours of exposure, 5% of the embryos experienced coagulation. This is because apart from coagulation, which is an indicator of embryo death, there are also abnormalities. Most of the embryos in the positive control group (+) had abnormalities which could lead to more deaths. Meanwhile, in the negative control group (-), no zebrafish embryos had coagulation. Therefore, these results are in line with one of

the validity criteria from the OECD 2013, in which the hatching in a negative control should be ≥80% until the end of the 96-hour exposure [15].



(a) (b)

Fig 1. (a) Graph of the percentage of zebrafish embryo mortality in the group of pegagan leaf ethyl acetate fraction, (b) Graph of the percentage of zebrafish embryo mortality in the group of pegagan leaf ethanol fraction.

Table 2. Development of coagulated zebrafish embryos after the administration of ethyl acetate and ethanol fractions of pegagan leaves for 96 hours.

Ethyl acetate fraction	% Embryos with coagulation				Ethanol fraction	% Embryos with coagulation			
	24 h	48 h	72 h	96 h		24 h	48 h	72 h	96 h
100 ppm	10	10	70	80	**250 ppm**	10	15	35	35
50 ppm	5	10	15	40	**125 ppm**	5	15	20	20
25 ppm	30	30	30	30	**62.5 ppm**	0	0	0	10
12.5 ppm	10	10	10	10	**31.25 ppm**	0	0	0	10
6.25 ppm	0	0	5	5	**15.625 ppm**	5	5	5	5
Control (+)	5	5	5	5	**Control (+)**	5	5	5	5
Control (-)	0	0	0	0	**Control (-)**	0	0	0	0

Note: Control (+) = 3,4-*dichloroaniline* (DCA); Control (-) = *dilution water*

The incidence of coagulation increased with the increasing of fraction concentration. The high number of active compounds in the fraction will cause coagulation. Based on coagulation parameters in Table 2, Ethyl acetate fraction is more potent in toxicity level than ethanol fraction.

Somite is the tissue which can develop into the spine and skeletal muscles; therefore, inhibited somite development can lead to abnormalities in the body axis and in the hatching process of zebrafish embryos. Somite formation can occur after 24 hours of fertilization.

Based on Table 3, the percentage of somite formation in the ethyl acetate and ethanol fractions of pegagan leaves decreased at each concentration. For example, in the ethyl acetate fraction with a concentration of 50 ppm, the percentage of somite formation reduced during the 24-hour to 96-hour observation because some

embryos coagulated, thereby affecting the percentage of somite formation at each hour. In addition, the higher the concentration of the test compound, the lower the percentage of zebrafish embryos with somite formation. In the positive control group (+), the percentage of embryos with somite formation was 95% since 5% of the embryos experienced coagulation from 24th to 96th hour. Meanwhile, from the observation of the negative control group after 24-96 hours of exposure, of the total live embryos there were 100% that experienced somite formation.

Table 3. Development of zebrafish embryos with somite formation after the administration of ethyl acetate and ethanol fractions of pegagan leaves for 96 hours.

Ethyl acetate fraction	% Embryos with somite formation				Ethanol fraction	% Embryos with somite formation			
	24 h	48 h	72 h	96 h		24 h	48 h	72 h	96 h
100 ppm	90	90	25	0	**250 ppm**	90	85	65	65
50 ppm	95	90	85	55	**125 ppm**	95	85	80	80
25 ppm	70	70	70	70	**62.5 ppm**	100	100	100	90
12.5 ppm	90	90	90	90	**31.25 ppm**	100	100	100	90
6.25 ppm	100	100	95	95	**15.625 ppm**	90	90	90	90
Control (+)	95	95	95	95	**Control (+)**	95	95	95	95
Control (-)	100	100	100	100	**Control (-)**	100	100	100	100

Tailbud is part of the tail of a zebrafish embryo and yolk is part of the egg of a zebrafish embryo which has a round shape with a tail attached to it to provide nutrients for the embryo. Detachment of the tailbud from the yolk can affect the egg hatching and the development of zebrafish embryos.

Table 4 shows that as the concentrations of the ethyl acetate and ethanol fractions of pegagan leaves decreased, there was a corresponding drop in the percentage of tailbud detachment from the yolk. Therefore, the higher the compound concentration, the lower the percentage of zebrafish embryos with tailbud detachment. In addition, some concentrations experienced a percentage decrease every hour due to embryo coagulation during 24th - 96th hour which therefore affected the percentage of tailbud detachment every hour. In the positive control group (+), the percentage of embryos that experienced tailbud detachment from the yolk was 95% because 5% of the embryos experienced coagulation from 24th hour to 96th hour. Meanwhile, from the observation after 24 hours to 96 hours of exposure in the negative control group (-), the zebrafish embryos with tailbud detachment from the yolk reached 100% of the total live embryos.

According to the OECD 236 (2013), the heartbeats of zebrafish embryos can be observed in 48 hours after fertilization. Heartbeat observation is also one of the parameters to determine the mortality rate of zebrafish embryos. The heartbeat of zebrafish embryos can be observed in 48 hours after fertilization. Heartbeat observation is also one of the parameters to determine the mortality rate of zebrafish embryos [15]. Based on Table 5 in the positive control group (+) after 48 - 96 hours of observation, the percentage of embryos with visible heartbeats decreased from 95% to 15%. Meanwhile, the negative control group (-) observed from the 48th hour to the end of the 96th hour showed 100% zebrafish embryos with visible heartbeats.

Table 4. Development of zebrafish embryos with tailbud detachment from the yolk after the administration of ethyl acetate and ethanol fractions of pegagan leaves for 96 hours.

Ethyl acetate fraction (ppm)	% Embryos with tailbud detachment				Ethanol fraction (ppm)	% Embryos with tailbud detachment			
	24 h	48 h	72 h	96 h		24 h	48 h	72 h	96 h
100	90	90	25	0	**250**	90	85	65	65
50	95	90	85	55	**125**	95	85	80	80
25	70	70	70	70	**62.5**	100	100	100	90
12.5	90	90	90	90	**31.25**	100	100	100	90
6.25	100	100	95	95	**15.625**	90	90	90	90
Control (+)	95	95	95	95	**Control (+)**	95	95	95	95
Control (-)	100	100	100	100	**Control (-)**	100	100	100	100

Table 5. Development of zebrafish embryos with visible heartbeats after the administration of ethyl acetate and ethanol fractions of pegagan leaves for 96 hours.

Ethyl acetate fraction (ppm)	% Embryos with visible heartbeat				Ethanol fraction (ppm)	% Embryos with visible heartbeat			
	24 h	48 h	72 h	96 h		24 h	48 h	72 h	96 h
100	0	90	25	0	**250**	0	85	65	65
50	0	90	85	55	**125**	0	85	80	80
25	0	70	70	70	**62.5**	0	100	100	90
12.5	0	90	90	90	**31.25**	0	100	100	90
6.25	0	100	95	95	**15.625**	0	95	95	95
Control (+)	0	95	55	15	**Control (+)**	0	95	55	15
Control (-)	0	0	0	0	**Control (-)**	0	0	0	0

A decrease in heartbeats is influenced by some factors, including the concentration of the test compounds, the duration of the exposure, and the active compounds in the plant. Flavonoids in pegagan (C. *asiatica* (L.) Urb) are thought to have a reducing effect on heartbeats by inhibiting the activity of sympathetic nerves while increasing the activity of parasympathetic nerves [23].

Decreased heart rate in fish embryos zebra is thought to be due to the presence of active compounds quercetin and flavonoids. Decreased heart rate is caused by due to the action of muscarinic fiber receptors (negative chronotropic effect). Fiber receptors muscarinics bind to acetylcholine so it will increase the influx of K+ towards intracellular which will be followed by a decrease in efflux Ca+2 from extracellular. It will make hyperpolarization and decreased depolarization [23].

Based on Table 6 in the positive control group (+), there were 95% embryos with abnormalities since the administration of 3,4-dichloroaniline compound affects embryo development, thus leading to abnormalities in the form of bent somite, somite deformity after 24 hours, tail end deformity, edema of the yolk sac, and edema of the pericardium. Meanwhile, in the negative control group (-), all the zebrafish embryos experienced no abnormalities after 96 hours of observation.

Table 6. Development of zebrafish embryos with abnormalities after the administration of ethyl acetate and ethanol fractions of pegagan leaves for 96 hours.

Ethyl acetate fraction (ppm)	% Embryos with abnormalities	Ethanol fraction (ppm)	% Embryos with abnormalities
100	80	250	25
50	35	125	10
25	0	62.5	10
12.5	0	31.25	10
6.25	5	15.625	0
Control (+)	95	Control (+)	95
Control (-)	0	Control (-)	0

4. Conclusions

The LC_{50} of the ethyl acetate fraction of pegagan leaves is 26.61 ppm which is classified as medium toxicity, while the LC_{50} of the ethanol fraction is 808.81 ppm which is categorized as low toxicity. The administration of the ethyl acetate and ethanol fractions of pegagan leaves to zebrafish embryos results in abnormalities in the form of bent somites, tail end deformities, edema of the yolk sac, and pericardial edema.

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