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Acute toxicity of jalawure (*Tacca leontopetaloides* (L.) Kuntze) and gadung tikus (*Tacca palmata* Blume) using zebrafish (*Danio rerio*) embryos as a model

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ABSTRACT

Tacca is a genus of several species reported to have potential as a new therapeutic agent with toxic properties for several cells. Jalawure and gadung tikus are two species from the Tacca genus that can be found in Indonesia. The aim of this study was to determine the level of acute toxicity of jalawure and gadung tikus by using zebrafish *(Danio rerio)* embryos as a model. The sample was extracted by maceration using ethanol 96%. The acute toxicity test was carried out by using fish embryo acute toxicity (FET) test protocol No. 236, *Organisation for Economic Co-Operation and Development (OECD)*, 96 hr of static exposure using the negative control group (medium embryo), the test solution group (extract) and the positive control group (3,4-dichloroaniline 4 μg/ml). The result of acute toxicity of extract against zebrafish embryos demonstrated that LC_{50} of jalawure leaf extract 26.06 $\pm$ 0.31 μg/ml, jalawure stem extract 251.52 $\pm$ 6.15 μg/ml, jalawure corm extract 463.24 $\pm$ 9.68 μg/ml, gadung tikus leaf extract 15.04 $\pm$ 0.15 μg/ml, gadung tikus stem extract 263.73 $\pm$ 6.58 μg/ml, and gadung tikus corm extract 17.71 $\pm$ 0.12 μg/ml. Embryo coagulation was the endpoint that was most commonly found in almost all extracts. Gadung tikus leaves extract, gadung tikus corm extract and jalawure leaves extract expressed moderate toxicity level.

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INTRODUCTION

Various plants have been widely used as medicine and therapy in most parts of human life. Research has succeeded in obtaining information about the therapeutic effects, use and development of plants into herbal medicine (Yin *et al.*, 2013; Jamshidi-Kia *et al.*, 2018).

In 1963, Scheuer purified a compound called taccalin, which was isolated from the jalawure corm. Taccalin is a compound with bitter yellow powder and has a tetracyclic structure. Then in 1988, several taccalonolides compounds were isolated from the Tacca genus. Taccalonolide is a group of highly oxidized steroid-derived compounds. In addition, the withanolides compounds and their glycosides were also isolated. These compounds have been shown to have cytotoxic effects on several cancer cell line by different mechanisms. The compound group was isolated from *Tacca plantaginea* (Hance) Drenth, *Tacca subflabellata* PP Ling & CT Ting and *Tacca integrifolia* Ker Gawl (Jiang *et al.*, 2014; Risinger and Mooberry, 2010).

Based on the concept of Linnaeus in the 18^{th} cen-

tury, which stated that plants, especially those that have similar morphological characteristics, generally also contained similar chemical substances. The discovery of these compounds from several types of Tacca with cytotoxic effects is possible for several other types, such as *Tacca leontopetaloides* and *Tacca palmata*, which belong to the same family also contain compounds that have the potential to have toxic effects.

Zebrafish (*Danio rerio*) is now widely used as a powerful research model in biology and medicine. Zebrafish could be used as a model organism for toxicity screening before the more expensive mammalian models will use. Zebrafish embryos and larvae are small in size and transparent, so they are very easy to test using multi-wells and observe embryo and larval development (Belyaeva *et al.*, 2009; Kanungo *et al.*, 2014). Based on the zebrafish genome sequencing of TuAB strains, in 2001 (Sanger Institute) informed that more than 71% of the genes encoding proteins in humans have at least 1 gene that is orthologous in zebrafish (Avdesh *et al.*, 2012; Howe *et al.*, 2013). So, this study aims to determine the acute toxicity of the jalawure and gadung tikus using zebrafish embryos as a model.

MATERIALS AND METHODS

Jalawure (*Tacca leontopetaloides* (L.) kuntze) was obtained from the BP3K Cikelet Garut, West Java and was determined at the Bandungese Herbarium, School of Biological Science and Technology - Bandung Institute of Technology. Gadung Tikus (*Tacca palmata* Blume.) was obtained and was determined at Bogor Botanical Garden, West Java, meanwhile zebrafish (*Danio rerio*) was obtained from Laksana aquarium Bandung and was determined at the Museum of Zoology, School of Biological Science and Technology - Bandung Institute of Technology.

Extraction process

One hundred gram crude drug of jalawure and gadung tikus parts (leaves, stem and corm) were macerated with one liter of 95% ethanol for 24 hr at room temperature, the macerate was filtered and the residue was re-extracted twice using the same method. The macerate was collected and then concentrated with a rotary evaporator.

Zebrafish maintenance and embryos handling

This study used zebrafish (*Danio rerio*) embryos less than 3 hpf (hours post fertilization), which were cultured in the laboratory. These embryos were obtained from adult male and female zebrafish aged 4-5 months, healthy and normal. The male and female zebrafish were separated for at least 1 week before mating.

The water circulation in the aquarium was continuously regulated by means of an aerator and filter system. Room temperature and water temperature were around 26-28.5°C with a pH between 6.8-7.5 and the lighting conditions 14 hr light and 10 hr dark. If necessary, the pH can be adjusted by adding sodium bicarbonate. Aquarium was cleaned regularly about 1-2 times a week, with a replacement of water 2/3 the volume of the aquarium. Fish were fed about 1-2 times a day and if the fish are to be spawned, they were fed 2-3 times a day about 1-5% of the bodyweight of the fish (Avdesh *et al.*, 2012; Harper and Lawrence, 2011).

Zebrafish embryos were produced through spawning groups. Male and female zebrafish (2:1) in the spawning group were placed in the spawning aquarium several hours before the dark phase. Embryos were collected from a minimum of three spawning groups, mixed and randomly selected to avoid a genetic bias. If deemed necessary, artificial plants made of an inert material (e.g. plastic or glass) can be attached to the spawning aquarium as a stimulus. Mating, spawning and fertilization take $\pm$ 30 min after the light phase and the spawning nets were carefully removed. The collected embryos can be cleaned carefully. It is recommended to rinse embryos with medium after collection from the spawning aquarium (Busquet *et al.*, 2013).

Preparation of medium zebrafish embryos

The embryo medium (E3) was used as a medium to deliver the test extract solution during testing. Prepare E3 medium by making a stock solution (60x concentration) consisting of 34.8 g NaCl; 1.6 g KCl; 5.8 g $CaCl_2.2H_2O$ and 9.78 g $MgCl_2.6H_2O$ were dissolved in 2 liters of distilled water and adjusted the pH to 7.2 with NaOH solution. E3 60x medium stock solution was stored at 4°C. The solution was diluted to E3 1x solution by taking 16.5 ml of stock solution 60x with distilled water to 1 liter and added 100 μl 1% methylene blue as a fungicide (Cold Spring Harbor Protocols, 2019).

Acute toxicity test using zebrafish embryos

The test referred to the protocols from the Organization for Economic Co-operation and Development (OECD) No. 236, 2013. Acute toxicity test of zebrafish embryos using five test concentration. The highest concentration should give a 100% mortality after 96 hr of exposure and the lowest concentration should give a 0% mortality. The finding range test aims to determine the appropriate concentration.

Zebrafish embryos (0.5-2 hpf) were collected and acclimatized for 2-3 hours in E3 1x medium at room

temperature 28°C. Selection of zebrafish embryos, 20 zebrafish embryos were inserted into well plates-24 (capacity 2.5 - 5 ml). The medium was carefully exchanged with the test sample (1 concentration/well plate-24), covered the well plate-24 with aluminum foil then incubated at 28°C. The observation was carried out for 96 hr (static exposure) and observed every 24 hr using a stereomicroscope (Olympus CKX41) at 4-10X magnification. Four endpoints were observed,

1. Coagulated embryo,
2. Lack of somite formation,
3. Non-detachment of the tail, and
4. Lack of heartbeat.

After 96 hr of observation, data from the four endpoints above were recorded and calculated to determine the LC_{50} value. 3,4-dichloroaniline 4 μg/ml per well plate-24 with 20 embryos was used as a positive control. Meanwhile, E3 medium with 24 embryos per well plate-24 as a negative control (Busquet *et al.*, 2013).

Data analysis

A probit analysis was performed on the percent embryo mortality data. The probit value of percent mortality is plotted as the Y and the log of concentration as the X. From this data, a line plot is obtained with the equation

$$Y = aX + b \; and \; linearity \; (R^2)$$

RESULTS AND DISCUSSION

Phytochemical screening

Phytochemical screening was carried out to see the presence of secondary metabolites in ethanol extract (jalawure leaves extract (JLE), jalawure stem extract (JSE), jalawure corm extract (JCE), gadung tikus leaves extract (GTLE), gadung tikus stem extract (GTSE) and gadung tikus corm extracts (GTCE), such as alkaloids, flavonoids, saponins, quinones, phenols, tannins and steroids/triterpenoids (Table 1).

All extracts positive contained saponins. It was similar to the previous study by (Abdel-Aziz *et al.*, 1990), with identified the presence of steroidal sapogenin compounds from acid hydrolysis in jalawure leaves extract. Saponins had toxic properties to the embryo based on Hassan *et al.* (2008) research that saponin isolated from *Quilajja Saponaria* at a concentration above 5 μg/ml caused 100% zebrafish embryo mortality.

Finding range

Finding range is used to determine the five test concentration. The highest concentration should give a 100% mortality after 96 hr of exposure and the lowest concentration should give a 0% mortality (Figure 1). The finding range test aims to determine the appropriate concentration. Five test concentrations were calculated by arithmetic methods from the highest and the lowest concentration.

Lethal concentration 50% (LC_{50}) of extract

Previous studies presented that several plants from the Tacca family have the potential to be developed as anticancer agents (Tinley *et al.*, 2003; Peng *et al.*, 2011; Li *et al.*, 2014), but the safety effect of the compounds has not been reported. Exposure jalawure extracts and gadung tikus extracts at certain concentrations caused embryotoxicity, as assessed from the hatching rate and defects in the zebrafish embryo. In this study, the effects of exposure of some jalawure extracts and gadung tikus extracts will be exposed, which cause death and malformation of zebrafish embryos as a test model. The percentage of zebrafish embryo mortality increased with increasing concentration of the test sample (Figure 2).

Four endpoint observations were made to determine lethality. If one of these four endpoints was found, it indicated embryonic death. Coagulation is characterized by the embryo appearing white in color and black clumps under the microscope. Damage to the somite form of the embryo can be characterized by the absence of somite formation after 48 hr of exposure. Somite deformities and non-detachment of the tail are almost never found in this test. Heart rate can be observed after 48 hr of exposure, normal embryo heart rate > 80 times per min. If no heart rate or less than 80 times per min, it indicated embryo death.

The LC_{50} value indicated the concentration of the extract that caused 50% mortality in zebrafish embryos, which was obtained from the results of the probit regression analysis during 96 hr of exposure (Table 2). Kovriznych *et al.* (2013) referred to the guideline ON 46 6807 (1988) classified the level of toxicity based on the LC_{50} value of a substance namely, concentration <1 μg/ml (high toxic), concentration 1-10 μg/ml (toxic), concentration 10-100 μg/ml (moderate toxic), concentration 100-1000 μg/ml (low toxic).

The results of probit regression analysis revealed that LC_{50} of JLE 26.06 $\pm$ 0.13 μg/ml, GTLE 15.04 $\pm$ 0.15 μg/ml, and GTCE 17.71 $\pm$ 0.12 μg/ml were moderate toxicity level. Meanwhile, LC_{50} of JSE 251.52 $\pm$ 6.15μg/ml, JCE 463.24 $\pm$ 9.68 μg/ml and GTSE 263.73 $\pm$ 6.58μg/ml were weak toxicity

Table 1: Phytochemical screening of extracts

	Flavonoids	Phenols	Tannins	Quinones	Saponins	Alkaloid	Steroids/ Triterpenoids
JLE	+	+	-	+	+	-	+
JSE	+	+	-	+	+	-	+
JCE	-	-	-	+	+	-	+
GTLE	+	+	-	-	+	-	+
GTSE	+	+	-	+	+	-	+
GTCE	+	+	-	+	+	-	+

+ Detected, - Not detected

Table 2: Lethal concentration (LC_{50}) of extracts

	JLE	JSE	JCE	GTLE	GTSE	GTCE
LC_{50} (μg/ml)	26.06 ± 0.13*	251.52 ± 6.15**	463.24 ± 9.68**	15.04 ± 0.15*	263.73 ± 6.58**	17,71 ± 0,12*

*Moderate toxic, **Low toxic

Table 3: Moderate toxicity extracts characterization

	Water content (%v/w)	Total ash content (%w/w)	Acid insoluble ash content (%w/w)
JLE	9.98 ± 0.01	6.68 ± 0.18	0.29 ± 0.18
GTLE	9.98 ± 0.01	8.41 ± 0.45	1.54 ± 0.82
GTCE	7.48 ± 0.01	3.08 ± 0.01	0.97 ± 0.22

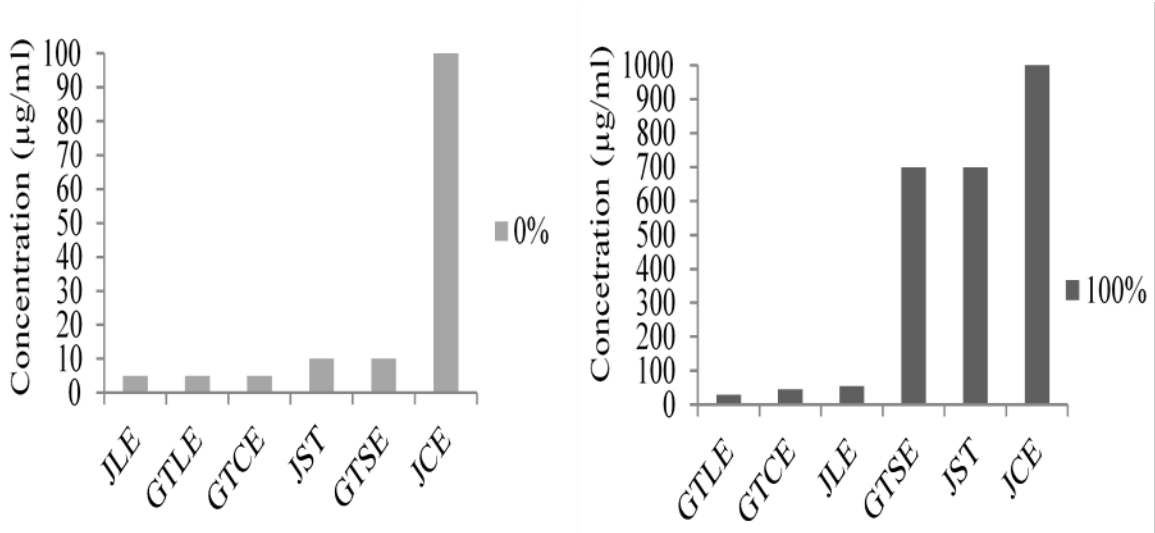

Figure 1: Finding range of extracts 0% and 100% mortality

level (Cold Spring Harbor Protocols, 2019).

JLE has a more active toxicity level (minimum 5 μg/ml) than JCE (minimum 100 μg/ml) against zebrafish embryos. This was similar with previous research by Elsheikh *et al.* (1990), which stated that the JLE has a toxicity level (minimum 100 μg/ml) against cerceriae larvae and miracidia larvae from *Schistosoma mansoni*, while its corm extract has a toxicity level (minimum 500 μg/ml) against cerceriae larvae and a toxicity level (minimum1000 μg/ml) against miracidia larvae.

Coagulated embryo

Embryo coagulation was the endpoint that was most commonly found (Figure 3). Some extracts also provided an endpoint in the form of cessation of embryo development due to weakness and even loss of heart rate and failure of embryo hatching. Lack of pigment, swelling of the abdomen and malformation of

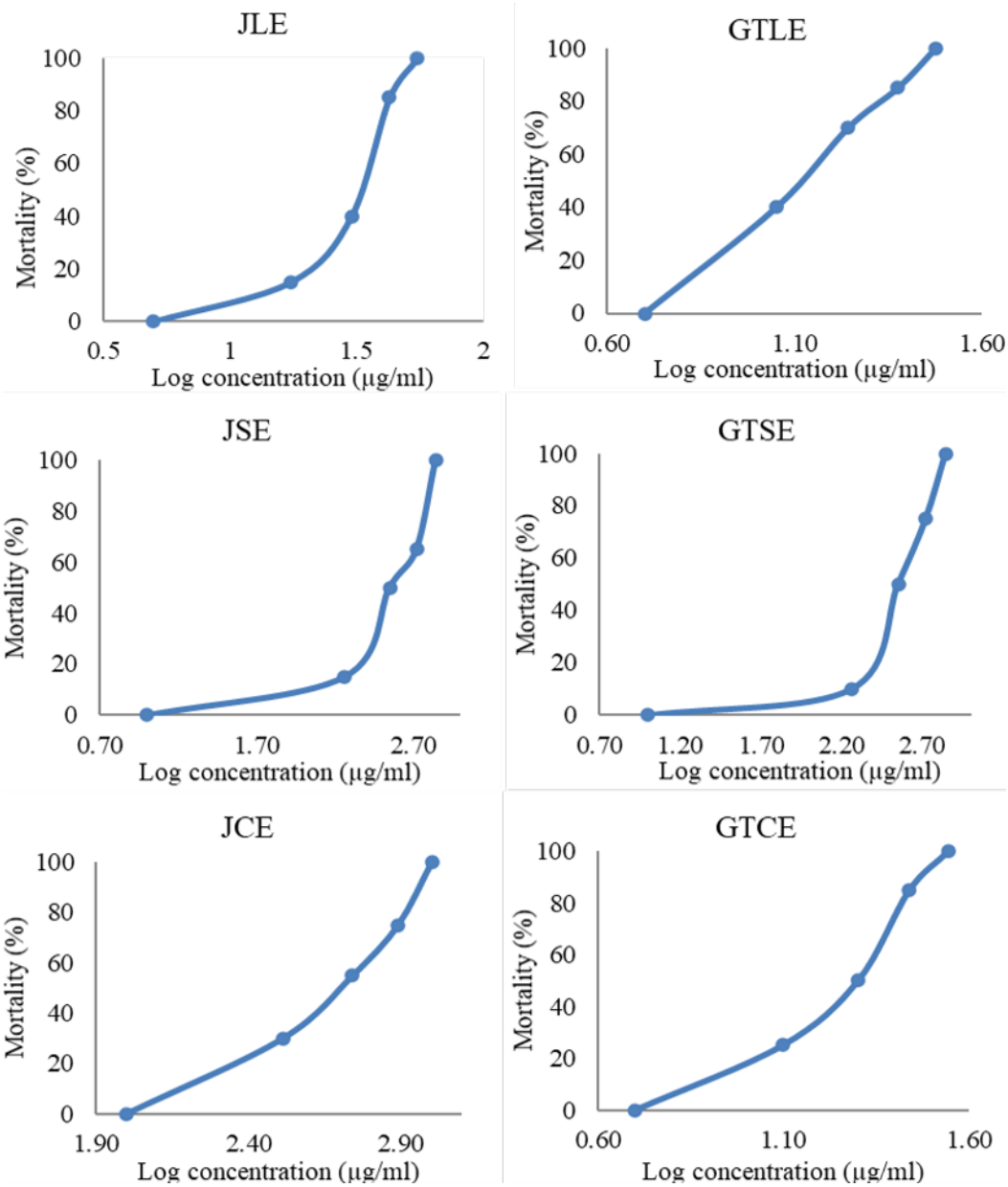

Figure 2: Curve probit regression analysis of extracts

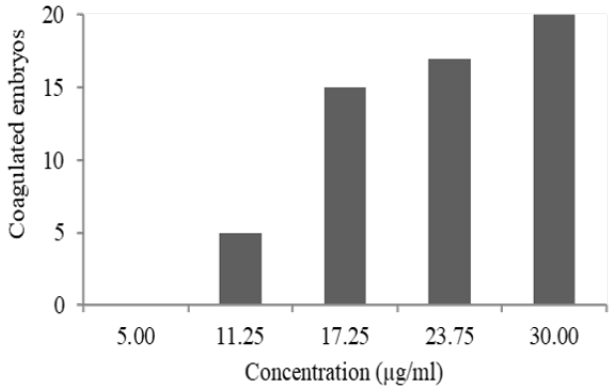

Figure 3: Coagulation effect of GTLE at 96 hr of exposure

the tail also occurred at a certain concentration, but these data were not calculated as the endpoint for the lethal concentration of 96 hr of exposure.

Extract characterization

The three extracts with moderate toxicity then were tested for characterization. The characterization was included, determination of water content, total ash content and acid insoluble ash content (Table 3).

Characterization was carried out in order to ascertain the character of each extract with a specific pharmacological effect. The total ash content indicated minerals from combustion either as natural minerals needed by plants or as environmental contaminants. GTLE, as the sample with the smallest LC_{50} value, had the highest total ash content and acid

insoluble ash value than the other two samples. The possibility of mineral oxides as insoluble acid ash also affected the level of extract toxicity. In addition, differences in plant parts also affect the amount of extract water content. Where both leaves extracts had higher water content compared to corm extract.

CONCLUSIONS

Based on the LC_{50} value, the six extract samples had toxic properties to zebrafish embryos. The jalawure stem extract (JSE), jalawure corm extract (JCE) and gadung tikus stem extract (GTSE) gave low toxicity level, meanwhile gadung tikus leaves extract (GTLE), gadung tikus corm extract (GTCE) and jalawure leaves extract (JLE) had a moderate level of toxicity.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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