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Dosage Determination of Galangal Extract (Alpinia purpurata) Through LC50-96 Method on Zebrafish Larvae (Danio rerio) as a Model

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Abstract

Galangal (*Alpinia purpurata*) is a herb and medicine plant that is found in Southeast Asia including Thailand, Malaysia, and Indonesia. It is known to have health benefits for its flavonoid, phytosterol, and phenols that are anti-oxidant, anti-cancer, and anti-fungal properties. The phytosterol also can be used for sex hormones production.The exploration of its uses in aquaculture are not yet fully understand. In this LC50-96 experiments were tested for its optimum dosage used in aquaculture by using zebrafish larvae (Danio rerio) as a model. The optimum dosage in the experiments were between 1 – 31.83 ppm for ethanolic extract of galangal and < 0.83 ppm for methanolic extract of galangal. Its uses in aquaculture must be further explored by its effect in fishes behaviors or by the histology of the organs.

Keywords: LC50-96, Galangal Extract, Danio rerio

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1. Introduction

Galangal (*Alpinia purpurata*) is a spice plant found in the highlands (± 1,200 m) of Southeast Asia including Thailand, Malaysia, and Indonesia (Indriani, 2021). Galangal has a stem height of 1-1.5 m with red cylindrical roots, and a spicy aroma at the roots when cut (Silalahi *et al*., 2018). In Asia galangal are commonly used as spices and traditional medicine. Previous research stated that galangal has anti-bacterial, anti-oxidant, anti-cancer, and anti-fungal properties based on the active ingredients in it, such as flavonoids, phytosterols, tocopherols, oryzanol, and phenols (Manurung, 2016) (Manasa, Chaudhari, & Tumaney, 2020). Other content such as phytosterols in galangal (*Alpinia purpurata*) can be used as a cell-forming material, building blocks of cells, and precursors of sex hormones such as androgens and estrogens (Law, 2000).

Galangal (Alpinia purpurata) used as spices and medicines in humans also has the potential for its uses as a substitute for anti-fungal and anti-bacterial as well as other uses as supplements in aquaculture. Aquaculture businesses generally rely on the use of antibiotics to control diseases (Romero, Feijoó, & Navarrete, 2012). Synthetic hormones also commonly used to increase the growth rate or used in fish masculinization (Singh, Saini, & Sharma, 2018). The use of antibiotics and synthetic material has carcinogenic properties for cultivators, fish and fish consumers. The use of antibiotics and synthetic materials in aquaculture can accumulate in the environment and in fish bodies (Organization, 2000). This situation encourages the use of natural materials as an alternative to the use of synthetic materials in fish farming.

In addition to its high potential as an alternative form antibiotics and synthetic materials, it is also necessary to optimize the dosage of use. Overdose in the use of extracts can result in high production cost or even reducing the effectiveness the use of the natural ingredients (Harikrishnan, Balasundaram, & Heo, 2011). Determination of the dose used can be done using the LC50 method. LC50 is a dose determination method by utilizing fish 50% mortality which stated as the maximum dose limit used in fish farming. The use of this method is generally carried out for 96 hours (4 days) or until the fish reaches a mortality of 50% (Wijaya, Junior, Soelistyowati, & Widanarni, 2017). Zebrafish (*Danio rerio*) is a fish from the family Cyprinidae originating from India, Bangladesh, and neighboring countries (Spence et al., 2006). The use of zebrafish as a model fish is commonly used in the medical and toxicological fields (Teame et al., 2019). This

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fish is a frequent spawner, which means it is easy to get offspring all year long, sensitive to environmental changes, and is a teleost model fish that can describe the possible impacts due to influence of the extract describes in its behavioral and histological (Yamani, 2011).

2. Methodology

2.1. Time and Place

The research was conducted at the Exploration and the Reproduction Laboratories at the Faculty of Fisheries and Marine Sciences, Brawijaya University, Malang, East Java, Indonesia. Implementation time is carried out in January-March 2022.

2.2. Research Design

The research was conducted using an experimental method where it is a quantitative methods to obtain data using causal-effect relationship and comparing it between control and experiments (Payadnya dan Jayantika, 2018). The treatments were 5 treatments with different doses. The doses used in this study had multiples of log 10, including: 0, 1, 10, 100, and 1000 ppm for the galangal extract using ethanol, while for the galangal extract using 0, 0.1, 1, 10, and 100 ppm methanol. Repetition was carried out 2 times with each repetition using 10 fish (total fish used during the study were 200 fish larvae).

2.3. Working Procedure

2.3.1. Preparation of Galangal (Alpinia purpurata) Extract

Extracts were made according to Tambun et al. (Tambun, Limbong, Pinem, & Manurung, 2016) with minor modifications. Galangal (Alpinia purpurata) was washed and sliced thinly before being dried in an oven at 30°C for 3 days. The dry galangal is then blended using a blender. The powder was then macerated using 90% ethanol and 90% methanol with a ratio of 1:2 powder and solvent. Maceration was carried out for 72 hours. The extract obtained was then evaporated at a temperature of 60°C to obtain a thick paste. The extract is then stored in the refrigerator until the time of use.

2.3.2. Zebrafish (Danio rerio) Spawning and Larvae Rearing

The zebrafish (Danio rerio) breeding procedures refers to Adyaksa et al. (Adyaksa, 2016). Spawning was carried out using 5 month old adult fish with a male to female ratio of 2:1. Spawning is done by using a shelter to separate the brood and eggs to prevent cannibalism. The eggs then hatch at 48 hours after fertilization. The hatched larvae were then given natural food in the form of algae cultures of Branhcionus and Nannochloropsis sp. at 3 to 4 days old after hatching for 7 days. The fish larvae were then fed commercial feed MS Prima Feed PF0 (protein 40%, lipids 6%, fibers 3%, and Ash 12%) until the fish were 20 days old. The fish larvae can then be used in the LC50 study.

2.3.3. LC50 Aquarium Preparation

The aquarium preparation used in this research refers to Agung et al. (Agung, Herjayanto, Ningsih, Solahudin, & Widiyawan, 2021) in which the aquarium was washed with detergent and rinsed thoroughly with water before drying and later used in the experiments. The aquarium used in the experiments were 30x15x15 cm (6.75 litres) filled with 6 litres of water as the medium for rearing fish larvae. The aquarium was then filled with water and aeration. The water used in the experiments was not added or replaced in the 96 hours of LC50 (closed system). The aquarium was marked and given a dose according to the treatment.

2.3.4. LC 50-96 Procedures

The fish used were 20 days old fish which were acclimatized in the aquarium for 3 days prior to the experiments. The extract used in this study was galangal (Alpinia purpurata) extract using ethanol 90% and methanol 90%. The dose used for ethanolic galangal extract was 0.1,10,100,1000 ppm while the dose used for methanolic galangal extract was 0;0,1;1;10;100 ppm. Immersion is done by dissolving each extract according to each dose listed on the aquarium. Fish larvae were observed every 12 hours for larvae mortality.

2.3.5. Water Quality

Water quality measurements were carried out on a daily basis. Water quality was observed 2 times in a day, in the morning at 9:00 and in the afternoon at 15:00. The water quality observed during the study was temperature, dissolved

oxygen (DO), and water PH. Water quality measurements were carried out using a thermometer for water temperature, DO using a DO meter, and Ph using a PH meter.

2.4. Data Analysis

2.4.1. Survival Rate (SR)

SR calculations were carried out to determine the survival of the fish during the experiments. The calculation is done by counting the number of fish at the beginning of the rearing minus the number of fish at the end of the study. SR calculation refers to Soumokil et al. (Soumokil, Lumamuly, & Laimeheriwa, 2020) which can be seen in the eq. 1.

$$SR = (Nt / No) \times 100\% \quad (1)$$

where:

SR = Survival Rate (Survival of Fish)
Nt = Number of fish at the end of the study
No = Number of fish at the beginning of the study
100% = Percent, unit

2.4.2. LC50-96

The LC50 calculation was carried out to determine the maximum limit of the use of galangal extract on the survival of fish. Calculations were made after the fish were treated for 96 hours. The LC50 calculation refers to the calculator from AAT Bioquest (2022) which can be seen in the equation below:

$$Y = Min + \frac{Max - Min}{1+\left(\frac{X}{LC50}\right)^{\text{Hill coefficient}}} \quad (2)$$

where:

Y = Value at Y coordinate (fish larvae mortality in %)
X = Value at X coordinate (extract dose in ppm)
Min = Minimum fish larvae mortality (0% / no death)
Max = Maximum value of fish larvae mortality (100% / total mortality)
1 = Coefficient
LC50 = Extract dose that causes 50% mortality
Hill coefficient = interaction between extract and fish survival

2.4.3. Data Analysis

The data collected during the study included: survival rate (SR) of fish in each treatment and supporting data on water quality including temperature, DO, and pH. Analysis of the data used in this research is statistical test using SPSS application using probit regression analysis.

3. Result and Discussions

3.1. While Phytochemical Analysis of Galangal (Alpinia purpurata) Extract

The extracts obtained in the two solvent media had different results and could have different impacts on the survival of fish larvae as well as on their application in aquaculture. The results of the phytochemical analysis of the extract can be seen in the table 1.

The use of different solvents in the maceration of plants extracts can draw a certain material. The active ingredients contained in galangal (*Alpinia purpurata*) and other plants have different charges, including: polar and non-polar. Methanol is a polar solvent, while ethanol is a non-polar polar solvent (Troung et al., 2018). Polar solvents in methanol have OH bonds where oxygen has covalent bonds that allow it to bind other hydrogen (H) present in active ingredients in the plants (Kaur et al., 2011). Meanwhile, ethanol has OH bonds which are polar bonds and C-H which are non-polar bonds, this situation allows ethanol to attract polar and non-polar active ingredients. Ethanol is also known as the universal solvent (Credo, Masimba, Machumi, & Heydenreich, 2018).

Table 1. Phytochemical Analysis of Etanolic Extract Galangal (Alpinia purpurata)

No.	Compound	Result
1.	Saponin	+
2.	Tanin	+
3.	Flavonoid	+
4.	Steroid	+

Table 2. Phytochemical Analysis of Methanolic Extract Galangal (Alpinia purpurata)

No.	Compound	Result
1.	Saponin	+
2.	Tanin	+
3.	Flavonoid	-
4.	Steroid	+

3.2. Survival Rate (SR) and Mortality

The results of the experiment using LC50 for 96 hours showed that with increasing time, the survival rate of fish would decrease and the mortality increased. The SR results of LC50 on galangal extract using methanol showed 100% and 35% at concentrations of 0 and 0.1 ppm, while 100% mortality was found at doses of 1, 10, and 100 ppm. Meanwhile, the SR results of LC50 on galangal extract using ethanol showed 100% at concentrations of 0.1, and 10 ppm, meanwhile 100% mortality was found at concentrations of 100 and 1000 ppm. SR results from fish can be seen in the table 3.

Table 3. Survival Rate and Mortality of Zebrafish (Danio rerio) Larvae (Ethanolic Extract)

Dosage (ppm)	Number of Larvae	SR %	Mortality (%)
0	20	100	0
1	20	100	0
10	20	100	0
100	20	0	100
1000	20	0	100

Table 4. Survival Rate and Mortality of Zebrafish (Danio rerio) Larvae (Ethanolic Extract)

Dosage (ppm)	Number of Larvae	SR%	Mortality (%)
0	20	100	0
0,1	20	35	65
1	20	0	100
10	20	0	100
100	20	0	100

After the extract was mixed with the aquarium water, there was an immediate reaction in the fish larvae, especially in its behaviours. The fish that were experimented in high dosage of methanol or ethanol extract of galangal goes to the surface of the water quickly and proceeding to swim erratically. The operculum of the larvae was observed moving faster than the control. In the experiments higher dosage of extract was observed the formation of foam, which we led

to believe that it was caused by saponin. Larvae mortality was observed after 15 minutes of mixing the extract (100—1000 ppm) and before reaching 12 hours were observed total mortality in the higher dosage of extract.

This can be caused by the methanol residue that is still left from the evaporation. Ethanol, which is a universal solvent, is more tolerated in fish than the use of methanol where its breakdown produces acetaldehyde, acetic acid, and acetyl Co-A. Meanwhile, methanol that enters the bloodstream will enter the liver where oxidation will occur so that it converts methanol into formaldehyde and formic acid which have toxic properties on organisms (Pohanka, 2016). Methanol with a concentration of 0.1% can be tolerated by zebrafish (Danio rerio) by showing behavioral changes in fish, but higher concentrations can cause damage to the nervous system in fish (Audira et al., 2020). The saponins found in the extract can cause disturbances or damage to the respiratory system of fish, where saponins produce foam on the surface of the water which may cause death in fish (Makkar, Francis, & Becker, 2007).

3.3. LC50-96

Calculation of LC50 was carried out using the program in AAT Bioquest (2022). The LC50 found in the galangal extract using ethanol was 31,83 ppm while that in the methanol extract was 0.83 ppm. LC50 results can be seen in the Figure 1.

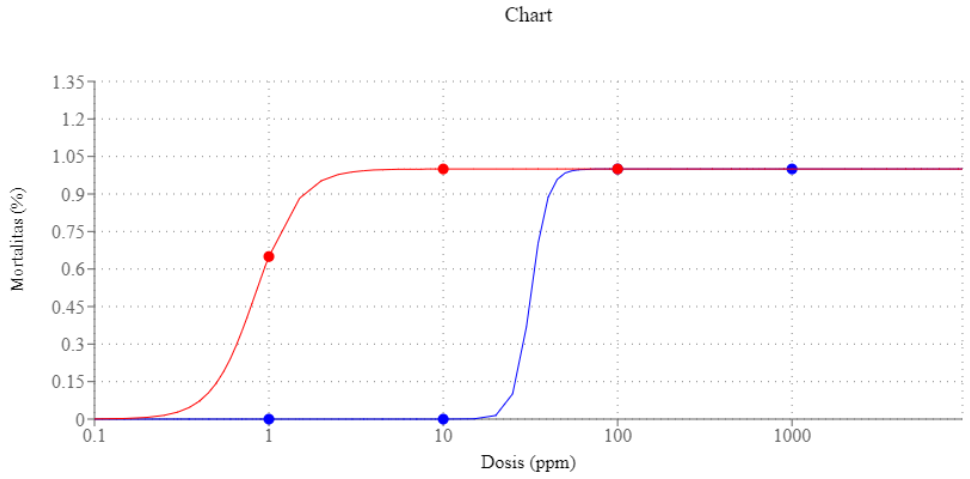

Figure 1. Mortality Chart of Zebra Fish Larvae LC50-96 Ethanol Extract (Blue) and Methanol Extract (Red) Galangal Red

Table 5. Formula for LC50-96

No.	Extract	Formula
1.	LC50-96 Formula for Ethanolic Galangal Extract	$Y = 0 + \frac{1-0}{1+\left(\frac{X}{31.8322}\right)^{-9.0585}}$
2.	LC50-96 Formula for Methanolic Galangal Extract	$Y = 0 + \frac{1-0}{1+\left(\frac{X}{0.8363}\right)^{-3.4634}}$

The higher the mortality and the faster the influence affect the survival rate, it is considered more toxic to the fish. If the mortality reach 50% after prolong periode (example: after 40—50 days) it can be conclude that the substance is non toxic to the fishes health. The LC50-96 shows that if mortality reaches 50% in the 96 hours period, the substance maybe toxic and could potencially threaten the health or habits of the fishes (Boyd, 2005). The experiments were carried out after 96 hours and the mortality stayed the same, it can be concluded that the alcoholic extract of galangal is non toxic (1—10 ppm) and for methanolic extract were non toxic under 0,1 ppm.

4. Conclusion

The eficacy of galangal extract application in aquaculture must be further research especially in vivo application for immunostimulant or as hormon supplements for increasing growth rate or used in fish masculinisation. The dosage of used based on LC50-96 proved that the lower dosage would be more effective and caused less mortality ini zebra fish larvae. In this research could be concluded that the dosage between 1—31,83 ppm of ethanolic extract and under 0,1 ppm for methanolic extract were save to use in 20 days old larvae zebrafish (Danio rerio). The application in other species or its effects on its behavior and histology must be further reaseach before the application in commercial aquaculture.

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