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Phytochemical Analysis from Three Different Methanolic Extracts of Red Ginger (*Zingiber officinale* var. Rubrum) Against LC_{50} Treament of Zebra Fish as Model Fish

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Abstract

Red ginger (*Zingiber officinale* var. Rubrum) as one of the Indonesian spices has so many important roles, especially in the health sector as a medicinal plant, which has many active compounds including phenols, alkaloids, flavonoids, steroids, and tannins. From some research before, we can know that plants growing in different places or locations have different tolerances generally. That statements can show that they have different content in their constituent metabolites. Therefore, phytochemical analysis is needed to analyze the content of each red ginger from three locations as Red ginger from Batu, Malang (RGB), Plaosan, Magetan (RGP), and Simalungun, Medan (RGS) to analyze each content contains. Also, LC50 performance was needed for analyzing its effect on Zebra fish (*Danio rerio*) as a model fish. For this research, we use an experimental study with a Completely Randomized Factorial Design (CRFD) method that has two independent variables such as different dosages and types of red ginger. In this study, from five dosages (10 mg/l; 20 mg/l; 30 mg/l; 40 mg/l; 50 mg/l) and three types of red ginger treatment, it found that the highest mortality and lowest survival rate were at the highest dosage (50 mg/l) in RGB and extreme increase or decrease in the curve is found in RGP treatment.

INTRODUCTION

Red ginger (*Z. officinale* var. Rubrum) is an Indonesian spice that can be easily found in markets and can have an important role in everyday life, especially in the health sector as a medicinal plant. Red ginger rhizome itself is also much sought after because it has properties as herbal medicines (traditional). Utilization of this medicinal plant has also been tried and used to prevent or cure a disease. Red ginger is also known to have several active compounds in it (Kamtchouing *et al*., 2002). The active compounds include alkaloids, flavonoids, steroids, and tannins (Bashir *et al*., 2015).

From some previous research, it is known that plants with different places of growth have different tolerances. Plants outdoors can grow well generally. However, there are some plants that in a certain range of environmental conditions can grow well as well. This can be influenced by soil pH, soil moisture, soil type,

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and light intensity which is stable relatively. Plants that grow and develop properly will get benefit from environmental conditions that suit the needs of that plants based on the interplay between internal factors (genetic) and external factors (environment). However, the existence of environmental conditions that vary widely on various surfaces of the earth makes plants that grow from one location to another location not the same, even though they have similar types. Environmental conditions in the same location can also change from time to time. The cause of this was supposed because of ecological changes in the location where the plant grows (Tarakanita *et al*., 2019). Therefore, three species were selected from three different locations in this study, namely Red ginger from Batu (Malang), Plaosan (Magetan), and Simalungun (Medan).

In this research also, phytochemical analysis is needed to analyze the content of each chosen red ginger. Qualitative phytochemical analysis is an initial analytical method to research and detect the content of chemical compounds present in a plant. The results are expected to provide information in the search for compounds with certain pharmacological effects and can spur the discovery of new drugs (Sangi *et al*., 2008). In this analysis, methanol compounds were chosen as solvents because methanol has a large extraction power, so it can attract polar and non-polar compounds contained in the sample (Simanjuntak, 2020). Methanol is also used as a solvent in maceration extraction because it is polar and able to dissolve polar bioactive elements in medicinal herbs such as Red ginger (Sumihe *et al*., 2014). In addition, methanol is also known to have a higher dielectric constant than ethanol solvents, so the amount of metabolite charge that it attracts tends to be larger than ethanol solvents (Riniati *et al*., 2021).

An example of fish that can be used as a model for this research, in particular, is the Zebra fish (*D. rerio*). Zebra fish are considered a good teleost model for developmental and molecular studies (Han *et al*., 2021). Zebra fish was chosen because it is a model animal that is considered to have similarities in physiological functions and genes with most individuals (Santoriello and Zon, 2012; Ali *et al*., 2021). Zebra fish are also being used for scientific research as a test animal model for vertebrates increasingly. This happens because this fish has several advantages such as being easy to obtain and observe, and is small in size, so it is easy to maintain on a laboratory scale. Another way, this fish has a sensitivity to laboratory treatment and also has a fairly high DNA similarity to humans, mice, and rats (Nugroho, 2018).

Therefore, to check the effect of secondary metabolite content in each of these red ginger LC_{50} treatment was applied. LC_{50} treatment is needed to determine the effect of dosages from three different types of red ginger on mortality and survival rate. The model fish used were Zebra fish which were already exposed to methanol extract of red ginger from Batu (Malang), Plaosan (Magetan), and Simalungun (Medan).

METHODOLOGY

Place and Time

This research started from October 2021 to January 2022 at the Fish Cultivation Laboratory in Fish Reproduction Division, D Building, 1st floor, Laboratory of Exploration of Fisheries and Marine Resources, A Building, 1st floor, Faculty of Fisheries and Marine Sciences, Universitas Brawijaya Malang, Microbiology Laboratory in Central Laboratory of Life Sciences, 3rd floor, Brawijaya University Malang and Biochemistry Laboratory in Halal Center Laboratory, 1st floor, Islamic University of Malang.

Research Materials

The tools used in this study included a glass jar (3 liters), Blender (Miyako-101-GS, Indonesia), Rotary Evaporator (IKA 10, Germany), dropper, micropipette (Denshine-10-100ul-MikroTransfer, US),

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test tube (Pyrex, US), Oven (IKA-125 Basic Dry, Germany), aerator set, Blower (Hi-blow Takatsuki-HP-200, Japan) and Shaker (IKA 8017400, Germany). The materials used in this study included red ginger from Batu (Malang), Plaosan (Magetan), Simalungun (Medan), 90% methanol, and 30-day-old Zebrafish.

Research Design

This research is an experimental study with two independent variables such as dosage and type of red ginger from three different locations. This study used 5 treatments, 3 levels, and 2 replications (Table 1).

Table 1. Research design.

Treatments	Dosage	Red Ginger Type		
		RGB (a)	RGP (b)	RGS (c)
A	10 mg/l	1Aa	1Ab	1Ac
		2Aa	2Ab	2Ac
B	20 mg/l	1Ba	1Bb	1Bc
		2Ba	2Bb	2Bc
C	30 mg/l	1Ca	1Cb	1Cc
		2Ca	2Cb	2Cc
D	40 mg/l	1Da	1Db	1Dc
		2Da	2Db	2Dc
E	50 mg/l	1Ea	1Eb	1Ec
		2Ea	2Eb	2Ec

Notes: RGB (red ginger from Batu), RGP (red ginger from Plaosan), RGS (red ginger from Simalungun).

Work Procedure

Identification of Red Ginger

Each sample of red ginger was grouped and analyzed for data including rhizome size, color, texture, soil pH, soil texture, and height of the growing place.

Red Ginger Simplicia Preparation

Each sample of red ginger as much as 2 kg was brushed, cleaned from the soil then rinsed with clean water and dried at room temperature in an oven at 30-40°C after being cut into thin slices with a thickness of about 0.1-0.2 cm. Each sample of dried red ginger is then ground into a fine powder using a blender. After that, the refined simplicia was weighed to determine the water content in wet ingredients. The calculation formula is as follows:

$$\text{Water content} = \frac{\text{wet weight} - \text{dry weight}}{\text{initial weight}} \times 100\%$$

Red Ginger Extraction

The weight of the powder samples (250 g/sample) was extracted by maceration method using a ratio 1:1 between the simplicia sample and 2 L 90% methanol solvent. Maceration of each sample for 48 hours (2 days), started by shaking and stirring each sample with a shaker for 30 minutes to proper extraction process. The macerated herbal samples which obtained and then concentrated using a rotary evaporator with a pressure of 1 atm, a temperature of 50°C, and a rotary rotation of 75 rpm. The extract that has thickened then can be cooled to a temperature conducive to further use (Benjamin *et al*., 2020). Extracts can also be stored in the refrigerator. However, do not put it in the freezer, because temperatures that are too cold will damage the quality of certain compounds found in the extract of Red ginger (Wulansari *et al.*, 2020).

Phytochemical Analysis of Red Ginger Extract

Extracts of Red ginger (0.5 g/ sample) were prepared in a test tube for testing several phytochemicals such as alkaloids, flavonoids, triterpenoids/ steroids, saponins, and tannins (Mahmiah *et al*., 2017).

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Phenol Examination

A total of 0.5 grams of the extract was added and dropped with 3-4 drops of 1% $FeCl_3$. The presence of phenolic compounds is indicated by a bluish-black color change to dark black in the solution (Azizah *et al.*, 2018).

Alkaloid Examination

A total of 0.5 g of the extract was put in a test tube and then dissolved with 2 mL of chloroform, 10 mL of ammonia, and then dropped with 10 drops of H_2SO_4. The mixture is then shaken and allowed to form 2 layers. The H_2SO_4 layer which formed then transferred into 3 test tubes with a volume of 2.5 ml/sample. And then, the solution in 3 tubes was tested with Meyer, Dragendorf, and Wagner reagents. A positive solution for alkaloid compounds in Meyer's reagent will be indicated by the presence of a white precipitate, in Dragendorf's reagent it will be indicated by a change in the solution to a red-orange color, while in Wagner's reagent, it will be indicated by a change in the color of the solution to brown (Rante *et al*., 2020).

Flavonoid Examination

A total of 0.5 grams of the extract was put in a test tube, then dissolved with 5 mL of ethanol. The solution was in a test tube and then heated for 5 minutes using a water bath. After that, added 10 drops of HCl and put 0.2 g of Mg powder. The presence of flavonoid compounds will be indicated by the appearance of a brownish-red color (Rante *et al*., 2020).

Steroid/Triterpenoid Examination

A total of 0.5 g of the extract were dropped with 10 drops of glacial acetic acid and 2 drops of H_2SO_4. The solution is then shaken slowly and left for 2 minutes. A positive solution for triterpenoid compounds will turn red or purple, while a positive solution for steroid compounds will turn blue or green (Rante *et al*., 2020).

Saponin Examination

A total of 0.5 g of extract was dissolved with 10 mL of distilled water or aquadest, then shaken vigorously for 1 minute. The sample was then allowed to stand for 10 minutes and observed the foam which formed. The presence of saponin compounds in the sample will be marked by the formation of a stable foam for 10 minutes with a height of 1-3 cm (Rante *et al*., 2020).

Tannin Examination

A total of 0.5 g of extract was dissolved in 10 mL of hot water, then dropped with 3 drops of 1% $FeCl_3$. The presence of tannin compounds in the sample will be marked by the appearance of a green-black color (Rante *et al*., 2020).

Zebrafish Test Animal Preparation

Thirty aquariums were prepared with size 50x20x20 cm as a place for fish to live, then fill the water with a height of about 15 cm. Prepare the aerator set and set it in each aquarium and turn it on at medium speed. Then, put in Zebra fish aged 30 days with a size of 2-3 cm in each aquarium as many as 20 fish/treatment.

LC_{50} Treatment

Add the extract of each red ginger to each treatment aquarium according to the specified dosage already determined (10 mg/l, 20 mg/l, 30 mg/l, 40 mg/l, 50 mg/l). The extract is then dissolved by stirring slowly. This LC_{50} treatment will be carried out for 96 hours (4 days). Previously, Hedayati and Jahanbakhshi (2012) have revealed that the duration of the lethal concentration test which is 96 hours is considered optimal and the organism mortality rate can be calculated at 24, 48, 72, and 96 hours. The calculation formula according to Sumihe *et al*. (2014) is as follows:

$$\text{Mortality (\%)} = \frac{\text{number of dead fish}}{\text{number of test fish}} \times 100\%$$

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Survival Rate

The surviving fish were removed from the LC_{50} treatment aquarium and count the survival rate of the remaining fish in each LC_{50} treatment using the calculation formula (Kumar *et al*., 2018) as follows:

$$\text{Survival Rate (\%)} = \frac{\text{living fish}}{\text{total fish}} \times 100\%$$

Data Analysis

Statistical analysis and quantitative data collection in this study were carried out by (ANOVA) using the Completely Randomized Factorial Design method using Ms. Excel 2013. Meanwhile, qualitative data collection in this study was carried out using a descriptive method.

RESULTS AND DISCUSSION

Identification of Red Ginger

Identification of each Red ginger is certainly needed for supporting data according to the statement of Tarakanita *et al*. (2019) which states that internal (genetic) and external (environmental) factors can affect the quality of an herbal ingredient, especially red ginger. It is known that plant growth is largely determined by various factors, both internal factors (hormones and genes) and external factors (soil pH and soil texture) (Mpapa, 2016). The geographical location of a place is also one of the factors that cause differences in the results of a phytochemical analysis of a plant (Achmadita, 2021). In addition, altitude and soil chemical conditions are also able to affect the phytochemical content of a plant, because the geographical conditions of one location and another are certain to be different (Istiawan and Kastono, 2019). The identification of the three red gingers respectively is as follows (Table 2).

Table 2. The result of three red ginger identifications.

Red Ginger Type	Red Ginger Size	Red Ginger Surface Texture	Red Ginger Color	Soil pH	Soil Texture	Height of Growing Place
Batu (Malang)	Small	Dry	Brownish yellow	7	Clay	871 masl
Plaosan (Magetan)	Big	Wet	Pale yellow, still brown	8	Clay	874 masl
Simalungun (Medan)	Medium	Wet	Yellowish-brown	7	Clay	900 masl

Red Ginger Simplicia Water Content

The water content can be shown to be the concentration of water contained in the material. The value of the water content in this material can be volumetric or gravimetric (mass) (Kristina, 2018). The water content in a material is considered to affect the quality and shelf life of the material. Determination of the water content of a material is very important in the processing process, because if there is improper handling in processing and determining the wrong water content it will cause damage to the material. Good water content is known to help improve the quality of the material because it avoids the substance or material from being contaminated by fungi and bacteria (Prasetyo *et al*., 2019). The water content of the three simplicias of red ginger was found to be different (Table 3).

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Table 3. The result of three red ginger water contents.

Types of Red Ginger	Wet Weight (kg)	Dry Weight (kg)	Water Content (%)
Batu (Malang)	4	0,550	86,25
Plaosan (Magetan)	4	0,456	88,60
Simalungun (Medan)	4	0,754	81,15

Identification of Red Ginger Extract

The difference in the extracted material can certainly produce different colors and odors. This is because the chemical compounds contained in the extracted material are different, even though the material is categorized as the same type (Astawan, 2008). The aroma or odor and color produced from an extract generally depend on the concentration of the ingredients used. The higher the concentration of the extract, the distinctive aroma or odor of the material will increase and the color of the extract will tend to be more concentrated (Juwita *et al*., 2013). Therefore, the results of the identification of the methanol extract of Red Ginger from each of these ingredients are described as follows (Table 4).

Table 4. The result of three red ginger extracts identification.

Types of Red Ginger	Methanol Extract Color	Methanol Extract Odor
Batu (Malang)	Reddish brown, thick	Like the smell of mint, pungent
Plaosan (Magetan)	Brown, thick	Like the smell of sulfur, pungent
Simalungun (Medan)	Reddish brown	Like a mint smell, a little pungent

Phytochemical Analysis of Red Ginger

Phytochemical screening is an early stage that is considered to be able to provide an overview of the content of certain compounds in natural materials to be studied. The group of these compounds will be illustrated from the results from the phytochemical screening of the material by the visual observation of changes in the color of the solution or sample (Dewatisari, 2020). It is known, for the phytochemical content of plants that grow in different locations, they can also face different environmental stress conditions. This allows the plant to contain different special compounds, including such as alkaloids, flavonoids, and terpenoids. This is also one of the reasons why a similar material grown in different locations has different biological properties and effects (Nguyen *et al*., 2019).

In this case, RGM was found to be positive for saponins, while RGS contains phenols and tannins. Koirewoa and Raunsay (2016) argue that metabolites such as saponins, phenols, and tannins can be influenced by human activities through the waters around which these plants grow. In this case, it was also conveyed that waters containing a lot of metals in it will help speed up the process of transporting phenol into plants. Where, if phenol levels are present in a plant, it is possible to find the presence of tannins in it as well. This is because tannins are derivatives of phenolic compounds. The results of the phytochemical analysis of the three red gingers can be seen in (Table 5).

Table 5. The result of three red gingers phytochemical analysis.

Types of Red Ginger	Phenol	Alkaloid	Flavonoid	Triterpenoid /Steroid	Saponin	Tannin
Batu (Malang)	-	+	-	++	+	-
Plaosan (Magetan)	-	+	-	++	-	-
Simalungun (Medan)	+	++	-	++	-	+

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LC_{50} Extract of Red Ginger against Zebra Fish

To determine the toxicity of a chemical in the aquatic environment, it is first necessary to estimate the mean lethal concentration (LC_{50}) of the chemical in the water. This is done through acute toxicity testing on organisms exposed to these chemicals (Hedayati *et al.*, 2012). In the LC_{50} treatment, if the mortality of fish is high, it implies that the death of fish that occurs can be caused by the harmful effects of several toxic substances in the extract and related materials (Gu *et al.*, 2021). In the graph (Figure 1) it can be seen that the mortality rate of Zebra fish seems to increase when each dosage is increased. However, a significant increase in mortality occurred in red ginger originating from Plaosan (Magetan). However, the highest mortality rate was shown in the 50 mg/l dosages of red ginger from Batu (Malang). Red ginger originating from Simalungun (Medan) so far, its mortality is still below the other two red gingers.

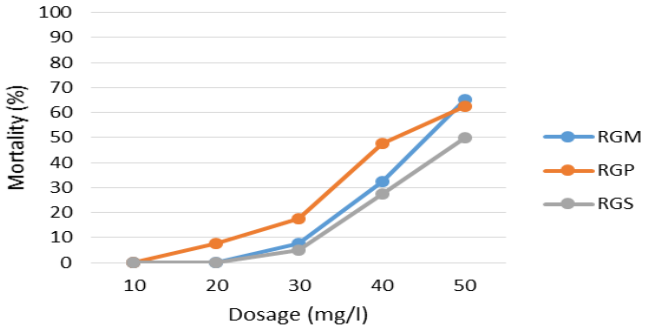

Figure 1. The results of LC_{50} with red ginger extract from against zebra fish.

High mortality rates and low fish survival are known to go hand in hand. Generally, the high mortality rate will increase along with increasing the dosage given to fish. It can be attributed to the failure of ionic and osmotic regulation that happened. The mechanism may happen due to higher blood plasma osmotic concentrations as a result of increased ion concentrations and the inability of fish to face a sudden increase in dosage (environmental stress) so that organisms tend to go into shock and find it difficult to return their body ion concentrations to normal levels (homeostasis).

This may occur because most fish try to withstand osmotic shock when suddenly exposed to water with different osmotic pressures through osmotic and ionic regulation mechanisms that result in a state of balance between body fluids and the external environment (Alkhshali and Alhilali, 2020). The provisional assumption that can be concluded from this, the highest cause of mortality obtained based on the 50 mg/l dosage of Red ginger originating from Batu (Malang) may be due to the presence of saponin compounds in Red ginger extract exposed to the Zebrafish. Lilis and Adawiyah (2021) revealed that saponins which are a type of glycoside are secondary metabolites that are widely found in nature.

However, this compound is toxic to cold-blooded animals, especially fish like small fish such as Zebrafish. This is because the toxic content of saponins (sapotoxins) is considered capable of destroying blood grains or triggering hemolysis in the blood. In addition, if fish are exposed to saponins for too long, saponins can also cause disruption of the respiratory system in fish and maybe in a certain time, these substances are also capable of damaging the gills which can eventually lead to death. It should be noted that fish gills are the most sensitive organs when exposed to strange substances, especially harmful substances (poisons). For an illustration of this can be seen in (Figure 2).

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Figure 2. Zebrafish experiencing seizures and environmental stress after being exposed to methanol extract of red ginger in the highest dosage (50 mg/l). Fish have difficulty breathing and then die with gills that look a little red for just a minute.

The presence of other metabolites like phenols, alkaloids, flavonoids, triterpenoids/steroids, and tannins are not the main agent causing death in fish. It is because the metabolites are the type of antibacterial metabolite, that have the task and the function of killing bacteria that infect fish or bacteria that are in the environment, where the fish live (Maharani *et al*., 2021). However, according to Irawan *et al*. (2019), if the saponin content is higher in an extract, this metabolite will cause death in fish. Especially in the family Cyprinidae such as Zebrafish. One of the side effects of saponin is able to decrease the dissolved oxygen. Saponins will enter the bloodstream through the gills. Then, when the fish takes the oxygen from the water, saponins will enter the body and bind with hemoglobin. Because of that, saponin will be causing the fish to lack blood and cause death.

Survival Rate of Zebra Fish

The survival rate is the percentage of fish that live from the number of fish kept during LC_{50} treatment in one rearing container (Mendefa *et al*., 2022). In the graph (Figure 3) it can be seen that the survival rate of Zebra fish began to decrease at a dosage of 30 mg/l for each Red Ginger. This is in line with the high mortality rate in the LC_{50} treatment that has been carried out. Dedi *et al*. (2018) argue that mortality must be in line with survival. If the mortality of an organism is high, the survival of that organism will be low. And also, if the mortality of an organism is low, the survival of the organism will be high.

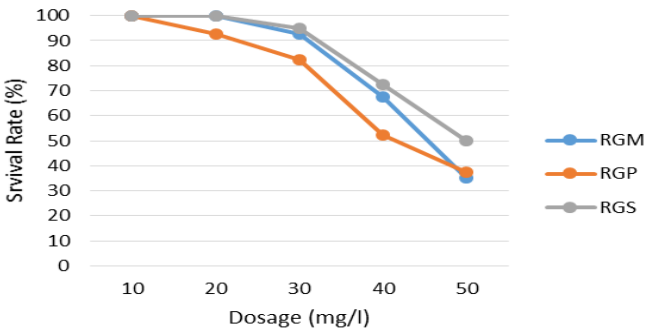

Figure 3. The result of zebra fish's survival rate that treat with red ginger extract.

CONCLUSION

From this research, it can be proved qualitatively that the content of the three red gingers was different. In the phytochemical analysis of red ginger from Batu (Malang), the material contains alkaloids, triterpenoids/steroids and saponins. Red ginger from Plaosan (Magetan), these ingredients contain alkaloids and triterpenoids/steroids. Meanwhile, Red ginger

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from Simalungun (Medan) contains phenols, alkaloids, triterpenoids/steroids and tannins. The LC_{50} results showed that the increase in mortality and decrease in survival occurred simultaneously starting from the administration of a dosage of 30 mg/l in all types of red ginger extract. However, the highest mortality was obtained by treatment with methanol extract of Red Ginger from Batu (Malang) in the fifth treatment, which was a dosage of 50 mg/l. Thus, it can be concluded that the maximum safe dosage of red extract used for Zebra fish generally is 20 mg/l.

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