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Toxicity Test Of N-Hexan Fraction, Ethyl Acetate, And Chloroform Red Gedi Plant (*Abelmoschus Manihot* L. Medik) With Brine Shrimp Lethality Test (Bslt) Method

Andi Atirah Masyita[1], Nurlina Ibrahim[2], Magfirah[3]
[1,2]Departement of Pharmay, Faculty of Pharmaceutical, Tadulako University, Central Sulawesi 94111, Indonesia.
[2]College of Pharmaceutical Scinces Pelita Mas Palu, Center Sulawesi 94111, Indonesia.
*Email Korespondensi : s.atyrah.almahdy@gmail.com

Abstrak

Red gedi (Abelmoschus manihot L. Medik) is a tropical plant in the family Malvaceae that is used empirically by the community of Palu City as an anti-cancer. Red gedi leaves contain flavones that have high antioxidant activity. This study aims to determine the toxicity potential by means of the LC50 value of red gedi leaf extract within n-hexane fraction, ethyl acetate fraction, and chloroform fraction using the Brine Shrimp Lethality Test (BSLT) method. The BSLT method used Artemia salina Leach larvae tested with all fractions at the concentration of 25ppm, 500ppm, 100ppm, 200ppm, and 500ppm. The extraction process began with the maceration method of red gedi leaf simplicia with ethanol 96% solvent. The extract obtained from the maceration process was concentrated with a rotary evaporator, then 30 ml of water was added to the extract for further partitioning purposes. The solution from the previous stage was first partitioned using n-hexane solvent to obtain the n-hexane fraction, then the insoluble extract in n-hexane was partitioned with ethyl acetate so as to obtain ethyl acetate fraction, then finally the extract which was insoluble in ethyl acetate was further partitioned with chloroform, so the chloroform fraction is obtained. The final toxicity test, performed using the BSLT method, yielded LC50 values of 81.563 ppm, 333.199 ppm, and 104.591 ppm for n-hexane, ethyl acetate, and chloroform fractions respectively. It can be concluded that the n-hexane fraction of red gedi leaves exhibits a stronger toxic effect compared to the chloroform and ethyl acetate fractions of red gedi leaves.

Keywords*: BSLT, LC50, red gedi leaf fraction, toxicity test*

INTRODUCTION

Cancer is a chronic disease that leads to fatalities in human populations worldwide. In 2012, roughly 8.2 million people died of cancer. The most death of cancer each year are caused by breast, cervical, lung, liver, stomach, and colorectal cancers (RI Ministry of Health, 2015)[1].

The use of conventional drugs in cancer therapy can cause very adverse side effects, one of which is the death of healthy body cells (Kowalak, 2011)[2]. Therefore, sometimes people preferred alternative treatments using natural ingredients that are believed to be safer with minimum side effects (Siahaan, et al 2017)[3]. One of the natural ingredients that is well-known among the community of Palu city is the red gedi leaf which is used as an anti-cancer. Some people only recognized red gedi leaves as vegetables and pulp ingredients (due to their small pink-colored fruit). However, this plant apparently has a cytotoxic effect as well.

Cytotoxic substances are drugs that damage and kill both normal and cancer cells, and are utilized to inhibit tumor growth. Brine Shrimp Lethality Test (BSLT) is one of the toxicity testing methods to test cytotoxic substances. This method uses *Artemia salina* Leach larvae as laboratory animals. The toxicity test with BSLT method is an acute toxicity test where the toxic effect of a compound is determined in a short time after administering the test dose (Siregar, 2008)[4]. Besides, *Artemia salina* L. can also be used in laboratory tests to detect the toxicity of a compound from plant extracts (Kanwar, 2007)[5].

RESEARCH METHODOLOGY

This research is a quantitative laboratory experiment by performing cytotoxic tests using the BSLT (Brine Shrimp Lethality Test) method on *Artemia salina* L. The parameter used is LC50 (Lethal Concentration 50) value. The research was conducted at the Pharmacognosy-Phytochemical Laboratory of the Pharmacy Department, Pelita Mas College of Pharmacy, and the Faculty of Mathematics and Natural Sciences, Tadulako University.

The apparatus used in this research are knives, scissors, ruler, tweezers, glassware, thermometer, dropper, blender, sifter, vacuum rotary evaporator, analytical balance, filter paper, aluminum foil, flannelette, pH meter, shrimp eggs incubator, aerators, vials, 1 ml and 5 ml spoilers, capillary tubes, incandescent lamps, pieces of newspaper, maceration containers, tube racks, stirring rods, funnels, desiccators, 254 nm and 366 nm UV lamps, chamber, and GF254 silica Gel TLC plate. Meanwhile, the materials used in this research are pieces of newspaper, fresh red gedi leaves (*Abelmoschus maniho* L.) obtained from Minti Makmur Village, Rio Pakava District, Donggala Regency, Central Sulawesi Province, ethanol 96%, *n*-hexane, chloroform, ethyl acetate, distilled water, yeast, seawater, and *Artemia salina* Leach larvae.

RESEARCH PROCEDURE

Sample Collection and Preparation

Fresh red gedi (*Abelmoschus manihot* L.) leaves samples were obtained from Minti Makmur Village, Rio Pakava District, Donggala Regency, Central Sulawesi Province. Red gedi leaves that have been picked were washed and then dried for 3 days. The dried red gedi leaves are then pulverized by blending and then passed through a 60-mesh sieve.

The identification was carried out at the Biodiversity Laboratory, Department of Biology, F-MIPA, Tadulako University. Identification is carried out in addition to looking at the morphology of plants to ensure that the obtained red gedi leaves are the correct *Abelmoschus manihot*.

Production of Extracts

The extraction began with macerating 450 grams red gedi (*Abelmoschus manihot* L) simplicia using 2 liters or more of ethanol 96% until the simplicia submerged completely. The content was kept for 4 × 24

hours and stirred occasionally. After 4 days the filtrate was collected and then evaporated with a rotary evaporator until an extract was obtained. The rendemen were then calculated (Sulastra et al, 2016)[6].

$$\%\ \text{Rendemen} = \frac{thick\ extract\ mass\ (g)}{sample\ mass\ (g)} \times\ 100\%$$

Fractionation

As much as 55 grams of red gedi ethanol extract (*Abelmoschus maniho* L.) was fractionated using the liquid-liquid partition method. At first, the extract was dissolved in 30 ml of water and put into a separating funnel. Then 100 mL of *n*-hexane was added (Firdaus et al, 2015)[7]. The mixture is gently shaken until a distributed mass is obtained, then the separating funnel is left on a stative clamp until separation occurs.

Once separated, the *n*-hexane layer is accommodated and the water layer is put back into the separating funnel for further partitioning. The *n*-hexane solvent partitioning process was repeated 5 times (Hanani, 2015)[8]. The residual layer of water is then repartitioned using 100 ml of ethyl acetate solvent with a similar treatment as *n*-hexane solvent until ethyl acetate fraction is obtained. The fraction which is insoluble in ethyl acetate is partitioned again with chloroform until a chloroform fraction is obtained. The remaining *n*-hexane, ethyl acetate, and chloroform partitions were evaporated using a rotary evaporator at 40-50°C (Hanani, 2015)[8].

The LC50 test was then performed on each obtained fraction. The fraction with the highest LC50 value will be analyzed using thin-layer chromatography. It is performed to determine the class of chemical compounds contained in the fraction.

Brine Shrimp Lethality Test (BSLT)

Preparation of Shrimp Larvae

Shrimp eggs are hatched in a cone-shaped hatcher filled with seawater. The system is equipped with a light bulb as a source of light and aerators as a source of oxygen to keep the eggs from settling. After 48 hours the larvae are ready to use for testing.

Preparation of Control and Sample Solutions

The control solution was made without adding the extract. Ten ml of artificial seawater and 10 larvae of shrimp *Artemia salina* Leach are added into the vial (Dumitrascu, M, 2011)[9].

Acute Toxicity Test with BSLT

Ten *Artemia* larvae that were 48 hours old were taken using a dropper pipette and inserted into a vial containing a sample with a certain concentration that had been evaporated in the open room, as well as 3 ml of seawater. Then a single drop of yeast suspension (3 mg of yeast in 5 ml of seawater) is added as food along with seawater to 5 ml. Each test is always conducted along with the control and is done in 5 replications for a specific extract concentration. The vial is maintained to get continuous illumination. After 24 hours, the number of dead larvae was calculated to determine the probit value and analyzed to determine the LC50 value (Mayer et al, 1982)[10].

Identification of chemical compounds by thin-layer chromatography

TLC plates were made rectangular with ± 2 cm × 8.5 cm elution distance. The plates were cut according to the number of test samples. After that, the most active fraction was put on the TLC plate as a little spot using a spotter and dried for a while. Elution was carried out using an eluent comparison obtained from the TLC profile. Furthermore, the plates were sprayed using stain-view detection fluid to determine the class of their compounds. The class of compounds to be tested are as follows:

1. Flavonoids

The plates were sprayed using AlCl3 reagents. The presence of flavonoid compounds is characterized by fluorescent colors of yellow, green, and black-blue (Waksmundzka et al, 2008)[11].

2. Alkaloids
   The plates were sprayed using Dragendorf reagents. The presence of alkaloid compounds is marked in orange (Waksmundzka et al, 2008)[11].
3. Steroids
   The plates were sprayed with concentrated sulfuric acid and left to dry. Then plates were heated at a temperature of 100 – 105°C for 10 minutes or until perfect color formed (Harborne, 1987)[12]. The presence of steroid compounds is indicated by green and yellow patches (Effendi, 2019)[13].
4. Phenolic
   The plates were sprayed using FeCl3 reagents. Positive results are indicated by the presence of green or blackish blue (Waksmundzka et al, 2008)[11].
5. Terpenoids
   The plates were sprayed with concentrated sulfuric acid and left to dry. Then the plates were heated at a temperature of 100 – 105°C for 10 minutes or until the perfect color formed. The presence of brown, yellow, violet, pink, and green patches indicates a positive containing terpenoids (Harborne, 1987)[12].

Data analysis

The obtained data as the resulting value of the toxicity test on shrimp *Artemia salina* leach larvae was analyzed to figure out the LC50 value using probit analysis with the help of SPSS 23 software

RESEARCH RESULTS

a. Red Gedi leaf extraction

In this research, the yield of red gedi leaf extract (*Abelmoschus manihot* L) was 12.22% (55 grams of thick extract from 450 grams of red gedi leaf powder).

b. Fractionation

The results of liquid partition – solid ethanol extract of red gedi leaves (*Abelmoschus manihot* L.) can be seen in Table 1.

Table 1. Results of liquid partition - solid ethanol extract of red gedi leaves

o	Extract	Extract Weight
l	Soluble n-hexane	20 grams
2	Soluble ethyl acetate	2 grams
3	Soluble chloroform	13 grams

c. Brine Shrimp lethality test (BSLT)

The results of BSLT test of red gedi leaf extract can be seen in Table 2.

Table 2. Acute toxicity tests of red gedi leaf extract in various fractions with varying concentrations

Fraction	Concentration (ppm)	Avg. living larvae	Avg. dead larvae	%Death	LC50 Score
Ethyl acetate	25	20	10	33.3	81.563
	50	17	13	43.3	
	100	15	15	50	
	200	10	20	67	
	500	8	22	73	

Hexane	25	17	13	43.3	333.199
	50	16	14	46.6	
	100	15	15	50	
	200	13	17	56.5	
	500	14	16	53.3	
Chloroform	25	24	6	20	104.591
	50	23	7	23.3	
	100	23	7	23.3	
	200	20	10	33.3	
	500	16	14	46.6	

d. Identification of chemical compounds by thin-layer chromatography

The results of the identification of chemical compound classes contained in the red gedi leaf extract can be seen in Table 3.

Table 3. Identification of secondary metabolite compounds in red gedi by TLC

No	Type of compound	Type of compound	Results	RF value
1	Flavonoids	$AlCl_3$	+	0.91
2	Alkaloids	Dragendorf	+	0.80
3	Terpenoids	Concentrated H2S04	-	-
4	Tannin	$FeCl_3$	+	0.87

DISCUSSION

In this research, the sample used is red gedi leaves extracted with 96% ethanol with the maceration method. The method's selection is driven by ease and simple production as well as less-complicated instrument usage. The extraction process yields 12.2% or 55 grams of thick extract from 450 grams of red gedi powder.

The fractionation was then carried out using *n*-hexane, ethyl acetate, and chloroform solvent in the liquid-liquid partition. The process is done using a separating funnel. Thick red gedi leaf extract was added along with 30 ml of water and then partitioned using 100 ml *n*-hexane solvent yielding an *n*-hexane fraction.

The *n*-hexane insoluble fraction was then added with 100 ml ethyl acetate to obtain the ethyl acetate fraction. Furthermore, the acetate insoluble fraction was partitioned with 100 ml of chloroform solvent so the chloroform fraction was obtained.

The treatment was replicated 5 times for each fraction. The fractions of all subsequent partitions are concentrated with a rotary evaporator. The results of the fractionation process can be seen in Table 1. Furthermore, the three fractions obtained *n*-hexane, ethyl acetate, and chloroform fraction were then tested for their toxicity using the BSLT (Brine shrimp Lethality Test) method each at the concentrations of 25ppm, 50ppm, 100ppm, 200ppm, and 500ppm within 3 replications. The variation of concentrations is intended to obtain the LC50 value of each of the most toxic fractions. The number of deaths of artemia salina leach larvae can be seen in Table 2. From this table, it can be seen that variations in the concentration of red gedi leaf fraction give a different effect on the average number of shrimp larvae deaths. There were 10 larvae used in this study for each glass test and the test was carried out in 3 replications for each concentration. The larvae used were 48 hours old, as at this age, their limbs are fully developed

compared to when they hatch (Muaja, DA. 2013)[14]. Observing the development of larvae up to the stage when they are ready to use for the toxicity test of the red gedi fraction leaves, a magnifying glass is needed.

The total number of deaths from each fraction is calculated by adding all the number of dead larvae at each concentration within each replication. It can be seen that the response of the shrimp larvae death is linear in each fraction where at high concentrations of 500ppm the death percentage or the total number of dead larvae is greater than those at 200ppm, 100ppm, 50ppm, and 25ppm. However, it does not indicate the exact value of the larvae death in a variety of fractions. For this reason, probit analysis is needed to determine the LC50 of larvae in each red gedi fraction.

The probit analysis was performed using SPSS. The obtained results are LC50 values for *n*-hexane, ethyl acetate, and chloroform fraction of red gedi leaf extract which are 81.563ppm, 333.199ppm, and 104.591ppm respectively. According to Anderson (1991)[15], LC50 values of 0-250 are highly toxic, 250-500 are toxic, 500-750 are moderate and 750-1000 are non-toxic. Based on this category, LC50 values of red gedi leaf fractions are highly toxic, very toxic, and toxic where the most toxic LC50 value belongs to the *n*-hexane fraction of 81.563 ppm.

Furthermore, the identification of secondary metabolite compounds contained in red gedi leaves using the TLC method was conducted. The results of the identification can be seen in Table 3 which shows that positive red gedi leaf extract contains compounds including alkaloids, flavonoids, and tannins. Flavonoids and tannins are the strongest oxidant compounds that function as precursor capturers of free radicals. Tannin can function as a collector that binds dangerous metals while flavonoids function as antioxidants that can donate hydrogen atoms or have the ability to act as a chelating agent.

The highest LC50 value belongs to the *n*-hexane fraction. This is because flavonoids and tannins are soluble in *n*-hexane solvent which is non-polar and can attract non-polar compounds such as steroids, terpenoids, sterols, and phenyl propanoids. Meanwhile, the smallest LC50 value is in the ethyl acetate fraction because ethyl acetate is a polar solvent that can dissolve non-polar substances such as flavonoids and tannins. (Harbone, 1987)[12]

CONCLUSION

The results showed that the red gedi leaf fraction has cytotoxic potential with LC50 values of *n*-hexane, ethyl acetate, and chloroform fraction are 81.563 ppm, 333.199 ppm, and 104.591 ppm respectively. Based on the LC50 value, the categories of the red gedi leaf fractions are highly toxic, very toxic, and toxic where the most toxic LC50 value belongs to the *n*-hexane fraction of 81.563 ppm

SUGGESTION

It is hoped that the research can be carried out further to the stage of isolating compounds that have cytotoxic activity.

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