Toxicity test of stem bark extract of banyuru (Pterospermum celebicum miq.) using BSLT (brine shrimp lethality test) and cream irritation test

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Abstract. A study to observe toxic effect of ethanol extract of Pterospermum celebicum Miq. by using Brine Shrimp Lethality Test (BSLT) has been carried out. The method to do this study was irritation test of cream formula of the extract. The aim of this study was to collect data about toxicity of fractionated extract of P. celebicum Miq. on Artemia salina by using BSLT method according to LC50 value obtained from probit analysis. The extract was extracted by maceration method using ethanol as solvent which was then formulated as cream containing 3% of P. celebicum Miq. extract. The toxicity test revealed that the value of LC50 was 15.15 µg/mL, while irritation test stated that no erythema and edema observed.

1. Introduction

Indonesia is a country located in tropical region. It possesses the second largest biodiversity after Brazil. Approximately 25,000 – 30,000 plants have been spotted in Indonesia. The number are equal to 80% of global plant species and 90% of plants found in Asia. Many tropical plants have been utilized for curing diseases. The therapy using plant has been recognized as traditional medication.

In Indonesia, traditional medicines have been used for thousand years before the use of modern medicine. One of the plants that has been utilized is Pterospermum celebicum Miq. This plant is a family of sterculiaceae. It has been identified that the bark of this plant contains flavonoids, terpenoids, polyphenols, and steroids. Flavonoid contents of P. celebicum Miq. have antimicrobial properties [1]. Ethanol extract of P. celebicum Miq bark show potency as anti-infective agent [1],[2].

There are many projects that have been carried out to observe potency of natural resources as medicine. To guarantee the safety of the medicine, the government has released Decree of Minister of Health Affairs Number 760/Menkes-/Pet/IX/1992 about traditional medicine and phytopharmaca. Another regulation is Decree of Minister of Health Affairs Number 381/Menkes/SK/III/2007 signed on 27 March 2007 regulating policies related to the use of traditional medicines. These regulations are
expected to become basis, direction, and guideline in the efforts to develop and improve the best quality, safe, and efficacious traditional medicines.

To fulfill the criteria as a good medicine, then a medicine must show effective therapeutic activity with minimum toxicity. Toxicity test is carried out to evaluate the safety of a certain material developed as medicine [3].

One of the methods used to test toxicity aspect of a plant is Brine Shrimp Lethality Test (BSLT) [4]. This method is an easy, quick, and cheap method so that it can be used as Bioassay Guided Fraction from natural resources. This method was introduced first by Trapley to determine insecticide residues, to observe anesthetic compounds, and to see toxicity level of sea water. Meyer developed the method for screening the active compounds by utilizing larvae of *Artemia salina*. To determine the toxicity level, value of LC$_{50}$ is counted through probit analysis. The potency of the extract is determined by comparing the LC$_{50}$ value which is less or equal to 1,000 ppm [5].

This recent study aimed to observe whether the fraction of ethanol extract of *P. celebicum* Miq. produced toxicity on larvae of *Artemia salina* by using method of BSLT. Another problem is whether the cream produces irritation.

2. Methods

2.1 Tools and Materials

The tools used were flasks, scissors, aluminium foil, desiccator, UV light 254 nm and 366 nm, a set of vacuum rotary evaporator (Buchi®), micropipette (BIOHIT®), a set of thin layer chromatography, capillary pipes (Nesco®), a set of vacumm liquid chromatography, analytical balance (Sartorius®), pH meter (Lutron®), thermometer, viscometer (Brookfield®), autoclave (All American®), climatic chamber (Climacell Sartorius®), freeze dryer (CoolSafeTM), homogenizer (Ultra Turax®), incubator (Memmert®), Laminary Air Flow (Envirco®).

The materials used in this study were extract of *P. celebicum* Miq, *Artemia salina* larvae, ethyl acetate, ethanol 96%, methanol, n-hexane, sulphuric acid 10%, formic acid 1%, stearic acid, α-tocopherol, Phytocream® emulgator, blue methylen, methyl paraben. The animal used in this study was the health rabbits.

2.2 Sample Preparation and Extraction

Bark sample of *P. celebicum* Miq. was collected from Kompang Village located in Sinjai Regency, South Sulawesi Province. The samples were then sorted, washed, and chopped. The samples were dried in oven at the temperature of ±50°C. The dried samples were powdered and then macerated by using ethanol 96% as solvent. The extracts were then filtered and put in rotary evaporator to evaporate the residue of solvent. This process was conducted at 50°C and 75 rpm to obtain dense extract [6].

2.3 Liquid-Liquid Extraction

As many as 52.34 gram of ethanol extract of *P. celebicum* Miq. bark was dissolved in 50 mL of methanol. 100 mL of n-hexane was added in separating funnel. The funnel was then shaken to generate 2 layers containing two groups of compounds which were dissolved either in methanol or n-hexane [7]. The extracts collected from this process were then tested by using TLC and BSLT methods.
2.3.1 Fractionation
Fractionation was carried out by TLC method. Methanol-dissolved fraction was balanced as many as 9.01 gram, dissolved in 10 mL methanol, mixed and dried by using silica gel GF254. The column for fractionation was packed by putting 80 gram of dried silica gel in vacuum condition. The dried extract was sprinkled homogenously on the pressed silica gel in the column. Elucidation of the column was done by using mobile phase of ethyl acetate – methanol with comparison of 32:1; 16:1; 1:1; 1:4; 1:16; and methanol only. The fractions collected from this fractionation were tested by using TLC and BSLT. LC$_{50}$ was then determined [8].

2.3.2 Preparation of Artemia salina Larvae
The eggs of *Artemia salina* were hatched in a transparent container containing sea water. A light bulb with power supply of 40-60 watt was provided in hatching process to prevent the temperature within 25°C-30°C. Oxygen was supplied by using blower. The larvae with 48-hours life span were used in toxicity test [5].

2.3.3 Preparation of the Tested Samples
The concentrations used in BSLT were 20 μg/mL, 40 μg/mL, 60 μg/mL, 80 μg/mL, 100 μg/mL, and negative control. Stock solution of dense extract was prepared by balancing 5 mg of the extract which was then dissolved in 5 mL ethanol to produce 1,000 ppm. The stock solution was then pipetted and put in vials. Each concentration has 3 replications [9].

The vials were aerated to dry the samples put in them. The vials were added DMSO 1% for water-undissolved fractions. Five milliliters of sea water were added to the vials. The process was followed by adding samples to the vials to make concentrations of 20 μg/mL, 40 μg/mL, 60 μg/mL, 80 μg/mL, and 100 μg/mL. The control had no sample in it.

2.3.4 Toxicity Test
Ten larvae of *Artemia salina* were added to each vial. The observation was conducted for 24 hours. Toxicity test was determined according to the number of death larvae [8].

2.4 Cream Formulation

| No. | Materials                       | Weight (g) |
|-----|---------------------------------|------------|
| 1.  | Extract of *P. celebicum* Miq.  | 3.00       |
| 2.  | Cetyl alcohol                   | 2.50       |
| 3.  | Stearic acid                    | 2.00       |
| 4.  | Propylene glycol                | 12.00      |
| 5.  | Methyl paraben                  | 0.20       |
| 6.  | Propyl paraben                  | 0.02       |
| 7.  | Phytocream®                     | 5.00       |
| 8.  | α-tocopherol                    | 0.05       |
| 9.  | Aquadest                        | 68.23      |
All of the materials were weighed as shown in table 1. Oil phase was made by melting cetyl alcohol, stearic acid, prophyl paraben, α-tocopherol, and Phytocream® in porcelain cup. The cup was put on water bath at the temperature of 70°C. Stirring the mixture until getting homogenous mixture. Water phase was prepared by heating water until 70°C in flask which was then followed by methyl paraben addition. Stirring the mixture until getting homogenous mixture. The oil phase was poured to water phase and stirred with electric stirrer. Cream basis was stirred until the temperature of 30°C. The extract was added the cream basis slowly until homogeneous.

2.5 Irritation Test
Albino rabbits were used to observe primary irritation test in this study. The primary irritation test was conducted by using patch test method on rabbit skin (Draize, 1959). As many as 0.5 gram or 0.5 mL irritant was applied on the skin of rabbit which have been shaved with size of 1 inch square. Sterile gauze was put on the treated skin and let it for 24 hours. After 24 hours, the gauze was peeled off to see the treatment reaction on the skin. The assessment was carried out by looking at the erythema and edema formed. The reaction was observed again 72 hours after the treatments. The score obtained after observation in both times (24 hours and 72 hours) was calculated to get primary irritation index [10].

2.6 Data Analysis
Toxicity test was assessed by determining LC$_{50}$ score. To obtain LC$_{50}$, mortality rate of larvae after 24 hours exposure was assessed first.

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\text{% mortality rate} = \frac{\text{number of death larvae}}{\text{Total number of larvae}} \times 100\%
\]

Having that mortality rate, probit analysis was carried out to find LC$_{50}$ score. LC$_{50}$ score is defined as the concentration where the compound produced 50% death. After doing probit analysis, LC$_{50}$ was counted by using linear regression equation $y = a + bx$.

Toxicity level of a compound is classified according to Mayer (1982) classification. LC$_{50}$ score in range of 1 – 10 µg/mL is defined as highly toxic. LC$_{50}$ in range of 10 – 100 µg/mL is classified as medium toxic, while 100 – 1,000 µg/mL as low toxic. Erythema assessment was carried out qualitatively according to the certain score [11]:

1. Erythema
   a. No erythema = 0
   b. Mild erythema (almost unobservable) = 1
   c. Mild erythema (very clear) = 2
   d. Moderate erythema (very red) = 3
   e. Severe erythema (would formation) = 4

2. Edema
   a. No edema = 0
   b. Mild edema (almost unobservable) = 1
   c. Mild edema (very clear) = 2
   d. Moderate edema (swelling about 1 mm in height) = 3
   e. Severe edema (swelling > 1 mm in height) = 4
Irritation index is counted by summing the score of each rabbit 24 hours and 72 hours after irritant exposure. The criteria are as follow:

- 0.00 = No irritation
- 0.04 - 0.99 = Less irritation
- 1.00 - 2.99 = Mild irritation
- 3.00 - 5.99 = Moderate irritation
- 6.00 - 8.00 = Severe irritation

3. Results and Discussion

Maceration was used to extract the components since it does not damage chemical entities of the plant. The powder of *P. celebicum* Miq. was extracted by using 96% ethanol as solvent. The use of this solvent since it is not associated with toxic effect and the polarity of ethanol is high. This property is related to the types of extracted compounds. Maceration method was carried out for three days which was followed by filtering and evaporating using rotary evaporator at the temperature of 50°C. This extraction produced dried powder as many as 74.6 gram.

LC\textsubscript{50} obtained through maceration method was 59.02 µg/mL with deviation standard was 2.14. This result showed that this initial extract of *P. celebicum* Miq. had toxic effect since it has LC\textsubscript{50} score below 1,000 µg/mL. This extract was followed by liquid-liquid extraction.

**Table 2.** LC\textsubscript{50} score of initial extract (maceration method)

| Extract            | Replication | LC\textsubscript{50} (µg/mL) | Mean ± SD (µg/mL) |
|--------------------|-------------|-------------------------------|-------------------|
| Initial extract    | 1           | 57.02                         |                   |
|                    | 2           | 62.43                         |                   |
|                    | 3           | 59.03                         | 59.02 ± 2.14      |

**Table 3.** LC\textsubscript{50} score of fractions

| Fractions          | Replication | LC\textsubscript{50} (µg/mL) | Mean ± SD (µg/mL) |
|--------------------|-------------|-------------------------------|-------------------|
| Methanol-dissolved | 1           | 44.65                         | 49.51 ± 4.59      |
|                    | 2           | 51.57                         |                   |
|                    | 3           | 52.32                         |                   |
| Hexane-dissolved   | 1           | 71.45                         | 86.01 ± 8.72      |
|                    | 2           | 90.34                         |                   |
|                    | 3           | 96.25                         |                   |

Partition of *P. celebicum* Miq. resulted in methanol-dissolved fraction as many as 17.9 gram and 4.23 gram of n-hexane-undissolved fraction. Those fractions were tested for their toxicity through BSLT method. The results showed that methanol-dissolved fractions had higher toxicity than hexane-dissolved fraction. This result directed us to continue the test by using methanol-dissolved fraction.
Table 4. LC_{50} score for methanol fraction by using some different eluents.

| Fractions | Replication | LC_{50} (µg/mL) | Mean ± SD (µg/mL) |
|-----------|-------------|-----------------|-------------------|
| A         | 1           | 310.44          |                   |
|           | 2           | 315.12          |                   |
|           | 3           | 312.25          | 312.6             |
| B         | 1           | 51.19           |                   |
|           | 2           | 53.42           |                   |
|           | 3           | 52.75           |                   |
| C         | 1           | 11.20           |                   |
|           | 2           | 15.65           |                   |
|           | 3           | 18.62           |                   |
| D         | 1           | 16.88           |                   |
|           | 2           | 32.43           | 24.54             |
|           | 3           | 24.32           |                   |
| E         | 1           | 85.86           |                   |
|           | 2           | 87.34           | 85.18             |
|           | 3           | 82.34           |                   |

Describe:
A: Ethyl acetate:methanol (32:1)  D: Ethyl acetate:methanol (1:4)
B: Ethyl acetate:methanol (16:1)  E: Ethyl acetate:methanol (1:16)
C: Ethyl acetate:methanol (1:1)

As many as 9.01 gram of methanol fraction was fractionated by using ethyl acetate:methanol eluent at ratio 31:1; 16:1; 1:1; 1:4; 1:16; and methanol only. The weights obtained were fraction A 0.67 gram; fraction B 0.94 gram; fraction C 1.15 gram; fraction D 2.86 gram; fraction E 1.88 gram. TLC test was then carried out for each fraction by using ethyl acetate:methanol (1:4) as mobile phase and silica gel as stationary phase.

The LC_{50} of those fractions were determined by using BSLT method. Before the test, 1% DMSO was added to improve solubility of the water-undissolved fractions. At this concentration, DMSO also produces no toxic effect on *Artemia salina*. As shown in table 4, LC_{50} scores of each fraction were 312.60 µg/mL; 52.45 µg/mL; 15.15 µg/mL; 24.54 µg/mL; and 85.18 µg/mL, respectively. These results showed that fraction C was the most active fraction than the others.

The mechanism by which the larvae are death during the test is associated with the role of flavonoids contained in bark of *P. celebicum* Miq. These metabolites are suspected to diminish the ability of larvae to eat. The metabolites act as stomach poisoning.

4. Cream preparation
Irritation test was initiated by shaving the back hair of the rabbits (*Oryctolagus cuniculus*). Four rabbits were used in this study. The area observed was as wide as 2.54 cm^2. As many as 0.5 gram of cream were applied to the observed area. One area had no treatment. After cream application, the treated areas were covered by sterile gauze. Twenty-four hours after cream application, the gauze was peeled off. The
irritation was observed based on Draize (1959). The treated areas were covered again with the same gauze and peeled off 72 hours after cream application. The observation of this irritation study was conducted based on 2 parameters i.e. erythema and edema. Erythema is caused by vasodilation, while edema is associated with plasma enlargement in irritative spots. Edema condition can be expedited by formation of fibrous tissues. The primary irritation index showed by cream of *P. celebicum* Miq. extract was negative (IP = 0). These results confirmed that the cream was not associated with dangerous effect (Balsam, M.S., Sagarin, E., 1974).

### Table 5. Cream Irritation Test

| Samples | Observation Time | Rabbits | Erythema | Edema |
|---------|------------------|---------|----------|--------|
|         | 24 hours | 48 hours | 72 hours | 24 hours | 48 hours | 72 hours |
| Control (-) |         |         |          |         |         |          |
| I       | 0        | 0        | 0        | 0        | 0        | 0        |
| II      | 0        | 0        | 0        | 0        | 0        | 0        |
| III     | 0        | 0        | 0        | 0        | 0        | 0        |
| Primary irritation index = 0 |

| Cream  |         |         |          |         |         |          |
|--------|---------|---------|----------|---------|---------|----------|
| I      | 0        | 0        | 0        | 0        | 0        | 0        |
| II     | 0        | 0        | 0        | 0        | 0        | 0        |
| III    | 0        | 0        | 0        | 0        | 0        | 0        |
| Primary irritation index = 0 |

| Basis  |         |         |          |         |         |          |
|--------|---------|---------|----------|---------|---------|----------|
| I      | 0        | 0        | 0        | 0        | 0        | 0        |
| II     | 0        | 0        | 0        | 0        | 0        | 0        |
| III    | 0        | 0        | 0        | 0        | 0        | 0        |
| Primary irritation index = 0 |

5. **CONCLUSION**

Fraction C of methanol-dissolved extract of *P. celebicum* Miq. stem bark is the most toxic fraction among other tested fractions with LC$_{50}$ of 15.15 µg/mL. When being formulated, the cream produces no irritation. In the future, isolation, purification, and further identification of the active compounds of *P. celebicum* Miq. must be carried out.

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