Brine Shrimp Lethality Test on Aqueous Extract of *Caesalpinia Sappan* L.

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Artikel Penelitian

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Abstract: *Caesalpinia sappan L. is a plant in the Fabaceae family and has long been used as a medicinal plant. The wood of this plant contains active compounds including brazilin, flavonoids, tannins, alkaloids, saponins, and terpenoids, which have antioxidant, anti-inflammatory, antibacterial, antiviral, and anticancer properties. The purpose of this study was to find out the lethal concentration (LC_{50}) in the aqueous extract of C. sappan wood using the brine shrimp lethality test (BSLT). LC_{50} is the concentration value of the compound that causes up to 50% death in larvae of Artemia salina. This study used four concentration treatments of 1000 ppm, 500 ppm, 100 ppm, and 50 ppm, and negative control repeated three times. Each concentration and the control negative used 10 Artemia salina larvae. The larval mortality was observed after 24 hours of treatment. An LC_{50} value of 322.54 ppm indicates an aqueous extract of C. sappan. Therefore, it was moderately toxic in this category.*

Keywords: *Caesalpinia sappan extract, BSLT, Artemia salina, toxicity, LC_{50}*

Abstrak: *Caesalpinia sappan* L. merupakan tanaman dari famili Fabaceae dan telah lama digunakan sebagai tanaman obat. Kayu tanaman ini mengandung brazilin, flavonoid, tannin, alkaloid, saponin dan terpenoid yang mana memiliki aktivitas antioksidan, anti-inflamasi, antibakteri, antivirus dan antikaker. Tujuan Penelitian ini adalah untuk mengetahui *lethal concentration* (LC_{50}) ekstrak air dari kayu *C. sappan* menggunakan metode brine shrimp lethality test (BSLT). LC_{50} adalah harga konsentrasi suatu senyawa dimana menyebab 50% kematian larva *Artemia salina*. Pada Penelitian ini konsentrasi pengujian dilakukan dengan konsentrasi 1000 ppm, 500 ppm, 100 ppm, and 50 ppm dan control negatif. Sebanyak 10 larva digunakan untuk masing-masing konsentrasi sampel dan kontrol negatif. Mortalitas larva dihitung setelah 24 jam perlakuan. Harga LC50 yang diperoleh yaitu 322,54 ppm yang mana ini menunjukkan bahwa ekstrak air *C. sappan* termasuk kategori tingkat toksisitas sedang.

Kata Kunci: extract *Caesalpinia sappan*, BSLT, *Artemia salina*, toksisitas, LC_{50}

Introduction

Caesalpinia sappan L. is one type of plant that grows in Indonesia and is often used for wood. *C.sappan* L. is a plant from the Fabaceae family, commonly known as Sappan wood. The wood part of the *C.sappan L.* . is often used as a traditional drink because it has many health benefits (1). Many compounds in this wood, such as phenolics, saponins, flavonoids, tannins, and others, function as antioxidants, antiviral, antibacterial, anti-fungal, anti-inflammatory, and anticancer (2).

One method to determine the level of toxicity of compounds in plant extracts can be done using the Brine Shrimp Lethality Test (BSLT) method, which uses *Artemia salina* Leach larvae as the bioindicators (3). Toxicity is a term in toxicology that means the ability of a compound to cause damage or injury. This toxicity depends on the amount of the toxic compound that is absorbed (4). In the BSLT method, observations on the number of deaths that occurred in larvae were observed after being given plant extracts incubated for 1x24 hours. After getting the number of deaths from the larvae, the LC_{50} (Lethal Concentration) value was calculated, making the concentration of compounds in the plant extract causing death in the larvae of 50% of the population (5)(6).

There have been many studies using *Caesalpinia sappan* L. wood, and it was reported that *C.sappan L.* . wood has potential as an antioxidant, but there has been no research showing the safety of an aqueous extract of *Caesalpinia sappan* L. by knowing the LC_{50} value from this extract. Therefore, the safety of the aqueous extract concentration of *C. sappan* wood using the BSLT method was carried out as an initial study by knowing the LC_{50} value as an initial screening for the bioactivity of compounds from this extract.

Material and Methods

1. Plant Materials

C. sappan L. plants used in this study were obtained from Bantul, Jogjakarta, Indonesia, and determined beforehand at the Herbarium Bogoriense, Research and Development Center for Botany, Research and Development Center for Biology, LIPI Bogor for authentication. *C. sappan* plants determined is then sorted for parts of the wood, weighed as much as 1 kg, then dried and ground into a fine powder using a grinder.

The *C. sappan* plants wood powder was extracted using the maceration method. First, the powder was put into a maceration vessel and then soaked for three days using distilled water. At this immersion stage, stirring is carried out once a day so that the water permeates the entire surface of the powder and the concentration is evenly distributed. Next, the dregs from the powder were taken and soaked again in water to be macerated again using water solvent until the *C. sappan* wood solution was close to clear. Finally, the filtrate was collected and concentrated at a temperature of 45^{o}C using a rotary evaporator to produce an aqueous extract of *C. sappan* wood. The thick extract of *C. sappan* wood was obtained after the extract was put into a vaporizer cup and placed in an oven at 45^{o}C.

2. Brine Shrimp Lethality Test

Before the brine shrimp lethality test was carried out, the hatching of the larvae of *Artemia salina*, the test animal in this study, was carried out first in a plastic container previously divided into dark and light sections using styrofoam as a barrier. At the bottom edge of the styrofoam, a hole is made so that the larvae hatched out of the hole on the bottom edge of the styrofoam. Next, seawater is filled into the container until the hole in the styrofoam is submerged. Next, the dark part is covered by aluminium foil where 1 gram of *A. salina* Leach egg is inserted. Finally, the bright part of the container is illuminated in fluorescent lamps to stimulate hatching. After 24 hours, the eggs will hatch and form larvae and then be transferred to another container until the larvae are 48 hours old.

Preparation of the concentration of the extract to be tested was carried out after hatching *A. salina* larvae. First, the thick extract of *C. sappan* wood was weighed using an analytical balance until 4 grams. The extract was then put into an Erlenmeyer flask and then dissolved in 2 mL of DMSO and reacted with 98 mL of distilled water to reach a volume of 100 mL. Next, the solution was stirred using a hot plate stirrer until smooth, and a standard solution concentration of 4000

ppm was obtained. The standard solution was then diluted to obtain the desired concentration of the test solution.

The standard solution was first diluted to 2000 ppm. After that, each microplate was filled with a micropipette containing 1 mL of the test solution and a micropipette containing 1 mL of seawater to a final volume of 2 mL. The concentration in the microplate was halved to be 1000 ppm and 500 ppm because 1 mL of seawater was added. This study also made a solution that was diluted to 200 ppm, and then each microplate was filled with a micropipette containing 1 mL of the test solution and a micropipette containing 1 mL of seawater to a final volume of 2 mL. The concentration in the microplate was halved to be 100 ppm and 50 ppm because 1 mL of seawater was added. Ten larvae of *A. salina* were inserted in each microplate using a dropper. The negative control contained ten larvae of *A. salina* and 2 mL of seawater. This experiment was repeated three times to get accurate data. The number of dead larvae was counted on each microplate after the microplate was placed outside for 24 hours. Calculations were carried out by observing the larvae in a microplate. Observe the larvae with the help of a light and a magnifying glass. The larvae of *Artemia salina* Leach died when the larvae had no movement for a few seconds of observation.

3. Significance of Brine Shrimp Lethality Test

Toxicity in the compound in *C. sappan* can be tested using the brine shrimp lethality test, which measures toxicity in plant extracts on its bioindicator, *A. salina* larvae (3). The level of trust in 95% caused the brine shrimp lethality test to separate toxic substances in plant extracts as initial stages (7)(8).

The brine shrimp lethality test requires a small amount of test material and is done quickly (5). This method is widely used because compounds with certain biological activities often have toxic properties to *Artemia salina* Leach larvae. Therefore, as an initial test that does not take long and uncomplicated time, the brine shrimp lethality test can be done to determine a compound's biological activity *in vitro*. The larvae used as their bioindicators will be incubated for 1x24 hours after being given plant extracts that will be tested for toxicity. The LC_{50} (lethal concentration) value is calculated from the brine shrimp lethality test. LC_{50} is a large concentration of compounds or extracts that can turn off animal tests up to 50% (9)(10). The LC_{50} value is classified according to the Meyer or Clarkson toxicity category (9). Classification of LC_{50} values according to the Meyer and Clarkson toxicity category is shown in **Table 1** and **Table 2** below:

Table 1. LC_{50} value according to the Meyer toxicity category

Meyer Toxicity Category	
LC_{50} Value	**Category**
LC_{50} < 1000 ppm	Toxic
LC_{50} > 1000 ppm	Non-toxic

Table 2. LC_{50} value according to the Clarkson toxicity category

Clarkson Toxicity Category	
LC_{50} Value	**Category**
LC_{50} 0-100 ppm	Highly Toxic
LC_{50} 100-500 ppm	Medium Toxic
LC_{50} 500-1000 ppm	Low Toxic
LC_{50} > 1000 ppm	Non-toxic

A. salina larvae were used in the brine shrimp lethality test because compounds in plant extracts could be detected using these animals. In addition, the toxicity test on these animals also showed a correlation with anticancer activity. The National Cancer Institute (NCI) in the United States has proven a significant correlation between testing using Artemia and inhibition of tumour cell growth in humans in vitro to be used as a pre-screening test for anticancer drug research (11). After 24 hours, the survival rate of Nauplii was calculated, and the mortality percentage was determined using the equation:

$$\textbf{Percentage of deaths} = \frac{\textbf{Number of dead larvae}}{\textbf{Total Number of larvae}} \times \textbf{100\%}$$

After obtaining the percentage of mortality from *Artemia salina* Leach larvae, the LC_{50} value was calculated using the probit analysis method on SPSS 25.0.

Result

Prior to the brine shrimp lethality test, the standard solution of this extract was diluted so that the concentrations of the test solutions of 1000 ppm, 500 ppm, 100 ppm, and 50 ppm were obtained. In addition to testing the diluted solution, negative controls were also tested to determine if there was an influence from seawater or other factors other than the extract tested on 48-hour-old *A. salina* larvae.

In this study, the negative control and each concentration were given 10 Artemia salina Leach larvae. Brine shrimp lethality test was performed three times, and each concentration was repeated three times (triple) to obtain accurate data. Counting the number of dead larvae with *C. sappan* wood extract was started after 24 hours of intervention.

Table 3 shows that the effect of giving each concentration of aqueous extract of *C. sappan* wood was different in each group of *Artemia salina* Leach larvae. Based on **Table 3**, it can be seen that the most significant number of larval deaths was at a concentration of 1000 ppm, while the smallest number of deaths was found at a concentration of 50 ppm. An increase in the number of larval deaths coincided with an increase in the concentration of the tested extracts. In addition, mortality in larvae was not found in the negative control, so it can be concluded that mortality was only affected by the concentration of this extract.

The calculation of the LC_{50} value was carried out after obtaining the percentage value of mortality from larvae. The concentration of this extract influenced the mortality of larvae. The LC_{50} value was calculated using the SPSS 25.0 probit analysis method. Calculation of the LC_{50} value using the SPSS application to avoid human error. The results obtained using the SPSS 25.0 probit analysis method were 322.54 ppm. This value indicates that the concentration of aqueous extract of *C. sappan* wood at 322.54 ppm can kill *A. salina* larvae up to 50% of the population.

Table 3. Effect of each concentration of aqueous extract of *C. sappan* wood on *Artemia salina* larvae

Microplate	***Artemia salina* Leach Larvae Mortality Rate**				**Negative Control**
	Concentration (ppm)				
	1000	*500*	*100*	*50*	
1	10	5	6	3	0
2	8	7	7	2	0
3	8	6	1	2	0
Average	8,7	6	4,7	2,3	0
Mortality Percentage (%)	87%	60%	47%	23%	0

According to the study of (8), the LC_{50} value is classified according to the Meyer or Clarkson toxicity category. So, it is categorized as medium toxic according to the Clarkson toxicity category because the LC_{50} value in this extract is between 100-500 ppm and the toxic category according to the Meyer toxicity category. After all, the LC_{50} value is <1000 ppm.

Discussion

Research on the brine shrimp lethality test on *C. sappan* wood extract has also been carried out previously by (11). The solvent used by (12) was

methanol. In the study conducted by (11), the concentrations tested were ten ppm, 100 ppm, and 1000 ppm. In addition, tests on negative controls that functioned to determine other influences outside the test extract that caused the larvae to die were also carried out. The percentage value of larval mortality showed that the highest number of deaths was at a concentration of 1000 ppm, while the lowest number of deaths was at a concentration of 10 ppm, and in the negative control test, there was no larval death at all. The LC_{50} value in this study was calculated using the probit analysis method, and it was found that the LC_{50} value in the methanol extract of *C. sappan* wood was 493.04 ppm.

The difference in the LC_{50} value between the brine shrimp lethality test on *C. sappan* wood extract conducted by (12) and the research conducted by the author could occur due to the influence of biological factors such as differences in the location where the *C. sappan* plant grows, chemical factors such as different quality and quantity of active compounds, and also differences in solvents used (11). For example, the research conducted by (12) used the *C. sappan* plant from Sambas, West Kalimantan, while for the study conducted by the author, the same type of plant was obtained from Bantul, Jogjakarta. Differences in where *C. sappan* grows can cause differences in soil quality, weather, and humidity. In addition, in the research conducted by the author, the solvent used was water, while the solvent used in the research of (11) was methanol. The methanol solvent causes the active compound drawn to be different from the water solvent.

Conclusion

The LC_{50} value of the brine shrimp lethality test on the aqueous extract of *C.sappan L.* . wood using the probit analysis method is 322.542 ppm. According to the Clarkson category, the aqueous extract of *C. sappan* wood is included in the moderately toxic category because the LC_{50} value in the aqueous extract of *C. sappan* wood is between 100-500 ppm.

Therefore, for further research, it is advisable to continue with the acute toxicity test in vivo, subchronic toxicity test, and chronic toxicity test of the aqueous extract of *C. sappan* wood to determine the value of LD_{50} (lethal dose).

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