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LETHAL CONCENTRATION OF *Saussurea costus* WITH *BRINE SHRIMP LETHALITY TEST* METHOD

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ABSTRAK

Keamanan penggunaan ekstrak tanaman herbal sangat penting untuk pasien COVID-19 yang mengonsumsi setiap hari dengan tujuan meningkatkan imunitas dan mengatasi gejala penyakit. Salah satu tanaman herbal yang digunakan masyarakat dalam terapi COVID-19 yaitu Qusthul hindi *(Saussurea costus).* Masyarakat perlu mengetahui keanaman penggunaan obat herbal tersebut ketika mengonsumsi, oleh karena itu penting untuk dilakukan uji toksisitas dari ekstrak Qusthul hindi. Senyawa-senyawa metabolit sekunder yang ada pada ekstrak tumbuhan dapat diuji toksisitasnya secara in vitro menggunakan metode *Brine shrimp lethality test (BSLT*) menggunakan larva Artemia salina Leach untuk menentukan nilai LC_{50}. Metode BSLT merupakan salah satu metode yang digunakan untuk mengetahui aktivitas biologis pada ekstrak tumbuhan seperti aktivitas sitotoksis, fototoksis, inhibisi enzim, dan regulasi ion menggunakan larva udang *Artemia salina Leach* sebagai *bioindicator*. Metode BSLT dilakukan dengan cara mengamati tingkat kematian yang timbul pada larva udang *Artemia salina Leach* yang diberi ekstrak dari tumbuhan lalu diinkubasi selama 1x24 jam. Hasil yang diperoleh dari penelitian ini sebagai nilai LC_{50} (*Lethal Concentration*) sebesar 532,78 ppm. Nilai LC_{50} (*Lethal Concentration*) sebesar 532,78 ppm yang menandakan konsentrasi senyawa tersebut dapat menyebabkan kematian pada *Artemia salina Leach* sebanyak 50%.

Kata kunci: LC_{50}, *Saussurea costus.* Qusthul hindi, Brine Shrimp Lethality Test

ABSTRACT

The safety of using herbal plant extracts is essential for COVID-19 patients who consume herbal plants daily to increase immunity and overcome the symptoms of the disease. One of the herbal plants used by the community in COVID-19 therapy is Qusthul Hindi (Saussurea costus). The public needs to know the safety of these herbal remedies when consuming them. Therefore it is essential to conduct a toxicity test of Hindi Qusthul extract. Secondary metabolite compounds present in plant extracts can be tested for toxicity in vitro using the Brine shrimp lethality test (BSLT) method using Artemia salina Leach larvae to determine LC_{50} values. The BSLT method is used to determine biological activities in extract plants, such

as cytotoxic activity, phototoxic, enzyme inhibition, and ion regulation, using Artemia salina Leach shrimp larvae as bioindicators. The BSLT method is carried out by observing the mortality rate in Artemia salina Leach shrimp larvae which are given extracts from plants and then incubated for 1x24 hours. The results obtained from this study as an LC_{50} (Lethal concentration) value of 532.78 ppm. LC_{50} (Lethal Concentration) value of 532.78 ppm indicates that the concentration of the compound can cause death in Artemia salina Leach by as much as 50%.

Keywords: *LC_{50}, Saussurea costus, Qusthul Hindi, Brine Shrimp Lethality Test*

INTRODUCTION

The World Health Organization (WHO) designates COVID-19 as a pandemic (WHO, 2020). COVID-19 is caused by SARS-CoV-2 (Severe Acute Respiratory Syndrome-related Coronavirus 2). COVID-19 is a highly infectious disease. The clinical manifestations of patients infected with SARS-CoV-2 are diverse. Some have no symptoms (asymptomatic), mild symptoms, or severe pneumonia with damage to organ function (Ge et al., 2020). The number of deaths due to COVID-19 in Indonesia is 143,592, with a Case Fatality Rate (CFR) of 3.4% (WHO, 2021). Previously, the CFR had touched 8.9% as of March 31, 2020, although in the end, it decreased to 3.4% as of November 4, 2021 (Khifzhon Azwar and Setiati, 2020). The CFR of Covid-19 in Indonesia is relatively high compared to the world CFR of 2.0% (Emerging Infection of the Ministry of Health of the Republic of Indonesia, 2021). The most Covid-19 subjects were women (56%), productive age with comorbid hypertension (29%) (Brisbane, 2022).

The current control of SARS-CoV-2 transmission is by vaccination following the emergency use authorization approval of the World Health Organization (WHO, 2022). Vaccines can effectively prevent the transmission of SARS-CoV-2 and reduce the risk of the progression of COVID-19 symptoms. Even so, individuals who have been fully vaccinated can still be infected with SARS-CoV-2, but the symptoms experienced will usually be milder (CDC, 2022). COVID-19 containment continues to be carried out with supportive, invasive, non-invasive oxygen treatments, antibiotics, antiparasitics, anti-inflammatory compounds, plasma healing and

antiretrovirals. Antiviruses used in Indonesia during the pandemic meet BPOM and Emergency Use Authorization (EUA) requirements. Several drugs are available, although the scientific evidence is debatable (Ardiani, 2022).

Alternative treatments for COVID-19 continue to be carried out until now, one of which uses natural ingredients. Recent research shows the potential of Indian wood or Qusthul Hindi (*Saussurea costus*). as an alternative treatment for COVID-19 (Jubayer, Kayshar and Mazumder, 2020). In addition to its good effectiveness for COVID-19 therapy, it should also be necessary to see safety in its use for an extended period because if it is effective and turns out to be toxic to the human body, it should be considered for its use at the right level or dosage. There have been many studies using *Saussurea costus*, and it is reported that the infused *Saussurea costus* has potential as a COVID-19 therapy, but no studies have shown the safety of using *Saussurea costus* by knowing the LC50 value of the Infuse *Saussurea costus*. Therefore, the safety of the concentration of infused *Saussurea costus* using the BSLT method is essential. The toxicity test carried out using the BSLT (Brine Shrimp Lethality Test) method is intended to determine a toxic compound's potential by knowing the toxicity level (Sepvina, 2022).

RESEARCH METHODS

Sample Preparation

In the preparation stage, before conducting a brine shrimp lethality test in the chamber is to divide the chamber into dark and light parts using styrofoam as a barrier. First, a hole is made at the styrofoam bottom edge if the larvae hatch from the hole. Next, the seawater is filled into the chamber until the hole in the styrofoam is submerged. Next, it is covered by aluminium foil in which 1 gram of artemia salina leach eggs is inserted on its dark part. Finally, the bright chamber illuminates by fluorescent lamps to stimulate hatching. After 24 hours, the eggs will hatch and form larvae and then be transferred to another chamber until the larvae are 48 hours old.

Sample Testing

Preparation of the infusion concentration *of Saussurea costus* to be tested with the larvae *of artemia salina leach* first, infused *of Saussurea costus* is made a standard solution concentration of 2,000 ppm. The standard solution is then diluted to obtain test solution concentrations of 1,000 ppm, 500 ppm, 100 ppm and 50 ppm. Each *microplate* is given an infusion test solution of *Saussurea costus* and seawater up to a final volume of 2 ml. The concentration in *the microplate* is 1,000 ppm, and 1 ml of seawater is added.

Ten larvae *of artemia salina* leach are inserted into each *microplate* using a pipette. The negative control contained ten larvae *of artemia salina* leach and 2 ml of seawater. In this study, replication was carried out three times.

The number of dead larvae is calculated on each *microplate* after the larvae *of artemia salina* leach are placed on *the microplate* for 24 hours. The calculation is carried out by observing the larvae in *the microplate*. First, observe the larvae with the help of light and a magnifying glass. The dead *larva of artemia salina* leach is when the larva has no movement for a few seconds of observation.

Sample Assessment

Compounds toxicity in *Saussurea costus* can be tested using *the brine shrimp lethality test*, which measures toxicity in plant extracts in its bioindicators are larvae *of artemia salina* leach (Veni, T and Pushpanathan, T., 2014). The confidence level of 95% causes *the brine shrimp lethality test* to separate toxic substances in plant extracts as an initial stage (Soliha *et al.,* 2019).

The brine shrimp lethality test requires a small amount of test material and is carried out quickly (Liukonas A. et al., 2019). This method is widely used because compounds with a specific biological activity often have toxic properties for the larvae *of artemia salina* leach. Therefore, as a preliminary test that does not take long and is not complicated, *a brine shrimp lethality test* can be carried out to determine the biological activity of the compound in vitro. The larvae used as bioindicators will be incubated for 1x24 hours after being given a plant extract that will be

tested for toxicity. The lethality test calculates the LC_{50} (lethal concentration) from the *brine shrimp lethality test*. LC_{50} contains several compounds or extracts that can kill test animals by up to 50%(Azizah,n., et al.,2018). Therefore, LC_{50} is classified according to *Meyer or Clarkson toxicity category* (Mshelia E et al.,2016). The classification of LC_{50} according to *Meyer and Clarkson toxicity category* is shown in table 1 and table 2 below:

Table 1. LC_{50} based on *Meyer toxicity category*

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

Table 2. LC_{50} based on *Clarkson toxicity category*

Clarkson Toxicity Category	
Rated LC_{50}	**Category**
LC_{50} 0-100 ppm	High Toxicity
LC_{50} 100-500 ppm	Moderate Toxicity
LC_{50} 500-1000 ppm	Low Toxicity
LC_{50} > 1000 ppm	Non-Toxic

The larvae *of artemia salina* leach were used in deadly tests of saltwater shrimp because the compounds in the plant extract can be detected using these animals. In addition, toxicity tests in these animals also showed a correlation with anti-cancer 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 for a pre-screening test for anti-cancer drug research (Anderson L *et al.*, 2018). After 24 hours, the survival rate of the larvae is calculated, and the percentage of death is determined using the equation:

$$\textbf{Percentage of death} = \frac{\textbf{Number of Dead Larvae}}{\textbf{Total Number of Larvae}} \times \mathbf{100}\%$$

After obtaining the percentage of mortality from the larvae of *Artemia salina* Leach, the LC_{50} was calculated using the probit analysis method at SPSS 22.0

RESULT AND DISCUSSION

Results

Before the brine shrimp lethality test, a standard solution of this extract is prepared so that the concentration of the Saussurea costus test solution is obtained 1,000 ppm, 500 ppm, 100 ppm and 50 ppm. In addition to testing Saussurea costus extract, negative controls were also tested to determine

whether there was any influence from seawater or other factors other than Saussurea costus extract tested at 48 hours of Artemia salina Leanach larvae.

In this study, on negative control and each concentration of the test solution were administered, ten larvae of Artemia salina Leach. The brine shrimp lethality test was performed by replication three times, and each concentration was repeated three times (three times) to obtain accurate data. After that, the number of dead larvae tested for *Saussurea costus* extract was calculated 24 hours after the intervention.

Table 3 shows that the effect of administering each concentration of *Saussurea costus* extract differs in each Artemia salina Leach larvae group. Table 3 shows that the most significant number of larval deaths is at a concentration of 1. 000 ppm, while the smallest number of deaths was found at 50 ppm. The increase in larval deaths was in line with the increased concentration of *Saussurea costus* extract tested. In addition, mortality in larvae is not found in negative controls, so it can be concluded that mortality is affected only by the concentration of *Saussurea costus* extract.

Table 3. Effect of Any Concentration of *Saussurea costus* Extract Against Larvae *Artemia Salina* Leach

Micro-plate	**Average Number of Deaths of *Artemia salina* Leach**				**Negative Control**
	Concentration (ppm)				
	1000	500	100	50	
1	7	8	2	0	0
2	8	6	3	1	0
3	8	5	2	0	0
Average	7,6	6,3	2,3	0,3	0
%Death	76%	63%	23%	3 %	0%

The calculation of the LC_{50} value is carried out after obtaining the value of the percentage of death of the larvae of *Artemia salina* Leach. This concentration of *Saussurea costus* extract affects the death of larvae. The LC value of 50 is calculated using the SPSS 2 2.0 probit analysis method to avoid human error. The result obtained using the SPSS 2 2.0 probit analysis method is 532. 78 ppm. This value indicates that the concentration of *Saussurea costus* extract is 532. 78 ppm can kill *Artemia salina* Leach larvae to 50% of the population. According to the study (Mshelia E et al.,2016), the LC_{50} is classified according to *the Meyer* or *Clarkson*

toxicity category. So, it is categorized as low *toxicity according to the Clarkson* toxicity category because the LC value of 50 in *Saussurea costus* extract is between 500-1.00 0 ppm, and the toxicity category according to *the Meyer toxicity category* LC value of 50 is toxic due to the LC value of $_{50}$ in *Saussurea costus* extract <1000 ppm.

Discussion

Indian wood, or Saussurea costus, is an herbal plant widely found in India. The genus Saussurea consists of about 300 species in the world, of which 61 species are found in India. The most famous species of Saussurea is S. costus. This plant (its roots and essential oils) has been widely used in traditional medicine since ancient times to treat various diseases, namely asthma, inflammatory diseases, ulcers and other stomach problems. *Saussurea costus* has a typical growth of 1–2 m by 1 m (3.3 ft) broad. It has irregular pattern lyrate (melira) leaves with an average length of 0.50–1.25 m. The plant's roots are sturdy, 8-12 cm long, with a diameter of 1-3 cm (Ali and Venkatesalu, 2022). (Nadda *et al*., 2020) Unfortunately, there have been no studies on the safety of *Saussurea costus* extract, so the LC_{50} research data is essential. The toxicity test of the compound is carried out by the Brine Shrimp Lethality Test (BSLT) method. This method is used as an initial screening for the search for compounds that have the potential to be anticancer (Husnul, 2019).

Based on the results of the *brine shrimp lethality* test, it was found that the percentage of larval deaths 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 a 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} in The *Saussurea costus* extract was 532. 78 ppm, meaning at a dose of *Saussurea costus* extract concentration of 532. 78 ppm can kill 50% of *Artemia salina* Legach larvae.

Saussurea costus root has long been used in China, India, and Japan as a traditional medicine to treat asthma, viral infections, arthritis, paralysis, diarrhoea, indigestion, cholecystitis,

cough, and hepatitis. The roots of such plants contain the active compounds costunolide, dehydrocostus lactone, cynaropicrin, and flavonoids. Various studies have shown that the three active compounds have anti-inflammatory, hepatoprotective, anti-ulcer, anti-cancer, anti-convulsant, antimicrobial, and many more activities. (Ali and Venkatesalu, 2022).

Root powder extract, oil and constituents isolated from *Saussurea costus* such as Costunolids, *Saussureamine* B and Dehydrocostus lactone in gastric ulceration and lesions; inhibition of antigen-induced degranulation, mucin production, immune cell count, eosinophils, and expression and secretion of Th2 cytokines (IL-4 and IL-13) in asthma. Cynaropicrin, Alantolactone, Caryophyllene, and costic acid were also reported to inhibit pro-inflammatory mediators. Also, sesquiterpene lactones profoundly affect the inhibition of inflammatory stages and cascades of apoptosis induced in cancer. Very little data on the safety and toxicity of plant parts needs to be scientifically evaluated. (Lee *et al.*, 2018) .

The lethal concentration in *Saussurea costus* extract occurs because, in *Saussurea costus* extract, there is a relatively high content of flavonoid compounds in a plant extract affecting the death of *Artemia salina* Leach shrimp larvae. Flavonoids can cause the rupture of cell membranes due to uncontrolled intake of Na^+ ions. The death of *Artemia salina* Leach shrimp larvae is because the OH^- group in flavonoids binds to the integral proteins of the cell membrane so that the active transport of Na^+ and K^+ is contained. The stopped active transport causes an uncontrolled intake of Na^+ ions into the cell so that the cell membrane breaks. The rupture of cell membranes leads to cell death (Sumihe *et al*., 2014).

CONCLUSION

The LC_{50} value of the brine shrimp lethality test on *Saussurea costus* extract using the probit analysis method was 532.78. Therefore, according to the Clarkson category, *Saussurea costus* extract belongs to the low toxicity category because the

LC_{50} value in *Saussurea costus* extract is between 500-1000 ppm. The higher the concentration level, the higher the toxicity produced (Nindiati, 2021).

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