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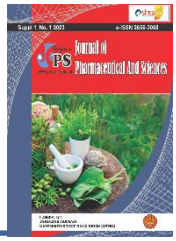

# Toxicity test of red palm (*Cyrtostachys renda* Blume.) using the brine shrimp lethality test (BSLT) method

# Uji toksisitas buah palem merah (*Cyrtostachys renda* Blume.) menggunakan metode *brine shrimp lethality test* (BSLT) method

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**ABSTRACT**

The red palm plant (*Cyrtostachys renda*) is a species of the Areca genus that grows widely in Jambi Province, making it an affordable plant to research for its medicinal properties. The Brine Shrimp Lethality Test (BSLT) is a toxicity test to screen for anticancer bioactive substances, with the test subject being Artemia salina Leach larvae, which are highly sensitive when exposed to toxic substances. The fruit and roots of C. renda were extracted using the maceration method by using a methanol solvent and partitioning using n-hexane, dichloromethane, ethyl acetate, and water. Based on the extract toxicity evaluation results, all extracts were toxic. Meanwhile, C.renda root dichloromethane extract had the highest toxic properties, with an $LC_{50}$ value of 43.42 ± 0.659 ppm. However, the positive control's toxicity, potassium dichromate, was more toxic, with an $LC_{50}$ value of 29.93 ± 0.668 ppm. The dichloromethane extract of C. renda roots can potentially be a poisonous agent. Further toxicity tests on cancer cells need to be carried out to be developed as an anticancer agent.

***Keywords:*** *Artemia salina; $LC_{50}$ value; Areca genus; crude extract; partitionates extract*

**ABSTRAK**

Tanaman palem merah (*Cyrtostachys renda*) merupakan salah satu spesies dari tanaman genus Areca yang tumbuh di Provinsi Jambi sehingga menjadi salah satu tanaman yang mudah didapat dan diteliti manfaatnya dalam bidang kesehatan. Brine Shrimp Lethality Test (BSLT) merupakan salah satu metode uji skrining toksisitas senyawa yang berpotensi sebagai antikanker menggunakan larva udang *Artemia salina* Leach yang sangat sensitif terhadap senyawa toksik. Metode d. ekstraksi buah dan akar C. renda menggunakan metode maserasi dengan pelarut methanol kemudian dilakukan partisi menggunakan pelarut n-heksan, diklorometan, etil asetat, dan air. Hasil penelitian ini menunjukkan bahwa seluruh ekstrak memiliki efek toksik. Ekstrak diklorometan akar C. renda memiliki nilai toksisitas yang paling tinggi dengan nilai $LC_{50}$ 43.42 ± 0.659 ppm. Akan tetapi, jika dibandingkan dengan kontrol positif yang digunakan yaitu kalium dikromat, memiliki efek jauh lebih toksik dengan nilai $LC_{50}$ 29.93 ± 0.668 ppm. Ekstrak diklorometan akar C.renda berpotensi menjadi agen toksisitas dan dapat dilakukan penelitian lebih lanjut terhadap sel kanker untuk dikembangkan sebagai antikanker.

***Kata Kunci:*** *Artemia salina; nilai LC50; genus Areca; ekstrak kasar; ekstrak partisi.*

## INTRODUCTION

The majority of anticancer medications currently are derived from plants with anticancer properties. Aside from its potential as an anticancer treatment, herbal plant therapy is one of the methods used for preventing the occurrence of multidrug resistance (MDR) because it has been suggested that using herbal plants is safer than using chemical drugs because they have fewer side effects (Mutiah et al., 2017). According to information from the 2020 Cancer Country Profile, out of Indonesia's 2019 population of 270,625,567, there were 348,809 new cases of cancer and 207,210 cancer-related deaths in 2018 (*Cancer Indonesia 2020 Country Profile*, 2020).

Cancer is a condition where cancer cells continue to develop uncontrollably due to faulty cell division. The cancer will then multiply and have the potential to spread to nearby tissue. Many researchers who have found plant species with anticancer potential have concentrated on species used in traditional medicine in developing nations. Plants are a significant supply of materials for a variety of therapeutic uses, making their utilization very promising.

The Simalungun Batak people of North Sumatra empirically use the red palm (Cyrtostachys renda) using the part of roots as a traditional medicinal that can treat fever, asthma, and even kidney disease (Silalahi et al., 2015). It is essential to examine C. renda for the presence of secondary metabolite chemicals and toxicity testing the plant; C. renda can be developed into a natural chemopreventive agent. In this study, phytochemical screening must be done to determine which secondary metabolites are present in red palm the presence of secondary metabolite chemicals in plants has biological activity, one of which is its cytotoxic properties against cancer cells. The Brine Shrimp Lethality Test (BSLT) is used to screen for toxicity at the beginning of the search for natural cytotoxic drugs against cancer cells. Using a bioindicator called Artemia salina Leach shrimp larvae, this method evaluates the cytotoxic effect or quantifies the toxicity capacities caused by plant extracts or bioactive chemicals on cells.

This evaluation is carried out to determine the Lethal Concentration 50 ($LC_{50}$) value during a 24-hour exposure to the test solution (Zulia et al., 2021). The quantity or concentration of an active substance at which 50% of Artemia salina Leach test larvae die due to that substance is the $LC_{50}$ value. This study aims to determine the possible toxicity of the secondary metabolites present in C. renda.

## METHODS

### Materials

The study used the following materials: dark red palm fruit and roots (Cyrtostachys renda Blume), which were obtained from the Regency Tebo in Jambi Province. DMSO (Sigma Aldrich) dissolved red palm fruit and root extract. Solvents used in extraction include methanol, ethyl acetate, dichloromethane, and n-hexane. The phytochemical screening reagents are Dragendorff reagent, Liebermann Burchard reagent, Mayer's reagent, Wagner's reagent, and several other components in between chloroform, distilled water, ammonia, acetic acid, $FeCl_3$, concentrated $H_2SO_4$, and magnesium powder. Toxicity testing materials include Artemia salina eggs, mineral salts, and potassium dichromate as a positive control.

### Preparation of Extract

Five hundred grams of red palm fruit and root powder were put in a maceration jar and immersed in 5 liters of methanol for 48 hours, stirring as often as possible. The macerate is then filtered using a paper filter. The remaining maceration residue is remacerated with the same solvent until clear maceration is achieved. The macerate is then collected and evaporated using a rotary evaporator at temperatures 50$^{o}$C until a thick methanol extract is obtained.

A separating funnel was used to do liquid-liquid extraction of a thick methanol extract of red palm roots. N-hexane, ethyl acetate, dichloromethane, and water were utilized as solvents. Thirty grams of palm root methanol extract red is dissolved in methanol, then partitioned with water and n-hexane in a separate funnel with a solvent ratio of water and n-hexane, precisely (1:1). Shake it with an occasional tap on the separated funnel to expel the gas created during the shaking procedure. The shaken liquid is then funneled and allowed to split into two layers: the water phase (bottom layer) and the n-hexane phase (top layer). The n-Hexane Phase was then separated by a layer of water. The water layer is then partitioned once more with n-hexane until the solution is clear. All of the collected n-hexane

phases are then mixed into evaporation using a rotary evaporator. The water produced is then partitioned into layers using the same approach, namely using a dichloromethane and ethyl acetate solvent. When partitioned with dichloromethane, it forms two layers: the phase dichloromethane (bottom layer) and the water phase (water layer). When it is partitioned with ethyl acetate solvent, two layers form the water phase (bottom layer) and the ethyl acetate phase (top layer). Using a rotary evaporator, concentrated n-hexane, dichloromethane, ethyl acetate, and water extract will be obtained (Figure 1).

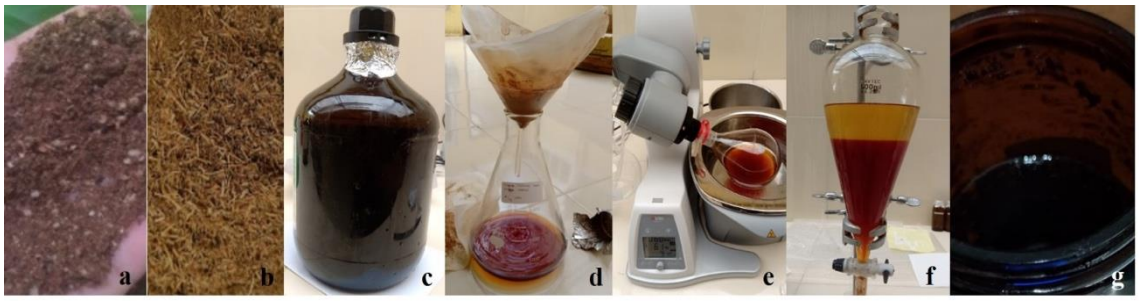


**Figure 1.** Cyrtostachys renda fruit and roots extraction process (a) C. renda fruit powder; (b) C. renda root powder; (c) maceration process; (d) filtration process; (e) evaporation process; (f) process of liquid-liquid separation; (g) extract.

## Characteristics of the Extract

The extract's specific and non-specific parameters characterized it. Identity and organoleptic are examples of particular parameters, while water content, ash content, and phytochemical screening are examples of non-specific parameters. The extract characterization process refers to FHI (Anonim, 2017).

## Phytochemical Screening

Making a stock solution with water and chloroform acetate is used to perform phytochemical screening assays. A total of 0.25 mg of sample was added to the tube reaction, followed by the addition of 5 mL of chloroform acetate and 5 mL of distilled water. The mixture was then shaken until homogenous. The mixture is allowed to separate after it has reached homogeneity until it forms two layers, one of water and the other of chloroform. Layer Alkaloids, terpenoids, and steroids are screened for chloroform acetate, while flavonoids and saponins are screened using the water layer. The process of identifying secondary metabolite in extracts refers to Harborne(Harborne, 1987). Alkaloids. The sample was mixed with 0.05 N chloroform and a few drops of H2SO4 shaken gently. Added with Dragendroff, Wagner, and Mayer reagents before. White mist to white lumps indicated the presence of alkaloids (Martinus, 2020). Flavonoids. The sample is heated in a bath until it boils, and Mg powder and a few drops of concentrated HCl are added (Harborne, 1987). Saponins. In a test tube with 2 mL of hot distilled water already in it, 2 mL of sample was added. Shaking rapidly for 15 seconds when it has cooled, then leave it up for 15 minutes. Positive if the foam that has been produced is stable(Ahmed et al., 2022). Tannin. 2 mL of sample was added with 2 mL of distilled water and 10% $FeCl_3$. Positive if a greenish gray or dark blue-black color forms. Steroids/Terpenoids. $H_2SO_4$ solution was concentrated and added to the sample. If a reddish-orange ring forms, the substance is terpenoid positive; nevertheless, if a blue-green color results from the addition of 2 drops each of concentrated sulfuric acid and anhydrous acetic acid, the substance is steroid positive (Muharrami et al., 2020)

## Toxicity Evaluation uses the Brine Shrimp Lethality Test (BSLT)

The toxicity evaluation process with the BSLT method refers to Sarah et al. (Sarah et al., 2017). 3000 mL of water is placed in the aquarium to cultivate Artemia salina. After that, add 30 grams of iodine-free salt to a watered-down aquarium and stir until homogeneous. The air pump's air outlet end is positioned near the aquarium's base to maintain sufficient aeration. Then Stir well after you've added enough Artemia salina eggs to the

aquarium's surface. A 50-watt light bulb should be turned on and positioned close to the tank. After 20 to 24 hours, the larvae will hatch, gather, and separate the larvae from the empty eggs. After that, use a Pasteur pipette (dropper pipette) to transfer ten larvae into the vial. Evaluation for toxicity requires exposing larvae to various extract concentrations and observing the results of partitioning. After 24 hours, count the number of alive larvae and the proportion of deaths. This Cytotoxicity test's death endpoint is indicated by the absence of monitored forward movement for around 30 seconds after observation. The following formula was used to determine the percentage of larval death for the control and each concentration (Lalat al 201life6):

$$\% \text{ death} = \frac{\text{Total Dead Larvae}}{\text{Number of dead larvae} + \text{Number of live larvae}} \text{ x } 100\%$$

## RESULT AND DISCUSSION

The red palm used in this study is Cyrtostachys renda Blume., a member of the Arecaceae family, according to the findings of determination number 48/HB/04/2022 carried out at the Jatinangor Herbarium, Plant Taxonomy Laboratory, Biology Department, FMIPA, Padjadjaran University. The yield of the red palm fruit and roots Simplicia after preparation and making was 59.38 and 25%, while the yield of the extract after maceration was 13.97% and 11.89%. The outcomes of the specific and non-specific parameter characteristics present in the extract will be shown in Table 1.

If the water content is greater than 30%, the extract still retains a significant amount of water and is said to have a high water content. This could be due to the drying process not being completed sufficiently. Water is a medium for the growth of microbes and also a medium for enzymatic processes that might break down the active chemicals; therefore, if an extract's water content does not match the criteria, it could result in microbial growth (Supriningrum et al., 2019). Until the remaining extract is formed after annealing, the ash content can be used to present an overview of the internal and external mineral content as well as the inorganic content arising from the initial procedure. The amount of minerals in an extract increases as it gets higher ash concentration. These minerals can exist as inorganic salts (e.g., phosphates, carbonates, chlorides, sulfate nitrates, and alkali metals), organic salts (e.g., as salts of malic acid, oxalate and pectate), or minerals that have been transformed into complex organic compound (Supriningrum et al., 2019). Table 1 shows the characteristics of the water and ash content of fruit and root extract.

**Table 1.** Characteristics of Cyrtostachys renda extract

| Parameters | Cyrtostachys renda Extract Plant Parts | |
|---|---|---|
| | **Fruit Extract** | **Root Extract** |
| Specific Characteristics of Cyrtostachys Renda Extract | | |
| Color | Blackish red | Blackish red |
| Aroma | Typically, red palm aroma | Naturally, the red palm aroma |
| Flavor | Chelate | Chelate |
| Extract form | Thick | Thick |
| Non-specific Characteristics of Cyrtostachys renda Extract | | |
| Water content | 27.33% ±1.08 | 23% ±0.57 |
| Ash content | 2.3% ±0.41 | 4% ±0.76 |

Based on the phytochemical screening in Table 2, it is possible to determine that the extract and each partition are positive for having alkaloids using at least two types of reagents; however, in the partition, only one test reagent demonstrates a positive result for alkaloids. The formation of a precipitate indicates positive results in the alkaloid test; for reagents Dragendroff's, this is characterized by an orange-brown or brick-red precipitate (Yanti & Vera, 2019). The resulting formation of a white, yellow, or brown precipitate distinguishes Mayer's reagent from Wagner's reagent (Syahadat et al., 2020).

**Table 2**. Secondary metabolites of Cyrtostachys renda in fruit and root extracts

| Secondary Metabolites | Fruit Extract | | | | | Root Extract | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Crude | n-hexane | DCM | Ethyl Acetate | Water | Crude | n-hexane | DCM | Ethyl Acetate | Water |
| Alkaloids | | | | | | | | | | |
| - Dragendorff | + | - | + | + | + | - | + | + | + | + |
| - Mayer | + | + | + | + | + | + | + | + | + | + |
| - Wagner | + | - | + | + | + | + | - | + | + | + |
| Flavonoids | + | + | - | + | + | + | + | + | + | + |
| Saponins | + | - | - | - | + | + | - | - | - | + |
| Tannins | + | - | - | + | - | + | - | - | - | + |
| Steroids | - | - | - | - | - | + | - | - | - | - |
| Terpenoids | + | - | - | - | - | + | + | - | - | - |

A red, yellow, or orange color change reaction during the flavonoid testing process showed that all extracts and each partition contained flavonoids (Yanti & Vera, 2019). Only crude extract and water partitions from tannin tests yielded positive results, indicated by a bluish-black or blackish-green color change when reacted with $FeCl_3$ solution (Muthmainnah B, 2019). The ethyl acetate partition of the saponin test was characterized by the formation of foam 1–10 cm high in no less than 10 minutes, whereas the crude extract only showed more robust positive results. A drop of 2 N HCl was added, but it did not make the foam that had formed remain off. In the terpenoid test, positive results are only found in the crude extract, which is marked by the formation of a red ring indicating the presence of terpenoids when added with $H_2SO_4$. The foam formed is not affected by acid, so when 2 N HCl is added, the foam formed will remain stable and not disappear.

The $LC_{50}$ value following exposure to the extract solution and partition results with each concentration, which is 5, 10, 20, 40, and 80 ppm for 24 hours, are used to determine the acute toxicity of a compound or extract using 48-hour-old Artemia salina Leach larvae as test animals in the BSLT. The crude extract and all partition results have the potential to be developed as anticancer, according to the BSLT test results (Table 3), but the dichloromethane partition of root C. renda is the most active partition and has the lowest $LC_{50}$ value of 43.42±0.659 ppm, followed by the ethyl acetate partition with an $LC_{50}$ value 46.01±0.518 ppm. Meanwhile, the C. renda fruit extract sample that has the highest toxicity is ethyl acetate extract with an $LC_{50}$ value of 76.25±0.745 ppm. The dichloromethane partition is dangerous based on the concentration of toxicity observed because its $LC_{50}$ value is less than 1000 ppm. This shows the potential for the ethyl acetate partition to be developed as an anticancer. According to Meyer et al. (1982), a substance that causes the death of 50% of artemia larvae within 24 hours at an $LC_{50}$ concentration of 1000 ppm is toxic when tested using the BSLT method. These findings suggest that the secondary metabolites in the C. renda extract may have anticancer properties.

The components in the extract or partitions of red palm root have a poisonous or gastrointestinal effect on the body as their mode of action. Therefore, the digestive systems of shrimp larvae will be influenced if these substances enter their bodies. Additionally, this substance can block taste receptors in the larvae's mouth area, preventing the larvae from receiving taste sensations and from recognizing their food, ultimately leading to their starved death. Through the mouth of A. salina, toxic substances in the extract or partition can enter and be absorbed into the digestive system through the cell membrane. Following the distribution of poisonous substances into A. Salina is the body through absorption; the metabolic reaction causes damage. The body of A. salina has a fundamental anatomical structure at the nauplii stage, comprising layers of skin, mouth, antennae, and digestive tract.

Toxic substances spread widely throughout A. salina's body due to the sharp differential in concentration between the inside and outside the cell. 50% of A. salina die as a result of the resulting metabolic damage, which manifests themselves

rapidly and can be identified within 24 hours (Neves et al., 2017; Ntungwe N et al., 2020)

**Table 3.** The $LC_{50}$ values of Fruit and Root C. renda Extracts in the BSLT test. Percent mortality was reported as mean values ± SD and $LC_{50}$ was reported as mean values ± SEM of three independent assays.

| Sample | Type of Extract | Mortality values of A. salina (%) | | | | | $LC_{50}$ (ppm) |
|---|---|---|---|---|---|---|---|
| | | 5 | 10 | 20 | 40 | 80 | |
| Fruit extracts | Crude extract | 26±1.304 | 32±2.191 | 40±1.789 | 42±1.817 | 44±0.894 | 135.71±0.715 |
| | n-hexane | 28±1.871 | 32±0.548 | 34±0.894 | 42±1.517 | 42±1.140 | 248.06±0.534 |
| | Dichloromethane | 28±2.550 | 34±1.342 | 34±1.517 | 44±1.673 | 48±0.707 | 111.06±0.697 |
| | Ethyl acetate | 22±2.074 | 32±2.608 | 38±1.414 | 42±1.140 | 50±1.095 | 76.25±0.745 |
| | Aqueous | 34±1.949 | 38±2.345 | 40±1.095 | 44±0.894 | 48±1.000 | 133.93±0.652 |
| Root extracts | Crude extract | 2±1.304 | 10±2.000 | 14±2.302 | 28±2.387 | 12±1.304 | 92.43±0.832 |
| | n-hexane | 8±1.095 | 0±1.924 | 20±1.304 | 12±1.095 | 10±0.000 | 134.02±0.485 |
| | Dichloromethane | 0±1.414 | 14±0.894 | 2±1.643 | 16±1.140 | 28±2.280 | 43.42±0.659 |
| | Ethyl acetate | 6±1.140 | 14±1.140 | 32±0.447 | 42±2.168 | 66±0.894 | 46.01±0.518 |
| | Aqueous | 16±1.517 | 2±0.837 | 14±1.342 | 10±1.871 | 24±0.894 | 285.19±0.578 |
| Potassium chromate | | 10±1.304 | 28±1.871 | 38±1.581 | 48±1.871 | 80±0.837 | 29.93±0.668 |

## CONCLUSION

A. salina is intolerant of the entire extract and partitioning of C. renda fruit and roots. Dichloromethane root extract, with an $LC_{50}$ value of 43.42±0.659 ppm, is the extract with the most potent toxic effects. This activity is no longer harmful compared to the toxicity of potassium dichromate, which was used as a positive control and has an LC50 value of 29.93±0.668 ppm.


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