Toxicity Test LC$_{50}$ of Pineung Nyen Teusalee Seeds (*Areca catechu*) Extract by Brine Shrimp Lethality Test (BSLT) Method

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**Abstract.** Utility of natural ingredients as traditional medicines is growing with increasing in exploration of the types of natural ingredients that can be used as traditional medicines to prevent and treat a disease. However, the use of inappropriate levels can cause a risk of health problems. One of the ingredients used to treat diabetes is young areca nut which is known by the people of Aceh as Pineung Nyen. Pineung Nyen, which is consumed by the community, is not only in the raw form but also has experienced a process of smoking or disalee. Excessive consumption of Pineung Nyen can cause symptoms of poisoning. For this reason, an analysis is needed to determine the safe limits of concentration that can be consumed without causing side effects. Toxicity tests can be carried out in vivo by using the Brine Shrimp Lethality Test (BSLT) method which uses Artemia salina Leach shrimp larvae as test animals. From the phytochemical test results, it was found that Pineung Nyen Teusalee methanol extract contained flavonoids, tannins and saponins. In addition, the methanol extract of Pineung Nyen Teusalee is toxic with an LC$_{50}$ value of 347.86 mg / L.

**Keywords:** Pineung nyen teusalee, toxicity test; BSLT

1. **Introduction**

Betel nut or Areca (*Areca catechu*) has wide range of utility. One of which is used as traditional medicine because it has antidiabetic compounds [1]. In Aceh, young Areca seeds are used as traditional medicine, processed by fumigation (or disalee in Acehnese) known as Pineung Nyen Teusalee. Acehnese people usually use Pineung Nyen Teusalee to treat various disease, including diabetics.

Areca seed extract contains arecoline and able to improve glucose tolerance in mice induced by hyperglycemia as evidenced by decreasing in blood glucose level [2]. Besides having an antidiabetic effect, Areca sed is also reported to have carcinogenics effect due to the presence of arecoline compounds [3]. However, incorrect dosage of consumption can affect the consumers body.

Study on Areca seed extract had been carried out by Petrina et al. (2017) which showed that antioxidant activity in the highest LC$_{50}$ rate was about 5.481 ppm in methanol fraction [4]. However, there are not many data reported in the activity of the Pineung Nyen Teusalee (fumigated young Areca) methanolic extract. It needs to be supported by the scientific information about its efficacy and side effect caused.
The use of any compounds including natural compounds has potential to be toxic depending on its dosage threshold in the body. Therefore, more study is needed to understand the safety standard of certain commodity by detecting its acute toxic to determine the LC$_{50}$ rate, various toxic symptoms, the toxic spectrum and death effect [5].

The aim of this study was to understand the content of polyphenol compounds and to prove the presence or absence the toxicity potential in the methanolic extract of Pineung Nyen Teusalee seeds by Brine Shrimp Lethality Test (BSLT) method. The BSLT method was chosen based on the preliminary test of the toxicity of an extract or compound that has the advantage of being simple, fast, cheap and reliable.

2. Methods

2.1. Time and location
Research was done at Basic Laboratory and Laboratory of Agriculture Faculty Teuku Umar University about 6 months.

2.2. Type of research
This research was an experimental study with a Post Test-Only Control Group Design approach. Pineung Nyen Teusalee extract was treated to the larva of *Artemia salina* Leach.

2.3. Sample and population
The population of this research was *Artemia salina* Leach larvae. The sample used consisted of two criterias, which was Inclusion Criteria (the larvae of *Artemia salina* L was 48 hours old as a sample test and it did not show any anatomically deformation under the macroscopic observation) and exclusion criteria (*Artemia salina* Leach larvae did not show any activity or movement before treatment).

Ten *Artemia salina* Leach larvae were used in each group of treatment. This study consisted of 5 treatments and 5 replications for each group, with the total number of larvae used is about 250 larvaees. Sample was prepared by Simple Random Sampling Method. The type of *Artemia salina* Leach larvae were homogen and prepared by the same preparation method. It means that each larva had the same opportunity to be selected as sample.

2.4. Variable of research

2.4.1 Independent variable
Independent variable of this research was Pineung Nyen Teusalee seed extract with 100% concentration. Extract was obtained by using maceration method using 70% methanol as solvent. Extract was then used in toxicity test to *Artemia salina* Leach larvae with Brine Shrimp Lethality Test (BST) method. After 24 h treatment, the mortality of larvae at 50% was used as indicator of LC$_{50}$ value determination.

2.4.2 Dependent variable
The toxicity potential of Pineung Nyen Teusalee seed extract to *Artemia salina* Leach larvae would be the dependent variable. It would be declared as toxic if the value of LC$_{50}$ was < 1000 µg/ml. Mortality criteria of *Artemia salina* Leach larvae was determined if *Artemia salina* Leach larvae did not show any movement for a few seconds of observation.

2.5. Tools and materials
Tools used in this research were beaker glass, knife, analytical balance, pipette, spatula, loop, vial, black flannel cloth, muslin cloth, waterbath, aquarium, aerator air pump, and light.

Materials used were Pineung Nyen Teusalee, methanol, aquadest, *Artemia salina* Leach larvae, yeast as larvae feed, and saline water.
2.6. Methods

2.6.1. Sample preparation (Pineung Nyen Teusalee seed extract) [6]

Pineung Nyen Teusalee seed extract as much as 1 kg was collected from Bireun, Aceh. Seed was washed, chopped and mashed until fine. Dry sample was made into powder and extracted with maceration method. Pineung Nyen Teusalee powder was soaked into 70% methanol for 24 h, filtered with muslin cloth. The filtered sample was soaked again into 70% methanol until perfectly extracted which was marked by the transparent color of solvent after 48 h.

The remaining solvent was evaporated using waterbath then air-dried until it formed concentrated extract in 100% concentration. Pineung Nyen Teusalee extract was weighed to obtain its pure extract weight. Preliminary test at concentration of 1%; 0.5%; 0.25% and 0.1% was done to determine the effective concentration to larvae mortality. Preliminary test result showed that the lowest concentration which causing mortality for almost all of the larvae was 0.1%. Therefore, the concentration for treatment was determined to be at 0.01%; 0.02%; 0.05% and 0.1%.

2.6.2. Phytochemical test

2.6.2.1. Flavonoid test

Flavonoid test was done refer to Harborne [7]. Extract was weighed at 50 mg, mixed with 100 mL hot water, boiled for 5 mins and filtered out. Magnesium powder at 0.05 mg was added into 5 ml filtrate, then added 1 ml concentrated HCl and mixed thoroughly. The forming magenta red color showed the presence of flavonoid.

2.6.2.2. Tannin test

Tannin test was done refer to Harborne [7]. Extract was weighed for 50 mg and dissolved in 2 mL water. Two drops of FeCl3 1% was added. The forming of blackish blue and blackish green color showed the presence of tannin.

2.6.2.3. Saponin test

Saponin test was done refer to Harborne [7]. Extract was weighed for 50 mg, dissolved in 10 mL water and mixed for 1 min. Then 2 drops of HCN 1N were added. The formed foam (stable for 7 mins) showed the presence of saponin.

2.6.3. Determination of treatment group

The selection of shrimp eggs was done by soaking the eggs into aquadest for 1 h. Good eggs would settle while the bad ones would float. The preservation of larvae was done by hatching the shrimp eggs at 48 h before treatment. Hatching was done by soaking the eggs into saline water and illuminating the part of the container that was not occupied by shrimp eggs with lamp. In this study, larvae divided into 5 groups of treatment randomly:

a) Control group: 10 larvae was treated with Pineung Nyen Teusalee extract at concentration 0 mg/L in the media

b) C1 group: 10 larvae was treated with Pineung Nyen Teusalee extract at concentration 50 mg/L in the media

c) C2 group: 10 larvae was treated with Pineung Nyen Teusalee extract at concentration 100 mg/L in the media

d) C3 group: 10 larvae was treated with Pineung Nyen Teusalee extract at concentration 500 mg/L in the media

e) C4 group: 10 larvae was treated with Pineung Nyen Teusalee extract at concentration 1000 mg/L in the media

2.6.4. LC50 Determination

Ten Artemia salina larvae was added into solution test at concentration 1000, 500, 250, 100, 50 mg/L and control. Each treatment was prepared at 5 replications and then compared to control. The observation of Artemia salina larvae was done after 24 h. The percentage of larvae mortality was calculated by using equation:
\[ \text{% Mortality} = \frac{\text{Number of dead larvae}}{\text{Number of tested larvae}} \times 100\% \quad (1) \]

Abbot’s formula was used if there was dead larvae at Control:

\[ \text{%Mortality} = \frac{\text{% mortality at treatment} - \text{% mortality at control}}{100 - \text{% mortality at control}} \times 100\% \quad (2) \]

2.6.5. Data collected
The primary data was collected from the number of dead larvae at 24 h after treatment in every concentration of Pineung Nyen Teusalee seed extract.

2.6.6. Data analysis
Data was arranged in table and graphic. The determination of Lc50 value was conducted by using SPSS.

3. Results and Discussion

3.1. Sample Preparation
Pineung Nyen Teusalee was peeled until the seed was obtained. The seeds were then mashed and sifted until the powder of Pineung Nyen Teusalee was obtained. The purpose of powder making was to enlarge the contact between the powder and solvent to speed up the extraction process [8] The powder was then extracted with methanol by using maceration method. Maceration method was used because it was easy to adopt and had smaller risk of damage and disintegration of natural extract [9]. The sample extraction was done 3 times to obtain the good separation results. After soaking for 3x24 h, 34.48 g extract from 50 g sample was obtained with 68.96 % yield. The use of methanol as solvent in maceration method was possible to produce the large amount of extract [10].

3.2. Phytochemical test
Phytochemical test of various fractions is shown in Table 1.

| Fraction     | Phytochemical test | Reagent   | Change with Reagents | Result |
|--------------|--------------------|-----------|----------------------|--------|
| Methanolic   | Flavonoid          | Mg-HCl    | Magenta red          | +      |
|              | Tanin              | FeCl3     | Blackish blue        | +      |
|              | Saponin            | Aquadest  | Foam formed          | +      |

3.2.1. Flavonoid test
Flavonoid test of Pineung Nyen Teusalee seed extract showed positive result to Mg-HCl by forming magenta red color solution. Mg and HCl reduced benzospiron nuclei in the flavonoid structure to form red color [11]. The addition of HCl in the flavonoid test was intended to hidrolyze the flavonoid into its aglycone, by hydrolyzing O-glycosyl. Glycosyl will be replaced by H+ from acids because of its electrophilic characteristic. Glycosides in the form of sugars commonly found are glucose, galactose and ramanose. Reduction with concentrated Mg and HCl produce red or orange complex compounds in flavonol, flavanon, flavanonol dan xantone [12].

3.2.2. Tanin test
Tannin test of Pineung Nyen Teusalee seed extract showed positive result to FeCl3 0.1% by forming blackish blue color solution. Tannin is amorphous compounds that produce acidic colloidal solution. Tannin forms compounds that are indigestible and insoluble in protein. This is the basis for the use of tannins in the leather tanning industry and for diarrhea medicine [13].

3.2.3. Saponin test
Saponin test of Pineung Nyen Teusalee seed extract showed positive result to aquadest by forming the consistent foam for 7 mins. The formation of foam was caused by the presence of glycoside groups that can form froth in water. Glycoside forms polar groups that are active on the surface. Saponin forms micel that look like foam when shaken with water [14].

3.3. Toxixity test by using BSLT method

LC50 toxicity test used *Artemia salina* Leach larvae assample from each tested fraction. It is because *Artemia salina* Leach larvae has high sensitivity to the changes in environmental condition and contamination of chemicals in the environment.

The number of dead larvae from each fraction was counted by using SPSS analysis probit. LC50 is the value that shows the concentration of toxic compounds causing organism mortality up to 50%. LC50 focuses on the total mortality of tested animal, rather that the specific damage of the organs. Therefore, the LC50 is used in the short-term test, because *Artemia salina* Leach larvae has simple digestion system [15].

Toxixity test to the larvae mortality showed the LC50 value was about 347,86 mg/L (Figure 1). LC50 value of showed that the extract caused the mortality of the larvae up to 50%. Based on LC50 value, methanolic extract of Pineung Nyen Teusalee was toxic because it was lower than 1000 mg/L. Meyer *et al* (1982) stated that the toxixity level ot the extract was categorized as follows: LC50 ≤ 30 mg/L = Very toxic; LC50 ≤ 1.000 mg/L = Toxic; LC50 > 1.000 mg/L = Non toxic.

![Figure 1. Toxixity of Pineung Nyen Teusalee Extract to *Artemia salina* Leach Larvae](image)

The highest mortality rate of *Artemia salina* larvae up to 100% was obtained from the maximum concentration used. The mortality rate of larvae was not only caused by the concentration of the extract given against *Artemia salina* larvae. However, it was also caused by the chemical interactions of the other secondary metabolites, such as triterpenes and polyphenols which work synergistically in one another. Areca seed contains polyphenol compounds, such as flavonoid dan tanin [16]. Polyphenol compound affects the mortality rate of *Artemia salina* larvae. Phenol can act as toxin for plasma at high concentrations by damaging the cell wall system and collecting proteins in cell, while it can act in inhibiting the multiplication of enzymes in vitro at low concentration [17].

4. Conclusion

Phytochemical test of Pineung Nyen Teusalee methanolic extract with maceration method showed it had phenolic compound, such as flavonoid, tannin and saponin. Pineung Nyen Teusalee methanolic extract had toxicity shown by low LC50 value about 347,86 mg/L, with 100% mortality to *Artemia salina* at concentration 1.000 mg/L.

Acknowledgements
The author would like to thank The Ministry of Higher Education, Technology and Research (Kemenristekdikti) for funding (Research for Beginner Lecturer), LPPM-PM and Agricultural Faculty of Teuku Umar University for support.

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