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Cytotoxic Activity Test of Ethanolic Extract Of Berenuk Fruit (*Crescentia Cujete* L.) On *Artemia Salina* Leach Shrimp Larva Using Brine Shrimp Lethality Test (BSLT) Method

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ABSTRACT

Introduction. Cancer is one of the leading causes of human death in the world. One of the therapies given to cancer patients is chemotherapy which has cytotoxic effects. One of the plants that have the same effect is the berenuk or calabash (*Crescentia cujete* L.) plant, from the family of *Bignoniaceae***,** which grows wild and is often referred to as a poisonous plant. However, some people traditionally use this plant as a medicinal plant such as for antifungal, anthelmintic, analgesic, and anti-inflammatory. This plant contains naphthoquinones as one of the cytotoxic chemical compounds and another metabolite secondary such as alkaloids, flavonoids, saponins, tannins, terpenoids, and anthraquinones. **Methods.** The BSLT (Brine Shrimp Lethality Test) method was used for this study. The powder of berenuk fruit flesh was extracted using ethanol. The obtained solvent was evaporated using a rotary evaporator to obtain a thick extract. The extract was tested on *A. salina* larva that has been prepared and the value of mortality was observed**. Results.** The result of the cytotoxic activity test of ethanolic extract of berenuk fruit on *A. salina* larva had the LC50 value of 529.386 ppm which is categorized as toxic. **Conclusion.** Extract ethanolic of berenuk fruit on A. salina had cytotoxic activity.

1. Introduction

One of the many diseases in the world that is the main cause of death is cancer. Cancer occurs due to the growth of abnormal cells that can damage body tissues. More than 40% of cancer deaths are caused by preventable cancer risk factors.[1] GLOBOCAN data (Global Burden of Cancer) states that the death rate that occurred worldwide was 10 million deaths with 19.3 million cases in 2020, while in Indonesia in the same year there were 234,511 deaths.[2] These treatments are very expensive and usually always cause side effects for the patient. The differences in the drugs used, the specific agents, individual responses, doses, duration of treatment, and the patient's health status affect the severity of the side effects of these drugs. Because this chemotherapeutic agent still has limitations such as resistance events or side effects, it needs to be developed again to find an effective and efficient chemotherapeutic agent.

Currently, the development of plants as medicine has been carried out by many researchers. One of the medicinal plants is berenuk (Crescentia Cujete L.). Berenuk, also known as bitter mojo, and its plant parts, namely the flesh, roots, leaves, and bark have been used for a long time as traditional medicine.[3] Berenuk fruit is green with a round shape with a diameter of up to 25 cm, the flesh of the berenuk contains white grains, and the shape of the seeds is small and flat white.[4] Berenuk fruit flesh is usually used traditionally by the community for anthelmintic, antifungal, analgesic, and urethritis.[5–7] Berenuk fruit also contains several secondary metabolites, namely alkaloids, phenols, terpenes, naphthoquinones, saponins, flavonoids, and tannins, anthraquinones, and cardenolide.[6,8–10] The nutritional content of berenuk fruit is carbohydrates and simple sugars such as sucrose, fructose, and galactose, protein, fiber, vitamins A, B, C, and E, and mineral content such as calcium, magnesium, zinc, potassium, sodium.[10–1210] The presence of chemical compounds in the form of secondary metabolites and nutrients makes Berenuk fruit can be used as a treatment.

The presence of cytotoxic activity contained in berenuk is caused by the presence of a chemical compound contained in it. Cytotoxicity is the ability of

a potential compound to induce cell death. A cytotoxic test is a standardized method used to determine whether a compound contains toxic substances and also to see the potential of a compound used as an anticancer.[13] The method that will be used for cytotoxic testing in this study is the Brine Shrimp Lethality Test (BSLT) method. This BSLT method is a method that uses marine animals, namely Artemia Salina shrimp larvae to screen for the toxicity of a plant extract. In this test, it is possible to describe the level of toxicity of an extract to Artemia salina Leach larvae by looking at the number of larval deaths caused by the test compound.[14] Based on the description above, this research will test the cytotoxic activity of the ethanol extract of berenuk fruit flesh using the BSLT method. This research hopes that data from phytochemical test results and data on the most potential LC50 (Lethal Concentration) value of the tested cytotoxic activity can be obtained.

2. Methods

Materials

The tools used are rotary evaporator, desiccator, calibrated vial, test tube, baker glass, Erlenmeyer, Artemia Salina Leach culture medium, lamp, filter paper, water bath, tube clamp, test tube rack, separating funnel, and container. larva hatching. The main ingredient used in this study was the fleshy part of the berenuk plant (*Crescentia Cujete L.*) in the form of young, green fruit obtained from community plantations in the Parungpanjang area, Bogor Regency, West Java. The test animal used was Artemia Salina Leach Shrimp Larva. The chemicals used in this study were 70% and 96% ethanol, distilled water, DMSO (dimethyl sulfoxide), chloroform, sulfuric acid, Meyer's reagent, anhydrous acetic acid, concentrated sulfuric acid, Dragendorph's reagent, Mg metal powder, HCl(p), FeCl3, benzene, and NaOH.

Preparation Of Sample

Prepare tools and materials needed for testing. Clean the flesh of the berenuk from impurities then chop it. After that, dry the flesh of the berenuk using an oven for 48 hours at a temperature of 70°C. After drying, puree the simplicia flesh until a smooth simplicia is obtained. The simplicity of dried berenuk fruit flesh was made into extract by maceration extraction method using 70% ethanol solvent for 3 days while stirring occasionally. After that, filter and soak the dregs again with 96% ethanol for 3 days. The third maceration was also soaked for 3 days using the same solvent. All filtrate was mixed and stirred evenly, then evaporated using a rotary evaporator until a thick extract was obtained.

Cytotoxicity testing of Berenuk Pulp (Crescentia Cujete L.) Extracts Using Brine Shrimp Lethality Test

In the cytotoxic test, the artemia larvae were hatched first to obtain shrimp larvae that were ready to be used for testing. The test itself consisted of six treatment groups, namely group 1 as a negative control that was not treated and group 2,3,4,5,6 as a positive control that was given treatment. Prepare vials, calibrate and label vials for the test solution with each concentration of 10 g/ml, 50 g/ml, 100 g/ml, 500 g/ml, 1000 g/ml, and one vial for control. The mother liquor for the test was prepared by dissolving 80 mg of extract in 8 ml of 96% ethanol, then shaking until dissolved. Take 5 l, 25 l, 50 l, 250 l, and 500 l of the mother liquor that has been prepared using a micropipette and put it into the vial. The vial containing the test solution is then evaporated in a desiccator until the solvent evaporates. Add 50 l of DMSO to the vial including the control vial to redissolve the sample. After that add 3.5 ml of seawater into all test vials. Enter the 10 larvae of Artemia Salina Leach that have been hatched into each vial including the control vial and then increase the volume to the calibration limit with seawater, then place it under the light for 24 hours. After 24 hours, then make observations and count the number of larvae that died as a result of the treatment that had been given. Perform 3 repetitions on each test. The number of existing larval deaths was calculated using the data analysis method, namely by performing statistical calculations using Probit Regression Analysis. This method is carried out by calculating the larval mortality so that it can produce the percentage of mortality which can then be seen in the probit table. From these data, it can be determined the LC50 value be entered into the regression equation.

The percentage of larval mortality by probit analysis can be obtained using the formula:

$$\text{Mortality (M)} = \frac{\text{Number of mortality larvae}}{\text{Number of larvae test}} \times 100\%$$

Description :

M = percentage (%) of mortality animal test.

3. Results

The results of the chemical examination of the flesh of the berenuk fruit (*Crescentia Cujete L.*) are shown in table 1. The results of the chemical examination showed that the flesh of the berenuk fruit (*Crescentia Cujete L.*) contained chemical compounds in the form of phenolic compounds, saponins, alkaloids, flavonoids, tannins, anthraquinones.

The results of the cytotoxic test of the berenuk fruit (*Crescentia Cujete L.*) flesh are shown in table 2.

Table 1. Phytochemical Test Results Berenuk Fruit Extract (Crescentia Cujete L.)

Test Type	Reagent	Description	Result
Phenol	Extract + Hot Aquadest + FeCl3 1%	Dark green, slab	(+) Positive
Saponin	Extract + Hot Aquadest	Foamy	(+) Positive
Flavonoid	Extract + Mg powder + Concentrated HCl	Red	(+) Positive
Alkaloid	Extract + HCl 2N + Dragendorf	Brown sediment	(+) Positive
Tannin	Extract + FeCl3 1%	Blackish green	(+) Positive
Anthraquinones	Extract + FeCl3 1% + HCl + Benzene + NaOH	Red	(+) Positive

Table 2. Result of Calculation of Mortality Percentage of Larvae of Artemia Salina Leach After Giving Berenuk Fruit Flesh Extract

Concentration (µg/ml)	Repetition	Animal Total	Number Of Mortality Animals	% Mortality
10	1	10	0	6,66%
	2	10	1	
	3	10	1	
50	1	10	1	6,66%
	2	10	1	
	3	10	0	
100	1	10	2	13,33%
	2	10	1	
	3	10	1	
500	1	10	4	43,33%
	2	10	3	
	3	10	6	
1000	1	10	10	100%
	2	10	10	
	3	10	10	

The results obtained show that higher percentage of larval mortality is caused by the higher concentration of extract used. Mortality concentrations of 10 g/ml and 50 g/ml get the same results (6.66%) with the same number of deaths. This can occur due to several factors such as testing process factor. When taking the ethanol extract using a micropipette with a concentration of 50 g/ml, there is a possibility not all of the extract solution entered the vial so that the total concentration was reduced.

4. Discussion

Phytochemical Test Results of Berenuk Fruit (Crescentia Cujete L.)

These results indicate that the flesh of the berenuk fruit has the potential to have cytotoxic activity. Judging from several studies of phytochemical tests on the flesh of the berenuk fruit that have been carried out previously, it can be seen that there are differences in the results of the phytochemical tests. Several factors can influence this occurrence, one of them is environmental factors. The difference in environmental conditions where a plant grows causes differences in the number and types of secondary metabolites contained in plants from one area to another. Differences in environmental conditions such as temperature and light intensity affect the yield of plant metabolites because the metabolic process of a plant will adjust the conditions in which the plant lives where the results of plant metabolism are used as protection by the plant itself.[15]

Cytotoxic Test Results Berenuk Fruit (Crescentia Cujete L.)

The test was carried out to determine the cytotoxic activity using the BSLT (Brine Shrimp Lethality Test) method which is a preliminary test to see the toxicity of the sample and using Artemia salina larvae as test animals.[16] There is a significant relationship between samples that are toxic to artemia larvae so that the test animals of artemia larvae can be used for toxicity testing, the relationship between samples that have toxic properties has a significant or significant role in larval mortality due to the presence of these toxic

properties. The number of larvae that have died is then counted respectively and the LC50 value is seen. This value is a value that shows the concentration of toxic compounds that can cause death up to 50% and focuses on the total death of the animals tested.[17,18]

Testing the cytotoxic activity of the fruit flesh using five concentrations, namely 10 g/ml, 50 g/ml, 100 g/ml, 500 g/ml, and 1000 g/ml which was then repeated three times (triple) so that the results obtained not biased. Then the number of test animals used as many as 10 tails per test to facilitate the process of calculating larvae so that the results obtained are accurate calculations.[8] The results of the number of deaths of Artemia Salina Leach larvae treated with the extract of the fruit flesh of the berenuk which were repeated three times (triple) can be seen in table 2.

Then the LC50 value was calculated using the probit regression analysis method and it can be seen the relationship between the concentration and the probit value of larval mortality.[18] This probit analysis is in the form of a relationship between the logarithmic value of the concentration of the toxic compound tested, namely berenuk fruit extract, and the probit value of the mortality percentage of the test animals, namely larvae.[19] The results of the calculation of the LC50 value that have been carried out have a value of 529.386 ppm. The toxicity level of an extract is categorized if the LC50 value 30 ppm = Very toxic, if the LC50 value 1,000 ppm = Toxic, and if the LC50 value 1,000 ppm = Non-toxic.[20] The LC50 value in this study showed that the extract showed larval mortality up to 50% because the resulting value was lower than 1,000 ppm and was categorized as having toxic properties. The presence of this toxic nature causes death in larval test animals.

Another study that has been conducted in the Philippines by Billacura et al. (2017) on the extract of berenuk fruit using several solvents, namely 95% ethanol, equates, ethyl acetate and hexane which was tested on shrimp larvae with concentrations of 10, 100 and 1000 ppm showed that the value of The LC50 produced with ethanol solvent is 0.529 ppm and equates solvent produces a value of 4.64 ppm, the value is less than 30 ppm, which means that the result is highly toxic.[5] Then Sagrin, et al (2019) in Malaysia also conducted a study on cytotoxic tests on berenuk fruit using the BSLT method using two types of solvents, namely equates and ethanol with concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15,625, 7,813, 3,907, and 1,953 g/mL which resulted in the LC50 value with distilled water being 38.74 ppm, 50% ethanol solvent at 133.15 ppm and 100% ethanol solvent at 292.17 ppm. The results obtained are less than 1000 ppm so it can be said to have toxic properties.[21] Another test was also carried out by Pastor PJB and Almadin FJF (2017) in the Philippines who conducted a test to see toxicity using the CAM Assay method with the results obtained in this study that there was no development in duck embryos with concentrations of 0.35 g/mL and 0, 47 g/mL which means it has antiangiogenic activity and is highly toxic at high concentrations.[22]

The use of solvents used in extracting simplicia can vary. One of them is in a study conducted by Sagrin, et al. (2019), extracts using equates as a solvent obtained a smaller LC50 value than those using ethanol as a solvent.[21] Then the research conducted by Billacura, et al (2017) using ethanol as a solvent obtained a smaller LC50 value than using equates as a solvent.[5] Therefore, the use of the solvent used in the extract will affect the LC50 value produced. In addition to the fruit, other parts of the berenuk fruit, namely leaves, seeds, bark, and root bark also have toxic properties.[8,23,24] Supported by research conducted by Billacura and Pangcoga (2017) tested the cytotoxic activity of berenuk leaves using the BSLT method with equates and ethanol solvents to get the LC50 tilapia results of 0.184 ppm in distilled water and 6.74 ppm in ethanol solvent, which means both are very toxic.[23] Cytotoxic activity testing was also carried out on the seeds of the berenuk fruit tested by Arel (2018) using the BSLT method which obtained an LC50 value of 82.30 ppm so the results were said to be toxic because the value was less than 1000 ppm.[8] Then another study was conducted by Aboaba and Fasimoye (2018) which tested the cytotoxic properties of the bark and root bark of berenuk using the BSLT method to obtain an LC50 value of 10.85 ppm in the bark and 16.54 ppm in the root bark. very toxic nature.[24] Supported by the results of this study, the part of the berenuk plant that has the most toxic properties is found in the fruit with an LC50 value of 0.529 ppm from the study by Billacura, et al (2017) in the Philippines.[5]

In this study, the results obtained were that the most deaths occurred in larvae that were given the extract with the highest concentration of 1000 g/ml while the least number of larval deaths occurred in the test given the extract with the lowest concentration of 10 g/ml. And supported by the results of other studies, it can be concluded that the level of concentration given affects the number of larval deaths, which means

that if the concentration is higher, the number of larvae deaths due to extracting administration will also increase. This is also related to the number of doses where the higher the dose of a substance, the higher its toxicity.

In addition, the chemical content contained in the extract also affects the number of larvae mortality. The chemical content contained in the flesh of the berenuk fruit may be toxic, namely alkaloids, flavonoids, saponins, tannins, naphthoquinones, and anthraquinones.[8] These chemical compounds interact synergistically, causing the larvae to die.[18] Saponin compounds contained in the flesh of the berenuk fruit can work to kill larvae by reducing the activity of digestive enzymes it will affect the process of food absorption. This saponin has a bitter and sharp taste and contains plant glycosides that are soluble in water and have a soap-like nature. In addition, the presence of alkaloids and flavonoid compounds in the flesh of this berenuk fruit works as a stomach poison so that the digestive tract of the larvae will be disturbed if these substances enter it.[17] With the presence of phenolic compounds in berenuk fruit, it can act as a toxin to plasma at high concentrations by damaging the cell wall system so that it can cause death in larvae.[18]

Then the flesh of the berenuk fruit contains naphthoquinone derivative compounds, namely 2-(l-Hydroxyethyl)naphtho[2,3-β]furan-4,9-dione and 5-Hydroxy-2-(l-hydroxyethyl) naphtho [2,3-β]furan-4,9-dione. The content of this compound has activity as an antitumor that works by inhibiting cell growth which can be in the form of apoptosis, oxidative stress, inhibition of topoisomerase II-α, and so on.[25]

Besides having cytotoxic activity, berenuk fruit also has antioxidant activity.[21] The presence of these antioxidants can protect cells from reactive oxygen and can damage DNA and inhibit mutagenesis.[5,26] With these toxic or toxic properties, berenuk fruit (*Crescentia Cujete L.*) is one of the plants that can be developed and has the potential for chemotherapy for cancer. The toxic properties are caused by the presence of chemical compounds in the flesh of the fruit that has been proven and can work on cancer by inhibiting cancer cell growth, and inducing apoptosis.[27] Currently, many anticancer agents are sought after with cytotoxic properties that only work on cancer cells and non-toxic properties on normal cells because many anticancer drugs that already exist with cytotoxic properties not only damage cancer cells but damage normal cells as well.[27] Research and development of anticancer agents from plants are still being carried out for the advancement and improvement of the side effects that exist in anticancer agents.[28] Therefore, this research is expected to be useful for the development of new anticancer agents.

5. Conclusion

From this study, it can be concluded that the calculation results of the LC50 value of the ethanol extract of the berenuk fruit pulp has a value of 529.386 ppm and is included in the toxic category so that it shows that the ethanol extract of the fruit flesh of this berenuk has cytotoxic activity against the larvae of Artemia Salina Leach which has the potential as an anticancer drug.

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