

EFFECT OF CONCENTRATION OF MANGROVE LEAF EXTRACT LUMNITZERA RACEMOSA ON HELA CELL VIABILITY

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ABSTRACT

Cervical cancer is a disease caused by a malignant process that occurs in the cervix or cervix. The cause of cervical cancer is not known for certain, but it is estimated that around 95% is caused by HPV (Human Papilloma Virus). Efforts to cure cancer with drugs (pharmacotherapy) or with chemical compounds (chemotherapy) in general have not been able to give satisfactory results, so alternative treatment methods are sought, including traditional medicine, namely by using mangroves. *Lumnitzera racemosa* is one type of mangrove plant that has been used in alternative medicine because of its potential as anticancer. The aim of this study was to determine the effect of *Lumnitzera racemosa* mangrove extract on hela cell viability. *Lumnitzera racemosa* leaf powder was extracted using graded maceration. The solvents used include n-hexane, ethyl acetate, and ethanol. The results showed that the LC₅₀ value was 56 ppm, it means that the ethanol extract has toxic properties. The results of the phytochemical test of the leaf extract of *Lumnitzera racemosa* contained alkaloids, steroids, triterpenoids and saponins. The test results showed that the extract yield was 11.58%, the water content of the extract was 22.17%, and the total phenol was 2742.17 mg GAE. The test results from the LC-MS test resulted in suspected compounds including pyrogallol, isoniazid and caffeine. The ethanolic extract of *Lumnitzera racemosa* leaf was cytotoxic to the viability of hela cells with the resulting IC₅₀ value of 493.33 µg/mL.

Keywords: cervical cancer, *Lumnitzera racemosa*, extract, hela cell, and cytotoxic

INTRODUCTION

Cancer is the second leading cause of death in the world and accounted for 8.8 million deaths in 2015. WHO estimates that the death rate from

cancer will increase significantly, around 13.1 million deaths per year worldwide by 2030. This number is 70% in low- and middle-income countries such as Indonesia (WHO, 2015). One of

the cancers that cause the most deaths is cervical cancer. This cancer is a disease caused by a malignant process that occurs in the cervix or cervix. The cause of cervical cancer is not known with certainty, but it is estimated that around 95% is caused by HPV (Human Papilloma Virus) (Ismail, et al. 2015). Cancer treatment can be done through surgery, radiation or chemotherapy. The ideal anticancer drug is one that has selective toxicity, meaning it destroys cancer cells without damaging normal tissue cells. Side effects of using these drugs include nausea, vomiting, hair loss, bladder irritation accompanied by the presence of blood in the urine. Efforts to cure cancer with drugs (pharmacotherapy) or with chemical compounds (chemotherapy) in general have not been able to provide satisfactory results, so alternative treatment methods are sought, including traditional medicine (Diastuti, et al. 2008). One source that can be used as an anticancer is herbal medicine. Treatment with herbal ingredients began to be widely used for reasons of lower costs and relatively smaller side

METHODS

Research Time and Place

This research was conducted in December 2019 – October 2020 at the Water Products Science Technology Laboratory (Fisheries Product Engineering Division) and Hydrobiology Laboratory (Environmental and Environmental Biotechnology Division), Faculty of Fisheries and Marine Sciences Universitas Brawijaya Malang, Materia Medika Kota Batu Malang,

effects. In terms of phytochemicals, the chemical compounds related to anticancer and antioxidant activity are alkaloids, terpenoids, polyphenols, flavonoids, and resins (Mills and Bone, 2013).

Lumnitzera racemosa is a mangrove plant from the Combretaceae family. In the results of the extract of the mangrove leaves, *Lumnitzera racemosa* is known to have bioactive compounds including phenols, flavonoids, sterols, tannins, carbohydrates, saponins and quinones. Total phenol content was higher than flavonoid content. The extract results also showed in vitro cytotoxicity and apoptotic ability in HepG2 cancer cells (Paul and Seenivasan, 2017). *Lumnitzera racemosa* leaves can be used for the treatment of snake bites, rheumatism, skin allergies, blood purifiers, asthma and diabetics (Pattanaik, et al. 2008). *Lumnitzera racemosa* was developed as an anticancer by knowing the cytotoxic activity of *Lumnitzera racemosa* mangrove leaf extract on hela cell viability by determining the IC_{50} value.

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Tools and Materials

The tools used in this study consisted of an oven, hot plate, digital scale, rotary evaporator, 1000 ml measuring cup, beaker glass, glass bottle, erlenmeyer, funnel, spatula, crushable

pliers, digital scale, desiccator, porcelain cup, furnace, tube. reaction, test tube rack, beaker glass, measuring cup, measuring flask, spatula, dropper, volume pipette, suction ball, and digital scale, measuring cup, measuring flask, volume pipette, analytical balance, suction ball, volume pipette, vial , spatula, and UV-Vis spectrophotometer, vials, pipettes and glass vials, LC-MS instrument, cryo tube apparatus, Laminar Air FlowI, water bath and CO₂ gas cylinder, inverted microscope, 0.2 m filter, spet, well, spirtus , centrifuge, micropipette, and reader analyzer.

The main material used was the Lumnitzera racemosa mangrove leaves obtained from the Clungup Mangrove Conservation (CMC) area, Sendang Biru, Malang, East Java. While the materials used in the test were n-hexane, ethyl acetate, ethanol, filter paper, plastic wrap, label paper, and aluminum foil, aquades, H₂SO₄, HCl, Meyer reagent, 1% FeCl₃,

magnesium powder, anhydrous acetic acid. , and label paper. gallic acid, Folin ciocelciu reagent 50%, Na₂CO₃ 5%, methanol, plastic wrap, filter paper, sea water, aquades, artemia salina, tissue, cultured hela cells, MC media, and heel cell staining.

Research Methods

The method in this study is an experimental method using a completely randomized design (CRD). Then analyzed using ANOVA (Analysis of variance) followed by Tukey's test.

Research Design

The research design was carried out using dried Lumnitzera racemosa leaves. The study was conducted with treatment doses of 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, and 62.5 µg/ml. Then processing was carried out using Completely Randomized Design (CRD).

Table 1. Research Experiment Design

Dosis Ekstrak	Ulangan		
	1	2	3
A (Konsentrasi 62,5 µg/ml)	A1	A2	A3
B (Konsentrasi 125 µg/ml)	B1	B2	B3
C (Konsentrasi 250 µg/ml)	C1	C2	C3
D (Konsentrasi 500 µg/ml)	D1	D2	D3
E (Konsentrasi 1000 µg/ml)	E1	E2	E3
F (Kontrol sel)	F1	F2	F3

Research Procedure

1. Sample Preparation

The leaves of the Lumnitzera racemosa mangrove were washed using running water and dried at room

temperature (6-7 days) to reduce the moisture content. Then it is ground using a grinding machine until it becomes powder and sieved.

2. Sample Extraction

Extraction of samples using multilevel meseration method with n-hexane, ethyl acetate, and ethanol as solvents. The extraction process begins with soaking the *Lumnitzera racemosa* mangrove powder in n-hexane solvent with a ratio of 1:2 (w/v) for 2x24 hours. Then the resulting filtrate is evaporated using a rotary evaporator. While the resulting residue was soaked again using ethyl acetate solvent for 2x24 hours. The resulting filtrate is evaporated using a rotary evaporator. Furthermore, the resulting residue was soaked using ethanol solvent for 2x24 hours. The resulting filtrate is evaporated using a rotary evaporator. After that obtained the results of the extract from each solvent.

3. Extract Yield Calculation

Yield calculation is the number of sample extracts from the extraction results. Calculation of the yield using the formula:

$$\% \text{ yield} = \frac{\text{Extract weight obtained (g)}}{\text{Simplisa weight before extraction (g)}} \times 100\%$$

4. Moisture Test

The extract was weighed as much as 2 grams, then put into a porcelain dish whose dry weight was known. Put in the oven for 16-24 hours at a temperature of 1050C. then remove from the oven and put in a desiccator for 30 minutes. Re-weigh the cup and sample weight as the

final weight. Calculation of % water content using the formula:

$$\% \text{ Water content} = \frac{B - C}{B - A} \times 100\%$$

Information:

A = weight of empty cup (g)

B = weight of cup and sample before oven (g)

C = weight of cup and sample after oven (g)

5. Phytochemical Test

- Alkoloid

0.05 grams of *Lumnitzera racemosa* leaf extract was put into a vial. Then a few drops of 2 N sulfuric acid and Meyer's reagent were added. Then observe the changes until a yellowish white precipitate is formed.

- Flavonoid

0.05 grams of *Lumnitzera racemosa* leaf extract was put into a vial. 0.1 mg of Mg powder and 0.4 ml of amyl alcohol were added. Then observe the changes until a dark red, yellow to orange color is formed.

- Steroid/Triterpenoid

0.05 grams of *Lumnitzera racemosa* leaf extract was put into a vial. Added 2 ml of chloroform. Then 10 drops of acetic anhydride and 3 drops of sulfuric acid were added. Observe the changes that occur until a blue or green color is formed for

steroids and orange or purple for triterpenoids.

- **Saponin**

1 ml of Lumnitzera racemosa leaf extract was put into a vial. Then 5 ml of hot distilled water was added. Shake vertically for 10 seconds. Observe the changes until 1-10 cm high foam is formed for 10 minutes.

- **Tanin**

0.05 grams of Lumnitzera racemosa leaf extract was put into a vial. Then add 3-4 drops of 1% FeCl₃. Observe the change until a blackish blue color is formed.

6. Total Phenol Test

A total of 0.01 grams of Lumnitzera racemosa leaf extract was put into a 10 ml volumetric flask and added 10% methanol to the mark. Then filtered and take the filtrate as much as 0.5 ml. Add 2.5 ml of Folin-Ciocalteau 10% reagent. then incubated at room temperature for 1 hour. Then measure the absorbance value using a UV-Vis spectrophotometer with a wavelength of 760 nm. Then put into the formula:

$$\text{Total Fenol} = \frac{x \text{ (ppm)} \cdot V \text{ sampel (ml)} \cdot FP}{\text{sampel (g)}}$$

7. Toxicity Test Using the BSLT Method

- **Preparation of Artemia salina**

The initial process was thawing freezing by removing the cells from the cryotube from the nitrogen tank

at -800C and put them in Lamiar Air Flow to avoid contamination. Previously spray 70% alcohol on the equipment to be used. Then the cryotube is thawed until the ice contained in it melts. Next, wash as much as 10 ml using RPMI medium. Centrifugation for 5 minutes at 3000 RPM to separate hela cells from RPMI media. Next, transfer the contents of the cryotube to a culture plate with a diameter of 9 cm which already contains 10-15 ml of RPMI media. Change of culture media is done after 2-3 days.

- **Solution Test Preparation**

A total of 200 grams of Lumnitzera racemosa leaf extract was dissolved in 100 ml of seawater, so that an initial concentration of 2000 ppm was obtained as the mother liquor. Subsequently, dilution was carried out to obtain concentrations of 1000, 100, 10 and 1 ppm. The solution used as a control was carried out without the addition of extract.

- **Toxicity Test**

Each concentration of the solution was taken as much as 5 ml, then put into a vial. Enter 10 Artemia salina tails that have been aged 24 hours. This study was carried out for 48 hours, then observed the number of dead Artemia salina.

8. Cytotoxicity Test with MTT Assay

Method

- HeLa Cell Preparation

The initial process was thawing freezing by removing the cells from the cryotube from the nitrogen tank at -80°C and put them in Lamiar Air Flow to avoid contamination. Previously spray 70% alcohol on the equipment to be used. Then the cryotube is thawed until the ice contained in it melts. Next, wash as much as 10 ml using RPMI medium. Centrifugation for 5 minutes at 3000 RPM to separate hela cells from RPMI media. Next, transfer the contents of the cryotube to a culture plate with a diameter of 9 cm which already contains 10-15 ml of RPMI media. Change of culture media is done after 2-3 days.

- HeLa . Cell Cytotoxicity Test

Hela cells were observed using an interved microscope, then subcultured if hela cells were attached and 80-90% confluent. Then the dosages of 2000, 1000, 500, 250, and 125 ppm were made. Take the heel cell culture flask into the LAF and withdraw all the media in the flask. Then put each dose into each well with 3 repetitions.

Incubate for 24 hours at 37°C with 5% CO₂ humidity for 24-48 hours. Discard the cell media and add 50-100 l of MTT reagent to each well, then incubate for 4 hours. Check the condition of the cells using an interved microscope, if formazan is clearly formed add a stopper solution of 10 l SDS 10% in 0.1 N HCl. Then read the absorbance value of each well using an Elisa reader with a wavelength of 595 nm. Calculate the percentage of live cells and analyze the IC₅₀ value.

% living cell

$$= \frac{\text{Abs. Treatment} - \text{Abs. Media control}}{\text{Abs. Cell control} - \text{Abs. Media control}} \times 100\%$$

Information:

OD = Optical Density (absorbance value)

9. Liquid Chromatography Mass Spectrophotometry (LC-MS) Analysis

1 ml of Lumnitzera racemosa leaf extract, dissolved in 1 ml of 95% methanol and 1 ml of 0.3% acetic acid. Take a 0.01 ml solution injected into the LC-MS system at a rate of 0.3 ml/min. Then the solution is pumped for 10-15 minutes and enters the selector column. Molecular weight was detected using a spectrophotometer.

1. Extract Yield

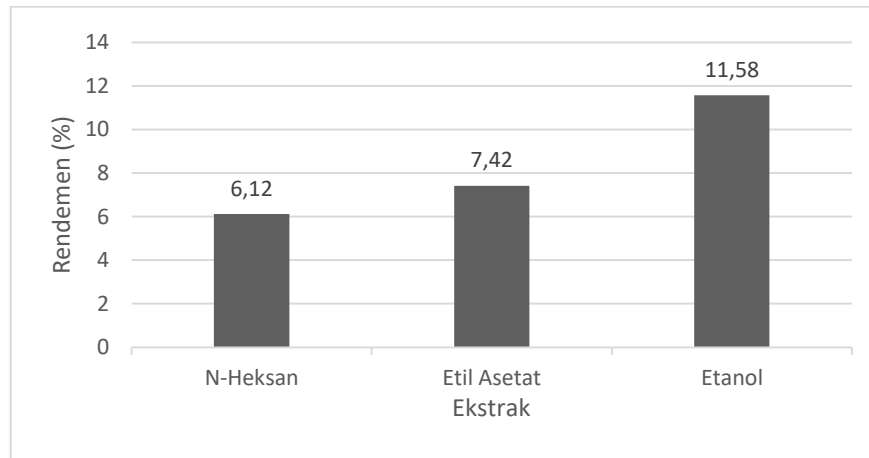
RESULTS AND DISCUSSION

Research Result

Yield calculations were carried out to determine the percentage of extract produced. The higher the yield value, the higher the extract value. The quality of the resulting extract is usually inversely

proportional to the amount of yield produced. The higher the yield value, the lower the quality obtained (Yati et al., 2014).

Figure 1. Extract Yield Graph



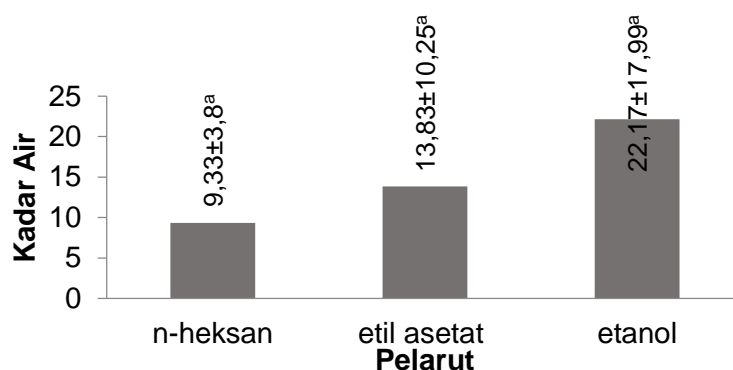
The highest yield of *Lumnitzera racemosa* mangrove leaf extract was 11.58% in ethanol extract and the lowest yield was 6.12% in n-hexane extract. This shows that the mangrove leaves of *Lumnitzera racemosa* are soluble in a polar solvent, namely ethanol. The amount of yield is influenced by the number of types of components that can be dissolved in the solvent used (Suryani, 2017). The polarity of the active compounds from various materials is different and the active components will only be extracted by solvents whose polarity is the same as that of the active components, this is confirmed by the results of research conducted by Salamah

et al. (2008), that the yield of macerated extracts with different solvents resulted in different yield percentages.

2. Extract Moisture Content

The water content of the extract was determined to maintain the quality of the extract. Determination of water content is useful for knowing the maximum limit or range of the amount of water content in the material. This is related to the purity and contaminants in the sample. Thus, the removal of water content up to a certain amount is useful for extending the durability of a material in storage and avoiding the influence of microbial activity (Handayani, et al. 2017).

Figure 2. Extract Moisture Content Chart



The results of the highest water content of *Lumnitzera racemosa* mangrove leaf extract with ethanol solvent of 22.17% and the lowest with n-hexane solvent of 9.33%. Based on the ANOVA results, it can be analyzed that the difference in the solvent used has no significant effect ($p>0.05$) on the moisture content of the *Lumnitzera racemosa* mangrove leaf extract.

From the results of the *Lumnitzera racemosa* mangrove leaf extract using

3. Phytochemicals

Phytochemical testing uses a qualitative method, namely a test by looking at the color change using the solvent reagent used. According to

both n-hexane, ethyl acetate and ethanol as solvents, it was categorized as slightly dry. The water content of the leaf extract using ethanol solvent was higher due to the longer extraction time. The longer the time used for extraction, the higher the water content produced. The resulting water content is higher because it gets a contribution of water from the solvent used (Yulianti, et al. 2014).

Hardoko (2011), the phytochemical test aims to determine the bioactive components contained in each mangrove extract.

Table 2. Phytochemical Test

No.	Test Compound	Extract N-Heksan	Extract Etil Asetat	Extract Etanol
1	Alkaloid	+	+	+
2	Saponin	+	+	+
3	Steroid	+	+	+
4	Triterpenoid	+	+	+
5	Flavonoid	-	-	+
6	Tanin	-	-	+

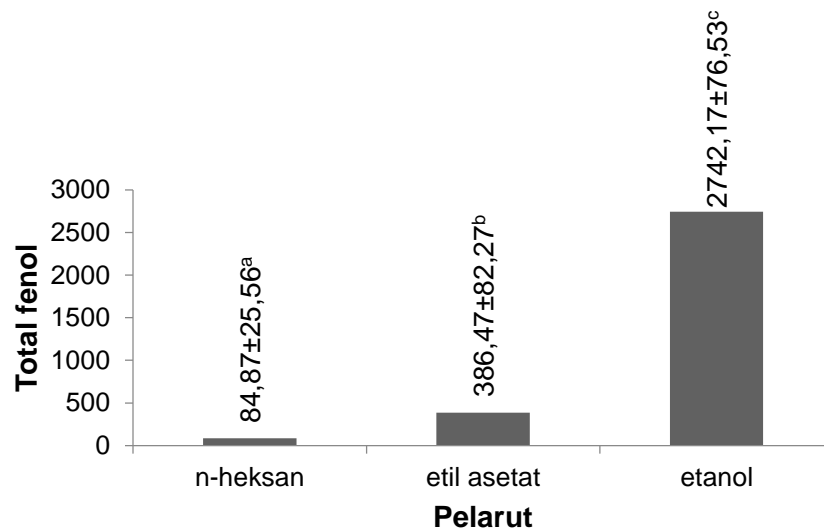
Phytochemical test results showed that the extract of the mangrove leaves *Lumnitzera racemosa* contains alkaloids, steroids, triterpenoids and saponins. Flavonoids according to Gafur et.al (2014), are polar compounds because they have a number of unsubstituted hydroxyl groups. Polar solvents such as ethanol or methanol are very good at detecting flavonoid compounds in plants.

4. Total Phenol

The total phenol test aims to determine the amount of phenol

contained in the sample being tested. The total phenol content test was carried out using the Follin-Ciocalteu method and then determined by visible light spectrophotometry. According to Amanah and Nurfina (2016), states that this method is based on the formation of a blue complex compound from phosphomolybdate-phosphotungstate which is reduced by phenolic compounds in alkaline conditions. The total phenol content was expressed as gallic acid equivalents.

Figure 3. Total Phenol Graph



The highest total phenol content of *Lumnitzera racemosa* mangrove leaf extract was found in ethanol solvent of 2742.17 mg GAE/100 g sample, while the lowest total phenol was found in n-hexane solvent of 84.87 mg GAE/100 g

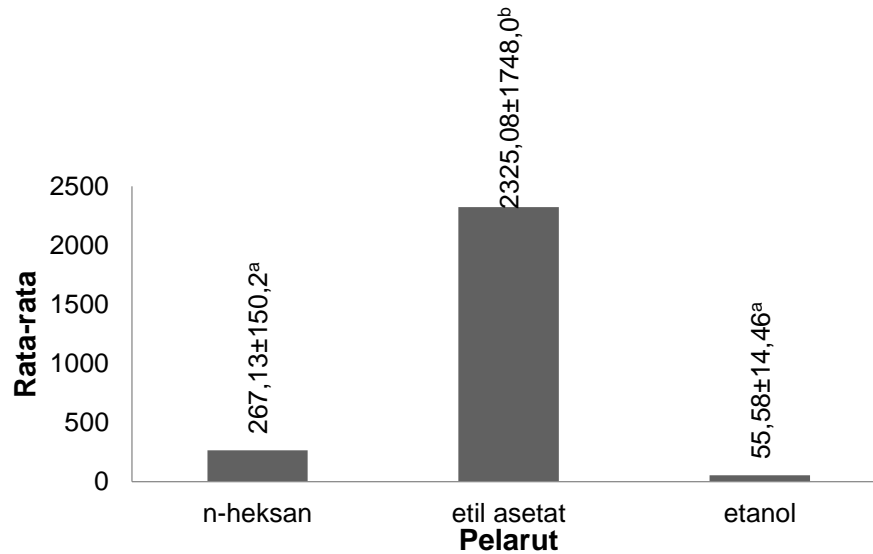
sample. These results are in accordance with the research of Santoso et al, (2011), which states that the solubility of phenol compounds will be different in each type of solvent. Phenol compounds tend to dissolve in ethanol compounds or other

polar compounds. The solubility of total phenol compounds is determined by the polarity of the solvent and which is used to extract and the degree of polymerization of phenol compounds compared to other components in a material.

5. Toxicity

Toxicity tests were carried out to determine the level of toxicity of the *Lumnitzera racemosa* mangrove leaf extract so that it could cause mortality in artemia larvae and to determine the LC_{50} value of each extract by calculating the log concentration against the probit value.

Figure 4. Toxicity Test Graph



The results of the toxicity test of *Lumnitzera racemosa* mangrove leaf extract showed that the toxic extract was obtained with ethanol solvent with an LC_{50} value of 56 ppm and the non-toxic extract obtained with ethyl acetate solvent with an LC_{50} value of 2325.08 ppm. Based on the results of ANOVA (Analysis of variance) showed that the extract treatment was significantly different ($p > 0.05$) against the toxicity test

of *Lumnitzera racemosa* mangrove leaf extract. The ANOVA results were followed by Tukey's further test.

The level of toxicity of plant extracts can be determined by looking at the LC_{50} value. The extract is considered very toxic if the LC_{50} value is below 30 ppm, toxic if the LC_{50} is 30-1000 ppm, and non-toxic if the LC_{50} is above 1000 ppm (Meyer et al. 1982). Based on the data above, it was shown that the ethanol

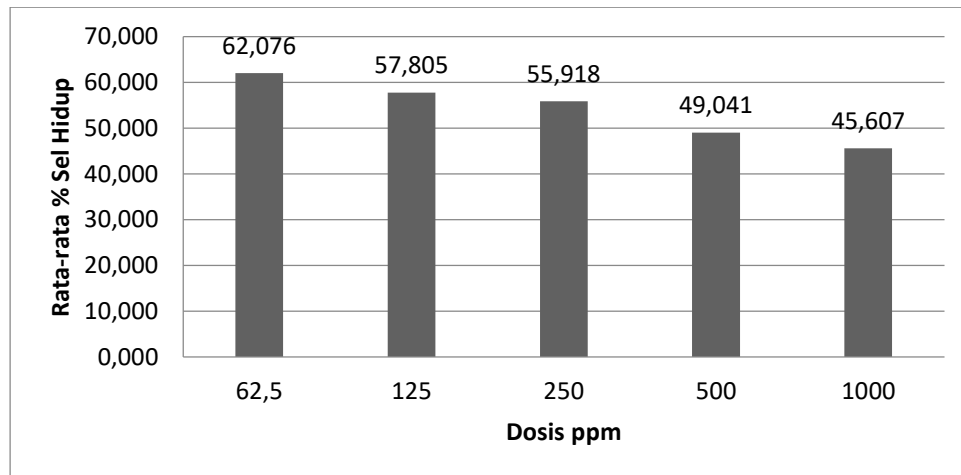
extract of the mangrove leaf *Lumnitzera racemosa* was declared toxic because the LC_{50} value was between 30-1000 ppm.

6. Hela Sel Cell Cytotoxicity

Cytotoxicity test of ethanol extract of *Lumnitzera racemosa* mangrove

leaves was carried out to determine its toxicity to hela cells. The parameter used in this test is IC_{50} (inhibitory concentration 50%).

Figure 5. Graph of Hela . Cell Viability



In the research results obtained, the percentage of the highest average value of viability in the treatment with a dose of 62.5 g/ml was 62.076%. While the percentage of the lowest average value of viability in the treatment with a dose of 1000 g/ml was 45.407%. The results of the ANOVA analysis showed that the dose variation treatment was significantly different ($p < 0.05$) on the viability of the hela cells. Then, it was continued with the Tukey test and showed different results between doses.

From the data generated, the viability of hela cells increased along with the increase in the dose given. This shows that the larger the dose given, the lower the percentage of cell viability. In a study conducted by Eswaraiah et al, (2019), it was stated that the extract of the mangrove leaves of *Lumnitzera racemosa* could inhibit the growth of 100% of the stem cells to 43.05% with a concentration of 100 mg/ml. the parameter used in the cytotoxicity test is the IC_{50} value.

Table 3. IC50 Value of Ethanol Extract

Dose (µg/ml)	% Live Cells	IC ₅₀
1000	45.607	493.33 µg/mL
500	49.041	
250	55.918	
125	57.805	
62.5	62.076	

The cytotoxicity of a substance is classified into several categories. The first category is if the IC₅₀ value is < 10 µg/mL in the very active category. The second category is if the IC₅₀ value is 10 – 100 µg/mL in the active category. The third category is if the IC₅₀ value is 100 – 500 µg/mL in the moderately active category. The last category is if the IC₅₀ value is > 500 µg/mL in the inactive category (Machana et al., 2011). So that the ethanol extract sample of *Lumnitzera racemosa* mangrove leaves is included in the fairly active category by getting an IC₅₀ value of 493.33 µg/mL. The content of flavonoids in the extract of the mangrove leaves *Lumnitzera racemosa* which can cause toxic effects on the hela cells. The content of these compounds is an anticancer compound because it can inhibit the growth of hela cells (Eswaraiah et al, 2019).

7. Liquid Chromatography Mass Spectrometry (LC-MS)

Identification of bioactive compounds from ethanol extract of *Lumnitzera racemosa* mangrove leaves

was carried out using the LC-MS (Liquid Chromatography-Mass Spectrometry) method. The results of the identification of bioactive compounds are displayed in the form of a chromatogram with a peak at a certain retention time. The results of the identification of bioactive compounds from the mangrove leaf extract *Lumnitzera racemosa* produced bioactive compounds pyrogallol, caffeine, and isoniazid.

Pyrogallol compounds have high toxicity and selectivity on HepG2 cells. In addition, pyrogallol is a superoxide anion generator because it has the potential to increase intracellular superoxide anion levels and reduce the value of glutathione content in hela cells (Weerapreeyakul et al., 2016). Caffeine is included in the alkaloid group that can be used as an anticancer with a mechanism of selectively increasing apoptosis in cancer cells (Ayuningtias et al., 2017). Regarding the use of caffeine with Cisplatin in the treatment to induce synergistic cytotoxicity in Hela cells, it was found that cisplatin has an IC50 of

8.93 M in Hela cells. In this study also demonstrated caffeine treatment with a low dose of 2 mM increased cisplatin cytotoxicity by inducing cell death. Therefore, caffeine treatment decreased the percentage of viable cells and increased dead cells (Aliwaini et al., 2017). In the compound Isoniazid is the

choice of drug content that has a function to inhibit the production of mycolic acid, a cell wall component in bacteria. The main mechanism of action of isoniazid is to focus on the formation of various reactive compounds, namely reactive oxygen species (Oliviera, 2016).

Table 4. Alleged Compounds of Lumnitzera racemosa Leaf Ethyl Acetate Extract

Treatment	Retention Time	Compound Period	Alleged Compound	Molecular Formula
Extract mangrove leaf ethanol	4.07	126.110	Pyrogallol	C ₆ H ₆ O ₃
Lumnitzera raseмосa	6.05	194.191	Cafein	C ₈ H ₁₀ N ₄ O ₂
	13.64	137.139	Isoniazid	C ₆ H ₇ N ₃ O

CONCLUSION

The IC50 value produced by the ethanol extract of the Lumnitzera racemosa mangrove leaves is 493.33 g/mL, so it can be seen that the Lumnitzera racemosa mangrove leaf extract has the potential as an anticancer, because the IC50

value produced is included in the moderately toxic category, namely IC50 100-500 g/mL. . Variation of dose of Lumnitzera racemosa mangrove leaf extract given to hela cells affected cell viability. The higher the dose given, the lower the cell viability.

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