

## **The Effect Of Dosage Of Mangrove Leaf Extract *Avicennia Marina* On The Viability Of Hela Cells**

**Mahbubur Rahman\*, Bambang Budi Sasmito**

Faculty of Fisheries and Marine Sciences Universitas Brawijaya.

\*Corresponding author: mahbub@student.ub.ac.id

### **ABSTRACT**

Cervical cancer is caused by infection with the Human Papilloma Virus (HPV) which attacks the reproductive organs of sexually active women. Treatment is done alternatively using natural materials such as mangrove plants. *Avicennia marina* is a type of mangrove plant that has been used in alternative medicine because of its potential as an anticancer. This study aimed to determine the effect of *Avicennia marina* mangrove leaf extract on the viability of HeLa cells. *Avicennia marina* mangrove 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 98.55 ppm, it means that the ethanol extract has toxic properties. Phytochemical test results of *Avicennia marina* mangrove leaf extract contain saponins, steroids/triterpenoids, flavonoids and tannins. The test results showed that the extract yield was 14.40%, the water content of the extract was 16.57%, and the total phenol was 1915.92 mg/g GAE. The results of the LC- MS test resulted in suspected compounds including Caffeine and Diosmetin. The ethanol extract of *Avicennia marina* mangrove leaves was cytotoxic to heLa cell viability with the resulting IC50 value of 115.345 g/mL.

**Keywords: Cervical Cancer, *Avicennia Marina*, Extract, HeLLa Cell, and Cytotoxic**

### **INTRODUCTION**

Cancer is one of the most feared diseases, this is because the healing process and treatment are very expensive. According to WHO (2015), it is estimated that 9 million people die from cancer and by 2030 it is estimated that this will increase to 11.4 million deaths caused by cancer cells. One type of cancer that attacks women is cervical cancer. This cancer is caused by the *Human Papilloma Virus* (HPV) infecting the reproductive organs of women who are sexually active. Factors that can affect cervical cancer include a history of cervical cancer, use of

contraceptives, sexual intercourse, and smoking (Rio & Suci, 2017).

Cancer treatment has been widely used chemotherapy. Chemotherapy is a method that is often used in the treatment of cancer patients by giving chemical compounds to reduce and inhibit the proliferation of cancer cells. Besides being expensive, chemotherapy treatment is not efficient because of the low selectivity of anticancer drugs, besides being antiproliferative against cancer cells as well as normal cells. Comotherapy also has side effects for patients such as weakness, nausea, vomiting, and drastic

weight loss due to normal cells being damaged (Sari et al., 2011). Thus, alternative treatment is carried out using more effective and selective natural ingredients derived from mangrove plants. The use of mangrove plants as natural medicine is due to the fact that mangroves have a high potential for bioactive content and one of them acts as an antioxidant (Paputungan et al., 2017).

Mangrove *Avicennia marina* is one type of mangrove plant that contains bioactive

## METHODS

### Research Time and Place

The research was carried out in March-October 2020 at the Water Products Science Technology Laboratory (Fisheries Product Engineering Division), Faculty of Fisheries and Marine Sciences, Brawijaya University, Malang. Central Police Forensic Laboratory, South Jakarta and Central Laboratory for Steam Cell Development, Airlangga University, Surabaya.

### Tools and materials

The tools used in this research are oven, digital scale, measuring cup, beaker glass, rotary evaporator, glass bottle, Erlenmeyer, funnel, spatula, porcelain cup, cruettable pliers, desiccator, test tube, test tube rack, measuring flask, spatula, pipette drops, measuring pipette, suction bulb, vial, UV-Vis spectrophotometer, aquarium aerator, serological pipette, LC-MS instrument, cryo tube, Laminar Air Flow, water bath, CO<sub>2</sub> gas cylinder, inverted microscope, 0.2 µm filter, spet , well, centrifuge, micropipette, multiple disk, and reader analysis.

compounds such as alkaloids, flavonoids, steroids/triterpenoids, saponins, and tannins that have the potential as anticancer (Danata & Yamindago, 2014). Plants that have the potential as anticancer will direct compounds that play a role in cell growth regulatory genes. *Avicennia marina* was developed as an anticancer by knowing the cytotoxic activity of *Avicennia marina* mangrove leaf extract on heLa cell viability by determining the IC<sub>50</sub> value.

The main material used is *Avicennia marina* mangrove leaves obtained from the Lembung mangrove area, Pamekasan, Madura, East Java. While the materials used in the test were n-hexane solvent, ethyl acetate solvent, ethanol solvent, filter paper, bottles, plastic wrap, label paper, aluminum foil, aquades, H<sub>2</sub>SO<sub>4</sub>, HCl, Meyer reagent, 1% FeCl, magnesium powder, anhydrous acetic acid, label paper, ascorbic acid, seawater, *Artemia salina*, cultured heLa cells, MC media, and heLa cell staining.

### Research methods

The method in this study is an experimental method using 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 *Avicennia marina* leaves that had been dried. The research was conducted with treatment doses of 1000 ppm, 500 ppm, 250 ppm, 125 ppm, and 62.5

ppm. Then processing was carried out using a Completely Randomized Design (CRD).

**Table 1.** Research Experiment Design

Sample	replay		
	1	2	3
P1 (1000 ppm)	P1.1	P1.2	P1.3
P2 (500 ppm)	P2.1	P2.2	P2.3
P3 (250 ppm)	P3.1	P3.2	P3.3
P4 (125 ppm)	P4.1	P4.2	P4.3
P5 (62.5 ppm)	P5.1	P5.2	P5.3

## Research procedure

### a. Sample Preparation

Avicennia mariana mangrove leaves 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.

### b. Sample Extraction

Extraction of samples using multilevel maceration method with n-hexane, ethyl acetate, and ethanol as solvents. The extraction process begins with soaking Avicennia marina mangrove powder in n-hexane solvent in a ratio of 1:2 (w/v) for 2x24 hours. Then the filtrate from the immersion is evaporated using a rotary evaporator. As for the residue, re-soaking was carried out using ethyl acetate solvent in a ratio of 2x24 hours. The filtrate from the immersion is evaporated using a rotary evaporator. Furthermore, the residue from the immersion was added with ethanol solvent for 2x24 hours. The filtrate from the immersion is evaporated using a rotary evaporator. after that, the extraction results from each solvent were obtained.

### c. Extract Yield Calculation

The yield calculation is the amount of the sample extract from the extraction. Calculation of yield results using the formula:

$$\% \text{ Yield} = \frac{\text{Final extract weight (g)}}{\text{Initial sample weight (g)}} \times 100\%$$

### d. Moisture Test

The water content test is in accordance with SNI 2354.2:2015 using the gravimetric method. The extract was weighed as much as 2 grams, then put it in a porcelain dish whose dry weight was known. Put it in the oven for 16-24 hours at 105°C. then remove from the oven and put in a desiccator for 30 minutes. Re-weigh the cup and sample as 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 the cup and sample after being in the oven, (g).

### e. Phytochemical Test

#### – Flavonoid

Avicennia marina leaf extract as much as 0.05 grams 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.

– **Alkaloid**

Avicennia marina leaf extract as much as 0.05 grams was put into a vial. Then add a few drops of 2 N sulfuric acid and Meyer's reagent. Then observe the changes until a yellowish white precipitate is formed.

– **Steroid/Triterpenoid**

Avicennia marina leaf extract as much as 0.05 grams was put into a vial. Add 2 ml of chloroform. Then, add 10 drops of acetic anhydride and 3 drops of sulfuric acid. Observe the changes that occur until a blue or green color is formed for steroids and orange or purple for triterpenoids.

– **Tanin**

Avicennia marina leaf extract as much as 0.05 grams was put into a vial. Then add 1% FeCl<sub>3</sub> as much as 3-4 drops. Observe the change until a blackish blue color is formed.

– **Saponin**

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

**f. Toxicity Test Using the BSLT Method**  
**Metode**

– **Preparation of Artemia salina**

Hatching is done by immersing 1 gram of Artemia saline eggs into 1 L of

seawater in a bottle. Then aerated for 48 hours.

– **Preparation of Test Solution**

A total of 200 grams of Avicennia marina leaf extract was dissolved in 100 ml of sea water, 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 conducted for 24 hours, then observed the number of dead Artemia salina.

**g. Total Phenol Test**

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

$$\text{Total phenol} = \frac{X(\text{ppm}) \cdot V \text{ sample (ml)} \cdot FP}{\text{Sample (gram)}}$$

#### h. Cytotoxicity Test by MTT. Method Assay

##### – HeLa Cell Preparation

The initial process was thawing freezing by removing the cells from the cryo tube from the nitrogen tank at -80°C and placing them in Laminar 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 with 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. Furthermore, making doses of 1000, 500, 250, 125, and 62.5 ppm. Take the heLa 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% CO<sub>2</sub> humidity for 24-48 hours. Discard the cell media and add 50-100 µl 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 IC<sub>50</sub> value.

$$\% \text{ live cells} = \frac{OD \text{ treatment} - OD \text{ medium}}{Cell \text{ control } OD - media \text{ OD}} \times 100\%$$

#### i. Liquid Chromatography Mass Spectrophotometry (LC-MS) Analysis

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

## RESULT AND DISCUSSION

### Research result

#### Extract Yield

Yield calculations were carried out to determine the comparison between the extract obtained and the initial weight (Wijaya et al., 2018).

**Table 2.** Calculation of Extract Yield

Solvent	Yield (%)
N-Heksan	3.96
Ethyl acetate	6.40
Ethanol	14.40

The highest yield of *Avicennia marina* leaf extract was 14.40% in ethanol extract and the lowest yield was 3.96% in n-hexane extract. These results are in accordance with the research of Prabowo et al., (2014), based on the solvent used, ethanol produces a higher extract yield than ethyl acetate and n-hexane solvents. This indicates that the compounds contained in mangrove leaves tend to be polar.

The yield of the extract with methanol solvent in the form of a paste extract which is very thick compared to extracts from ethanol and acetone solvents in the form of pastes or extracts from isopropyl alcohol and ethyl acetate solvents in the form of liquid extracts. This is because the compounds in the extract tend to be polar, polar compounds will dissolve with polar solvents. One example is wax compounds, wax compounds are dissolved, causing the extract produced with methanol to be more viscous than the extract using a solvent that has a polarity level below methanol (Savitri et al., 2017).

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The results of the calculation of the moisture content of *Avicennia marina* mangrove leaf extract were obtained from the oven for 16-24 hours at a temperature of 105°C.

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Solvent	Water content (%)
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Ethyl acetate	12,80±2,54 <sup>a</sup>
Ethanol	16,57±2,79 <sup>b</sup>

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The result of the highest water content of *Avicennia marina* leaf extract was in the ethanol

extract of  $16.57 \pm 2.79\%$ , while the lowest water content was found in the n-hexane extract of  $11.99 \pm 1.46\%$ . The water content yield of mangrove leaves was obtained from the comparison of the sample weight (extracted from each solvent) after treatment (oven; SNI 2354.2:2015) with the sample weight before treatment, then multiplied by 100%. The value of the water content in the extraction results shows the ability of the solvent to dissolve compounds in

### Phytochemicals

Phytochemical test is a qualitative test used to determine the bioactive compounds contained in *Avicennia marina* leaves.

**Table 4.** Phytochemical Test

Bioactive Compound	Ekstrak		
	n-heksan	Ethyl acetate	Ethanol
Alkaloid	+	-	-
Saponin	-	-	+
Steroid/ triterpenoid	+	+	+
Flavonoid	+	-	+
Tanin	-	+	+

Phytochemical test results showed that *Avicennia marina* mangrove leaf extract contains alkaloids (in n-hexane extract), saponins (in ethanol extract), steroids/triterpenoids, flavonoids (in n-hexane and ethanol extracts), and tannins (in ethyl acetate and ethanol extracts). ethanol).

mangrove leaves. The highest yield was shown in ethanol with a value of 16.57%. These results indicate that the compounds in *Avicennia marina* mangrove leaves tend to be polar (as evidenced by significant/not significant differences). This result is in accordance with the research of Dia et al., (2015) and Jacob et al., (2011), where the highest yield after extraction of *Avicennia marina* mangrove leaves was obtained in ethanol solvent.

Bioactive compounds have the ability for human health, among others, as a source of antioxidants, antibacterial, anti-inflammatory, and anticancer (Firdiyani et al., 2015).

### Toxicity

Toxicity test aims to determine the ability of a compound as a poison by knowing the toxic level of the plant (Puspitasari et al., 2018). The parameter used in the toxicity test is the  $LC_{50}$  value.

**Table 5.** Toxicity Test Results

Ekstrak	LC50 value (ppm)
Ethanol	$98.55 \pm 43.71a$
N-heksan	$441.39 \pm 150.18a$
Ethyl acetate	$1931.49 \pm 750.89b$

The results of the toxicity test of *Avicennia marina* leaf extract were highest in ethyl acetate extract of  $1931.49 \pm 750.89$  ppm and the lowest was found in ethanol extract of  $98.55 \pm 43.71$  ppm. Based on the test results of *Avicennia marina* extract, the extract which was toxic when tested using the Brine Shrimp Lethality Test (BSLT) method caused the death of 50% of artemia larvae at a concentration of  $LC_{50} < 1000$  ppm. In this

study, it indicated that the samples had potential as anticancer, antibacterial and antifungal agents (Ningdyah et al., 2015).

The ethanol extract of *Avicennia marina* mangrove leaves has a low LC50 value and has a high toxic ability, because the ethanol solvent is able to attract more bioactive compounds in plants than other solvents. Based on the phytochemical test, the compounds contained in the ethanol extract were saponins, steroids/triterpenoids, flavonoids, and tannins. These compounds have toxic properties, so that these compounds have the ability to kill *Artemia salina* Leach shrimp larvae which work as stomach poisons. Will cause the digestive process in *Artemia salina* Leach to be disrupted. In addition, the compounds in the sample extract will inhibit the taste receptors in the mouth of *Artemia salina* Leach, causing the larvae to fail to obtain a taste stimulus and causing the *Artemia salina* Leach shrimp larvae to starve to death because they cannot recognize their food (Rohmah et al., 2019).

### Total Phenol

Total phenol is a basic test carried out on antioxidant activity testing, because phenolic compounds play a role in preventing oxidation. Measurement of total antioxidants in foodstuffs of plant origin can be done by measuring total phenolic levels using Folin-Ciocalteu reagent (Toripah et al., 2014).

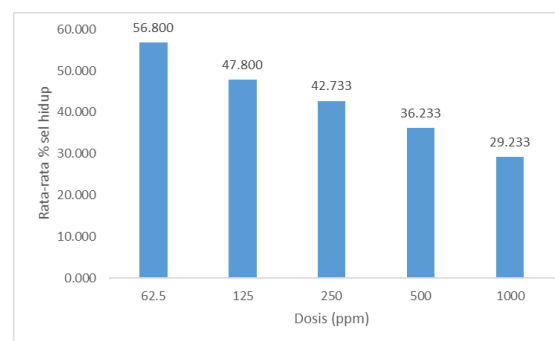
**Table 6.** Total Phenol . Results

Extract	Average (mg/g GAE)
N-Heksan	113.22±17.9 <sup>a</sup>
Ethyl acetate	752.97±80.3 <sup>b</sup>
Ethanol	1915.92±35.2 <sup>c</sup>

The highest yield was found in the ethanol extract of 1915.92±35.2 mg/g GAE, while the lowest yield was found in the n-hexane extract of 113.22±17.9 mg/g GAE. The content of phenolic compounds contained in the ethanol extract is greater because the phenolic compounds contained in plants are mostly polar. Phenol compounds will be more easily extracted using semi-polar and polar organic solvents. The total phenol produced by the n-hexane, ethyl acetate and ethanol extracts increased because the phenolic content in plants would increase along with the increasing polarity of the solvent used (Yanuarti et al., 2017).

### HeLa . Cell Cytotoxicity

Cytotoxicity test aims to determine the effect of the toxicity of ethanol extract of *Avicennia marina* leaves on heLa cells.



**Figure 1.** HeLa Cell Viability Graph

From the resulting data indicate that there is a decrease in the percentage of living cells along with the addition of the dose of ethanol extract of *Avicennia marina* mangrove leaves given. The results of the percentage of viability of HeLa cells were analyzed using a linear regression graph.



Meanwhile, the regression equation between dose and % of cell life can be seen in Figure 2.

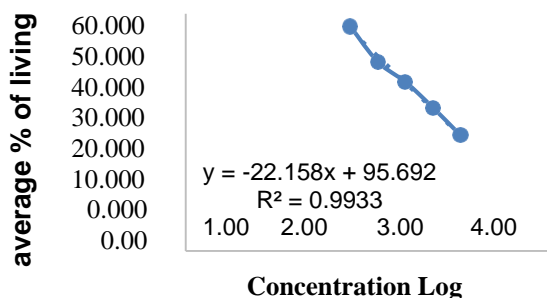


Figure 2. Interlog Regression Equation

With a linear equation between the log dose and % viability of HeLa cells in this study, the equation  $y = -22.158x + 95.692$  was obtained which was then entered into the formula  $y = 50$  to obtain a value for IC<sub>50</sub> of 115.345 ppm. These results can be concluded that the ethanol extract of *Avicennia marina* mangrove leaves caused the death of half the number of HeLa cells at a concentration of 115.345 ppm. The cytotoxic activity of a substance is classified into several categories. First, if the IC<sub>50</sub> value of an extract is below 50 ppm, it is declared very active. Second, the IC<sub>50</sub> value is between 50- 100 ppm is included in the active category. Third, the IC<sub>50</sub> value is

**Table 7.** Alleged Compound Ethanol Extract of *Avicennia marina* Leaves

Treatment	Retention Time	Compound Mass	Alleged Compound	Molecular Formula
Ethanol Extract	4.27	194.191	Caffeine	C <sub>8</sub> H <sub>10</sub> N <sub>4</sub> O <sub>2</sub>
	8.66	300.363	Diosmetin	C <sub>16</sub> H <sub>12</sub> O <sub>6</sub>

## CONCLUSION

The ethanol extract of *Avicennia marina* mangrove leaf samples given to HeLa cells with different doses had an effect on the level of cell

between 100-150 ppm which is categorized as quite active. Fourth, the IC<sub>50</sub> value is between 150-200 ppm categorized as inactive (Bahriul et al., 2014).

## Liquid Chromatography Mass Spectrometry (LC-MS)

LCMS testing aims to identify bioactive compounds found in plants. The identification results of the bioactive compounds will be displayed in the form of a chromatogram with a peak at a certain retention time. The results of the identification of bioactive compounds from *Avicennia marina* leaf extract resulted in the bioactive compounds caffeine and diosmetin. Caffeine compounds are included in the alkaloid group that can be used as an anti-cancer with a mechanism of selectively increasing apoptosis in cancer cells (Ayuningtias et al., 2017). Diosmetin compounds can be used as anticancer, because they produce anti-carcinogenic effects by increasing apoptosis and inhibiting cell proliferation (Martati et al., 2018).

viability. Where by increasing the dose of HeLa cells it causes a decrease in the percentage value of living cells. The IC<sub>50</sub> value of *Avicennia marina* mangrove leaf ethanol extract was

115.345 ppm, so it can be seen that *Avicennia marina* mangrove leaf ethanol extract has the potential as an anticancer. The lowest viability value at a dose of 1000 ppm was 29.233%.

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