In Vitro Anticancer Potential of *Avicennia marina* Leave Extract and Taurin on *HeLa* Cell Line: An Alternative Approach of Anticancer

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Keywords: *Avicennia Marina*, Cytotoxic, Antiproliferative, Taurine.

Abstract: The pharmacological activities of *Avicennia marina* tree leaves, often known as api, have been scientifically proven to include anti-inflammatory, analgesic, and toxicological effects. The *Avicennia marina* plant is thought to have anticancer qualities since it contains high levels of flavonoids, tannins, saponins, and alkaloids. Taurine's organic acids has antioxidant and anticancer properties, alongside mangrove plants. Cancer is caused by excessive cell growth, which damages surrounding cells and tissues. Currently, cervical cancer is the second most common cause of mortality among women globally. The MTT technique (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) on HeLa cervical cancer cell cultures showed that leaf extract and taurine effectively inhibited cancer growth. The findings indicated that the leaf extract of api-api and taurine had cytotoxic properties, with IC50 values of 206 ppm, 122 ppm, and 603 ppm, respectively. In contrast, the antiproliferation test demonstrated that the api-api leaf extract and taurine exhibited a longer period of cell division compared to the control cells, with doubling times of 72 and 19 hours, respectively. The utilization of api-api leaf extract and taurine exhibited a deleterious impact on HeLa cells as compared to untreated cells (control cells). Treatment also inhibited cell proliferation, as shown by the longer doubling time of treated cells compared to control cells.

1 INTRODUCTION

Cancer results from unregulated cell proliferation in dysfunctional tissues. Cancer ranks as the second most prevalent cause of mortality, trailing behind cardiovascular disease. The number of cancer-related fatalities in 2018 was roughly 9.6 million, according to the World Health Organization's report in 2020. It is projected that by 2030, there will be a rise of 11.4 million deaths attributed to cancer cells (Rio, S., Suci, 2017). Indonesia has the second largest number of cervical cancer cases globally, with a fatality rate of 50% (Kementerian Kesehatan 2020). HeLa cells are cervical cancer cells that have been infected with the HPV-18 virus. The current cancer treatment remains inadequate in achieving a complete cure, primarily due to the temporary effectiveness of chemical drug-based treatments. These medications lack selectivity for target cells, resulting in damage to normal cells in the body. Exploring diverse natural resources, such as the mangrove ecosystem, can facilitate the development of alternative medications (Albinhassan et al., 2021).

Mangroves are resilient plants that can adjust to shifting environments with erratic salinity and tidal patterns. The capacity is attributed to the production of unique chemicals by api-api leaves (*Avicennia marina*) for the purpose of adaptation. The objective of this study is to expand the availability of phytopharmaceuticals (medicinal plants) for the investigation of secondary metabolites from api-api leaves (*Avicennia marina*) and taurine as an anticancer agent, focusing on the components. The efficacy of this intervention will next be assessed on HeLa cells, namely those derived from cervical carcinoma (Rahman, 2021).

Andriani, S., Widiastuti, E. L., Kurniawaty, E., Suharyani, , Khairani, I. A. and Andeska, D. P.
In Vitro Anticancer Potential of Avicennia marina Leave Extract and Taurin on HeLa Cell Line: An Alternative Approach of Anticancer.
DOI: 10.5220/0013110000003873

In *Proceedings of the 1st International Conference on Medical Science and Health (ICOMESH 2023)*, pages 25-31
ISBN: 978-989-758-740-5

2 METHOD

2.1 Extract Preparation

The Api-api leaves were acquired from the Lampung Mangrove Center (LMC) located in Labuhan Maringgai, East Lampung. The Api-api leaves were rinsed with flowing water. Subsequently, the drying process is carried out by subjecting it to an oven set at a temperature range of 30-40˚C. Following the drying process, it is ground into a powder known as simplicia. Simplicia is macerated in 1:10 methanol solvent for 24 hours, 100 grams per liter. The macerate is further strained using filter paper. A rotary evaporator at 50°C evaporated the extract to a thick consistency (Nurfitri, W. A., Endang, L. W., & Endang 2019).

2.2 Hela Cell Cuture

A 10% solution of Fetal Bovine Serum (FBS) was prepared by adding 5 ml of the solution and 0.5 ml of Penicillin Streptomycin. This mixture was then combined with 50 ml of Rosewell Park Memorial Institute medium (RPMI 1640) according to CCRC (2009). For cell counting, 10 µl of HeLa cells were pipetted into a well plate, 10 µl of trypan blue was added, and the cells were counted on a hemocytometer. A living cell appears clear, while a dead cell is red. Hemocytometer calculations are done in 4 rooms. The subsequent calculations pertain to the quantity of cells to be cultivated (CCRC, 2009).

Preparation of a stock solution of 10 mg *Avicennia marina* extract with 1 ml 1% DMSO for taurine in 1 ml distilled water. The original solution was diluted to concentrations of 125 parts per million (ppm), 100 ppm, 75 ppm, 50 ppm, and 25 ppm (CCRC, 2009).

2.3 Cytotoxic Test Using the MTT Method*(3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazoliumbromida)*

For the cytotoxic test, 100 µl of cells were put to each well, with each well containing 20,000 cells (CCRC, 2009). After a 24-hour culture period, the cells are then washed with phosphate buffer saline (PBS). Extract and taurine were pre-concentrated and incubated for 24 hours in each well. The solution was discarded and subsequently washed with a PBS solution. Combine 10 µl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) with 5 mg/ml phosphate buffer saline. Subsequently, the sample was placed in a CO_2 incubator and kept at a temperature of 37°C for a duration of 2 hours. Living cells will undergo metabolic processes to convert MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium bromide) into a purple compound called formazan. The MTT reaction was halted by adding 100 µl of 100% dimethyl sulfoxide (DMSO) stopper reagent to each well. The absorbance was measured using an ELISA reader at a wavelength of 550 nm (CCRC, 2013).

2.4 Antiproliferative Test with the MTT Method *(3-(4,5- dimetiltiazol-2-il)-2,5-difenil tetrazoliumbromida)*

Antiproliferative assay involved 100 µl of HeLa cells each well, totaling 20,000 cells. The incubation process lasted for 24 hours at a temperature of 37°C in a CO_2 incubator (CCRC, 2009). After 24 hours of growth in well plates, cells were given 100 µl of extract and taurine at doses of 125, 100, 75, 50, and 25 ppm. The incubation process was carried out for 24, 48, or 72 hours at a temperature of 37°C in a CO_2 incubator. Incubation was followed by PBS rinsing of the wells. 10 µl of MTT solution (with a concentration of 5 mg/ml in PBS) was applied to the wells. The wells were then incubated for an additional 2 hours at 37°C in a CO_2 incubator. Active cells will transform MTT into a purple formazan compound. The process was halted by introducing 100 µl of 100% DMSO into each well. The measurement of absorbance for each well was conducted using an ELISA reader at a specific wavelength of 550 nm. Next, statistical analysis was used to compare viable cell counts during different incubation times (CCRC, 2013).

2.5 Data Analysis

Analysing cytotoxic test data on HeLa cells involves calculating the proportion of viable cells. The percentage is transformed into a probit number in order to obtain the IC50 value. Antiproliferative test data analysis was used to estimate the doubling time of extract and taurine at varied doses and incubation times. The estimates were derived using linear regression, which involved correlating the incubation time with the logarithm of the number of viable cells. A statistical analysis was performed using the One-way ANOVA test with a 95% confidence level to assess the influence of concentration on the average number of live cells. If there are substantial disparities between treatments, the analysis will proceed with the Least Significant Difference (LSD) test.

3 RESULT AND DISCUSSION

3.1 Cytotoxic Test of *Avicennia Maria* Extract

Cytotoxic studies using *Avicennia marina* leaf extract and taurine against HeLa cervical cancer cells yielded a graph connecting extract concentration to cell viability. Figure 1 and Figure 2 display these graphs.

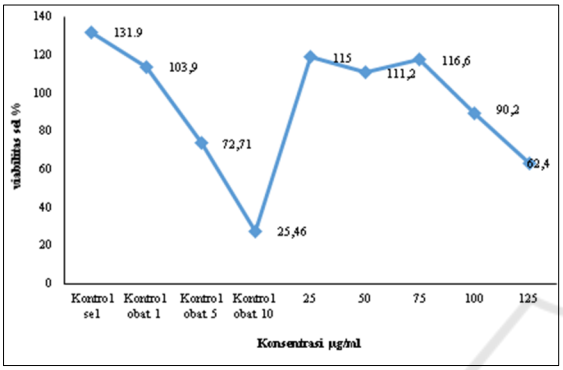


Figure 1: Comparison of extract concentration with percentage of Hela cell viability

As shown in Figure 1, *Avicennia marina* leaf extract affects cell viability compared to the control group. The leaf extract, when present at a concentration of 125 ppm, exhibited the lowest viability percentage of 62.4%. This value was significantly lower compared to the other concentrations and the control group of cells. Administration of *Avicennia marina* extract at a dosage of 125 ppm led to a greater degree of cell inhibition than the inhibitory effect of the control drug at a concentration of 5 ppm.

Taurine has distinct properties compared to *Avicennia marina* leaf extract. Evidence indicates that taurine elicits varying reactions based on the level of cell viability. Taurine had the lowest percentage of viability (74.92%) and cell inhibition (24.07%) at a dose of 100 ppm. However, the inhibition at 100 ppm was still below the control drug's 5 and 10 ppm effects. When compared to the drug control at a dose of 1 ppm, the inhibition value was still greater.

All treatments have shown considerable cytotoxic action, which reduces test cell viability relative to the control group. Figures 3–5 show how this cytotoxic action alters cell shape and structure. Under normal circumstances, HeLa cells often have a polygonal morphology and closely interact with their extracellular matrix. However, in the event of a disruption or the initiation of apoptosis, the cellular morphology and structure will undergo a transformation. Indications of these alterations comprise a reduction in cell dimensions and cell contraction. Hutomo et al. (2016) have provided additional clarification for this occurrence.

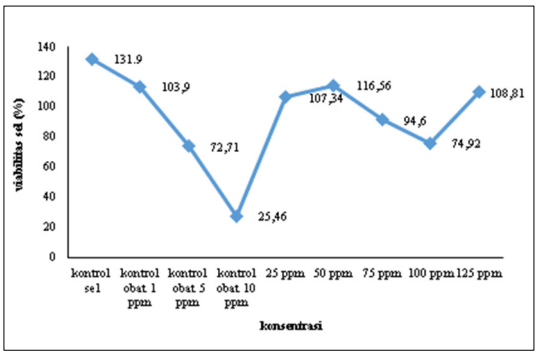
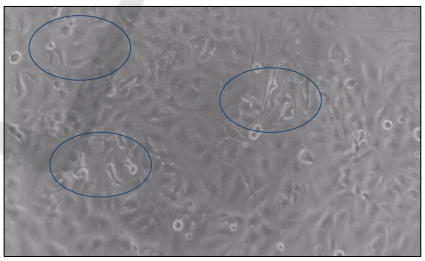


Figure 2. Relationship between taurine concentration and % cell viability

Figure 3 (A) illustrates a significantly increased density of viable HeLa cells in comparison to untreated control cells. Living cells have a high density due to cells that develop without impediments and meet nutritional needs. The cells have a flat epithelial morphology, with a spherical and compact nucleus placed centrally. They possess a basal lamina that serves to bind them to the substrate, and it is structurally intricate. Deceased HeLa cells exhibit an uneven shape and lack luminescence (Nurani 2011). In the pharmacological control group, cell density was observed to be low due to apoptosis, which resulted in cell death.



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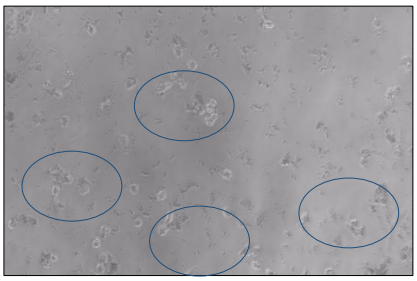
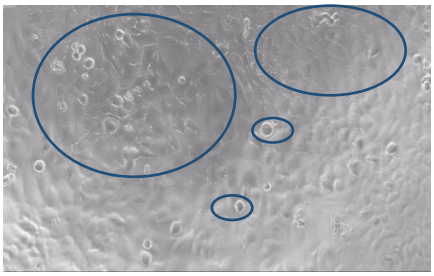


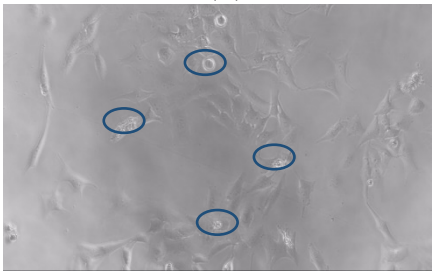
(B)

Note: Apoptosis

Figure 3: *HeLa* cell morphology (A) cell control and (B) drug control with *Doxorubicin.*



(A)

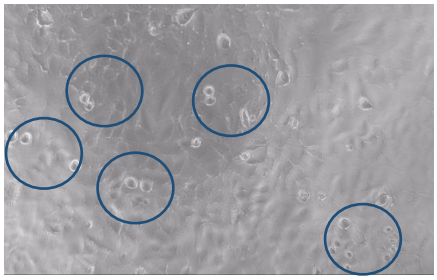


(B)

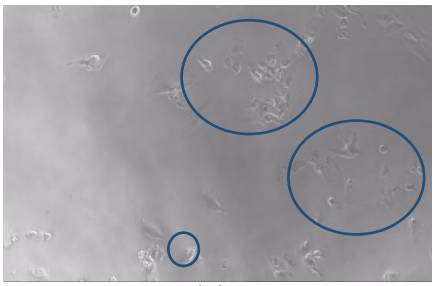
Note: ◯ Apoptosis

Figure 4: HeLa Cell Morphology in Api-api Leaf Extract Treatmen (A) 25 ppm; (B) 125 ppm.

Figure 4 indicates that 25 ppm and 125 ppm extracts significantly change cell density. The cell density was greater at a concentration of 25 ppm compared to 125 ppm. The cell density is relatively low when the api-api leaf extract is present at a concentration of 125 ppm. Certain cells undergo apoptosis, resulting in abnormal cellular morphology. The application of 125 ppm api-api leaf extract was deemed efficacious in suppressing the percentage of cell viability in comparison to other concentrations. These findings demonstrate the toxicity of api-api leaf extract towards HeLa carcinoma cells. At 25 ppm, cell density was lower than control cells, but at 125 ppm, cell density was rarely visible and color and shape changed, indicating Hela was undergoing apoptosis. At a dosage of 125 parts per million (ppm), it exhibits the highest level of effectiveness in suppressing cell development and is highly toxic to HeLa cells.



(A)



(B)

Note: ◯ Apoptosis

Figure 5: HeLa Cell Morphology in Taurin (A) 25 ppm dan (B) 100 ppm.

Cell density exhibited a significant decrease when treated with a concentration of 100 ppm, in contrast to a concentration of 25 ppm. However, viable HeLa cells were still detectable at this concentration. Among all the concentrations tested, taurine at 100 ppm had the best response in terms of inhibiting the development of HeLa cells. After calculating cell viability with the extract and taurine, the IC50 value is calculated using the reference (CCRC, 2013).

Table 2: Test Compounds' IC50 Cytotoxic Activity against HeLa Cervical Cancer Cells

Test compound	Concen-tration (ppm)	Cell viability (%)	IC_{50} (ppm)
	25	94,48	206
	50	89,52	
A.marina	75	80,55	
	100	73,76	
	125	61,44	
	25	106,4	603
	50	114,5	
Taurin	75	91,4	
	100	75,9	
	125	109,9	
	1	113,38	12,35
Doxorubicin	5	73,71	
	10	27,46	

IC50 is the concentration at which a drug inhibits test cell growth by 50%. A lower IC50 value of the test material indicates a higher level of toxicity and a better potential for use as a medication. The American National Cancer Institute (NCI) defines the cytotoxic activity criterion for crude extracts as having an IC50 value of less than 30 µg/ml, as stated by (de Oliveira et al. 2016).

Table 2 demonstrates that api-api leaf extract, taurine, and doxorubicin, at different doses, result in a

reduction in cell viability. This suggests that the test chemical exhibits cytotoxic action against HeLa cells. The regression calculations indicate that the IC50 value for api-api seed extract is 206 ppm, taurine is 603 ppm, and doxorubicin is 12.35 ppm. The IC50 value of the api-api leaf extract plus taurine is significantly higher when compared to doxorubicin.

Table 3 indicates the doubling time values varied across different treatment concentrations of api-api leaf extract and taurine. Cell proliferation is measured by linear regression equation slope in the doubling time test. The control cell yielded a slope value of 0.0042. This value functions as a point of reference for clusters of cells undergoing therapy. Treatment slope lower than control cell slope increases doubling time. However, the doubling time is reduced when the slope is higher than that of the control cells (Meiyanto et al., 2008). According to the information provided in Table 3, the slope values of all treatment cells are lower than the slope values of the control cell. According to Haryoto et al. (2013), the research shows that treated HeLa cells have a longer period of time between each cell division compared to untreated HeLa cells.

The doubling time of api-api leaf extract increases with extract concentration. At 25 ppm, api-api leaf extract doubles in time. Above 25 ppm, doxorubicin did not produce the same doubling time values as the control agent. This occurrence can be ascribed to the negative coefficient in the linear regression equation. A negative slope value indicates the absence of growth as a result of cellular mortality (Nurani 2011). The cells that were exposed to taurine had diverse doubling time values, which were significantly greater than those of the control cells. This fact implies that api-api leaf extract and taurine have the potential to function as anti-proliferative agents in HeLa cervical cancer cells.

Table 3. Doubling Time Value in Antiproliferation Test

Test compound	**Concentration (ppm)**	**Equation of incubation time line and log of cell number**	**Slope value**	***Doubling Time value* (hours)**
A.Marina	25	0,0019x + 4,194	0,0019	170
	50	0,0013x +4,232	0,0013	253
	75	0,0012x + 4,168	0,0012	325
	100	0,0007x + 4,145	0,0007	482
	125	0,0002x + 4,136	0,0002	1884
	25	0,0014x+4,2937	0,0014	218
	50	0,001x + 4,3142	0,001	285
Taurin	75	0,0013x+4,2752	0,0013	249
	100	0,0004x + 4,259	0,0004	852
	125	0,0016x+4,2878	0,0016	195
Cell control	0	0,0021x + 4,306	0,0042	73,17
	1	-0,0037x+4,3688	-0,0037	Tidak ada
Doxorubicin	5	-0,0187x + 4,462	-0,0187	Tidak ada
	10	-0,0208x+4,2854	-0,0208	Tidak ada

Table 4: Average Number of Cells in Test Compound Treatment

Test Compound	**Concentration (ppm)**	**Number of living cells (x 1000 Sel)**		
		24 hours	**48 hours**	**72 hours**
Extract *Avicennia marina*	25	18,9±1,48a	21,0±1,30a	26,3±0,52 a
	50	18,9±0,79a	19,2 ±0,72a	23,8±0,94 a
	75	16,5±0,60ab	15,6±0,92b	21,4±0,83 ab
	100	15,4±0,30b	14,7±1,21b	24,1±2,88 a
	125	15,0±1,34b	13,3±0,30b	18,9±0,69b
Taurin	25	21,3±0,30ab	21,8±1,42	25,7±0,60ab
	50	22,9±1,48a	22,9±1,60	24,2±0,35b
	75	18,3±0,66bc	22,1±0,99	23,6±1,80b
	100	15,2±1,99c	21,4±3,08	19,4±1,12c
	125	22,0±1,43ab	19,5±1,28	27,8±0,21a

The mean count of viable cells in all api-api leaf extract groups varied significantly, according to One-way ANOVA. Subsequent analyses utilizing the Least Significant Difference (LSD) revealed that the greatest quantity of viable cells was detected at a concentration of 125 ppm following a 72-hour incubation period. Conversely, the minimum value was documented at a concentration of 100 ppm for a duration of 24 hours. Different taurine dosages caused changes in 24-hour and 72-hour viable cell numbers. In addition, the taurine concentration reached its peak at 125 ppm after 72 hours, whereas the lowest level was measured at 100 ppm after 24 hours.

The anticancer action of the active chemicals in the methanol extract of api-api seeds (*Avicennia marina*) may be attributed to various probable pathways. *A. marina*, a natural source abundant in medicinal properties, has been recognized for its potential to function as an anti-cancer agent. Api-api seeds consist of a diverse range of chemical constituents, including cyclic triterpenoids, flavonoids, iridoids, naphthaquinones, polyphenols, polysaccharides, and steroids. The majority of these substances have demonstrated substantial anticancer efficacy, so validating the promise of A. marina as a natural agent in cancer treatment. The reference is from Tian et al. (2020). Studies conducted on various solid tumor models, both in vivo and in vitro, have demonstrated that the activity of the treatment is dependent on the dosage. Furthermore, the treatment exhibits selectivity towards cancer cells, hence minimizing the occurrence of adverse effects caused by non-specific distribution.

Antioxidant activity depends on extract phenolic and flavonoid concentration. Higher phenolic content increases antioxidant activity (Gaffar et al. 2022). The active chemical component acts as an inhibitor of signal transduction. Growth factor-induced signal transduction begins with external stimulation and is subsequently detected by receptors. Signal transduction cascades can be hindered by a variety of test compounds, including phosphatase inhibitors and kinase inhibitors. Flavonoids, like ATP, can interfere with the phosphorylation process, leading to its inhibition (Meiyanto et al., 2008). Saponin compounds possess the capability to inhibit the synthesis of Bcl-2. Bcl-2 is a protein with anti-apoptotic properties, which means it prevents cell death and promotes cell proliferation (Nitami, 2019). Research has shown that the use of *Avicennia marina* extract can trigger apoptosis in cancer cells and enhance the expression of p53 in these cells (Momtazi-Borojeni, 2013).

4 CONCLUSION

1. Methanol extract from api-api leaves and taurine have a cytotoxic effect on HeLa cervical cancer cells.
2. Methanol extract of api-api leaves and taurine inhibit HeLa cervical cancer cell growth, as evidenced by slower cell doubling rates compared to controls.
3. Test compounds showed variations in cytotoxic and antiproliferative activity. Api-api leaf extract stands out with higher activity against HeLa cervical cancer cells than taurine.

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