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S Andriani[1]), E L Widiastuti [1*]), N Nurcahyani [3]), E rosa[4]), H Busman[5]
[1,2,3,4,5] Fakultas Matematika dan Ilmu Pengetahuan Alam, Universitas Lampung

email: silviaandriani12@gmail.com[1],elwidi@yahoo.com[2],nuning.nurcahyani@fmipa.unila.ac.id[3], emantisrosa@gmail.com[4], hendri_busman@yahoo.com[5]
*Coresponden: Email : elwidi@yahoo.com

Abstract: The aim of study was to determine any anticancer potential from *Avicennia marina* leaf extract compare to taurine on cytotoxic and anti-proliferation activities against *HeLa* cervical cancer cell line by using the MTT method (3- (4, 5-dimethylthiazol-2-yl)) - 2, 5-diphenyltetrazolium bromide). The results indicated that methanol extraction of *A. marina* leaf as well as taurine did have cytotoxic and anti-proliferation effects on *HeLa* cell line with IC50 values of 321 ppm and 603 ppm 1000 ppm. While the doubling time value for anti-proliferation of *A. marina* leaf methanol extraction and taurine showed higher values than the control group (72.19 hours).
Keywords : *Avicenia marina*, taurine, cytotoxic,cytotoxic and anti-proliferative, *HeLa cells*

1. INTRODUCTION

The incidence of diseases of Indonesia is ranked 8th in Southeast Asia, and 23rd in the Asian Continent [1]. One of the diseases is cervical cancer which mostly caused by *Human Papillomavirus* (HPV 18) [2]. Currently cervical cancer is the second leading cause of mortality in women in the world with a new incidence of 570,000 new cases in 2018 representing 6.6% of all cancers affecting women [3]. Various attempts have been made to prevent and cure cancer. However, current cancer treatments are still not effective in healing, some are caused by using chemical drugs which only temporarily and cannot resist selective target cells, on the other hand, causes some damage on normal body cells. Efforts to find alternative natural cancer drugs is needed with high effect on target cells but not for normal cells by exploring natural sources, one of which is exploring potential drugs derived from of mangrove plants, namely *Avicenia marina*.

Avicenia marina is a type of mangrove plants in which with other mangrove plants have been widely used as traditional medicine in many different part of the world. *A. marina* belongs to Acanthaceae family with a height of 14 m and a specialized root structure known as pneumatophores. This *A. marina* is known to contain various bioactive compounds including alkaloids, saponins, triterpenoids, glycosides, tannins, flavonoids which act as drugs and high antioxidants [4]. Other studies also indicated that *A.marina* leaves have the potential to be

developed as phytopharmaca as well as other Avicennia plants [5, 6]. Therefore, in this study we aimed to investigate its function on *HeLa* cervical cancer cell lines compared to those of taurine.

On the other hand, Taurine, which part of free amino acids, known with numerous benefits to human health [7]. Taurine is also indicated to have the ability of antioxidants to prevent oxidative damage to the effects of paraquat induction [8]. Taurine also can be widely found in marine animals such as fish, squid, clams, snails and oysters.

2 METHODS

2.1 Sample Preparation

A. marina leaves was obtained from the Lampung Mangrove Center (LMC) in Labuhan Maringgai of Lampung Province, Indonesia. *A. marina* leaves extraction was carried out at the Biomolecular Laboratory of Biology, Faculty of Mathematics and Natural Sciences, University of Lampung. *A. marina* leaves dried in an oven at 30 °C, then ground to obtain simplicia powder. Maceration was applied to the simplicia powder by using a methanol solvent 1:10 for 24 hours followed by filtration using a bucher funnel and filter paper. The filtrate then was evaporated using a rotary evaporator at 50 °C until an extract was obtained in the form of a paste [9]. Cytotoxic and antiproliferative testing of *HeLa* cells was carried out at the Cell Culture and Cytogenetic Laboratory, Faculty of Medicine, Padjadjaran University [10].

2.2 HeLa Cell Culture Media Preparation

The media was made by dissolving 5 ml of 10% FBS solution, and 0.5 ml Pensterp (Penicillin-Streptomycin) which has been thawed in a sterile water bottle and then added with 50 ml of RPMI 1640 (Rosewell Park Memorial Institute)[10].

2.3 Harvesting and Calculation of HeLa Cell Count

Cells were transferred into a conical tube and 3 ml of media were added and centrifuged at 1500 rpm for 5 minutes. A total of 10 µl of mixed cells and trypan blue was used to calculate the number of cells by using hemocytometer. Living cells were marked with a clear color while those that were not blue. Calculation with a hemocytometer was done by selecting 4 counting rooms. Following was a series of calculations for the number of cells to be cultured [10]

Align Cell $= \frac{the\ cell\ count\ for\ all\ rooms\ is\ calculated}{4}$

Number of cells count / ml $= \text{Mean cell x dilution factor x } 10^4$

Total number of cell needed = total of well x total of sel well

Transfer volume sel $= \frac{\text{total number of sel needed}}{\text{number of cell count/ml}}$

2.4 Preparation for testing of the extract solution

A stock solution was made by dissolving each extract as much as 10 mg with 1 ml of 1% dimethyl sulfoxide (DMSO), while for taurine as much as 10 mg dissolved with 1 ml of distilled water. The stock solution was then diluted again to a series of concentrations of 125 ppm, 100 ppm, 75 ppm, 50 ppm and 25 ppm. *A. marina* leaf extract solution and taurine in various levels then were tested on *HeLa* culture cells.

2.5 Cytotoxic test by using MTT (3-(4,5- dimetiltiazol-2-il)-2,5-difenil tetrazoliumbromida)

Cells that have been cultured in a well plate for 24 hours were removed from the incubator then the culture media were removed and the cells were rinsed with phosphate buffer saline (PBS). Each well was then given each extract and taurine with a predetermined concentration and incubated again for 24 hours. The test solution was then discarded, and the wells were rinsed with a phosphate buffer saline (PBS) solution. To the well was added 10 µl MTT (3- (4,5-dimethyltiazol-2-il) -2,5-diphenyl tetrazolium bromide) with a concentration of 5 mg / ml phosphate buffer saline (PBS). Then incubated again for 2 hours at 37 °C in a CO_2 incubator. Living cells would metabolize MTT (3- (4,5-Dimethyltiazol-2-il) -2,5-Diphenyl Tetrazolium bromide) to formazan giving purple color. The MTT reaction (3- (4,5-dimethyltiazol-2-il) -2,5-diphenyl tetrazolium bromide) was stopped with 100% dimethyl sulfoxide (DMSO) stopper reagent as much as 100 µl per well. The uptake was then read with an ELISA reader at a wavelength of 550 nm [12]

2.6 Antiproliferative test by using MTT (3-(4,5- dimetiltiazol-2-il)-2,5-difenil tetrazoliumbromida)

Cells that had been cultured in hourly well plates were given 100 µl extracts and taurine with 125 concentration series, respectively; 100; 75; 50; and 25 ppm. Sampling was carried out for incubating with different times, namely, 24 hours, 48 hours, and 72 hours at 37 °C in a CO_2 incubator. The absorbance of each treatment was measured with a wavelength ELISA reader 550 nm. Then a statistical analysis was performed to determine the difference in the number of living cells at different incubation times [11]

2.7 Data Analysis

Cytotoxic tests on *HeLa* cancer cells were carried out using probit analysis to determine the IC_{50} value of each extract. The percentage of cell viability was obtained by the following formula [12]. Analysis of antiproliferative test for the doubling time value was obtained from the linear regression equation between incubation time vs. log number of living cells. To determine the effect of concentration on the average number of living cells, a statistical analysis of the One Way ANOVA test with SPSS was performed at a 95% confidence level followed by the Least Significant Difference (LSD) test at 5%.

3. RESULTS

3.1 Cytotoxic Test

Based on cytotoxic tests that have been carried out with *A. marina* and taurine leaf extracts against *HeLa* cervical cancer cells, it was obtained that the relationship of extract concentration with cell viability (%) which could be seen in Figure 1, Figure 2 and Figure 3. To understand the cytotoxic effect of the extract and taurine, Doxorubicin as commonly used for cancer drug was applied with 1 5 and 10 ppm (data for doxorubicin and taurine was shared with Widiastuti *et al*, 2020 unpublished) .

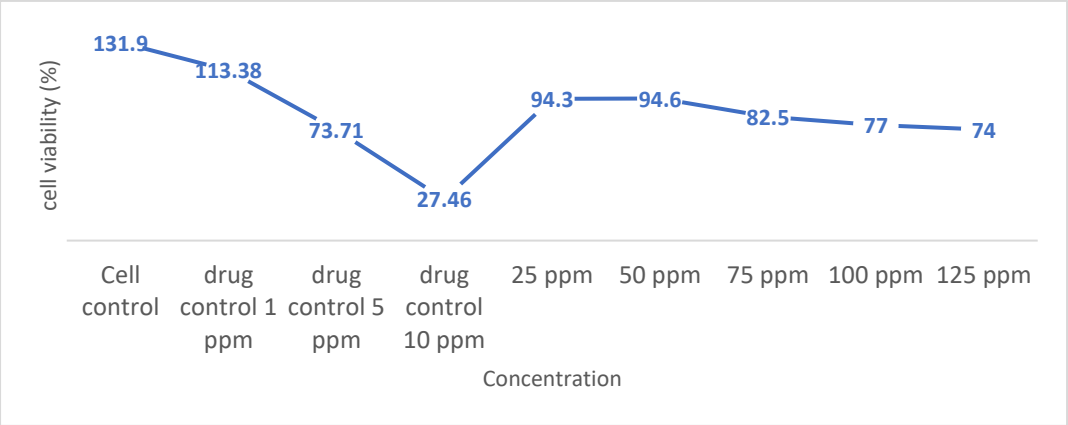

Figure 1. Effect of *A. marina* leaf extract on *HeLa* cell viability (%)

The result indicated that *A. marina* leaf extract with a concentration of 125 ppm showed the lowest viability percentage of 74 % among other concentrations and cell control. This concentration was almost close to those given with Doxorubicin (commonly used for cancer treatment) as much as 5 ppm.

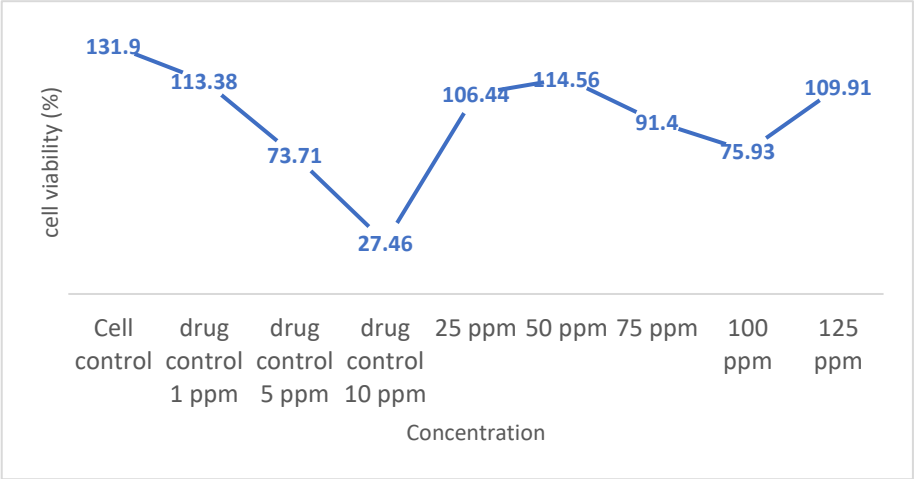

Figure 2. Effect of taurine on *HeLa* cell viability (%)

Similar to those, given Taurine treatment also showed the lowest cell viability percentage at a concentration of 100 ppm which was indicated for 75.93%, from which the percentage of cell inhibition was 24.07%. Similar to those in *A. marina* extract, this concentration of taurine reduced the viability of the cells indicated by those given doxorubicin at 5 ppm.

The effect of the *A. marina* extract as well taurine at different concentration on cytotoxic activity by looking at the IC_{50} values could be seen in Table 1 as followed. The IC_{50} against of *A. marina* lied in 321 ppm and taurine was in 603 ppm while cancer drug, doxorubicin, was in 12.35 ppm.

Tabel 1. Cytotoxic activity of *A. marina* and Taurine on *HeLa* cell line (IC_{50} value)

Compound	Consentration (ppm)	Cell Viability (%)	IC_{50} (ppm)
A. marina	25	94.3	321
	50	94.6	
	75	82.5	
	100	77	
	125	74	
Taurin	25	106.44	603
	50	114.56	
	75	91.4	
	100	75.93	
	125	109.91	
Doxorubicin	1	113.38	12.35
	5	73.71	
	10	27.46	

The *HeLa* cells morphology from different treatment groups could be seen in Figure 3. Some apoptotic cells indicated with shrinking and irregular shape, while the *HeLa* cell shape was polygonal and attaching to the matrix. Criteria for a cytotoxic activity for crude extracts according to the American National Cancer Institute (NCI) are IC_{50} <30 µg/ml [13].

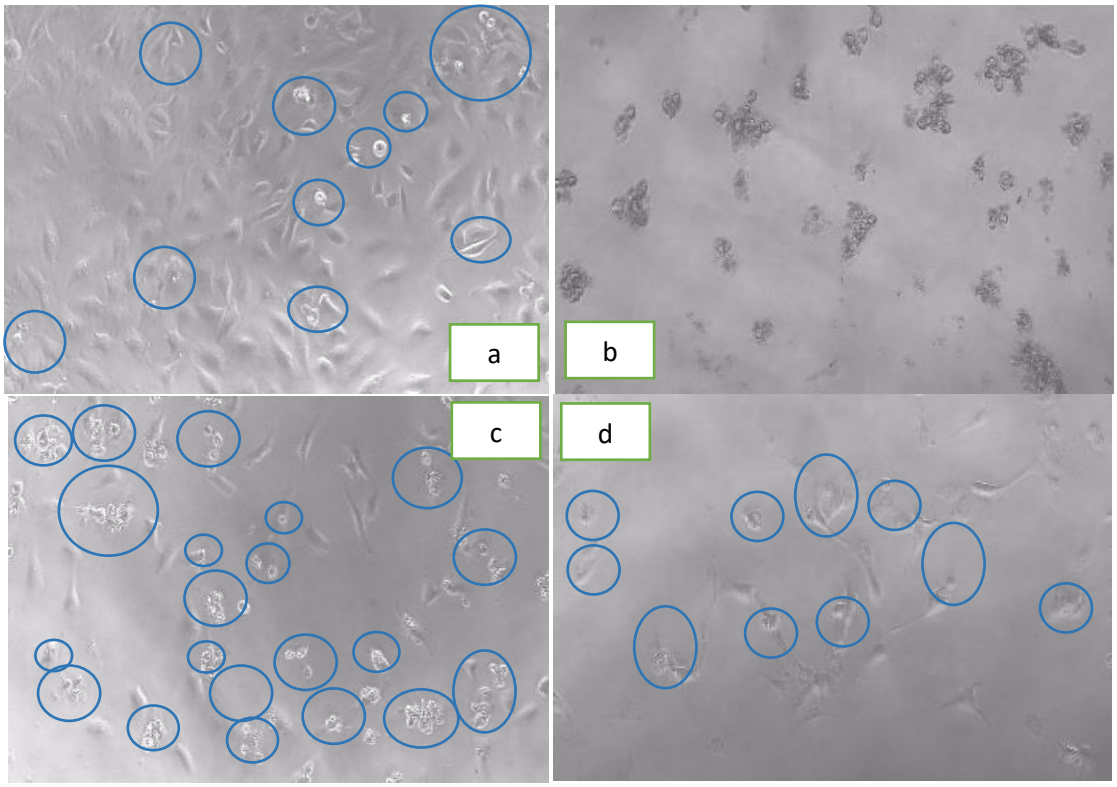

Figure 3. The appearance of *HeLa* cell mophology in cell control (a), drug control with doxorubicin (b), 75 ppm concentration *A. marina* methanol extract treatment (c), 100 ppm concentration taurine treatment (d). Note: ○ : apoptotic cells

The doubling time of the *HeLa* Cell line with different treatment groups could be seen in Table 2. Doubling time was determined to understand how much time was needed for the culture cells to increase their number or to proliferate.

Tabel 2. *Doubling Time* of *A. marina* and Taurine on *HeLa* cell line

Treatment groups	**Concentration (ppm)**	**Incubation time line equation and log of cell count**	**Slope**	***Doubling Time* (h) value**
A. marina	25	0.0721x + 4.1948	0.0721	562
	50	0.0348x + 4.2329	0.0348	1055
	75	0.0312x + 4.1683	0.0312	1384
	100	0.0262x + 4.1457	0.0262	1734
	125	0.0157x + 4.1368	0.0157	2950
Taurin	25	0.0014x+4.2937	0.0014	218
	50	0.001x + 4.3142	0.0010	285
	75	0.0013x+4.2752	0.0013	249
	100	0.0004x +4.259	0.0004	852
	125	0.0016x+4.2878	0.0016	195
Control	0	0.0041x + 4.304	0.0041	72.19
Doxorubicin	1	-0.0037x+4.3688	-0.0037	-
	5	-0.0187x +4.462	-0.0187	-
	10	-0.0208x+4.2854	-0.0208	-

Note: Cell control slope values = 0.0041

The doubling time of *A. marina* leaf extract indicated a positive correlation with the concentration of the extract, the greater the concentration of the extract the longer the doubling time. The doubling time values of the drug treatment (doxorubicin) could not be obtained, this related with negative value of the slope in the linear regression equation, presumably all the *HeLa* cells died or no proliferation occurred. Meanwhile, those treatment groups given taurine indicated their doubling time value was higher than the control groups. Therefore, both extract of *A. marina* and taurine were able to interrupt the proliferation of *HeLa* cervical cancer cells.

In order to elucidate how far the interruption of the *A. marina* extract and taurine on the *HeLa* cervical cancer cell line, determination of the number of surviving cells then was calculated and could be seen in Table 3 as followed.

The number of surviving *HeLa* cells varied for both treatment groups and from 24 hours up to 72 hours of observation time. The number of surviving cells decreased as the concentration of extract and taurine increased.

Tabel 3. Effect of *A. marina* and Taurine against average number of surviving cells

Treatment Groups	Concentration (ppm)	Number of living cells (x 1000 cells)		
		24 hours	**48** hours	**72** hours
Avicennia	25	18.9±1.48[a]	21.0±1.30[a]	26.3±0.35 [a]
	50	18.9±0.79[a]	19.2±0.72[a]	22.2±0.66 [b]
	75	16.5±0.60[ab]	15.6±0,92[b]	19.1±0.55[c]
	100	15.4±0.30[b]	14.7±1,21[b]	17.4±0.49 [d]
	125	15.0±1.34[b]	13.3±0,30[b]	16.1±0.29 [d]
Taurin	25	21.3±0.30[ab]	21.8±1.42	25.7±0.60[ab]
	50	22.9±1.48[a]	22.9±1.60	24.2±0.35[b]
	75	18.3±0.66[bc]	22.1±0.99	23.6±1.80[b]
	100	15.2±1.99[c]	21.4±3.08	19.4±1.12[c]
	125	22.0±1.43[ab]	19.5±1.28	27.8±0.21[a]

Note: superscript of a, b and c indicated significant different at 5% of LSD

4. DISCUSSION

Based on cytotoxic results carried out for methanol extract of *A. marina* leaves and taurine on *HeLa* cervical cancer cells, both of compounds (*A. marina* extract and taurine) were able to kill the *HeLa* cells indicated by lowering in cell viability (%). This cytotoxic ability was also showed by the destruction of cells by looking at the morphology (Figure 3 a, b, c, and d). *HeLa* cells that underwent apoptosis had a different morphology. Cells shrinked as a result of intracellular fluid loss and very low cell density [13]. Living *HeLa* cells are generally polygonal in shape and attached to the matrix. However, it will be separated from the matrix after administration of trypsin.

The IC50 values for *A. marina* leaf extract was 321 ppm, taurine was 603 ppm, and doxorubicin was 12.35 ppm (Table 1). When compared to those of doxorubicin, the IC50 values of the two test compounds were greater. Yet, the standard IC50 value was <1000 ppm, meaning that extract of *A. marina* and taurine belonged to potential cytotoxic compound for the *HeLa* cell lines [14]. Therefore, these two compounds indicated their potential for cytotoxic activity, from which then can be used as anticancer agents [15]

Table 2 shows that the doubling-time values obtained were different at each concentration of the methanol extract of *A. marina* leaf and taurine. In this value for their doubling-time, the slope value of the linear regression equation could be used as a parameter of cell proliferation kinetics. In which the control cells had the slope value of 0.0041. This value became a reference for the treatment groups. If the value of the treatment slope was smaller than the value of the cell control slope, the time required for doubling time was longer [16]. As seen in Table 3, all treatment slope values were lower than the cell control slope values, indicating that the time needed for cells with multiple treatments was longer than for those of the control cells. All the preparation for treated cells to proliferate needed much longer time. These results also indicated that the methanol extract from *A. marina* leaf and taurine had potential as an antiproliferative compound for *HeLa* cervical cancer cells [17].

Based on the treatment with *A. marina* extract it showed a significant difference in the number of living cells in all concentrations. The highest average number of living cells was shown by giving

extract concentration of 25 ppm with incubation time of 72 hours, while the lowest average living cells are indicated by extract concentration of 125 ppm with incubation time of 72 hours. In the treatment of taurine, known as a free amino acid, with various concentrations, the average number of living cells differed significantly at the incubation time of 24 hours and 72 hours. These two test compounds indicted that they had cytotoxic activation of *HeLa* cervical cancer cells, presumably this was due to the presence of bioactive compounds contained in the extract of *A marina*, such as steroid, flavonoids which known also as high levels of antioxidant, therefore, it could inhibit the process of oxidation and proliferation [18].

The cytotoxic activity of *A. marina* plants also showed strong anti-proliferation against L-929 rat fibroblasts and human leukemia cells K562 [19]. Against BT-20 breast cancer cells *A. marina* also showed moderate cytotoxicity [20]. *A. marina* leaf extracts had also proven cytotoxicity to breast cancer cells MCF-7 MDA-MB 231 through apoptosis [21]. *A. marina* as one of mangrove plants has the widest geographical distribution and function, other than as animal feed. Toxicological studies of *A.marina* extracts also indicated that *A. marina* could be developed into anticancer, antibacterial, and anti-arthritis medicinal plants [22].

5. CONCLUSION

a. *A. marina* leaf extract, and taurine are toxic to *HeLa* cervical cancer cells, evidenced by the percentage of treatment cell viability that is lower than cell control, and the acquisition of IC50 values for all test compounds less than 1000 ppm
b. *A. marina* leaf extract and taurine can inhibit the proliferation of *HeLa* cervical cancer cells indicating by their doubling-time which much longer than the cell control.
c. Based on the differences in cytotoxic and anti-proliferative abilities, extract of *A. marina* is more effective as a cytotoxic and anti-proliferative compound against *HeLa* cervical cancer cells compared to a free amino acid taurine

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