ANTICANCER POTENCY OF HOLLY MANGROVE LEAF *(Acanthus ilicifolius)* METHANOL EXTRACT WITH TAURINE BY *IN VITRO* TEST IN CELL CULTURE OF HELA CERVICAL CANCER

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Abstract. Holly mangrove which much abundance in mangrove area, known by locals as anti-inflammatory. This study was conducted to evaluate of its action as anticancer *in vitro*. Holly mangrove leaf methanol extraction was proven through cytotoxic and antiproliferation tests by MTT method (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) on HeLa cervical cancer cell culture. The results indicates that the extraction had a cytotoxic activity with IC50 values are 206 ppm, while the doubling time value in the antiproliferation test indicated much higher values than the control (72.19 hours).

Keywords : holly mangrove leaf, cytotoxic, antiproliferation, and HeLa cell.

1. Introduction

Cervical cancer is a malignancy originating from the cervix. The cervix is the lower third of the uterus, cylindrical, prominent and connects to the vagina through the external uterine ostium [1]. Cervical cancer is the fourth most frequent cancer in women with an estimated 570,000 new cases in 2018 representing 6.6% of all female cancers [2]. However, current cancer treatment methods such as chemotherapy and radiation are not yet fully effective in removing cancer and are still causing side effects for sufferers. So much research needs to be done to find treatment methods that are right on target and friendly for human body normal cells, one of which is by using natural materials

Holly mangrove (Jeruju in local name) is a coastal ecosystem plant that is potentially used as a source of phytopharmaca, due to the content of various bioactive compounds. Phytochemical Screening [3] in jeruju extracts, with various types of solvents showing the content of various secondary metabolites such as alkaloids, flavonoids, phenolic compounds, terpenoids/steroids, and saponins. Research related to the potency of jeruju as an anticancer compound has been

carried out one of them on MCF-7 and PA-1 cancer cells that produced Cytotoxicity concentration (CC50) values of 24.22 ± 0.2 μg / mL and 15.74 ± 0.1 μg / m [4].

In addition to jeruju, taurine which is mostly obtained from marine animals such as fish, squid, shellfish, snails, and oysters are proven to have various health benefits. The benefits of taurine are, regulating osmoregulation, nerve maturity, and brain development [5]. The study on proliferative inhibition of MCF-7 breast cancer cells and MDA-MB-231 breast cancer cells was carried out [6]. The results showed that taurine significantly inhibited cell growth at incubation times of 24 hours, 48 hours, and 72 hours with IC50 values of 284.50 mM, 133.61 mM, and 106.40 mM (MCF-7 cells) and 617.99 mM, 127.21 mM and 90.46 mM (MDA-MB-231 cells). Considering the various potentials of jeruju and taurine, this research was conducted to prove the cytotoxic and antiproliferation activities of jeruju leaves extract and taurine in HeLa cervical cancer cells.

2. Methods

Preparation of Plant Extracts

Jeruju leaves were obtained from Lampung Mangrove Center (LMC) in Labuhan Maringgai, East Lampung. Jeruju leaves were sorted out the best and cleaned using flowing water. Furthermore, jeruju leaves were dried using an oven at 30˚C. The dried jeruju leaves then were finely crushed and blended. The powders were then soaked with methanol in ratio 1:10 for 24 hours. Solutions were then filtered using a funnel glass and filter paper. The methanol extracts obtained were then evaporated using a rotary evaporator at 50°C until they formed crude extract [7].

Phytochemical Test

To determine the content of secondary metabolites contained in the methanol extract of jeruju leaves, phytochemical screening was carried out. The Phytochemical test procedure of jeruju leaves extract followed and modification from other studies [8][9].

1. **Saponins**
   5 ml of distilled water mixed into 0.5 ml of the extract then shaken for 10 seconds. The indicator of saponin existence is when the foam is formed
2. **Terpenoids**
   Three drops of Lieberman-Burchard reagent (glacial acetic acid + concentrated H2SO4) were dropped into 0.5 ml extract. A positive triterpenoid test gives a red or purple color.
3. **Tannin**
   3 drops of FeCl3 1% solution mixed with 1 ml of extract. Positive indicators of tannin are blackish-green or dark blue formed.
4. **Alkaloids**
   5 drops of chloroform and 5 drops of Mayer reagent were mixed into 0.5 ml of extract. The presence of alkaloids is characterized by the formation of white to brownish color.
5. **Flavonoids**
   0.5 g of Mg powder was mixed into 0.5 mL of extract, then added 5 mL HCl into the mixture drop by drop. A positive indicator of the presence of flavonoids is the formation of a red or yellow solution in the presence of foam.

HeLa Cell Culture Media Preparation

5 ml of Fetal Bovine Serum (FBS) 10%, and 0.5 ml Pensterp (Penicillin-Streptomycin) that has been thawed at room temperature are mixed in sterile bottles and then added with Rosewell Park Memorial Institute (RPMI 1640) to 50 ml [10].

HeLa Cell Count Calculation

10 µl HeLa cells and 10 µl trypan blue were pipetted into the well plate. 10 µl mixture of cells and trypan blue then pipetted on a hemocytometer to calculate the number of cells. Living cells are marked with a clear color and dead cells s blue. Calculation with a hemocytometer is done by selecting 4 counting rooms. Following is a series of calculations for the number of cells to be cultured [10].

$$\text{Cell average} = \frac{\text{The cell number of all rooms}}{4}$$

$$\text{The number of cells counted / ml} = \text{Average cell x dilution factor x } 10^4$$

$$\text{The total number of cells needed} = \text{Number of wells x number of cells per well}$$

$$\text{Transfer volume of cell harvesting} = \frac{\text{Total number of cells needed}}{\text{The number of cells counted /ml}}$$

Test Compounds Preparation

Preparation of the test compound is done first by making a stock solution which is dissolved each extract as much as 10 mg with 1 ml dimethyl sulfoxide (DMSO) 1%, while for taurine with the same mass dissolved with distilled water as much as 1 ml. The stock solution is then diluted again to a concentration of 125 ppm, 100 ppm, 75 ppm, 50 ppm, and 25 ppm [10].

Cytotoxicity Assay

Before the cytotoxic test is performed, the calculated HeLa cells are cultured for 24 hours at 37 ° C in a CO2 incubator. For cytotoxic tests, each well was filled with 100 µl of cell suspension, with each well there were 20,000 cells. The culture process aims to get cells attached to the flask wall [10]. Cells that have been cultured in a well plate for 24 hours are removed from the incubator then the culture media are removed and the cells are 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 is then discarded, and the wells are rinsed with a solution of phosphate buffer saline (PBS). 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 CO2 incubator. Living cells will metabolize MTT (3- (4,5-Dimethyltiazol-2-il) -2,5-Diphenyl Tetrazolium bromide) to purple formazan. 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 absorbance is then read with an ELISA reader at a wavelength of 550 nm [10].

Antiproliferation Assay

For the antiproliferative test, each well was filled with 100 µl HeLa cell suspension. Each well contained 20000 cells, then incubated for 24 hours at 37 ° C in a CO2 incubator [10]. Cells that had been cultured in a well plate for 24 hours were given 100 µl extract and taurine with a concentration of 125; 100; 75; 50; and 25 ppm. Then incubation was carried out with

different treatment time,24 hours, 48 hours, and 72 hours at 37 ° C in a CO2 incubator. After the treatment time is reached the test solution is then discarded, and the wells are rinsed with a phosphate buffer saline (PBS) solution. To the well then 10 µl MTT (3- (4,5-Dimethyltiazol-2-il) -2,5-Diphenyl Tetrazolium bromide are added with a concentration of 5 mg/ml Phosphate Buffer Saline (PBS). The results were then incubated again for 2 hours at 37 ° C in a CO2 incubator. Living cells will metabolize MTT (3- (4,5-dimethyltiazol-2-il) -2,5-diphenyl tetrazolium bromide) to formazan which is purple in 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 absorbance of each treatment was measured with a wavelength ELISA reader 550 nm. Then performed statistical analysis to determine the difference in the number of living cells at different incubation times [11].

Data Analysis

Data processing for cytotoxic testing of HeLa cancer cells is done by calculating the percentage of cell viability. The cell viability percentage values then changed in the form of a probit value to determine the IC50 value. IC50 value determination involves linear regression equations of log concentration vs. probit value of cell viability percentage using the Microsoft Excell program. The percentage of cell viability is obtained by the following formula [11].

$$\text{Percentage of cell viability} = \frac{(\text{The absorbance of treatment} - \text{Absorbance of media control})}{(\text{The absorbance of solvent control} - \text{Absorbance of media control})} \text{x} 100\%$$

Meanwhile, the processing of antiproliferative test data was carried out to determine differences in the number of living cells in the extract and taurine treatments with various concentrations over different incubation times. The number of living cells at each incubation time will then be used to determine the doubling time value of each treatment. The doubling time value is obtained from the linear regression equation between incubation time vs. log number of living cells. To find out 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. If there is a difference between treatments then it is tested further by testing *Least Significant Difference* (LSD).

3. Results and Discussion

Phytochemical Test of holly mangrove/jeruju leaf can be seen in table 1 as follows.

Table 1. Phytochemical Test of methanol extraction of holly mangrove/Jeruju Leaf

Phytochemical Test	Jeruju leaves
Saponins	+
Terpenoids	-
Tannins	+
Alkaloids	-
Flavonoids	+

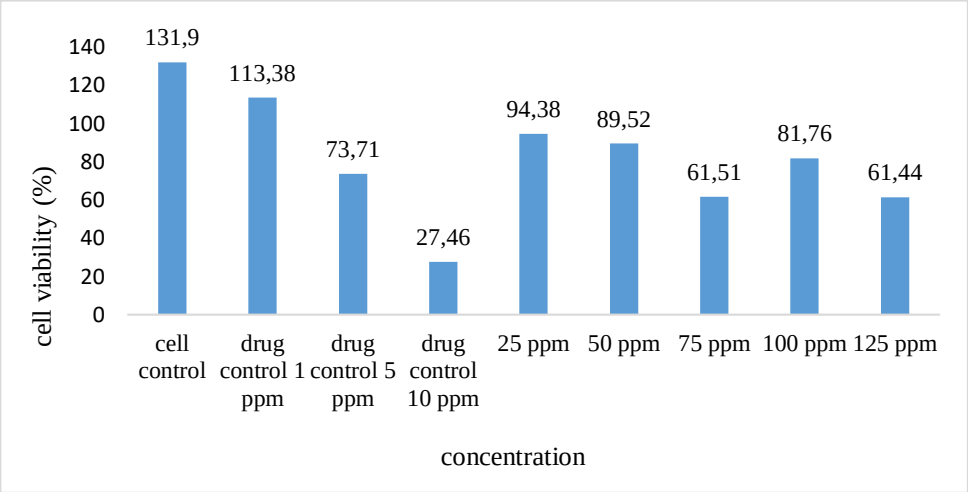
Note : (+) = Contain Test Compounds
(-) = Contain Test Compounds

From Table 1, it can be seen that methanol extraction of jeruju leaf contains of saponins, tannins, and flavonoids but do not contain terpenoids and alkaloids. Referring to other study [12] in the phytochemical test of jeruju leaf extract, there is no different and proves that jeruju contained chemical compounds in the form of alkaloids, resins, tannins, glycosides, saponins, sterols, terpenoids, phenols, and catechols. Similar to those obtained by other study [13] which also indicated phytochemical content of flavonoids, alkaloids, phenols, and steroids.

The content of secondary metabolites extracted is influenced by one type of solvent used in extraction. In this research the solvent used is methanol. Methanol is a solvent that has a CH3OH molecular structure, is polar because it has a hydroxyl group (-OH) and is also non-polar because it has a methyl group (-CH3). However, methanol is a very polar compound [13]. This is in line with the opinion of other study [14] which states that methanol is a polar solvent that can only dissolve polar compounds such as phenols. There are various reasons for choosing methanol as a solvent such as a selectivity, boiling point of solvents, solvents that are not soluble in water, solvents are inert so they do not react with other components, and the price of solvents [15].

Cytotoxic Test

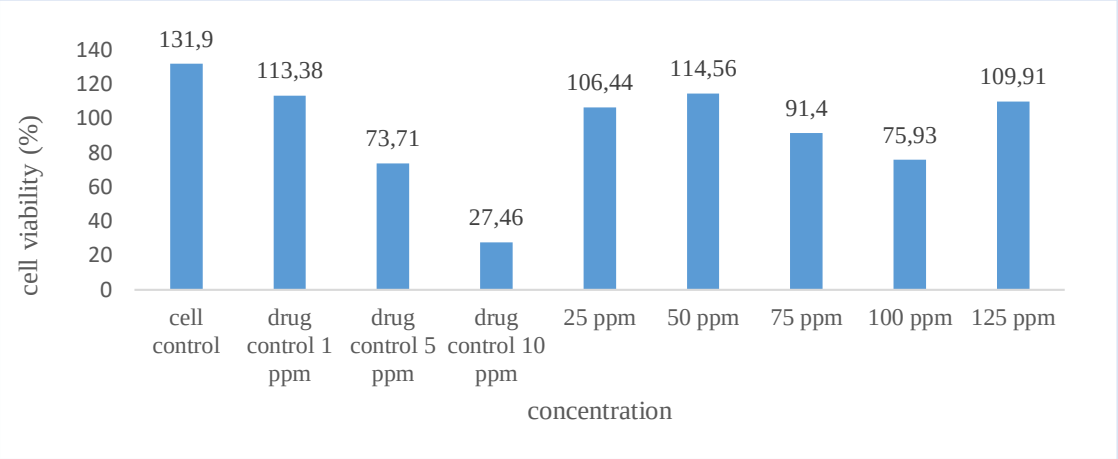
Based on cytotoxic tests that have been carried out with extracts of Jeruju (*Acanthus ilicifolius*) and taurine extracts against HeLa cervical cancer cells, graphs obtained the relationship of extract concentration with cell viability (%) as shown in Figure 1 and 2.



Note: Drug control is carried out with Doxorubicin

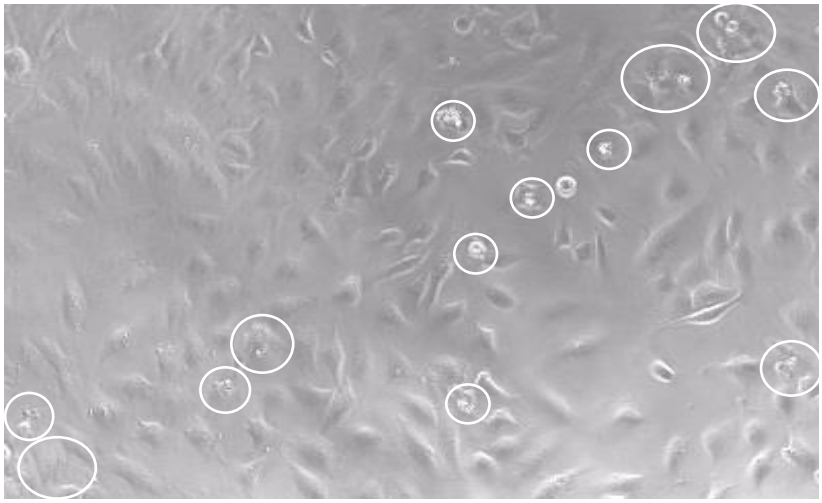
Figure 1. Relationship of Jeruju extract concentration with cell viability (%)

From Figure 1 above the treatment of jeruju leaf extract affects the percentage of cell viability compared to cell control and drug control using Doxorubicin. Doxorubicin is an anthracycline class of antibiotics that are widely used to treat various types of cancer such as breast cancer, acute leukemia, bone, and ovarian cancer [16]. Jeruju leaf extract with a concentration of 125 ppm showed the lowest viability percentage of 61.44% among other concentrations and cell control. Jeruju leaf extract concentration of 125 ppm also showed a higher percentage of cell inhibition than the percentage of inhibition by 5 ppm drug control.



Note: Drug control is carried out with Doxorubicin
Figure 2. Relationship of taurine concentration with cell viability (%)

Overall treatment with test compounds showed the presence of cytotoxic activity so as to reduce the percentage of test cell viability (compared to control cells). The ability of this cytotoxic activity can be seen from the cell morphology in Figure 3 and Figure 4. Living HeLa cells are generally polygonal in shape and attached to the matrix. However, it will float on the media if it is separated from the matrix (usually after trypsin treatment). HeLa cells in a phase leading to apoptosis or have undergone apoptosis have a different morphology. Cells will experience a reduction in size and shrinkage as an effect of loss of intracellular fluid as well as a loss of contact with neighboring cells [17].



Note: : ◯ apoptotic cells
Figure 3. The appearance of HeLa cell morphology in cell control

The picture above can be seen as the density of live HeLa cells in cell control without very dense treatment. The high level of density of living cells is possible because cells in the cell control experimental unit do not experience obstacles in their growth and are also balanced with nutritional needs that are met. However, some cells undergoing apoptosis can be found in this treatment.

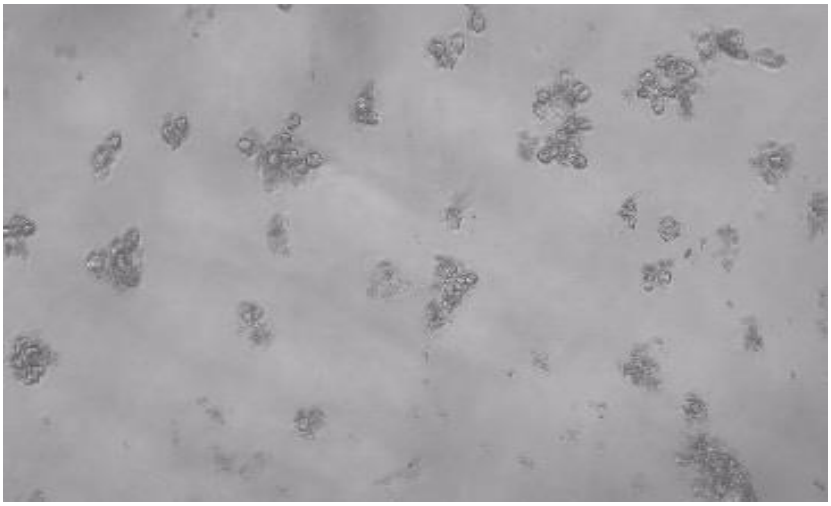


Figure 4. The appearance of HeLa cell morphology in drug control with Doxorubicin

In drug control treatment with doxorubicin (Figure 4), HeLa cells appear to have apoptosis even very low cell density. The shrinking shape of the HeLa cell and its smaller size and blackened color prove that doxorubicin as positive control is very effective as a cancer drug. Doxorubicin works through the mechanism of intercalation with DNA, inhibits the action of topoisomerase II, inhibits the relocation of DNA strands, and disrupts membrane fluidity [18].

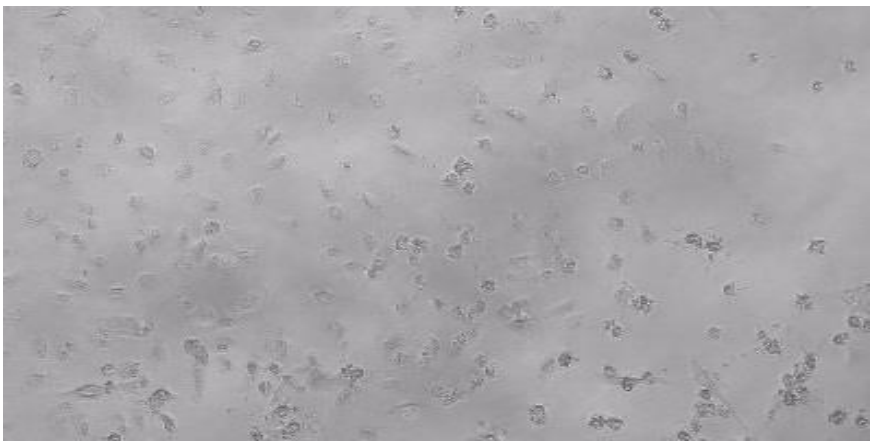


Figure 5. The appearance of HeLa cell morphology at 75 ppm jeruju leaf extract treatment

In the Jeruju leaf extract treatment, the concentration of 75 ppm (Figure 5) the density of HeLa cells is very low when compared to the cell control. The morphology and color of HeLa cells also indicate the cell has died.

In the treatment of taurine with a concentration of 100 ppm, cell density is very rare with a view that tends to be clean. However, in this concentration treatment can still be found HeLa cells in living conditions. The administration of taurine with a concentration of 100 ppm is the most effective level in this test in inhibiting the growth of HeLa cells when compared with other concentrations.

The percentage of cell viability obtained after the treatment of extracts and taurine is then used to calculate the IC50 value. The percentage of cell viability is changed into probit values

while the extract concentration is converted into log form from each concentration. Using log concentration and probit values then a log curve of concentration vs. probit value of cell viability percentage to get the linear regression equation y = bx + a. By entering the value of y = 5 (probit value of 50%) in the linear regression equation, the value of x will be obtained, which is then converted into antilog. The antilog value of x is the IC_{50} value of the test compound [11].

Table 2. Cytotoxic Activity of Test Compounds for HeLa Cervical Cancer Cells in Value IC_{50}

Test compound	**Concentration (ppm)**	**Cell Viability (%)**	**IC_{50} (ppm)**
Jeruju leaf	25	94,38	200
	50	89,52	
	75	61,51	
	100	81,76	
	125	61,44	
Taurine	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	

IC50 is inhibitory concentration or the ability of a substance to inhibit test cell proliferation by 50%. The smaller the IC_{50} value possessed by the test substance indicates that the toxic level is higher and more potential if used as a drug [19]. Criteria for a cytotoxic activity for crude extracts according to the American National Cancer Institute (NCI) are IC_{50} <30 µg / ml [20].

Based on Table 2 above, we can see extracts of jeruju leaf, taurine, and doxorubicin with various concentrations causing a decrease in cell viability. This indicates the presence of cytotoxic activity by test compounds on HeLa cells. The results of the regression calculations showed the IC_{50} value for the extract of Jeruju leaves was 200 ppm, taurine 603 ppm and doxorubicin 12.35 ppm. IC_{50} values of jeruju leaf extracts, seagrass extract and taurine are too large compared with doxorubicin and based on IC_{50} values by the American National Cancer Institute extracts of jeruju, seagrass, and taurine leaves are concluded to have no potential as anticancer compounds.

Table 3 shows that the doubling time value obtained is different at each concentration of the treatment of plant extract and taurine. In the doubling time test, the slope value of the linear regression equation is a parameter of cell proliferation kinetics. In the control cell, the slope value obtained was 0.0041. This value becomes a reference for the cell group with treatment. If the value of the treatment slope is smaller than the value of the cell control slope, the time required for doubling time is longer. If the slope value is greater than the control cell, indicating the time for doubling time is getting shorter [21]. Based on Table 3 above, all treatment slope values are lower than the cell control slope values. This indicates that the treated HeLa cells need more time to multiply than the HeLa cells without treatment.

Table 3. Doubling Time Values in Antiproliferation Test

Test Compound	Concentrastion (ppm)	The incubation time line equation and log the cell count	Slope value	Doubling Time Value (hours)
Jeruju leaf	25	0,0019x + 4,2754	0,0019	170
	50	0,0013x +4,270	0,0013	253
	75	0,0012x + 4,2095	0,0012	325
	100	0,0007x + 4,2625	0,0007	482
	125	0,0002x + 4,2232	0,0002	1884
Taurine	25	0,0014x+4,2937	0,0014	218
	50	0,001x + 4,3142	0,001	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
Cell 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	-

The doubling time of jeruju leaf extract shows a positive correlation with the concentration of the extract, where the higher the concentration the longer the doubling time. At concentrations higher than 25 ppm, doubling time values were not obtained as the drug control treatment with doxorubicin. This condition can be explained by the negative value of the slope in the linear regression equation. Negative slope values indicate no proliferation because the cell has presumably died. For the treatment of cells with taurine showed a doubling time value that varied, but far higher than the control cell. Referring to this fact, it means that the methanol extracts of jeruju leaf and taurine have potential as antiproliferative compounds in HeLa cervical cancer cells.

Table 4. Average Cell Counts in the Treatment of Test Compounds

Test Compound	Concentration (ppm)	Number of living cells (x 1000 Cell)		
		24 hours	48 hours	72 hours
Jeruju leaf	25	$18,9\pm0,98^{a}$	$24,2\pm1,00^{a}$	$26,3\pm0,52^{a}$
	50	$17,9\pm0,66^{ab}$	$21,8\pm0,84^{ab}$	$23,8\pm0,94^{a}$
	75	$12,3\pm0,76^{b}$	$19,5\pm1,37^{b}$	$21,4\pm0,83^{ab}$
	100	$16,4\pm0,61^{c}$	$20,5\pm1,34^{b}$	$24,1\pm2,88^{a}$
	125	$12,3\pm0,18^{c}$	$18,8\pm0,49^{b}$	$18,9\pm0,69^{b}$
Taurine	25	$21,3\pm0,30^{ab}$	$21,8\pm1,42$	$25,7\pm0,60^{ab}$
	50	$22,9\pm1,48^{a}$	$22,9\pm1,60$	$24,2\pm0,35^{b}$
	75	$18,3\pm0,66^{bc}$	$22,1\pm0,99$	$23,6\pm1,80^{b}$
	100	$15,2\pm1,99^{c}$	$21,4\pm3,08$	$19,4\pm1,12^{c}$
	125	$22,0\pm1,43^{ab}$	$19,5\pm1,28$	$27,8\pm0,21^{a}$

Note : a, b and c indicate the significant difference LSD ($p<0.05$)

Based on the results of the One Way ANOVA statistical analysis of the average number of living cells, there were significant differences in all treatment groups with jeruju leaf extract. The results of further tests with Least Significant Difference (LSD) showed the highest average number of living cells obtained at a concentration of 125 ppm with 72

hours incubation time, while the lowest average number of living cells was shown by treatment with a concentration of 100 ppm and 24 hours incubation time.

In the treatment of taurine amino acids with various concentrations, the mean number of living cells was significantly different at the incubation time of 24 hours and 72 hours. The highest average number of living cells is indicated by taurine treatment 125 ppm and incubation time 72 hours, while the lowest average number of living cells is indicated by taurine treatment 100 ppm and incubation time 24 hours.

There are various possible mechanisms of action of active compounds of methanol extract as anticancer. First, the content of active compounds acts as a barrier to signal transduction. Signal transduction in the form of growth factors that begin with stimulation from outside the cell and are captured by the receptor. It is known then receptor will then convey the proliferative signal to proteins in the cytoplasm. Activation of this signal transduction involves ATP and proteins are generally a type of protein kinase, through a process of phosphorylation [22]. This cascade signal transduction process can be inhibited by several test compounds including phosphatase inhibitors and kinase inhibitors. Like flavonoids that can compete with ATP in the phosphorylation process so that the phosphorylation process is inhibited [21]. Second, inhibition of oxidative processes that can cause cancer initiation by alkaloids. This mechanism is mediated by a decrease in the enzyme Lipooksigenase (LOX) and Xanthine Oxidase Cyclooxygenase (COX) needed in the peroxidation process thereby delaying the cell cycle [23]. Alkaloid compounds are also capable of binding to tubulin (a protein making up microtubules) so that it can inhibit protein polymerase and interfere with cell proliferation [24]. Third, blockade of the S phase or synthesis of the cell cycle by tannins. In the S phase, the cell will carry out DNA synthesis and the process of chromosome replication [25][26]. Besides inhibiting the synthesis phase, tannins can also increase the synthesis of p27 protein which inhibits the cell cycle [13]. P27 protein is a protein that binds cyclin and cyclin-dependent kinase (CDK) so that there is an obstacle to the S phase [27]. CDK is a protein that regulates the transition from G1 to S phases, termination of cells in the G1 phase will provide the opportunity for damaged cells to be recognized and continue the process of apoptosis. CDK suppression can cause the cell cycle to stop in the G1 phase so that the process of repair and apoptosis can take place [28]. Fourth, saponins inhibit the formation of Bcl-2. Bcl-2 is an anti-apoptotic protein that causes cells to proliferate [29]. Bcl-2 works by inhibiting cytochrome release c. Inhibition of Bcl-2 will not inhibit cytochrome release c. Then cytochrome c together with Apoptosis Protease Activating Factor-1 (Apaf-1) and pro-caspase 9 form caspase 9, this complex is called apoptosome. The formation of caspase 9 as the initial caspase will activate the exclusive caspase, namely caspase 3, 6, and 7 so that it can cause apoptotic cell death [30].

4. Conclusion

Jeruju leaves methanol extract and taurine are cytotoxic in HeLa cervical cancer cells, therefore it is possible to have the potential as anticancer compounds. While the methanol extract of Jeruju leaves and taurine can inhibit the proliferation of HeLa cervical cancer cells. This is evidenced by the doubling time of cells with the treatment of test compounds that are longer than control cells.

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