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Anticancer Potency of Seagrass *(Enhalus acoroides)* Methanol Extract in the *HeLa* Cervical Cancer Cell Culture

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ABSTRACT
Anticancer potential of methanol extracts of seagrass and taurine 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 showed that the methanol extract of seagrass had a cytotoxic activity with IC_{50} values are 122 ppm. While the doubling time value in the antiproliferation test by methanol extracts of seagrass and taurine showed higher values than cell control (72.19 hours).

***Keywords**: Seagrass, taurine, cytotoxic, antiproliferation, and HeLa cell*

1. INTRODUCTION

Cancer is a large group of diseases that can start in almost any organ or tissue of the body when abnormal cells grow uncontrollably, go beyond their usual boundaries to invade adjoining parts of the body and/or spread to other organs. 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 [1]. 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 [2].

Cancer treatments such as chemotherapy, radiation, and surgery often have bad effects for cancer sufferers, so many patients choose alternative treatments with natural ingredients because they feel minimal risk. Seagrass is a plant that has a variety of health benefits such as skin disease, fever-reducing, and abdominal pain and is also believed to have potential as anticancer compounds [3, 4, 5].

Besides seagrass, taurine is also known as a compound with various benefits for the human body. Taurine is the free amino acids find in the heart and brain muscle tissue [6]. Research showed that giving taurine decreased glucose and total cholesterol levels in mice [7]. Taurine has also been shown to have the ability of antioxidants to prevent oxidative damage to the effects of paraquat induction [8]. Based on the facts above, further research is needed to prove the ability of seagrass as anticancer.

2. METHODS

2.1. Preparation of Plant Extracts

Seagrass sorted out the best and cleaned using flowing water. Seagrasses were dried using an oven at 30°C. The dried Seagrasses then were finely crushed and blended. The powders 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].

2.2. Phytochemical Test

To find out the content of secondary metabolites contained in the seagrass methanol extract, phytochemical screening was carried out. The Phytochemical screening of seagrass extract included tannins, flavonoids, alkaloids, saponins, and terpenoids test [9,10].

2.3. 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 [11].

2.4. Hela Cell Count Calculation

10 µl mixture of HeLa cells and trypan blue pipetted ona hemocytometer. Calculation with a hemocytometer is done by selecting 4 counting rooms. The text below is series of calculations for the number of cells to be cultured [12].

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

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

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

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

2.5. Test Compounds Preparation

Preparation of the test compound is done by dissolved 10 mg methanol seagrass extract into 1 ml dimethyl sulfoxide (DMSO) 1%. For reference study, taurine (which also used for other study) for the same mass dissolved with 1 ml distilled water. The stock solution is then diluted again to a concentration of 125 ppm, 100 ppm, 75 ppm, 50 ppm, and 25 ppm [13].

2.6. Cytotoxicity Assay

Cells that have been cultured in a well plate for 24 hours are rinsed with phosphate buffer saline (PBS). Each well was given seagrass extract and taurine with 125 ppm, 100 ppm, 75 ppm, 50 ppm, and 25 ppm concentration then incubated for 24 hours. Seagrass methanol extract and taurine then discarded, and the wells are rinsed with a solution of phosphate buffer saline (PBS). Each well was added 10 µl MTT (3- (4,5-dimethyltiazol-2-il) -2,5-diphenyl tetrazolium bromide) and incubated for 2 hours at 37°C in a CO2 incubator. Then MTT reaction was stopped with 100 µl dimethyl sulfoxide (DMSO) 100% stopper reagent. The absorbance is then read with an ELISA reader at 550 nm wavelength [14].

2.7. Antiproliferation Assay

Cells that had been cultured in a well plate for 24 hours were given 100 µl seagrass extract and taurine with 125 ppm, 100 ppm, 75 ppm, 50 ppm, and 25 ppm concentration. Wells then incubation with different treatment time that is 24 hours, 48 hours, and 72 hours at 37°C in a CO_2 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. Into the well then added 10 µl MTT. Wells then incubated again for 2 hours at 37°C in a CO_2 incubator. The MTT reaction was stopped with 100 µl dimethyl sulfoxide (DMSO) 100% stopper reagent per well. The absorbance of each treatment was measured with an ELISA reader 550 nm wavelength [15].

2.8. Data Analysis

Data analysis for the *HeLa* cells cytotoxic test is done by calculating the percentage of cell viability. The cell viability percentage values then changed into probit value to determine the IC50 value. 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 treatment, then it is tested further by testing the *Least Significant Difference* (LSD).

3. RESULTS AND DISCUSSION

3.1. Phytochemical Test

Table 1. Phytochemical Test of Seagrass methanol extract

Phytochemical Test	Seagrass
Saponins	+
Terpenoids	-
Tannins	+
Alkaloids	-
Flavonoids	+

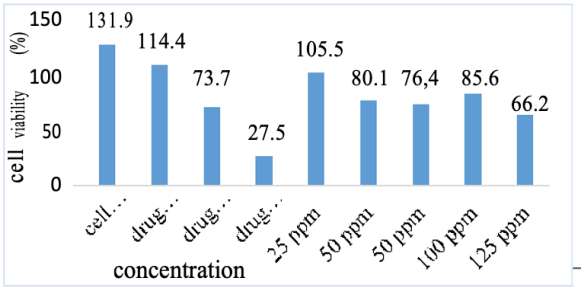
(+) = Contain Test Compounds
(-) = Contain Test Compounds

Based on Table 1, phytochemical screening of seagrass extract showed negative results on the presence of terpenoids and alkaloids. This can be caused by terpenoid compounds that are non-polar so that these compounds cannot be extracted perfectly in methanol solvents. Alkaloids are commonly found in various parts of plants but with levels less than 1% in the tissues, so phytochemical screening is often not identified [17].

3.2. Cytotoxic Test

Figure 1 showed that treatment with seagrass extract concentration 125 ppm results in the lowest viability percentage (66.22%), which means it also showed the highest percentage of growth inhibition (33.78%).

Based on cytotoxic tests did with seagrass methanol extract against *HeLa* cervical cancer cells, graphs obtained the relation of test compound concentrations with cell viability (%) as shown in Figure 1. Overall treatment with test compounds showed the presence of cytotoxic activity to reduce the percentage of test cell viability (compared to control cells). *HeLa* cells in a phase leading to apoptosis have a different morphology. Cells will reduction in size and shrinkage as an effect of loss of intracellular fluid and a loss of contact with neighboring cells [18].



Drug control treatment by Doxorubicin (which also used for other study Andriani et al 2020)

Figure 1 Relation of seagrass methanol extract concentration with cell viability (%)

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

Test compound	Concentration (ppm)	Cell Viability (%)	IC_{50} (ppm)
Seagrass	25	105.54	112
	50	80.09	
	75	76.35	
	100	85.92	
	125	66.22	
	1	113.38	
Doxorubicin*	5	73.71	12.35
	10	27.46	

[a] *Criteria for a cytotoxic activity for crude extracts according to the American National Cancer Institute (NCI) are IC50<30 µg/ml [16].*
* *Reference value*

The percentage of cell viability obtained after the treatment of seagrass methanol extracts and taurine is then used to calculate the IC_{50} value [14]. Criteria for a cytotoxic activity for crude extracts according to the American National Cancer Institute (NCI) are IC_{50}<30 µg/ml [16]. Based on Table 2, seagrass methanol extract and doxorubicin with various concentrations causing a decrease in cell viability. This indicates the presence of cytotoxic activity by test compounds on *HeLa* cells. Based on IC50 values by American National Cancer Institute, seagrass methanol extracts have no potential as anticancer compounds.

Table 3 shows that the doubling time value obtained is different at each concentration of the treatment of seagrass methanol extract and taurine. In the doubling time test, the slope value of the linear regression equation is a parameter of cell proliferation kinetics.

Table 3. Doubling Time Values in Antiproliferation Test

Test Compound	Concentration (ppm)	The incubation timeline equation and log of the cell count	Slope value	Doubling Time Value (hours)
	25	0.0011x+4.2949	0.0011	277
	50	-3E-05x+4.2534	-3E-05	-
Seagrass	75	-0.001x + 4.2572	-0.0010	-
	100	-0.0029x+4.2939	-0.0029	-
	125	-0.0029x+4.2609	-0.0029	-
	1	-0.0037x+4.3688	-0.0037	-
Doxorubicin	5	-0.0187x +4.462	-0.0187	-
	10	-0.0208x+4.2854	-0.0208	-
Cell Control	0	0.0041x + 4.304	0.0041	72.19

**Cell control slope values = 0.0041.*

In the control cell, the slope value obtained was 0.0041. 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. Referring to this fact, it means that the methanol extracts of seagrass have potential as antiproliferative compounds in *HeLa* cervical cancer cells. Negative slope values indicate no proliferation because the cell has died [19].

Table 4. Number of living *HeLa* cells in different concentration of seagrass methanol extraction

Test Compound	Concentration (ppm)	Number of living cells (x 1000 Cell)		
		24 hours	48 hours	72 hours
Seagrass	25	$21.1\pm0,6^{a}$	21.1 ± 0.5^{a}	24.6 ± 1.0^{a}
	50	16.0 ± 0.1^{b}	16.0 ± 1.2^{b}	19.9 ± 0.7^{b}
	75	15.3 ± 0.9^{bc}	15.0 ± 0.3^{bc}	16.7 ± 0.1^{c}
	100	17.2 ± 0.6^{b}	13.0 ± 0.7^{c}	12.9 ± 0.9^{d}
	125	13.2 ± 0.9^{c}	13.6 ± 0.3^{c}	11.5 ± 0.4^{d}

^{a}, ^{b}, ^{c}: superscript indicates significant difference at 5%

Treatment with seagrass methanol extract showed a significant difference in the number of living cells at all concentrations. The highest average number of living cells is shown by giving extract concentration of 25 ppm and incubation time 72 hours, while the lowest average number of living cells is shown by extract concentration of 125 ppm and incubation time 72 hours.

There are various possible action mechanisms 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. The receptor will then convey the proliferative signal to proteins in the cytoplasm [20]. 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 [21]. Third, blockade ofthe 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 [22, 23]. Fourth, saponins inhibit the formation of Bcl-2. Bcl-2 is an anti-apoptotic protein that causes cells to proliferate [23].

4. CONCLUSION

1. Seagrass methanol extracts are cytotoxic in *HeLa* cervical cancer cells but lack the potential as anticancer compounds.
2. Seagrass methanol extract 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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