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The Potency of β-Sitosterol Isolated From Hydroid Aglaophenia cupressina Lamoureoux as Antimitotic Agent towards Sea Urchin Tripneustes gratilla Linn.

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ABSTRACT

The potency of β-Sitosterol isolated from hydroid *Aglaophenia cupressina* Lamoureoux as antimitotic agent towards early cleavage of Sea Urchin *Tripneustes gratilla* Linn had been conducted. The study aimed to determine β-Sitosterol activity isolated compound from hydroid *A. cupressina* Lamoureoux as antimitotic compound towards early cleavage of zygote of Sea Urchin *T. gratilla* Linn. Antimitotic treatments were β-Sitosterol (concentration 1 µg/ml, 10 µg/ml and100 µg/ml), positive control vincristine (concentration 0.01 µg/ml, 0.1 µg/ml, and 1 µg/ml) and negative control. A protozoa-free seawater was used in this assay. The results revealed that β-Sitosterol compound had the highest inhibition activity at concentration of 100 µg/ml was 69.22 % with IC-50 on 0.95 µg/ml which could develop as cancer chemotherapy compound.

Keywords: *Antimitotic, β-Sitosterol, Hydroid Aglaophenia cupressina Lamoureoux, Tripneustes gratilla Linn.*

1. INTRODUCTION

Hydroids are sea invertebrate that easily found in shallow waters. Hydroids live in colonies, and attached to the sponge or dead coral. The abundance of hydroid in waters making it the source of wealth of new molecular. Hydroid *A. cupressina* L is known to have a variety of active compounds with β-sitosterol is one of them [1]. Active compound isolated from A cupressina L had been studied extensively, but not β-sitosterol compound [2, 3 & 4].

β-sitosterol is a sterol that has various benefits such as lowering cholesterol, cancer prevention and as a cosmetic raw material [5]. β-sitosterol compound as a cancer chemotherapeutic agent is to be tested through various stages of biopharmacology testing, both in-vivo and in-vitro. As for the in-vitro test include antimitotic assay [6].

Sea Urchin *T. gratilla* Linn is commonly used in trials because of its sensitivity to exposure in measuring the compound toxicity, the zygote of Sea Urchin *T. gratilla* Linnis often used as a test medium. Besides its abundance, its easy and inexpensive application in procedure makes it a test media that frequently used. It is important to comprehend the information on β-sitosterol as antimitotic material. Therefore, this study was undertaken.

The studies aimed to determine β-Sitosterol activity as the isolated compound from hydroid *A. cupressina* L as antimitotic

compound on first cleavage of zygote cell of Sea Urchin *T. gratilla* Linn. Thus, the study was expected to provide the information on mitotic inhibition by β-sitosterol.

2. MATERIALS AND METHODS

A. Materials

The tools used in this study were reaction tube (Pyrex), erlenmeyer 250 ml(Pyrex), Erlenmeyer 1000 ml (Pyrex), beaker (Pyrex), measuring cup 50 ml (Witeg), incubator (Memmert), analytical balance (Sartorius), calipers (Vernier), microtube, micropipette, light microscope (nikon), deck glass, object glass, cool box, mobile aerator, aquarium aerator, refrigerator, laminary air flow, bunsen burner, tweezer, tube rack, separating funnel, syringe stir bar, dilution bottle and horn spoon.

The materials used in this study was the isolated compound from hydroid *A. cupressina* L (β-sitosterol), Sea Urchin *T. gratilla* Linn, protozoa-free seawater, DMSO (dimetil sulfoxide), Amoxicillin (tablet), Formalin 10%, KCl 10%, acetocarmine solution, aquades, filter paper, label paper, cotton tissue paper.

B. Methods

The study was experimental by testing the mitotic inhibitory activity of β-sitosterol compound which isolated from hydroid *A. cupressina* at antimitotic assay. This compound tested and observed on the zygote cell of sea urchin *T. gratilla* Linn. Antimitotic assay conducted by using protozoa-free sea water as media, protozoa-free sea water without any compound as negative control and vincristine compound which dissolved in protozoa-free sea water as a positive control. Vincristine as a positive control was diluted at various concentration of 0.01 µg/ml, 0.1 µg/mland 1 µg/ml. The dilution of test compound and the positive control were using protozoa-free sea water and DMSO compound. The method was adopted from Rahman et al. [6] which has been modified.

C. Research Stages

C.1 Field

C.1.1 Hydroid A. cupressina L collection

Hydroid *A. cupressina* L was collected from Lae-lae waters of Makassar, at a depth of 1-2 meters. Samples hydroid *A. cupressina* L in adult-size (7-20 cm high).

C.1.2 Sea Urchin T. gratilla Linn collection

Sea urchin *T. gratilla* Linn sampling was collected from Bonebatang waters of Makassar, at depth of 1-2 meters. Sampling was done during the full moon. Samples sea urchin *T. gratilla* Linn in adult-size (diameter > 60 mm). Male and female were separated into different containers with the regularly aerated. The aeration was maintained using aerator during transportation as in laboratory.

C.2 Laboratory

C.2.1 Test Sample Preparation

A 1 mg isolates **(**β-sitosterol) hydroid *A. cupressina* L was diluted with 100 µl of DMSO. Dilution was conducted by employing ready to use protozoa-free seawater. Dilution is conducted in order to obtain a 1000 µg/ml concentration for a

stock. From this stock solution, three different stock test concentrations are produced 1, 10, and 100 µg/ml. Antimitotic test prepared in tubes with pipette100 µl of test compound.

Positive control vincristine was prepared at a concentration of 0.01, 0.1 and 1 µg/ml. Positive control was placed in micro tube by using a straw to get 100 µl of each concentration. As negative control, protozoa-free seawater without any compounds are utilized.

C.2.2 Test Media Preparation

Sea urchin *T. gratilla* Linn was washed with protozoa free sea water for two minutes. Sea urchin *T. gratilla* Linn was subsequently induced with 1 ml KCl 10 % solution by using syringe. The KCl solution induction caused a discharge of milky white sperm in males and eggs are golden yellow in females. Semen and liquid eggs were placed in different beakers. Sperm cells actively moved (motile) with smaller size compared to the egg cell. Semen and liquid eggs are then stored on the stock in the refrigerator (chiller) with a temperature around 14-15^{o} C.

Test medium zygote was obtained by in-vitro method through artificial fertilization. Artificial fertilization was conducted by mixing 1 ml of sperm cells and 5 ml of egg cells in a beaker that contained 50 ml protozoa-free seawater. Then it was left to stand for 10-15 minutes in the refrigerator (chiller) with temperature range between 14-15°C.

C.2.3 Implementation

C.2.3.1 Inhibitory Concentration-50

Microtubes that contained test samples (β-sitosterol) and positive control (vincristine) were added with protozoa-free seawater in accordance to make the final volume of 1 ml. Next, 100 µl of zygotes suspension was added into the tube (ten minutes after fertilization). This process was repeated three times for each test sample, the positive control and negative control. The tubes were stored in the refrigerator (chiller) with a temperature range between 14-15^{o}C and periodically treated with a stir.

Observations on antimitotic test held after two hours of incubation by using a camera equipped microscope. Fixation was then performed by putting10% formalin drops in each tube in order to stall all cell activity. For each tube, data was recorded on the amount of divided and undivided cells of the 300 observed cells. Counting was conducted manually by observing via a light microscope.

C.2.3.2 Antimitotic Activity Observation

Antimitotic activity observation was performed in the similar method of the procedure of determining the inhibitory concentration 50 (IC-50) from the beginning to the mixing stage zygote cells with a solution (compound) test or control. The concentration used in the implementation on this observation only employed the inhibitory concentration 50 (IC-50) from the test sample (β-sitosterol). That solution was stored at a temperature of 14-15^{o} C and periodically

treated with a stir. Antimitotic activity observation was carried out after two hours of incubation and staining. Observations were made by using microscope. Then photography of the subject was taken.

3. RESULTS AND DISCUSSION

A. Results

A.1 Negative Control (Protozoa-Free Seawater)

The result showed that the average percentage of undivided zygote cell is 30.67% (Table 1). The number average percentage of undivided zygote cell then used in the calculation of compounds inhibitory percentage of test compound (β-sitosterol) or the positive control (vincristine) with the following formula: % Compounds Inhibitory = % Undivided Zygote Cells - % Average Undivided Zygote Cells (negative control) = % Undivided Zygote Cells – 30.67%.

A.2 Test Substance (β-sitosterol)

Antimitotic test was repeated three times in three different levels of concentration. This repetition found the sample standard deviation of undivided zygote cells of 12.124 cells [1] µg/ml], 2.516 cells [0 µg/ml] and 0.577 cells [100 µg/ml] (Table 2). In addition, the repetition also found that the average inhibitory percentage was 50.33% [1 µg/ml], 59.77% [10 µg/ml] and 69.22% [100 µg/ml] (Table 1). The data was processed by probit analysis in order to obtain IC-50 (Inhibitory Concentration 50) at 0.95 µg/ml. The IC-50 then used in the antimitotic activity observation.

A.3 Positive Control (Vincristine)

Antimitotic test used vincristine as positive control. Antimitotic test was repeated three times in three different levels of concentration. The repetition found that the sample standard deviation of undivided zygote cells at 15.695 cells [0.01 µg/ml], 16.370 cells [0.1 µg/ml] and 15.534 cells [1 µg/ml] (Table 2). In addition, the repetition found that the average inhibitory percentage at 26.55 % [0.01 µg/ml], 41.33 % [0.1 µg/ml] and 59.77 % [1 µg/ml] (Table 1). The data was processed by probit analysis in order to obtain IC-50 (Inhibitory Concentration 50) at 0.28 µg/ml.

Table 1. Observation Results of Zygote Cell cleavage of Sea urchin *T. gratilla* Linn. (Two hours after test

Compounds	Concentration (µg/ml)	Amount of Undivided Zygote Cells	Amount of Divide Zygote Cells	Total Cells	% Undivided Zygote Cells	% Compounds Inhibitory
Protozoa-Free Seawater (Negative control)	0	95	205	300	31,67	0
		89	211	300	29,67	0
		92	208	300	30,67	0
	% Average Undivided Zygote Cells				30,67	
Beta-sitosterol (Test Compound)	1	241	59	300	80,33	49,66
		256	44	300	85,33	54,66
		232	68	300	77,33	46,66
	% Average Inhibitory					**50,33**
	10	274	26	300	91,33	60,66
		271	29	300	90,33	59,66
		269	31	300	89,67	59
	% Average Inhibitory					**59,77**
	100	299	1	300	99,67	69
		300	0	300	100	69,33
		300	0	300	100	69,33
	% Average Inhibitory					**69,22**
Vincristine (Positive Control)	0,01	184	116	300	61,33	30,66
		177	123	300	59	28,33
		154	146	300	51,33	20,66
	% Average Inhibitory					**26,55**
	0,1	212	88	300	70,67	40
		202	98	300	67,33	36,66
		234	66	300	78	47,33
	% Average Inhibitory					**41,33**
	1	284	16	300	94,67	64
		276	24	300	92	61,33
		254	46	300	84,67	54
	% Average Inhibitory					**59,77**

Table 2. Calculation Results of IC-50 Base on Log Probit – Concentration Graphical Method

Compound	Concentration (µg/ml)	Log Concentration (X Axis)	% Average Inhibitory	Probit Value (Y Axis)	Equation	IC-50 (µg/ml)
Beta-sitosterol (T est Compound)	1	0	50,33	50,099	Y= 0,2473X + 5,006	0,95
	10	1	59,77	52,454		
	100	2	69,22	55,044	R2= 0,9992	
	IC-50	-0,02426	50	5		
Vincristine (Positive Control)	0,01	-2	26,55	43,765	Y= 0,4344X + 5,235	0,28
	0,1	-1	41,33	47,799		
	1	0	59,77	52,454	R2= 0,9983	
	IC-50	-0,5412	50	5		

B. Discussion

The interval selection of test compound concentration (β-sitosterol) used in this test was based on the concentration log interval of 1 µg/ml at log concentration 0, 10µg/ml at log concentration 1 and 100 µg/ml at log concentration 2. The selection of this interval also refers to previous studies that showed IC-50 of fraction of β-sitosterol in the range of 1.033µg/ml [7].

The selection of testing interval concentration of positive control (vincristine) also used the interval of log concentration are 1 µg/ml atlog concentration 0, 0.1 µg/ml at log concentration 1 and 0.01 µg/ml at log concentration 2. The selection of this interval also refers to previous studies that showing IC-50 of vincristine compound in the range of 0.102µg/ml [7].

The zygote cells of sea urchin T. gratilla Linn were used in this test because of its sensitivity to exposure of the compound. The sensitivity of the zygote cells makes them frequently employed as a biological test (bioassay) medium in measuring the toxicity of a compound. The effect of the β-sitosterol compound on numbers of divided cells at various concentrations showed a decrease in the number of dividing cells. The similar effect also occurred on the vincristine compound. It was also discovered that the number of dividing cells was proportionally decreased in response to the increase in concentration. Thus, the conclusion is the β-sitosterol compound has similar inhibitory properties as the vincristine compound

B.1 IC-50 (Inhibitory Concentration 50)

The IC-50 was selected for this test in order to determine the levels of concentration of test compound or control compound in inhibiting the division process from half of test medium. IC-50 data of a compound then used as a comparison against cancer chemotherapy compounds that have been commonly used in the medical world.

The IC-50 according to Trianto et al. [8] was categorized in various categories. IC-50 < 3 µg/ml (very active), IC-50 > 5 µg/ml (moderate) and> 10 µg/ml (weak). While Syarifah *et al.* (2011) categorized with different values, IC-50 ≤ 10 µg/ml (very active), 10 < IC-50 < 50 µg/ml (moderate) and ≥ 50 µg/ml (weak). Meanwhile, United States National Cancer Institute (US-NCI), compounds classified as active when the IC-50 is ≤ 20 µg/ml (Boyed, 1997 in [9 & 10].

NCI also determines that a compound could be analyzed further if the value of IC-50 < 30 µg/ml [8]. The compound can be categorized as a potential anticancer agent when the IC-50 value < 25

µg/ml. This fact is different than the criteria set by Boik (2011) in [11], that a compound could be consider an anticancer potential when the IC-50 < 4 µg/ml.

The conducted observation showed that β-sitosterol had antimitotic properties with IC-50 at a concentration 0.95 µg/ml. The IC-50 value indicated that β-sitosterol compound was classified as highly active compound. Moreover, the IC-50 value was lower than previous study's value that showed the concentration of 1.033 µg/ml [7]. There was a difference of 0.083 µg/ml between the two studies.

Positive control used for comparison (vincristine compound) had IC-50 of 0.28 µg/ml. The discovered IC-50 value also indicated the vincristine compound can be classified as a highly active compound. The IC-50 value was higher than in previous studies that showed a concentration of 0.102 µg/ml [7]. Thus the difference between two studies was 0.178 µg/ml.

Based on the above explanation, it can be concluded that β-sitosterol compound is classified as highly active as vincristine compound that has been commonly used as a raw base material for anti-cancer. The IC-50 value of β-sitosterol meets the criteria for further analysis as defined by NCI [8 & 11].

B.2 Antimitotic Observation of β-sitosterol Substance

Antimitotic test on test compound showed a decrease in the number of dividing cells at three different levels (Figure 1). The decrease in the number of dividing cells showed an increase in the number of undivided zygote cell percentage. The highest average of inhibitory percentage for test compound reached 69.22% [100µg/ml].

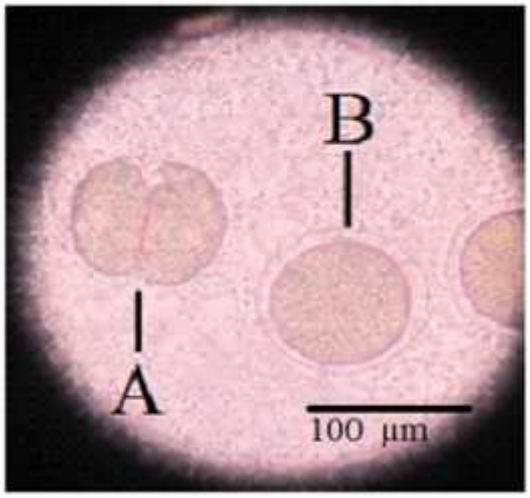

Fig 1. Antimitotic activity observation using light microscope (Nikon) with Acetocarmine Staining (Note: (A) Cell has been through first cleavage and (B) Undivided cell)

The value was relatively low when compared to previous studies that reached 86.5 % at the same concentration level (100µg/ml) (Khalid, 2009). The average of inhibitory percentage that exceeded half a zygote cell number and an increase the inhibitory percentage showed the inhibitory properties of the test compound (β-sitosterol) (Table 1).

Antimitotic activity observation of β-sitosterol compound used 0.95 µg/ml of IC-50 concentration. Visual observation on zygote cells with acetocarmine staining show divide and undivided cells clearly (Figure 1). The process of cell division in the test medium occurs normally in the absence of cell division abnormalities like mass cell

lysis, size differences of dividing cells, and others. Therefore it could be concluded that the test compound (β-sitosterol) as antimitotic compound was cytostatic (inhibit cell division) and non-cytocidal. In-vivo studies showed inhibition of proliferation and causes apoptosis of solid tumors in the colon and breast cancer. But in-vitro studies looked different. β-sitosterol was cytocidal in cultured breast cancer cells, but non-toxic in normal cells [5].

4. CONCLUSION

1. Antimitotic assay on β-sitosterol compound isolated from hydroid *A. cupressina* L showed the inhibition properties (antimitotic effect) on cleavage of zygote cells of sea urchin *T. gratilla* Linn.
2. The average value results of the highest inhibition of test compound (β-sitosterol) as the isolated compound from hydroid *A. cupressina* L obtained at concentration 100 µg/ml is 69.22 %.
3. β-sitosterol has antimitotic effect based on the value of IC_{50} at 0.95 µg/ml that has the similar properties to vincristine as positive control that has IC_{50} at 0.28 µg/ml.
4. β-sitosterol isolated from hydroid *A. cupressina* Lamoureoux has the potential to be used as the raw base material for anti-cancer based on IC-50.

REFERENCES

[1]. Johannes E. (2008). *Isolasi, Karakterisasi* and *Uji Bioaktivitas Metabolit Sekunder dari Hydroid Aglaophenia cupressina Lamoureoux Sebagai Bahan Dasar Antimikroba***.** Thesis. Makassar: Pascasarjana Program, Hasanuddin University.

[2]. Umriani, N. (2008). *Studi bioaktivitas Antibakteri Fraksi Protein Hidroid Aglophenia cupressina Dari Pulau Lae-Lae Sulawesi Selatan.* Indonesia Chimica Acta. 12: Vol. 1. No. 1.

[3]. Johannes E., Ishak E., Usman H., & Bilang M. (2013). Effectiveness of extracted antibacterial compound from hydroid *Aglaophenia cupressina* Lamoureoux against bacterial cell of *Escherichia coli*. International Journal of Scientific and Technology Research Vol.2. Issue 2. ISSN: 2277-8616

[4]. Johannes, E. Litaay, & M. Syahribulan. (2016). The Bioactivity of Hexadecanoic acid compound isolated from hydroid *Aglaophenia cupressina* Lamoureoux as antibacterial agent against *Salmonella typhi*. International Journal of Biological & Medical Research 7(2):5469-5472.

[5]. Phytochemicals. (2012). *Beta-Sitosterol*. Retrieved from www.phytochemicals.info/phytocemicals/beta-sitosterol.php.

[6]. Rahman, A.U., M.I. Choudhary & W.J. Thomson. (2005). *Bioassay Techniques for Drug Development.* Amsterdam: Harwood Academic Publisher.

[7]. Khalid A. (2009). *Pengaruh Uji Antimitotik Fraksi N-Heksan dari Hydroid AGLAOPHENIA cupressina Lamoureoux Terhadap Pembelahan Awal Sel Zigot Bulu Babi Tripneustes gratilla Linn.* Undergraduate Thesis. Makassar: Biology Department, Mathematics and Natural Sciences Faculty, Hasanuddin University.

[8]. Trianto A., Ambariyanto, & R. Murwani M. (2004). *Skrining Bahan Anti Kanker pada Berbagai Jenis Sponge* and *Gorgonian Terhadap*

L1210 Cell Line. Semarang: Ilmu Kelautan UNDIP, Vol.9 (3): 120-124.

[9]. Saetung A.A., Itharat C., Dechsukum, C., Wattanapiromsakul N., Keawpraduband P., & Ratanasuwan. (2005). *Cytotoxic Activity of Thai Medicinal Plants for Cancer Treatment.* Hat Yai: Songklanarin J.Sci.Technol. Vol.27 (suppl.2). Thai Herbs.

[10]. Malek, S.N.A., C.W. Phang, H. Ibrahim, N.A. Wahab and K.S. Sim. 2011. *Phytochemical and Cytotoxic Investigation of Alpinia mutica Rhizomes*. Basel: Molecules 16, 583-589; doi; 10.3390/molecules 16010583.

[11]. Syarifah, M.M.S., M.Y. Nurhanan, J.M. Haffiz, A.M. Ilham, K. Getha, O. Asiah,I. Norhayati, H.L. Sahira and S.A. Suryani 2011. *Potential Anticancer Compound from Cerbera*

[12]. *Odolam*. Kepong: Journal of Tropical Forest Science 23 (1): 89-96.