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Effect of *Sargassum filipendula* Fucoxanthin against HeLa Cell and Lymphocyte Proliferation

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ABSTRACT: This study aims to Obtain a pure extract of *Sargassum filipendula*, fucoxanthin, determine the effect of concentration fucoxanthin against HeLa cells and determine the effect of concentration fucoxanthin against cancer cell lymphocytes. The benefit of this research is to find out that fucoxanthin can be used as an anti-cancer treatment. The study was conducted in September 2013 at the Laboratory of Fishery Product Technology, Faculty of Fisheries and Marine Science and Medical Biomedical Laboratories, University of Brawijaya, Malang. Sampling brown seaweed (*Sargassum filipendula*) of the District Rural Padike Talango Sumenep Madura. Extracted isolated by column chromatography using silica gel stationary phase and mobile phase hexane : ethyl acetate (6:4, v/v) ± 100 ml, then identified by TLC using the stationary phase a silica gel F - 254 HPLC with ODS stationary phase (C-18) 5 ml with a mobile phase of methanol, acetone and ammonium acetate (1M) (80:10:10, v/v) andinjected a flow rate of 1.0 ml / min pigment solution of 20 mL compared with the standard of Japan. Research results indicate that, spectra maximum wavelength of 450 nm and 446 nm with a minimum of acetone solvent. HPLC has the same retention time = 10.12 standard, while *Sargassum filipendula* = 10.11. The best concentrations for lethal Hela cells at 100 ppm was 4.717% which means that fucoxanthin effect against HeLa cells have the potential fucoxanthin that can induce cell apoptosis that occurs in HeLa cells, it is possible because of the anti- carcinogenic fucoxanthin hasstructure unique. While the cell lymphocytes in the dosage of 100 ppm were dead at 9.543 %, the effect of the toxicity of a substance can be observed from how many dead lymphocytes when compared with the state charity by observing level of lymphocyte proliferation.
Key words: Fucoxanthin, Hela Cells, Lymphocytes, Mtt

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INTRODUCTION

Cancer is the one of the important health problems in the world. Number of cancer deaths increasing each year. Two types of cancer with the greatest incidence in Indonesia is cervical cancer and breast cancer. Each year about 8,000 Indonesian women died from cervical cancer [1].

The magnitude of the mortality rate due largely to the delay cancer treatment or medical treatment for various reasons. In addition, many cancer drugs available today have a low therapeutic indication, and the maximum dose given only give the minimum effectiveness. Not all types of cancer patients and or drug-responsive to existing cancer drugs. In fact, many cancer drugs that cause side effects and are resistant to the effects of patient [2]. To answer these problems, research to find potential candidates of new antitumor drugs is needed. Compounds from macroalgae natural ingredients have been proven to be one source of new bioactive compounds [3, 4]. Brown algae (P australis, T conoides, filipendula S, S and S echinocarpumcinereum) reported to contain large amounts of the dye, ie fucoxanthin and other substances, which act as an anti-obesity [5], anti-oxidant [6]. Fucoxanthin as the main part of the carotenoid in brown algae have certain Alenic bond, where 5,6 - monoepoxida play an important role in the structure of fucoxanthin [7, 8, 9], anti-coagulant [10], anti-diabetic [11], anti-cancer [9], anti-tumor and colon cancer [12].

Ideal model in search of potential antitumor drugs are bioactive compounds that can kill tumor cells but has low toxicity to normal cells. Three initial stages that must be passed to nutrasetikal product development include: extraction stage, biological screening stage, and testing stage of pharmacology and toxicology and safety testing wear [13].

This research aims to study the potential bioactivity of extracts of macroalgae *Sargassum filipendula* as antitumor HeLa (cervical tumors) as well as in vitro toxicology tests on human blood cells. An orange pigment fucoxanthin including xanthophyll group of carotenoids which have clusters of oxygen (O_2). The main framework of an isoprenoid polyene formed by the merger of eight isoprene units [14]. Fucoxanthin pigment found in brown algae, green and red, but not found in higher plants [15]. Poliene chain of carotenoids, containing conjugated double bonds which are responsible for the formation of carotenoid pigments and has the ability to absorb photons at a wavelength of visible light [16].

Pure fucoxanthin with spectrophotometer measurements indicate that the compound has a molecular weight of 658.92 gmol and consists of one molecule of acetyl groups and two hydroxyl groups [17]. Isoprene units connected by a regular way between the head of the tail, except at the center of the molecule, where the order

between the head and the tail inverted, so that the configuration of the molecule is symmetrical. Thus, the two methyl groups are located in the center of the molecule are separated by C-6 atoms and groups of atoms separated by metiI other C - 5 [18]. Its chemical formula is C42H58O6 and fucoxanthin has a unique structure with bond alenat and two heterocyclic group at both ends of the molecule, where one of its cyclic structure containing ester groups and the structure of 5,6 - monoepoksida the cyclic structure [17]. Fucoxanthin functional groups which are 2 hydroxyl group (OH) at C - 3 and 5', and a keto group at position C-8[19]. Moss [20] proposed the name of fucoxanthin in the International Union of Pure and Applied Chemistry (IUPAC) is (3S, 5S, 6R, 3'S, 5'R, 6'R)-3'-Acetoxy-5,6-epoxy-3,5'-dihydroxy-6',7'-didehydro-5,6,7,8,5',6'-hexahydro-β, β-carotene-8-one. Gross [21] suggested that based on the content of some of the hydroxyl groups attached to the ring structure, and variations in the structure , then fucoxanthin can be classified into three groups , namely :

1) Hydroxyl (example: manols, diols) hydroxyl substituent at C3
2) Epoxy (eg, methoxy, aldehyde, oxo, carboxy, ester)
3) Acetylene (example: C = C = C on one end of the chain, the C = C at position 7,8)

Based on the configuration of the molecule, fucoxanthin consists of three kinds , namely mono - cis , cis - and trans in - fucoxanthin. Fucoxanthin resulted in the trans configuration , associated with OH (C - 3) and the epoxide group, so that 3 - hydroxy - 5 ,6 - epoxide carotenoids have 3 'S configuration , 5'R , 6'R and configuration (3S,5S, 6R) epoxides can synthesized via epoxidation of the carotenoid -containing ring - β using perptalat acid or perbenzoat [22]. Allenic carotenoid fucoxanthin is a configuration that has the 3S , 5S , 6R , 3 'S , 5'R. Fucoxanthin found in brown algae are mostly trans-fucoxanthin [7]. Haugan [23] analyze stereomutasi using iodine catalyst and separated by semi-preparative HPLC resulted in eight forms molecular configuration of fucoxanthin isolated from Fucus serratus , which is 15 - cis , 13.9 ' - di- cis , 13 , 13' - di- cis , 9 ' , 13' - di- cis , 13 - cis , 13' - di- cis , 9' - cis , and trans.

Fucoxanthin melting point is at a temperature of 1640C , with a density of 1.09 and fucoxanthin soluble in ethanol and slightly soluble in carbon disulfide and ether , but insoluble in petroleum ether . In addition to these characteristics , fucoxanthin also be labile to bases [23]. Fucoxanthin has the green light spectrum, blue to yellow with a peak wavelength between 450-540 nm [25].

Synthesis of fucoxanthin according to Jeffrey[17] first derived from the synthesized lycopene into β - carotene through the process of cyclization or ring formation at the β position. β - carotene which has been formed into zeasantin undergo hydroxylation, and epoxidation process into the next stage which later became violasantin antesantin. Violasantin formed undergo structural arrangement into a new pigment called neosantin . In addition to forming neosantin , violasantin also formed diadinosantin which further leads to the formation diotasantin and fucoxanthin.Jeffrey [17] reported that the spectral pattern of brown algae fucoxanthin have a minimum absorption at a wavelength of 446 nm and maximum absorption at a wavelength of 468.3 nm .

MATERIAL AND METHODS

Sampling, Extraction, and Fractionation

Microalgae *Sargassum filipendula* taken from coastal tidal zone Padike, district. Talango, Madura Islands. Sample S. filipendula gained as much as 10 kg. Samples were washed with fresh water to remove dirt and attached epiphytes, then macerated in 10 L 95% ethanol for 3 days. The solution obtained was filtered with Whatman # 41 filter paper and evaporated with a Buchi Rotavapor. After all the ethanol evaporates, extract (freeze-dried) at a temperature of 280-430C x 10-3 mbar pressure to obtain a dry powder form of the extract (crude extract ethanol). After testing bioactivity, the ethanol crude extract further fractionated in solid-liquid. Fractionation process carried out in succession by an increase in solvent polarity using n-hexane, ethyl acetate, and methanol.

Cell Culture HeLa and T47D

HeLa tumor cells used in this fieldwork came from the U.S. NAMRU (United States Naval Medical Research Unit) Jakarta, whereas T47D cells derived from a collection of UB's Faculty of Medicine, Malang.

HeLa cells were grown in RPMI 1640 medium (Sigma) containing the complete fetal bovine serum (FBS) 10% (Gibco), fungizon 0.5% (Gibco), penicillin-streptomycin and 2% (Gibco). T47D cells were grown in DMEM medium (Gibco) containing complete 10% FBS (Gibco) and Penicillin-Streptomycin 2%. Both cells were cultured using 25 mL flasks (Nunc) in a CO2 incubator at a temperature of 370 C and 5% CO2 stream.

Isolation of Lymphocyte Cells

Isolation of lymphocytes was performed according to the method used by Wahyuni [25]. Blood was taken from healthy respondents are sterile and plus anticoagulants. Separation of cellular components is done by centrifugation of blood samples at a speed of 1,000 rpm vacutteiner for 10 minutes. Part severe blood (red blood cells) are under separate blood and plasma at the top. Buffy coat layer (containing lymphocytes) are located in between the two layers is taken then coupled with RPMI medium. Furthermore, the lymphocyte suspension is passed on Ficoll - Hypaque solution slowly to form two layers separated. Then, the tubes were centrifuged again with less speed of 1,500 rpm for 30 minutes. Lymphocytes, monocytes, and platelets are in the top layer of Ficoll surface and does not penetrate to the bottom, whereas granulocytes and red blood cells separated on the basis of

centrifuge tubes . Layer containing lymphocytes , monocytes , and platelets were washed with RPMI medium and centrifuged at 1500 rpm for 10 minutes. Supernatant was discarded and then the pellet was washed and centrifuged again at a speed of 1,500 rpm for 10 min to separate lymphocytes from platelets, monocytes and Ficoll (in the supernatant) . Cell pellet obtained was then added complete RPMI growth medium containing Fetal Bovine museum (FBS) 10%, 0.5% Fungizone, penicillin - streptomycin and 2 % and homogenized, then performed cell counts using a hemocytometer.

Cytotoxicity test against HeLa cancer cells and T47D

Cytotoxicity assay was conducted using MTT (3 - [4, 5-dimetilthiazol-2YL] -2, 5-diphenyl tetrazolium bromide) by Zachary [26]. Created series of concentrations of the extract of S. filipendula of 12.5, 25, 50, 100, and 200 ppm using the solvent as much as 3 replications growth medium. Also made 3 kinds of control, namely: control of tumor cell (100 ml + 100 ml of tumor cell media), media control (200 ml media), and the control sample (100 ml extract of *S. filipendula* + 100 ml media). A total of 100 ml extract solution *S. filipendula* of each concentration incorporated into microplate wells containing tumor cells that have as many as 2 x 10^4 cells (100 ml). Microplate and then incubated for 24 hours in a CO2 incubator, then added 10 ml of MTT into each microplate wells and incubated again for 4 hours in a CO2 incubator. MTT reaction was stopped by adding sodium dodecyl sulfate (SDS) 10% then back microplate was incubated for 12 hours in the dark at room temperature. After the incubation, the absorbance of each of the wells was measured by spectrophotometer microplate reader (DYNEX Technologies MRX type) at a wavelength of 570 nm.

Determination of the percentage of cell death was calculated based on the formula:

Mortality = $\frac{(A-D)(B-C)}{(A-D)}$ x 100 %

Description:

A = absorbance of control cells

B = absorbance of the sample

C = absorbance of control samples

D = absorbance of control media

Lethal concentration50 value calculation (LC50) was performed using the probit analysis in MINITAB program version 13.2 with 95% confidence interval.

Lymphocyte proliferation test

Lymphocyte proliferation test was also conducted using MTT (3 - [4,5-dimetilthiazol-2YL] -2,5-diphenyl tetrazolium bromide) by Zachary [26]. However, the number of lymphocytes used were as many as 2 x 105 cells in each of the wells.

Determination of the percentage of lymphocyte proliferation was performed using the formula:

Lymphocyte proliferation = $\frac{absorbance\ of\ sample}{absorbance\ of\ control}$ x 100%

RESULT AND DISCUSSION

The results of the study of brown algae fucoxanthin isolation (*Sargassum filipendula*) with the results of column parameters, test identification by TLC (thin layer chromatography), test pattern identification spectra with a spectrophotometer Shimadzu UV-Vis 1601 and yield calculations can be seen from Table 1.

Tabel 1. Identification of Pigment Fucoxanthin Test Data

No	Identification Test	Tool	Result		Literature
1.			0,029825%		
2	Column	Chromatography column	135 isolates of pigment in the reaction tube. Isolates fucoxanthin from 96-121 tube		Fucoxanthin coloured orange [17]
3.	TLC	TLC	Rf 0,28		Rf fucoxanthin 0,25-0,28 [27]
4.	Pattern Spectra	Spektrofotometer Uv-Vis 1601 Shimadzu	Solvent		In ethanol solvents [17] 446.3nm wavelength In Solvents Ethanol [28] Wavelength 450 nm
			acetone	ethanol	
			447 nm	451nm	

The ability of fucoxanthin as an anti-carcinogenic agent fregmented due to its ability to induce DNA that causes apoptosis [11]. Fucoxanthin role induce apoptosis in Hela cells. Anticarcinogenic mechanism was obtained through its ability to decrease the expression of the protein bcl-2 and bcl-xl in the cell wall of the mitochondria [33].

Fucoxanthin has the ability to increase catalase perokisom located in the cell that converts H_2O_2 (peroxide) into molecular oxygen and water. Fucoxanthin prominent effect on DNA and the damage, inhibit the activity of DNA associated with fucoxanthin's ability to increase catalase [30]. Figure 1 shows the percentage of cell death caused by the influence of the dose fukosantin againts hela and lymphocytes cell.

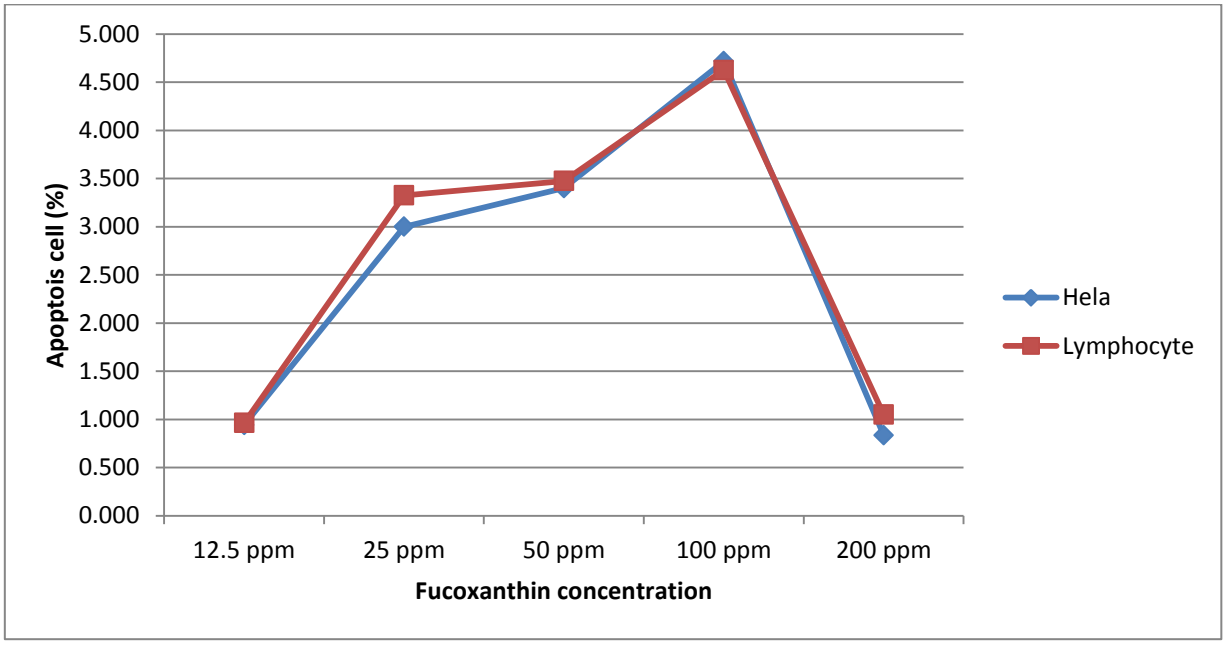

Figure 1. Fucoxanthin dose effect on hela cells and lymphocytes

Results of the study reported that the compound fucoxanthin with dosing more dead HeLa cells but at a dose of 200 ppm decreased HeLa cell death, so the medication/ administration of herbal effects when most would be toxic [29].This research report shows that the maximum dose at 100 ppm dosing fucoxanthin will turn off HeLa cells by 4,717 %, which means that fucoxanthin has the effect on HeLa cells. Fucoxanthin potential to induce cell apoptosis that occurs in HeLa cells. This is possible because of the anti-carcinogens of fucoxanthin that has a unique structure that is 5.6 epoxide and alenik bond. Figure 2 shows morphological observation on Hela Cell.

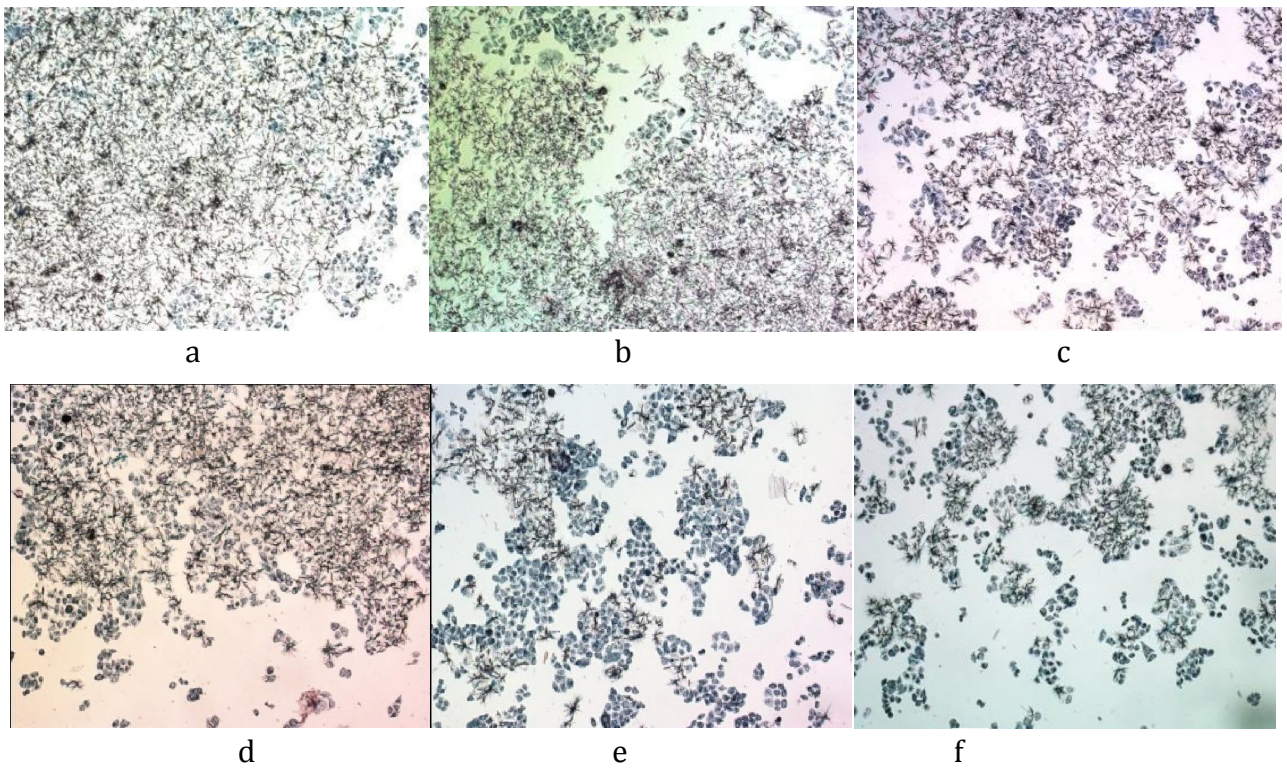

Figure 2. Morphological observation of Hela cell (1000x) (a. control; b. Fucoxanthin dose of 12.5 ppm; c. Fucoxanthin dose of 25 ppm; d. Fucoxanthin dose of 50 ppm; e. Fucoxanthin dose of 100 ppm; f. Fucoxanthin dose of 200 ppm).

Fucoxanthin has a bond alenik and 5.6 monoepoxide where these molecules play a role in the resistance of the structure [11]. The antioxidant activity of fucoxanthin has a very good effect in the inhibition of cancer cell molecules. Fucoxanthin is able to inhibit the growth of cancer cells by inducing cell cycle arrest and enhance gap junctional intercellular [30]. Dose of 100 ppm provide the best treatment. Fucoxanthin dose escalation to 200 ppm lead to a decrease in hela cell death. This may be due to a dose of 200 ppm fucoxanthin not be an antioxidant but as a prooxidant.From the results of the study reported that fucoxanthin compound capable of suppressing cancer cell proliferation [31]. Fucoxanthin effects on lymphocytes can be seen in Figure 3.

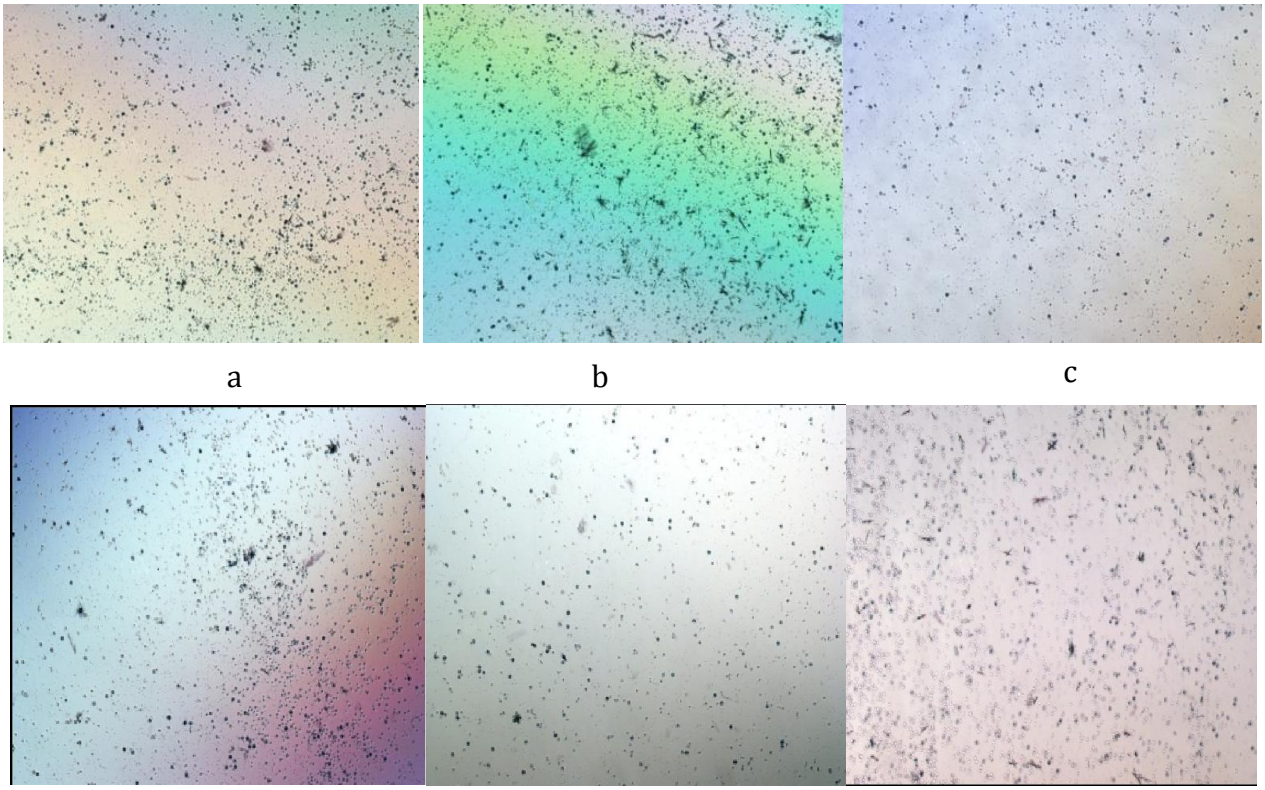

Figure 3. Morphological observation of Limphocyte cell (1000x) (a. control; b. Fucoxanthin dose of 12.5 ppm; c. Fucoxanthin dose of 25 ppm; d. Fucoxanthin dose of 50 ppm; e. Fucoxanthin dose of 100 ppm; f. Fucoxanthin dose of 200 ppm).

Figure 1 shows that the administration of a dose of 12.5 ppm fucoxanthin showed lymphocytes that die at 3.500% , 25 ppm dose of fucoxanthin lymphocytes die by 4,567%, fucoxanthin dose of 50 ppm lymphocytes die by 4.313%, 100 ppm dose of fucoxanthin lymphocytes are dead amounting to 9.543%, while for the 200 ppm dose of fucoxanthin lymphocytes die by 0.366% . This suggests that the use of fucoxanthin does not affect significantly on human lymphocyte. It is agree with Molina [34], that human lymphocyte treated with fucoxanthin did not present any significant alteration in the proliferation of T- and B-lymphocyte.

The use of human lymphocytes because lymphocytes are cells that are highly susceptible to chemicals . Advantages of in vitro testing is a very sensitive test used and the impact can be seen directly [25].The effects of the toxicity of a substance can be observed by how much the number of dead lymphocytes when compared with the initial state and the observed rate of cell proliferation of lymphocytes [32]. Lymphocytes are the cells with large nuclei and round and has little plasma. It has been calculated that in humans is about 3.5 x 1030 lymphocytes per day into the blood circulation.

Lymphocyte proliferation test was conducted to determine the bioactivity of fucoxanthin on cell lymphocytes are treated as normal human cells. If the extract is not toxic to lymphocytes, it can be hypothesized that the extract is not toxic to normal cells.Lymphocytes are the cells that are suspended in the blood , easily isolated , and is highly sensitive cell types. Therefore fucoxanthin in vitro testing can be carried out to test the properties of the bioactive component sitoktosis lymphocytes. Figure 1 shows the various concentrations of fucoxanthin showed fairly good activity against the proliferation of lymphocytes. According Miksusanti [32], a type of blood lymphocyte cells suspended in blood, easily isolated and is highly sensitive cell types. Therefore, testing the activity of bioactive components (fucoxanthin) to do.

CONCLUSION

Fucoxanthin with dosing more dead HeLa cells but at a dose of 200 ppm decreased HeLa cell death, so the medication/ administration of herbal effects when most would be toxic. Various concentrations showed fairly good activity against the proliferation of lymphocytes cells. Generally seen that the synergistic effect of the

molecules contained in extracts of brown algae (fucoxanthin) in improving the presentation of lymphocyte proliferation higher.

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