The Anticancer Activity of Marine Sponge *Cinachyrella* sp. (Family Tetillidae)

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***Abstract*— *Cinachyrella* sp. marine sponge produce many kinds of secondary metabolites. The purpose of this study was to examine the cytotoxic and anti-proliferative activity of marine sponge *Cinachyrella* sp. The sponge was extracted with 96 % ethanol. Ethanol extract cytotoxicity assay were performed with MTT method (Microculture Tetrazolium) against to cell lines of HeLa, T47D, WiDr and Vero. The results show that of the ethanol extract were only toxic to cell lines of HeLa IC_{50} 897.809 µg/mL but did not toxic to cell lines of T47D, WiDr and normal cell lines of Vero. Fractionation of the ethanol extract was conducted by a Vacuum Column Chromatography (VCC) and have 4 fraction that were F1, F2, F3 and F4. Cytotoxicity and cell proliferation inhibitory were tested of fraction F1, F2, F3 and F4 against cell lines of T47D. The values IC_{50} of F1; F2; F3 and F4 against cell lines of T47D were 82.744; 163.679; 66.522 and 333.026 µg/mL and fraction F3 concentration 31.5 µg/mL inhibits cell proliferation cell lines of T47D at 48 hours of incubation.**

Keywords—***Cinachyrella* sp., MTT, Cell Lines HeLa, T47D, WiDr and Vero.**

***Abstrak*— *Spons laut Cinachyrella sp. menghasilkan banyak metabolit sekunder. Tujuan penelitian ini mengkaji sitotoksisik dan antiproliferasi spons laut Cinachyrella sp. Spons diekstraksi dengan etanol 96%. Uji sitotoksisitas ekstrak ethanol dilakukan dengan Metode MTT (Microculture Tetrazolium) terhadap sel HeLa, T47D, WiDr dan Vero. Ekstrak ethanol menunjukkan sitotoksik terhadap sel HeLa dengan nilai IC_{50} 897,809 µg/mL, tetapi tidak toksik terhadap sel T47D, WiDr dan Vero. Ekstrak ethanol difraksionasi dengan metode kromatografi kolom vakum (KKCV). Hasil fraksionasi diperoleh 4 fraksi F1, F2, F3 dan F4. Semua fraksi dilakukan uji sitotoksik dan anti-proliferasi terhadap sel T47D. Fraksi F1, F2, F3 dan F4 menunjukkan sitotoksik terhadap sel T47D dengan nilai IC_{50} 82,744; 163,679; 66,522 dan 333,026 µg/mL dan fraksi F3 konsentrasi 31,5 µg/mL menghambat proliferasi sel T47D pada inkubasi 24 jam*.**

Keywords—***Cinachyrella sp., MTT, HeLa, T47D, WiDr, Vero.***

I. Introduction

Marine sponges *Cinachyrella sp.* (phylum Porifera, class Demospongiae, order Spirophorida, family Tetillidae) is sessile marine invertebrates mostly spherical to elliptical. The sponge are characterized by a spherical and spiraling growth form. They are frequently referred to as ‘‘golf ball sponges’’ and ‘‘moon sponges’ [1]. *Cinachyrella sp.* is dominant type of sponge in the intertidal beach Kukup [2].

They have been found to be a source of secondary metabolites with potential medical applications such hemagglutinating activity for papainized type Aerythrocytes [3], modulated the function of mammalian ionotropic glutamate receptors [4], and antiproliferative activity against HeLa, PC3 and 3T3 cell lines [5]. Marine sponge *Tetilla* sp. that was namely one family with Tetillidae have antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* [6] and anticancer activity showed G0-G1 phase arrest cells lines WiDr [7].

Cancer is a devasting disease with tremendous negative implications at the personal, health care, economical and social level [8]. It is one of the leading causes of death in the World, aflicting an estimated 7.9 million people in 2007 *World Health Organization* (WHO), and this number continues to increase almost 80 million per year [9]. In Indonesia cancer patients also tended to increased [10].

Cancer develops due to failures in the mechanisms that normally control cell growth and proliferation. Therefore, losses in the regulation of these cells are, in most cases, caused by genetic damage [11]. Changes in the cell genome due to errors in DNA replication, DNA instability, but it is also caused by exposure to chemical carcinogens such as ionizing radiation and UV [12].

Conventional cancer treatment can be done in several ways: surgery, radiotherapy, chemotherapy, or in some cases, it is necessary to combine more than one method for treating the cancer. Several distinct biological strategies might prove effective in eliminating established tumors or preventing the maintenance of its progression [13].

In America and Europe estimated 65% of cancer drugs derived from natural materials commercially [14]. Derivative compounds of bioactive natural products have specific targets and has no side effects [15]. Marine sponges (Porifera) are the oldest metazoan group having an outstanding importance as a living fossil. Biomass of sponges is the largest and most diverse in marine habitats [16].

Sponge and symbiont microorganisms produce a number of secondary metabolites [17]. Sponges have been considered as a gold mine for the chemists. More than 12,000 compounds have been isolated from marine sources with hundreds of new compounds still being discovered every year [18]. Thirty percent of the natural ingredients that have been isolated from the

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sponge derived [19]. Many of these compounds function as chemical weapons and have evolved into highly potent inhibitors of physiological processes in the prey, predators [20], or competitors of the marine organisms that use them [21, 22].

This work was taken to investigate the cytotoxic and antiproliferative properties from marine sponge *Cinachyrella* sp. against to human tumor cell lines of HeLa, T47D, WiDr and normal cell line of Vero. This investigated to some other types of cancer cells, because cancer cells have characteristic and sensitivity of different molecular.

II. METHOD

A. *Sponge Collection*

Samples of *Cinachyrella* sp. were collected along tidal zones the south coast of Kukup, Gunung Kidul, Yogyakarta in 2011. The specimen was identified as *Cinachyrella* sp. by Dr. Nicole J. de Voogd. The voucher number for this collection is RMNH POR.8636 and a voucher sample is maintained at the Marine Zoology Museum Darwinweg, Leiden.

B. *Extraction and Purification*

The sponge (15 kg) extracted with ethanol 96% to give 448.31 g extract ethanol [23]. Further the extract ethanol was partitioned by ethyl acetate. Fractionation ethyl acetate extracts were separated by vacuum liquid column chromatography with silica gel stationary phase and mobile phase combined organic solvent N-Hexane: Ethyl acetate (10:0; 9;1; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 2:8; 1:9; 0:10); Chloroform:Methanol=1:1. Fractions were evaporated and identified by thin layer chromatography/TLC with n-hexane=7:3 v/v as eluent [24]. Fractions with similarity TLC profiles are merged. The combined fractions (F) used to anticancer activity. Active isolate of fraction was isolation by preparative TLC (TLCP).

C. *Cell line and Culture*

The human servix cancer cell line HeLa (ATCC®CCL-2™), breast cancer cell line T47D (ATCC®HTB-133™ and colon cancer cell line WiDr (ATCC®CCL-218™) were cultured in RPMI 1640 medium (*Rosewell park memorian institute*). The normal cell line Vero (ATCC®CCL-81™) was cultured in M199 medium. The medium supplemented with 2 mL of penicillin-streptomycin 1% and 0.5% 1mL fungizon. The cell cultures were grown in a humidified 5% CO_2 at 37 C.

D. *Cytotoxicity and Cell Viability Analysis*

The colorimetric MTT assay was used to determine cytotoxicity of extract ethanol and fraction marine sponge *Cinachyrella* sp. on HeLa, T47D, WiDr and Vero cells [25]. Briefly, 10,000 cells/well were seeded in 96-well plates. Cells were exposed to different concentrations of extract (15.625; 31.25; 62.5; 125; 250; 500; 1000 µg/mL in 1 ml of sterile 10% DMSO). Control group was added 100 mL Doxorubicin (1.562; 3.125; 6:25; 12.5; 25; 50; 100 µg/mL) for 24 h. Cells were then incubated with MTT solution (4 mg/mL in PBS) for 3 h at 37 C in dark. Results of the MTT assays were obtained using a microplate reader (ELISA) at 595 nm. The IC_{50} value was defined as the concentration that caused a 50% inhibition of cell growth compare to control RPMI. Each experiment was in triplicate format.

Determination of the concentration of sponge for toxicity tests follow the guidelines the American National Cancer Institute [26]. The extract have significant antiproliferative effects when LC_{50} values ≤ 20 µg/mL. Cytotoxicity was determined using [{1 − (OD treated/OD control)} ×100] [27].

E. *Cell Growth Inhibition Assay*

Cell proliferation assay T47D cells ($1x10^3$ cells/well) were plated in 96-well tissue culture microplates in 100 µL of medium and treated 24, 48 and 72 h later with F in triplicate at 3 different concentrations 31.25; 62.5 and 125 µg/mL. The absorbance was monitored at 595 nm and results were expressed as the inhibition of cell proliferation calculated using [(1 − (OD595 treated/OD595 control) ×100]. Cell doubling time *(doubling time)* is obtained from equation curve relationship between incubation time and cell number (%).

F. *Statistical Analysis*

Data cytotoxicity assay and cell proliferation kinetics were analyzed statistically by analysis of variance (ANOVA) one way using SPSS 13. Value *Inhibitor Concentration* 50 (IC_{50}) was determined using probit analysis with the help of the statistical program SPSS 13 [28].

III. RESULT AND DISCUSSION

Cinachyrella sp. is globular sponges of max. 4-5 cm,with upper surface heavily covered by dark sediment and algae. Colour is orange, buds are yellow [29] Fig. 1. Sponges are usually found on firm surfaces such as rocks, but some sponges can attach themselves to soft sediment by means of a root-like base. The morphology and structure of the marine sponge *Cinachyrella* sp. is similar with *Cinachyrella arabica* [30].

A. *The Extract Ethanol and Fraction of Cinachyrella sp-induced Cytotoxicity in HeLa, T47D, WiDr and Vero Cells*

To investigate extract ethanol and fraction inhibited on HeLa, T47D, WiDr and Vero cells were treated with different doses (15.625-1000 µg/mL). These results were expressed as percent viability and as total number of viable cell as shown in Fig. 2. In the statistical analysis of Duncan's *one way* to T47D, WiDr and Vero cells showed no significant ($P < 0.05$) and inhibit weak in HeLa cells. The ethanol extracts *Cinachyrella* sp. is sensitive for Hela cells lines.

Test results of fraction against cell lines T47D, WiDr and Vero showed in Fig. 3 and Table 2. Cytotoxicity of the fraction (F1, F2, F3 and F4) *Cinachyrella* sp. on cell lines T47D at a 24-h incubation time. Cell viability was measured using the MTT assay and expressed as the percentage of treated samples to untreated control samples.The percent viability on cell lines HeLa, T47D, WiDr and normal cell lines Vero increased. Statistical analysis of the active fraction Duncan *two way* showed a significant ($P < 0.05$).

The ethanol is an organic solvent that general nature of all organic material. Identification by TLC of the extract

ethanol and fractions of F1, F2, F3 and F4 by *Dragendorf* reagents and compounds *of Cerium sulfate* showed alkaloids and terpenoids.

Test anticancer activity is influenced by the type of extract and cell lines. The ethanol is more toxic against cell lines HeLa however ethyl acetate is more toxic to T47D cells [31]. The extract ethanol was polar and non-polar compounds [32] while the extract ethyl acetate more interested semipolar and nonpolar compounds such as polyphenols group, alkaloids, flavonoids and glycosides. The results of this study indicate that the toxic compounds from the marine sponge *Cinachyrella* sp. is semipolar and nonpolar. In some other studies, nonpolar compounds more toxic than polar compounds. The ethyl acetate extract of the marine sponge *Petrossian* sp., *Jaspis* sp. and *Pericharax heteroraphis* have a higher cytotoxicity of the hexane extract and butanol [33]. The value IC_{50} of extract ethyl acetate of red seaweed *Gracilaria verrucosa* against cell lines HeLa was 220.09 ppm, 62.5% higher than the dichloromethane extract 37.5% [34]. The results of cytotoxicity assay ethanol extract marine sponge *Cinachyrella* sp. This is less toxic than the ethanol extract of the marine sponge *Aaptos suberitoides* Pasir Putih, Situbondo, East Java. The value IC_{50} ethanol extract of marine sponge *A. suberitoides* against cell lines HeLa, T47D and WiDr were 133.968; 153.109 and 144.540 µg /mL [35].

Alkaloids are microtubule interfere agents which can bind with beta tubulin, thus preventing the cell from making the mitotic spindle fibres necessary to move the chromosomes around as the cell divides [36], inhibiting topoisomerase [37], mitochondrial damage and inducing the release of cytochrome C and apoptosis inducting factor. Examples that the trifluoroacetat salt of Cinachyramine was isolated from the Okinawan sponge *Cinachyrella* sp. Cinachyramine is a novel alkaloid with an unprecedented cage system possessing a hydrazone and two aminals [38]. Enigmazole A, a noval phosphate-containing macrolide, was isolated from Papua New Guinea collection of the marine sponge *C. anigmatica*. The enigmazole are the first phosphomacrolides from a marine source and exhibited significant cytotoxicity in the NCI 60-cell line antitumor screen, with a mean GI_{50} of 1.7 µM [39].

Terpenoids display a wide range of biological activities against cancer, malaria, inflammation, and a variety of infectious diseases [40]. In marine waters is estimated there are hundreds of drugs derived from terpenoid compounds [41]. Triterpene B compound from marine sponge *Globostellifera stellate* and *Geodia stellate* were taken from ocean Fijian have anticancer activity against tumor cell lines A2780 and cell lines leukemia K562 [42]. Sesquiterpenes quinine isolated from sponges *Hippospongia* sp. have activity against human tumor cell lines H460, Hep G2, SF268, MCF7, HeLa and HL60 also caused arrest cycle cell lines HepG2 [43].

Different sensitivity of cell lines caused different molecular characteristics of cell lines. Jennei reported that cell lines TD7D expressed the estrogen receptor subtype β [44]. Cell lines Vero which had been known as a normal cell from a normal cell derivate African Green Monkey is also potentially lead to tumor phenotypes [45]. The cell lines Vero often used as vaccine trials [46]. Inhibitory proliferation potency based on American National Cancer Institute of extract IC_{50} values ≤ 100 µg/mL. Threshold set for natural ingredients that can be developed as anticancer ≤ 50 µg/mL [26]. Active isolate of fraction F3 of marine sponge *Cinachyrella* sp. at Kukup beach have potential as an anticancer agent.

B. *Antiproliferative effect of fraction Cinachyrella sp. on T47D Cell*

Logarithmically growing cell line T47D were seeded at a concentration 1 x 10^3 cells per mL and then incubated with increasing concentration of fraction Cinachyrella inhibited the growth of T47D cell. After 24 h, 48 h and 72 h of incubation *Cinachyrella* sp.inhibit the growth of T47D cells. The fraction F4 31.5 µg/mL inhibited proliferative T47D cells (Table 3 and Fig. 6).

Differented response of cell lines to various types of sponges from various regions thought to be caused due to contained different active ingredients which each extract and isolate have components of different compounds [44]. Besides the secondary metabolites of the sponge is also influenced by water conditions, and the type of sponge symbiont organisms [47, 48, 49]. The ethanol extract is a mixture of various compounds, while the isolate was isolated compounds that contain more specific compounds. The ethyl acetate extract contains many alkaloids and terpenoids compounds that are more toxic than the ethanol extract.

Cinachyrella sp. also contained fatty acids,such as. phospholipid fatty acid composition of the sponge *C. alloclada* Senegal are two new fatty acids hitherto not found in nature 10,13-octadecadienoic acid and 16-tricosenoic acid [50]. Class composition of phospholipids, fatty acids and sterols from the sponge *C. alloclada* and *C. kükenthali* of the Saudi Arabian Red Sea and in Senegal (East Atlantic) and New Caledonia (West Pacific) over 50 fatty acids were identified as methyl esters and N-acyl pyrrolidides. Comparison with *Cinachyrella* species from other geographical areas showed marked differences for both the fatty acid composition of phospholipids and sterols [51]. Lectin protein as the Cinachyrella galectin (CchGs) in aqueous sponge extract that modulated the function of mammalian ionitropic glutamate. There are significant correlations between high anticancer activity and the high phenolic contents [52].

IV. Conclusion

Marine sponges *Cinachyrella* sp. are potential sources of many unique metabolites, including cytotoxic and anticancer compounds to human tumor cell lines of T47D. The values IC_{50} of active isolate of fraction F3 66.522 µg/mL. Isolate active of fraction F3 concentration 31.5 µg/mL inhibits cell proliferation at 48 hours of incubation

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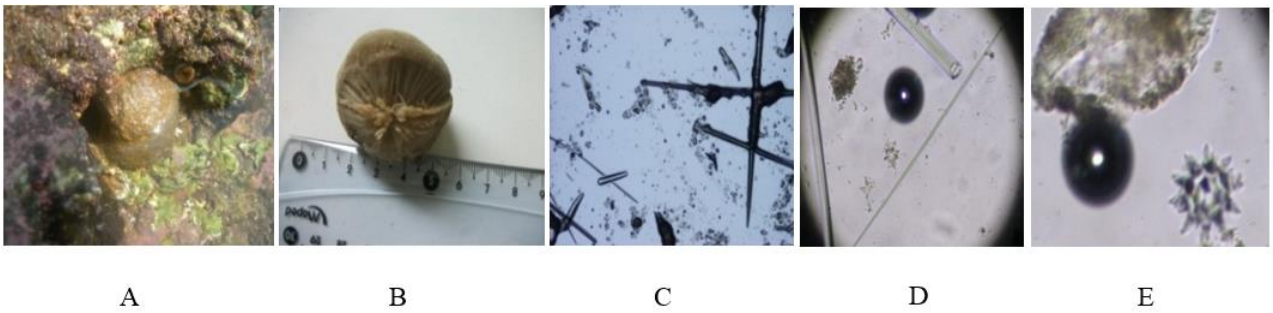

Figure 1. Characteristics of marine sponges *Cinachyrella* sp. a. Morphology of *Cinachyrella* sp. attached under a rock, b. Cross sections of *Cinachyrella* sp. viewed from below, c. Preparations microscopic of *Cinachyrella* sp., Magnification 10x; d. Style of *Cinachyrella* sp., Magnification 10x e. Megasclera of *Cinachyrella* sp. 10x magnification.

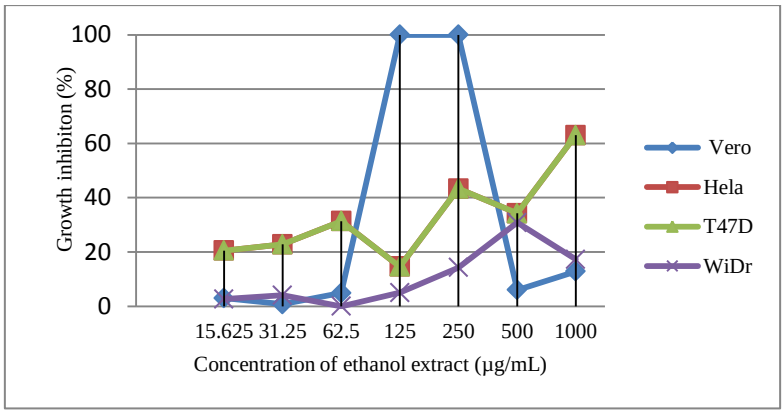

Figure 2. Cytotoxicity of ethanol extract *Cinachyrella* sp. on cell lines (Hela, T47D, WiDr and Vero) at a 24-h incubation time along with normal cell lines Vero. Cell viability was measured using the MTT assay and expressed as the percentage of treated samples to untreated control samples.

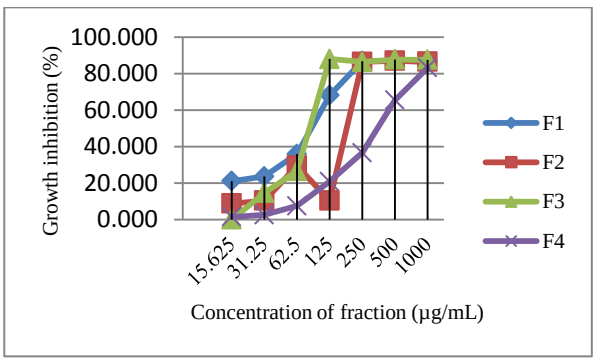

Figure 3. Cytotoxicity of the fraction (F1, F2, F3 and F4) *Cinachyrella* sp. on cell lines T47D at a 24-h incubation time. Cell viability was measured using the MTT assay and expressed as the percentage of treated samples to untreated control samples.

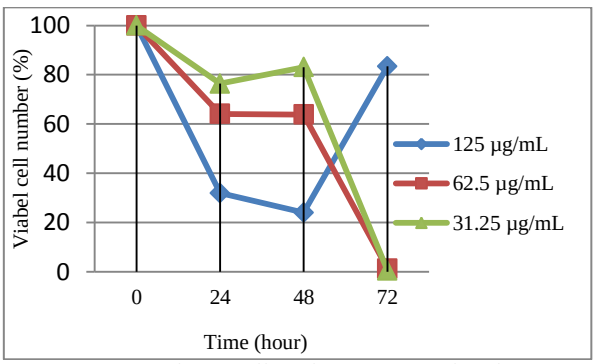

Figure 4. Antiproliferative effect of active isolate of the fraction F1 *Cinachyrella* sp. on cell lines T47D.

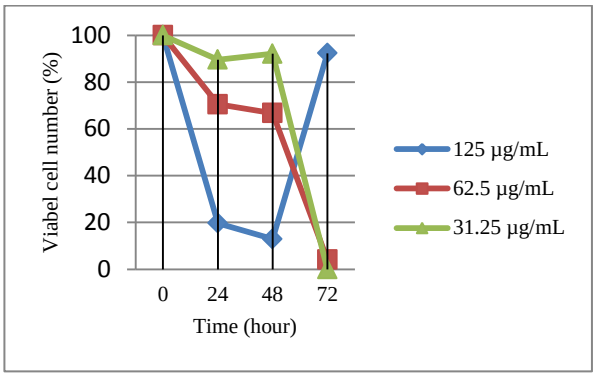

Figure 5. Antiproliferative effect of active isolate of the fraction F2 *Cinachyrella* sp. on cell lines T47D.

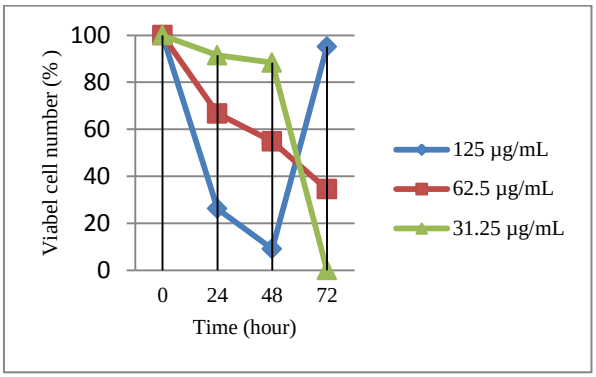

Figure 6. Antiproliferative effect of active isolate of the fraction F3 *Cinachyrella* sp. on cell lines T47D.

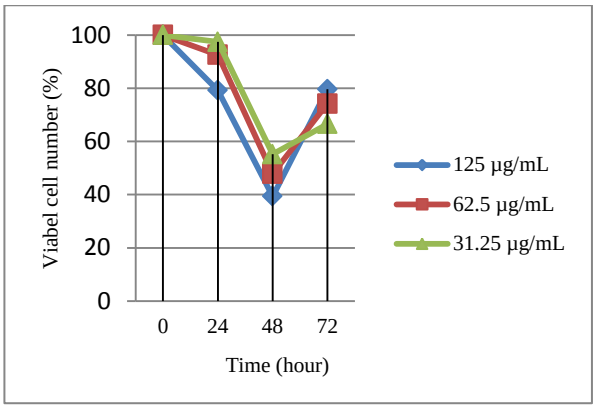

Figure 7. Antiproliferative effect of active isolate of the fraction F4 *Cinachyrella* sp. on cell lines T47D.

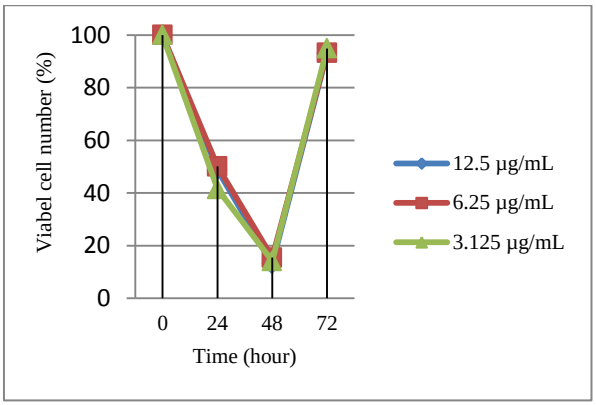

Figure 8. Antiproliferative effect of Doxorubicine on cell lines T47D.

TABLE 1.
THE CYTOTOXICITY OF ETHANOL EXTRACT OF THE MARINE SPONGE *CYNACHYRELLA* SP. AGAINST CELL LINES HELA, T47D, WIDR AND VERO

No	Cell lines	Type of cell	IC_{50} Ethanol extract (µg/mL)	IC_{50} Doxorubicin (µg/mL)
1	Vero	Normal cell line	>1000	>1000
2	Hela	Servix cancer cell line	897.809	10.062
3	T47D	Breast cancer cell line	>1000	32.974
4	WiDr	Colon cancer cell line	>1000	14.93

TABLE 2.
THE CYTOTOCITY OF ACTIVE ISOLATE OF THE FRACTION (F1, F2, F3 AND F4) *CINACHYRELLA* SP. ON CELL LINES T47D AT A 24-H INCUBATION TIME.

No	Treatment of fraction	IC_{50} µg/mL
1	F1	82.44
2	F2	163.679
3	F3	66.522
4	F4	333.026
5	Doxorubicin	9.33

TABLE 3.
REGRESSION EQUATION OF ANTIPROLIFERATIVE EFFECT OF ACTIVE ISOLATES (F1, F2, F3 AND F4) *CINACHYRELLA* SP. ON CELL LINE T47D.

Active isolate of fractionation	Concentration of the fraction (µg/mL)	Equation	Slope (α)	R^2 (The correlation of coefficient)
F 1	125	0.594 (x) + 20.385	0.594	0.637
	62.5	(-)0.576 (x) + 72.805	-0.576	0.753
	31.5	(-) 0.433 (x) + 71.077	-0.433	0.684
F 2	125	0.450 (x) + 29.266	0.450	0.681
	62.5	(-) 0.573 (x) + 74.977	-0.573	0.792
	31.5	(-) 0.286 (x) + 62.066	-0.286	0.735
F3	125	0.721 (x) + 3.748	0.721	0.981
	62.5	0.601 (x) + 17.530	0.601	0.992
	31.5	(-) 0.432 (x) + 64.366	-0.432	0.997
F 4	125	0.009 (x) + 47.385	0.009	0.000
	62.5	(-) 0.436 (x) + 79.200	-0.436	0.167

	31.5	(-) 0.776 (x) + 104.657	-0.776	0.498
Doxorubicin	12.5	0.332 (x) + 30.867	0.332	0.306
	62.5	0.341 (x) + 29.968	0.341	0.306
	3.125	0.380 (x) + 28.955	0.380	0.423