

Cytotoxic Activity of Ethanolic Extract and Fractions of the Marine Sponge *Cinachyrella* sp

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Abstract

The study was aimed at evaluating the anticancer activity on the *Cinachyrella* sp on Hela, T47D and W1Dr Cell lines. The ethanolic extract was fractionated by using vacuum-liquid column chromatograph with stationary-phase silica-gel and mobile phase *n*-hexane; *n*-hexane : ethylacetate (9;1; 8;2; 7;3; 6;4; 5;5; 4;6; 3;7; 2;8; 1;9 v/v); ethyl acetate; *n*-hexane : chloroform =1:1v/v); methanol. The percent viability of the cell lines was carried out by using colorimetric method. The cytotoxicity of *Cinachyrella* sp on T47D cell lines was evaluated by MTT assay. The research showed that ethanolic extract had an IC₅₀ value of 897,809µg/mL on Hela cell line. Meanwhile, fraction 5 showed an IC₅₀ value of 72,862µg/mL, higher than that of other fractions.

Key words:

Cinachyrella sp., cytotoxicity, extract ethanolic, fraction

I. INTRODUCTION

Cancer is cellular disease characterized by the loss of cell control function against circulating cell regulation and homeostasis of cell in multi-cellular organism. Because of the failure, the cells cannot proliferate normally. As a result, the cell will proliferate continuously and eventually lead to abnormal tissue growth (Ullah and Aatif, 2009). The cancerous cells may come from different tissues and organs, which are estimated to be more than 100 types (Kleinsmith, 2006).

There are many causes of the disease, which ranks the fourth after stroke, hypertension, and diabetes (Ullah and Aatif, 2009). According to the data released by World Health Organization (WHO), in 2000, there were about 6 billions of cancer cases, and the number keeps increasing by almost 80-million cases per year. In 2020, it is estimated that the cancer cases will have tolled 7,5 billions, and by 2050, it is projected to be 8.9 billions (Parkin, 2001). In 2012, there were about 1.638.910 newly identified cancer cases in the United States and led to 577.190 cases of death (Siegel *et al.*, 2012). In Indonesia, the incidence of cancer shows a steadily increasing trend with time (Tjindarbumi and Mangunkusumo, 2002). The 2007 report released by the Ministry of Health showed a cancer incidence of 10 per 100.000 populations.

Many efforts have been made to overcome cancer. In the beginning of the 20th century, cancer was treated using radiotherapy and surgical intervention. The methods showed a success rate of no more than 30%; therefore, additional or combined therapies are necessary. They include chemotherapy and chemo-preventive (DeVita and Chu, 2008). Chemotherapy has been used for many types of cancer (Morrison, 2010). Chemotherapy and chemo-

preventive have played an important role in cancer treatment. In the United States and Europe, about 65% of the commercial treatments for cancer have been derived from natural ingredients (Wei *et al.*, 2007). Anti-cancer treatments derived from natural ingredients like herbal plants, microbes, and sea organisms were more than 60%. Derivative compounds from natural ingredients have specific bioactive targets, which are free of side effects (Iwamaru *et al.*, 2007). Therefore, the efforts of identifying chemotherapeutic treatment from natural ingredients are still aggressively made (Nobili *et al.*, 2009).

Marine habitat is a huge source of natural ingredients, which have not been investigated in a maximum way. The greatest and most varied biomass in the sea habitat includes sponge (Hochmuth *et al.*, 2010). Sponges vary widely; in Australia, about 1400-1500 species of sponges have been identified. As a tropical area, Indonesia is estimated to have about 4000-6000 types of sponges (Hooper, 1997 and Van Soest, 1989). In phylogenetic point of view, sponge is the most primitive multi-cellular invertebrate, which lived during *Precambrian* period of more than 635 years ago (Hochmuth *et al.*, 2010). Sponge is a member of Metazoa, which has been in evolution for more millions of years. This is evident from the fact that it is widely distributed throughout the world, both in the seawaters and fresh waters (Utiti *et al.*, 1996). As a sessile animal, the filter feeder has a distinct physiology, reproduction, and an effective defensive mechanism against the infections of bacteria, fungi, viruses, and predators (Brackman and Daloz, 1986), spatial competition with other organisms (Schupp *et al.*, 2009), and defense against sea pathogens (Muller *et al.*, 2004). Sponges can produce different kinds of secondary

metabolites (Bell, 2008) and have pharmacological potentials (Faulkner, 2001). Sponges whose habitat is coral reefs can produce highly toxic secondary metabolite because of extreme water environment and pressures, competition and self-defense against nudibranch, sponge-eating gastropods, and carnivorous fish (Perdicaris *et al.*, 2013).

Research on natural ingredients from sponge began to be more aggressive since Bergmann and Feeney successfully isolated arabino-nucleoside from the sponge *Tectitethya crypta* in 1951. The efforts of finding and developing medicine from sponge began in 1970s (Yan, 2004). Sponge is the greatest source of secondary metabolites with new and unique structures, which are not founded in terrestrial organisms (Haufner, 2003). About 20.000 types of active compounds are produced from sponges, which have varied chemical classes. They include sterol, terpenoid, isoprenoid, nonisoprenoid, quinone, brominates, heterocyclic nitrogen, heterocyclic sulphur nitrogen (Bhakuni and Rawat, 2005), amino acid, porphyrin and peroxide (Tilvi *et al.*, 2004). The compounds derived from sponge also have varied functional groups (OH, OCH₃, OAc, OSO₃, Na⁺) (Wright, 1998). Each change in the functional group is potential to modify the polarity of each component in a radical way. An oncological study on the compounds from marine sponge found that it could interact with the important components in the cellular cycles, enzymes, and other targets (Tohme *et al.*, 2011).

More than 10% of the bioactive compounds from sponge have cytotoxic activities (Zhang *et al.*, 2003), anti-bacterial, anticoagulant, anti-viral, anti-fungal, anti-inflammatory, anti-tuberculosis, anti-malaria, anti-platelet, and anti-cancer properties (Faulkner, 2001); it is also hemolytic against erythrocytes, has anti-proliferative properties (Perdicaris *et al.*, 2013), and stimulates amino acid receptor (Ueda *et al.*, 2013). Penastrel derivative lanosterol, which was isolated from sponge *Penares* sp. in Okinawan sea was found to have anti-leukemia activity (Cheng *et al.*, 1988). *Aaptamine*, *isooaptamine* and *demethylate aaptamine* from the sponge *Aaptos* sp found in Menado sea, Indonesia was found to have anti-fouling activity (Jeffrey *et al.*, 2006). Sponge as a source of anti-tumor compound (Munro *et al.*, 1999) such as *calyculin A* isolated from the sponge *Discodermia calyx* (Kato *et al.*, 1986), *discodermide* and *discodermolide* isolated from the sponge *Discodermia dissoluta* (Gunasekera *et al.*, 1991), *mycalamides A* and *B* isolated from the sponge *Mycale* sp. (Perry *et al.*, 1990), halichondrin B isolated from the sponge *Lissodendoryx* sp., *Halichondria okadai* (Hirata and Uemura, 1986) and *Phakellia carteri* (Pettit *et al.*, 1993) and as an anti-neoplastic agent has been used in a clinical setting, such as Ara-A and Ara-C (Brown *et al.*, 2001). Until 2004, at least 12 anti-cancer compounds had been identified from natural ingredients. They included LAF389 (sponge amino acid derivative), Discodermolide (polyketide from sponge), HT1286 (tripeptide from sponges), and IPL512602 (steroid from sponges) (Haufner, 2003).

Cinachyrella sp. belongs to Demospongiae class, Spiroborida order, and Tetillidae family (Vacelet *et al.*,

2007). This type of sponge is dominant in every intertidal area, such as Kukup Beach, Gunung Kidul, DIY (Nurhayati *et al.*, 2011). Other species of sponge found at Kukup Beach from Tetillidae included the sponge *Tetilla* sp, which had been studied for anti-bacterial activity test by Perkasa and Budiyanto (2010). The test showed that symbion bacteria of sponge *Tetilla* sp. (Family: Tetillidae) had an anti-bacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Cytotoxic assay, which had been conducted on the sponge *Cinachyrella* sp. (Nurhayati *et al.*, 2011) revealed that ethanolic fraction of marine sponge *Cinachyrella* sp. from Gunung Kidul, DIY, had an arrest of G₀-G₁ phase against WiDr cancerous cell.

II. MATERIAL AND METHODS

2.1. Sampling of marine sponge *Cinachyrella* sp.

Marine sponge *Cinachyrella* sp. was taken from the beach of Kukup Desa Kemadang, Kecamatan Tanjungsari, Gunung Kidul Regency, Yogyakarta. The sample was taken from an intertidal area by means of direct collection (Wright, 1998).



Figure 1. The location of sample collection at Kukup Beach, coordinate : 8°08'1''S and 110°33'15''E

Sponge samples were taken from various microhabitats. Before the collection, the sponge samples were photographed in their natural conditions. The sponge data collected for the research included shape, color, and size. In addition, the samples were put into plastic bags and stored in icebox under a temperature of 5°C until the extraction time (literature)

2.2. Extraction of Marine Sponge *Cinachyrella* sp.

Sponge extraction was conducted by modifying the method proposed by Isnansetyo (2001). The sponge mass was cleaned and polluting particles were desiccated into the size of smaller than 2-3 mm, based on the method proposed by Orhan (2012). The sponge mass was macerated using 96% ethanol for 2x24 hours, while being stirred occasionally to prevent saturation. After being let for 2 nights, the sponge mass was filtered using a vacuum filter. The resulting filtration was collected (filtrate 1), while the sponge mass was re-extracted using ethanol in the same volume, let for 2x24 hours, and then

filtered to generate filtrate 2. The extraction process was repeated once more to generate filtrate 3. All of the filtrates were evaporated by using a rotary vacuum to generate thick extract. Extraction and fractionation of the marine sponge *Cinachyrella* sp. was conducted in the Pharmacy Biological Laboratory, Faculty of Pharmacy, Gadjah Mada University. AE and En of the marine sponge *Cinachyrella* sp was used for cytotoxicity assay against cancerous Hela, T47D and W1Dr, and Vero cell lines to determine the target cancerous cells for further assay. The cytotoxicity assay was conducted in Parasitology Laboratory, Faculty of Medicine, Gadjah Mada University, Yogyakarta.

2.3. Trituration of the extract of marine sponge *Cinachyrella* sp.

The ethanolic extract was partitioned using ethyl acetate in a funnel flask. The extract suspension was homogenated for about 15 minutes. The suspension was let to form 2 layers, then separated using a isolation funnel, to generate ethyl acetate soluble filtrate and ethyl acetate non-soluble filtrate. Each of the filtrates was thickened.

2.4. Fractionation of EA of the marine sponge *Cinachyrella* sp.

EA was fractionated using vacuum-liquid column chromatograph with stationery-phase silica-gel and mobile phase *n*-hexane; *n*-hexane : ethyl acetate (9;1; 8;2; 7;3; 6;4; 5;5; 4;6; 3;7; 2;8; 1;9 v/v); ethyl acetate; *n*-hexane : chloroform =1:1v/v); methanol. The resulting fraction was evaporated and then identified with thin-layer chromatograph (TLC). Fractions with similar TLC profiles were united. The united fraction was used as a sample of activity assay. The major fraction was derived and then isolated using preparative TLC (PTLC).

2.5. Cytotoxic Assay of the Extracts and fractions of marine sponge *Cinachyrella* sp.

Toxicity test was conducted using MTT method based on Stoddart (2011). About 100 μ L of cell suspension with a density of 2×10^4 cell/100 μ L media was distributed into 96-well plate and incubated for 24 hours. After incubation, 100 μ L of *Cinachyrella* sp. fractions in varied series of concentration (15,62; 31,25; 62,50; 125; 250; 500; 1000 μ g/mL) was introduced into the wells. As a positive control, 100 μ L of doxorubicin in varied series of concentrations was introduced into the

wells, which contained 100 μ L of cell suspension. As a cell control, 100 μ L of culture medium was introduced into the wells, which contained 100 μ L of cell suspension, and as a solvent control, 100 μ L of DMSO was introduced into the wells, which contained 100 μ L of cell suspension based on the delusion of test solution concentration. Then, it was incubated for 24 hours in an incubator with a flow of 5% CO₂ and 95% O₂. At the end of incubation, the culture medium was removed and added 110 μ L of MTT solution (5 mg/mL PBS), and then the cell was incubated for 3-4 hours. MTT reaction was terminated by adding SDS (100 μ L) as a reagent stopper. The micro-plates, which contained cell suspension, were sealed for \pm 5 minutes, then wrapped using aluminum foil and incubated overnight under room temperature. The living cells would react against MTT and turned into purple. The test results were read using ELISA reader at a wavelength of 595 nm. Percent cell death was calculated using the formula $\frac{\{(A-D)-(B-C)\}}{(A-D)} \times 100 \%$, in which A = absorbance of cell control, B = extract absorbance, C = absorbance of extract control, and D = absorbance of media control. The value of *Inhibitor Concentration 50* (IC₅₀) was determined using probit analysis and statistic software of SPSS.

III. RESULTS AND DISCUSSIONS

Marine sponge *Cinachyrella* sp. was taken from the beach of Kukup Desa Kemadang, Kecamatan Tanjungsari, Gunung Kidul Regency, Yogyakarta, by means of direct collection (Wright, 1998). The samples were determined in the Zoological Laboratory, Department of Biology, Faculty of Mathematics and Natural Science, Technological Institute of Surabaya. Then, the sample was confirmed by Dr Nicole J. de Voogd of the Museum of Marine Zoology, Darwinweg, Leiden, and then recorded as the specimen number RMNH POR.8635.

Habitat *Cinachyrella* sp. is commonly found in both deep and shallow sea. The sponge is attached to the substrates like coral in globular colony life form, massive globose colony, vertical and lateral growth directions, and leukonoid-type waterways (Fernandez *et al.*, 2011). The sponge is red, since it contains pigment found in amoebocyte. The color seems to function to protect its body from the sun light. The body part attached on the substrate was compared to the other extended body part (Figure 1).

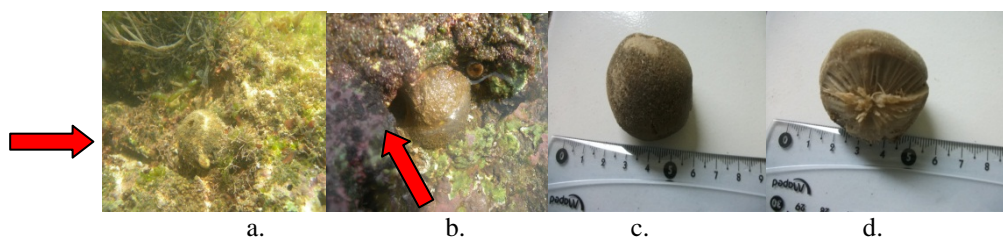


Figure 1. Morphology of sponge *Cinachyrella* sp. (arrow) a. Below the sea water; b. attached below the coral reef; c. upper surface of the sponge *Cinachyrella* sp; d. basal part of the sponge *Cinachyrella* sp.

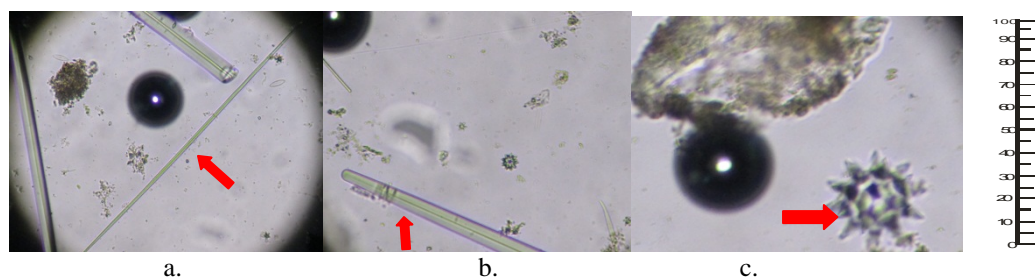


Figure 2. The spicule structure of marine sponge *inachyrella* sp. using fresh preparation method; a. Microsclera in 10x magnification; b. megasclera in 10x magnification; c. astrosphere. Then, 3 staining procedures were conducted upon the histological preparation with paraffine method. They included Hematoxilin and Mallory. Hematoxilin staining for the spicule turned in into bluish color, while with Mallory stain, it turned into soft blue (Figure 3).

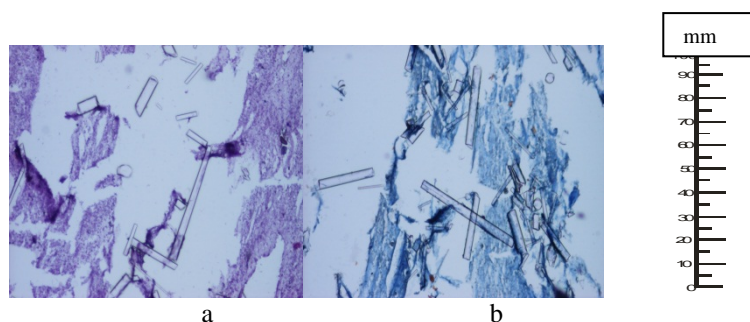


Figure 3. Spicule structure of marine sponge *Cinachyrella* sp. paraffin method, 10x magnification. A Hematoxilin staining; b. Mallory staining.

In the sea, the sponge is bright yellow, while in the ethanolic solution, it will turn into brownish color. The sponge has a tiny size, with a diameter of 2-3 centimeters. The skeletal structure is radial, with various *monaxons*, *protriaene*, *anatriaene* and *microsclera*, aperturo, and tiny porocalyx (Vacelet *et al.*, 2007). In addition to the identification with external morphology, identification was also conducted for spicule form of the wet/fresh preparation (Figure 2) (Ackers and Moss, 2007). The transparent spicule has *monoaxonemegasclera*, *style* and *microscleraeuaster* (the mark for *astrophoridae*) but it does not have *triaenaxone* and *microsclera sigma* (Fernandez *et al.*, 2011).

The results of sponge identification were:

Kingdom	: Animalia
Phylum	: Porifera
Class	: Demospongiae
Order	: Spirophoridae
Family	: Tetillidae
Genus	: <i>Cinachyrella</i>
Species	: <i>Cinachyrella</i> sp. (Vacelet <i>et al.</i> , 2007)

Identification of sponge *Cinachyrella* sp. was conducted for the first time by Uliczka in 1929 in Brazil under the name of *Cinachyra* sp. The name was then revised to *Cinachyrella* sp. *Cinachyrella* sp. belongs to Demospongiae class, Tetillidae family (Vacelet *et al.*, 2007). Demospongiae is the largest class of sponge, accounting for 90% of all Porifera. Demospongiae sponge has spicule made of spongin fibers. *Cinachyrella* sp. sponge is frequently known as *golf ball* and *moon sponges* (Szitenberget *et al.*, 2013).

In this research, identification was conducted up to three levels of genus axon. Identification up to the complicated species was conducted since the research did not include molecular analysis. For molecular analysis, DNA Barcode is necessary; it involved identification of sponge by comparing specific order of the specimen DNA, which was not recognized by DNA Barcode sequence of the identified specimen (Hebert *et al.*, 2003). DNA Barcode used in the research was cytochrome c subunit I oxydase (COI), which is a part of ribosomal 28 S gene fragment (domain C1-D2) (Cardenas *et al.*, 2009). DNA Barcode like sponge *cox1*, 18S rRNA and gene 28S rRNA (domain C1-D2) had also been used to sequence the phylogenetic type of marine sponge *Cinachyrella* sp. (Szitenberget *et al.*, 2013).

The extract served as the attractor of compound components in the sample by using appropriate solvent. In this research, extraction was conducted using maceration method, by using 95% ethanol as solvent to prevent the damage of thermolabile compound. Ethanol can attract polar and non-polar compounds. It is the most tolerant solvent among other (Sticher, 2007). The polar solvent is more potential to penetrate the cell membrane (Ruzicka *et al.*, 2009). Ethanol is also recommended by HBOI (*Harbor Branch Oceanographic Institute*) as a solvent in the extraction of marine natural ingredients (Wright, 1998). Fifteen kilograms of sponge was used for this research. Maceration procedures generated 448,31g (2,98%) of desiccated ethanolic extract. Loss on drying was huge (97,01%), indicating that sponge *Cinachyrella* sp. has high water content. The marine natural ingredients generally have more than 50% of water content (Wright, 1998). The cytotoxic effect of ethanolic extract was greatest for T47D cell line, with IC₅₀ value of .967,926 µg/mL, compared to

that of doxorubicine, with IC_{50} value of 32,974 μ g/mL. This effect is relatively weak; therefore, isolation of polar and non-polar compound was necessary to do by using ethyl acetate solvent and trituration technique.

Trituration was conducted to separate polar compounds from semi-polar ones (El-Naggar and Capon, 2009). Solvent used for trituration was ethyl acetate. Ethyl acetate is more polar than *n*-hexane is, but less polar than ethanol is. Ethyl acetate can attract polyphenol, alkaloid, flavonoid, and glycoside compounds. The trituration of 448,31 grams (10.26%) of dried ethanolic extract generated 46 grams of desiccated EA and 262.90 grams (58,64%) of desiccated En. EA is a non-polar extract, while En is polar. Trituration resulted in more polar extracts than non-polar or semi-polar ones.

TLC assay informed that in ethanolic extracts, ethyl acetate soluble and insoluble extracts had some compounds. Chromatographic profile of the extract is presented in Figure 4 and Table 1.

The compounds contained in the extract were assumed to belong to alkaloid and terpenoid group. Therefore, for TLC assay, Dragendrof reactor and serium sulfate were used.

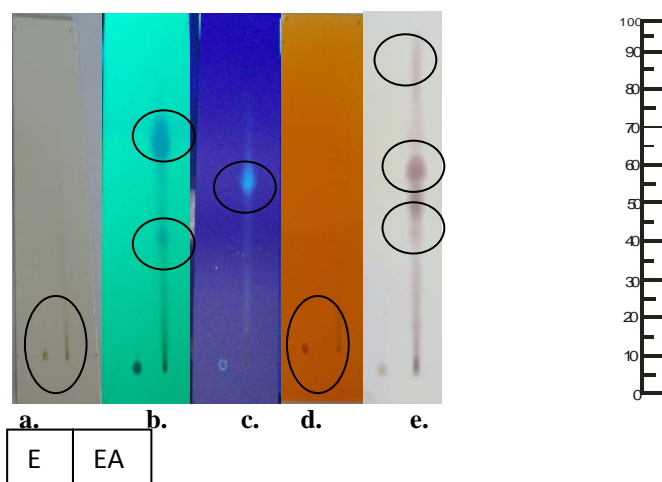


Figure 4. Chromatographic profile of ethanolic extract (E) and ethyl acetate extract (EA) of the marine sponge *Cinachyrella* sp. Mobile phase = *n*-hexane:chloroform:ethyl acetate = 8:2 v/v, 5:1 v/v. Stationary-phase silica Gel GF₂₅₄. a. Visible light, b. UV 254, c. UV 366, d. Dragendrof detector. e. CeSO₄ detector

Table.1 The HRF price of ethanolic and ethyl acetate extracts of marine sponge *Cinachyrella* sp., specimen no. RMNH POR.8635

Sample	Spot	HRf	Detection			
			UV ₂₅₄	UV ₃₆₆	Dragendorff	CeSO ₄
Ethanolic extract (E)	1	0	-	quenching	Reddish-Orange	Soft brown
Ethyl acetate extract (EA)	4 spots, 2 Tailing	4	Brownish blue	Fluorescence	Orange-brown	Brown
		5	Soft blue			
		6	Bright blue			
		6,5	Dark blue			
		0-4	Tailing	Fluorescence		
6-9	Tailing	Fluorescence	Brown			

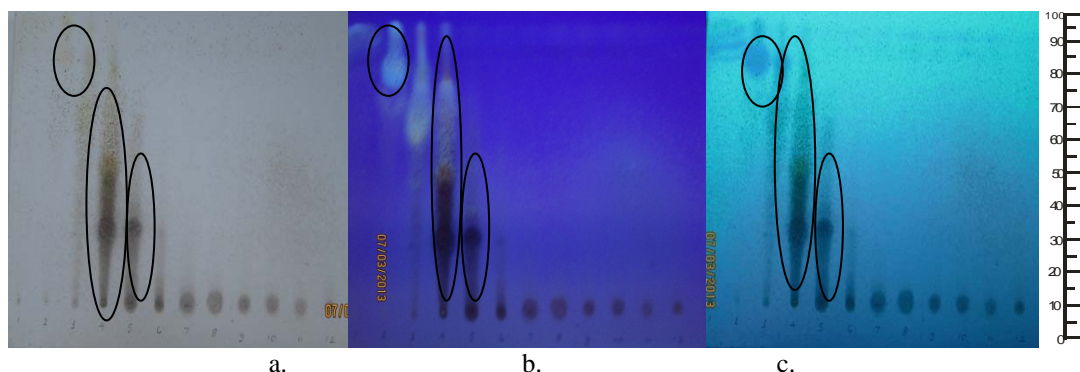


Figure 5. Chromatographic profile of the 12 fractions isolated from marine sponge *Cinachyrella* sp. Spot detector = CeSO_4 . Mobile phase = -hexane:ethyl acetate = 7:3 $\%$. Stationary phase = Silica Gel GF₂₅₄. a. visible light; b. UV 254, c. UV 366

Table 3. IC₅₀ values of the fractions (F1, F2, F3, F4, F5 and F6) against T47D cancer cell line

No	Samples	IC ₅₀ ($\mu\text{g/mL}$)
1.	Fraction 1	961,835
2.	Fraction 2	314,811
3.	Fraction 3	93,806
4.	Fraction 4	124,841
5.	Fraction 5	72,862
6.	Fraction 6	396,171
7.	Doxorubicin	86,660

In this research, fractionation was conducted to classify the non-polar, polar, and semi-polar solvent soluble compounds. Non-polar compound used in the research was n-hexane to attract non-polar compounds like fats and terpenoid. Its volatile character facilitated isolation of the solvent from the dissolved compounds without using high-temperature heating. Ethyl acetate is more polar than n-hexane, which is able to attract compounds like polyphenol, alkaloid, flavonoid, and glycosides. The chloroform solvent is more polar than ethyl acetate. Methanol is a polar solvent, which can attract compounds like glycoside saponin, carbohydrates, and glucose (Ruzicka *et al.*, 2009). Of the 12 isolated fractions, only 6 fractions was subject to cytotoxic effect assay against T47D cell line. Of the 6 fractions, only fractions 5 and 6 had IC₅₀ <100 $\mu\text{g/mL}$ (93,806 and 72,862 $\mu\text{g/mL}$), compared to the other fractions.

CONCLUSION

Factions of marine sponge *Cinachyrella* sp. F1, F2, F3, F4, F5 and F6 showed cytotoxic activities against T47D cancer cells, with IC₅₀ values of 961,835; 314,81; 93,81; 124,84; 72,86 and 396,17 $\mu\text{g/mL}$. IC₅₀ value of doxorubicin was 86,66 $\mu\text{g/mL}$.

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