CYTOTOXIC ACTIVITY AND APOPTOSIS INDUCTION OF T47D CELL LINES BY *Turbinaria decurrens* EXTRACT

Aktivitas Sitotoksik dan Induksi Apoptosis pada Sel T47D oleh Ekstrak Turbinaria decurrens

Muhammad Nursid*, Nurrahmi Dewi Fajarningsih, and Ekowati Chasanah[1]

[1] Research and Development Center for Marine and Fisheries Product Processing and Biotechnology, Jl. K.S. Tubun Petamburan VI, Jakarta Pusat 10260, Indonesia
*Correspondence Author: muhammadnursid@gmail.com

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ABSTRACT

Marine algae is known to contain a wide variety of biomedical compounds having pharmaceutical applications. The aim of this research was to evaluate cytotoxic activity and apoptosis induction of Turbinaria decurrens extract on T47D cell lines. Cytotoxic activity test was conducted by using MTT assay whereas detection of apoptosis was evaluated by DNA fragmentations and flow cytometry analysis. The MTT test showed that crude extract had medium cytotoxic activity to T47D, HepG2, and C28 cell lines with IC_{50} value of 172, againts 360 and 330 µg ml^{-1}, respectively. After solvent partition of crude extract, the cytotoxic activity of n-hexane and ethyl acetate fractions T47D cell increased, the cytotoxic activity of n. hexane and ethyl acetate fractions T47D cell increased with IC_{50} value of with IC_{50} 43.1 and 51.9 µg ml^{-1}, respectively, whereas IC_{50} value of methanol fraction was 383.0 µg ml^{-1}. Analysis of DNA fragmentation of T47D cell showed that both n-hexane and ethyl acetate fractions could not fragment DNA as a features of apoptosis. However, flow cytometry analysis by using annexin-V and propidium iodide staining revealed that n-hexane and ethyl acetate fractions could induce apoptosis in T47D cell. This research indicated that *Turbinaria decurrens* had potency to induce apoptosis in T47D cells.

Keywords: marine alga, *Turbinaria decurrens*, cytotoxic activity, apoptosis induction

ABSTRAK

Rumput laut diketahui mengandung senyawa biomedis yang memiliki potensi untuk diaplikasikan dalam bidang farmasi. Penelitian ini bertujuan untuk mengetahui aktivitas sitotoksik dan induksi apoptosis ekstrak rumput laut *Turbinaria decurrens* pada sel lestari T47D . Aktivitas sitotoksik diuji dengan metode MTT, sedangkan apoptosis diuji dengan uji fragmentasi DNA dan analisis flow cytometry. Hasil uji MTT memperlihatkan bahwa ekstrak kasar memiliki aktivitas sitotoksik sedang terhadap sel T47D, HepG2, dan C28 dengan nilai IC_{50} berturut-turut sebesar 172, 360 and 330 µg ml^{-1}. Aktivitas sitotoksik terhadap sel T47D meningkat setelah dilakukan partisi ekstrak kasar dengan nilai IC_{50} fraksi *n*-heksana dan etil asetat masing-masing sebesar 43,1 dan 51,9 µg ml^{-1} sedangkan fraksi metanol memiliki nilai IC_{50} sebesar 383,0 µg ml^{-1}. Analisis fragmentasi DNA sel T47D memperlihatkan bahwa fraksi n-heksana dan etil asetat tidak begitu jelas memperlihatkan terjadinya fragmentasi DNA, tetapi analisis *flow cytometry* dengan menggunakan annexin-V dan propidium iodida memperlihatkan bahwa kedua fraksi tersebut dapat menginduksi apoptosis pada sel T47D. Hasil penelitian ini memperlihatkan bahwa alga coklat *T.decurrens* memiliki potensi sebagai penginduksi apoptosis pada sel T47D.

Kata Kunci: rumput laut, *Turbinaria decurrens*, aktivitas sitotoksik, induksi apoptosis

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INTRODUCTION

Breast cancer is the third most tumor in the world. Around 1.15 million cases that was recorded in 2002, accounting for around 23 percent of all cancer in women (WCRF/AICR, 2007). In this case, novel breast anticancer drugs with new mechanism of action are essential for future chemotherapeutic treatment. Natural products have historically played an important role in the development of anticancer drugs and have potential to conduct research and development also in the future (Felt, 2011).

Marine algae are known to contain a wide variety of bioactive compounds, many of which have

commercial applications in pharmaceutical, medical, cosmetic, nutraceutical, food and agricultural industries (Kelman et al., 2012). Brown algae is one of seaweed recognized as a rich sources of biopharmaceutical compound especially fucoxanthin. Fucoxanthin is a carotenoid commonly distributed in brown alga (*Turbinaria, Padina, Sargassum*, etc.). It is confirmed to have an anticancer effect as well as antioxidant activity in some experiment tests. Fucoxanthin can be utilized as an excellent supplement like astaxanthin since it acts as an antioxidant and inhibits GOTO cells of neuroblastoma and colon cancer cells. Recently, the apoptosis activity against HL-60 and Caco-2 cells has been reported for fucoxanthin (Mori et al., 2004). Apoptosis induction in HeLa cell lines by *n*-hexane extract from *Turbinaria decurrens* had been reported previously (Nursid et al., 2007).

Apoptosis, or programmed cell death, is a major control mechanism by which cells die if DNA damage is not repaired. Apoptosis is also important in controlling cell number and proliferation as a part of normal development (Ghobrial et al., 2005). The process of apoptosis is morphologically distinct from necrosis in many of its characteristics such as cell shrinkage, cytoplasmic condensation, DNA fragmentation, chromatin condensation, nuclear fragmentation, cytoplasmic membrane blebbing and formation of apoptotic bodies (Rastogi et al., 2009). Many studies have indicated that an imbalance cell death and cell proliferation may result in tumor formation. In addition, the killing of tumor cells by diverse cytotoxic approaches such as anticancer drugs, gamma irradiation, or immunotherapy, is predominantly mediated through the induction of apoptosis. Apoptosis therapy has attracted many group of investigator to develop the first generation of apoptotic anticancer meditation (Matsushita et al., 2005).

The objectives of study were to evaluate cytotoxic activity (IC_{50}) and apoptosis induction of *Turbinaria decurrens* extract.

MATERIAL AND METHOD

Seaweed Sampling and Extract Preparation

Seaweeds *Turbinaria decurrens* (1 kg wet weight) was collected from the Binuangeun Beach, Lebak District, Banten, Indonesia in April 2012. After rinsed clean water, active compound were extracted from fresh seaweeds with methanol and concentrated in vacuum evaporator. Aqueous suspension of crude extract fractionated (100 ml) was extracted with *n*-hexane and ethyl acetate (each 100 ml x 3) respectively and both evaporated in vacuum to get *n*-hexane and ethyl acetate fractions. Methanol-aqueous residue was evaporated to get methanol fractions.

Cytotoxicity Test

T47D (breast cancer), HepG2 (liver cancer), and C28 (colon cancer), were cultured in RPMI 1640 medium (sigma), supplemented with 10 % fetal bovine serum, 1% fungizone and 2% penicillin-streptomycin. The cells were maintained at 37°C in a moisture-saturated atmosphere containing 5% CO_2. All of the cells were seeded at density of $2x10^4$ cells $well^{-1}$ in 200 µL of medium and allowed to attach overnight. After the cells were grown to about 80% confluence, treatments were initiated by supplementing to get 5, 10, 20, 50, and 100 µg ml^{-1} of final media concentration of compound. All treatments were conducted in three replicates. Cytotoxicity test was performed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma) assay according to Ebada et al. (2008). The MTT assay is a colorimetric assay based on the cleveage of yellow water-soluble tetrazolium salt, MTT, to form water-insoluble, dark blue formazan cristals. MTT cleveage occurs only in living cells by mitocrondrial enzyme succinate dehydrogenase. The cleavage product of formazan was measured spectrophotometrically at 570 nm using microplate reader (Dynex revelation). The inhibition percentage of cells growth were calculated with formula: (A-D) – (B-C)/(A-D) x 100 %, where A = control cell absorbance, B = compounds absorbance, C = controls compound absorbance, and D = control media absorbance. The inhibition concentration 50 (IC_{50}) value is defined as the concentrations of compound which inhibited 50% of the cell growth. IC_{50} value was determined by using Minitab probit analysis version 16.0.

Detection of Apoptosis Induction

Analysis of DNA Fragmentation: DNA fragmentation was detected by using Apoptotic DNA Ladder Kit (Roche). T47D cell was seeded at a final density of 700.000 cells/well in 6-well microculture and incubated for 12 h in CO_2 incubator (37°C, 5% of CO_2 flow). The extracts were added to cells at 40 and 50 µg ml^{-1} for 24 h. At the end of the incubation period, supernatant was collected and centrifuged at 200 g rpm for 5 min to collect detached cells. The remaining cells were detached using 0.025% trypsin EDTA solution for approximately 5 min and centrifuged at 200 g rpm. Pellet cells were washed twice with PBS. DNA samples obtained were analyzed by 1% agarose gel electrophoresis following the kit. After electrophoresis, the gel was stained with SYBER Green I Nucleic Acid Stain (Invitrogen) and visualized

as a DNA ladder with UV. Lyophilized apoptotic U937 cells was used as a positive control.

Detection using Flowcytometric Analysis: Discrimination of apoptosis and necrosis cell was conducted using Annexin-V-FLUOS staining kit (Roche) according to Elmore (2007). Briefly, the T47D cell was seeded at a final density of $7x100^5$ cells $well^{-1}$ in 6-well microculture and incubated for 12 h in CO_2 incubator (37°C, 5% of CO_2 flow). The extract were added to cells at 40 and 50 μg ml^{-1} for 24 h. The cells treated with the extract were collected by using the same method with analysis of DNA fragmentation. Finally, the cells were resuspended in 100 μl of Annexin-V-FLUOS staining kit (Roche) then incubated in dark room for 10 min at 20-25°C. Typical histogram of apoptotic and necrotic cells was performed using FACSCalibur (Becton-Dickinson) flow cytometer. Doxorubicin was used as a positive control.

RESULT AND DISCUSSION

Cytotoxicity Test

The MTT test was used for the evaluation of cytotoxic properties of the *T.decurrens* extract. Mortality of T47D, HepG2, and C28 cells after treated with crude extract were shown in Figure 1A. After 24 h incubation, the highest mortality caused by crude extract treatment was T47D cell followed by HepG2 and C28 cells. Probit analysis showed that crude extract had medium cytotoxic activity to T47D, HepG2, and C28 with IC_{50} values of 172, 360 and 330 μg ml^{-1}, respectively. For further study, T47D cells were used because it showed the most sensitive to crude extract. After solvent partition of crude extract, the cytotoxic activity increased (Figure 1B) with IC_{50} of *n*-hexane, ethyl acetate and methanol fractions were 43.1, 51.9 and 383.0 μg ml^{-1}, respectively.

Bioactivity of brown alga has been widely reported, but the bioactivity of these algae from Indonesian is still rarely published. Cytotoxic activity and apoptosis induction of T47D cells by *T.decurrens* collected from Indonesia was the first time reported. Nursid *et al.* (2007) reported that non polar fractions of *T. decurrens* had cytotoxic activity to HeLa cell with IC_{50} value of 15.1 μg ml^{-1} as well as induced apoptosis indicated by DNA fragmentation. This result indicated that *T. decurrens* was potential as sources of nutraceutical compound. This research suggested to use *T.decurrens* as nutraceutical resources since this species is abundant along the southern Java coast.

Detection of Apoptosis Induction

Analysis of DNA Fragmentation

The T47D cells were exposed to *n*-hexane and ethyl acetate *T. decurrens* extracts for 24 h and then the DNA was extracted. DNA agarose gel electrophoresis was performed and a typical DNA ladder pattern of apoptosis was observed. The gel showed that both *n*-hexane and ethyl acetate fractions could not fragment DNA as a features of apoptosis (Figure 2). In contrast, a clear DNA ladder was visible in the lyophilized apoptotic U937 cells as a positive control.

The formation of distinct DNA fragments is a biochemical hallmark of apoptosis, with

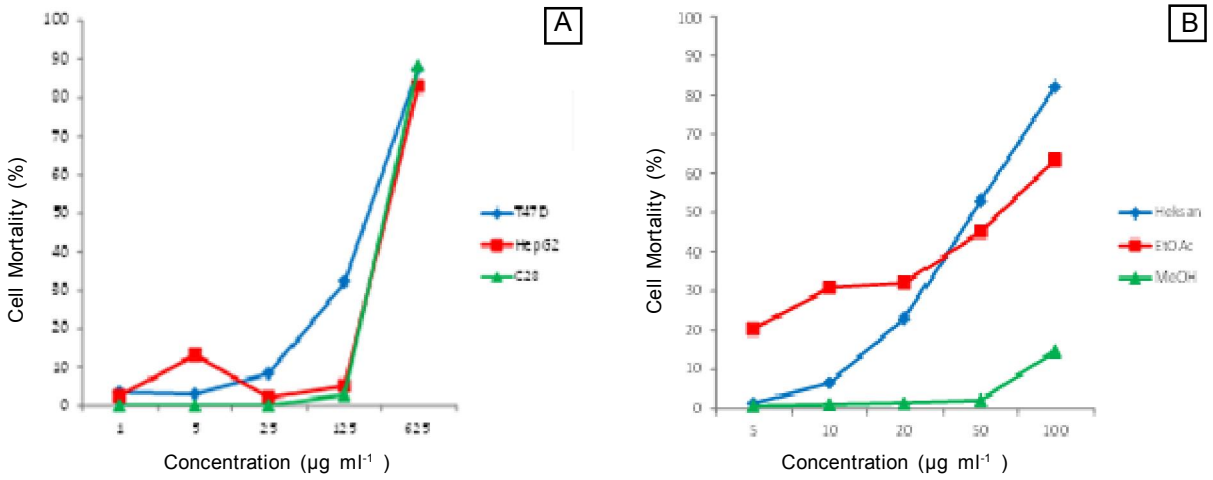

Figure 1. Mortality of T47D, HepG2 and C28 cells after treatment with crude extract of *T.decurrens* (A) and mortality of T47D cells treated with *n*-hexane, ethyl acetate and methanol fractions of *T.decurrens* (B).

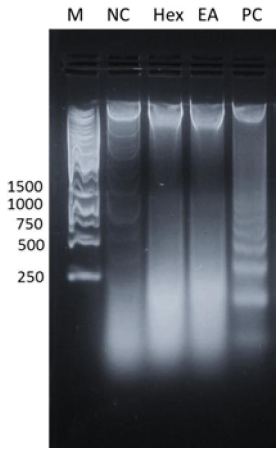

Figure 2. Analysis of DNA fragmentation in T47D cells treated with n-hexane and ethyl acetate extract of *T.decurrens*. Note: (M), DNA marker; (NC), negative control/untreated cells; (Hex), *n*-hexane fraction; (EA), ethyl acetate fraction; (PC), positive control/ lyophilized apoptotic U937.

internucleosomal DNA cleavage activity as a major characteristic. In many systems, this DNA fragmentation was from activation of an endogenous Ca^{2+} and Mg^{2+} dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA fragments. These DNA fragments reveal, upon agarose gel electrophoresis, a distinctive ladder pattern consisting of multiples of an approximately 180 bp subunit (Rannali et al., 2003; Yu et al., 2005).

Flow Cytometry Analysis

Because the induction of apoptosis by *T. decurrens* fractions of T47D cells was not observed by analysis of DNA fragmentation, other analysis were used to determine whether the growth inhibition of T47D cells by *n*-hexane and ethyl acetate fraction was associated with the induction of apoptosis. In this research flow cytometry analysis was performed by using annexin V-propidium iodide staining. According to this method, T47D cells were treated by *n*-hexane and ethyl acetate fractions for 24 hours. Doxorubicin was used to induce apoptosis in T47D cells as a positive control. Based on the flow cytometry analysis, *T. decurrens* extract could induce apoptosis in T47D cells, but the induction of apoptosis of these fractions was not as strong as that of doxorubicin. Percentage of apoptotic cells treated with *n*-hexane and ethyl acetate fractions were 16.0% and 14.9%, respectively whereas that treated with doxorubicin was 74.8% (Figure 3).

When a cell undergoes apoptosis, changes occur at the cell surface. One of plasma membrane alteration is the translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cells. PS exposure therefore represent a useful assay for the apoptosis. PS present on the outer layer can be detected using Annexin V (Elmore 2007). Annexin V is Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS. This protein can hence be used as a sensitive probe for PS exposure upon the outer layer of the cell membrane and is, therefore, suited to detect apoptosis cells. Necrotic cells also expose PS, and will therefore also bind Annexin V. To differentiate between apoptotic and necrotic cells, PI is often used in conjunction with Annexin V. PI will mark necrotic cells, but not apoptotic cells. In this assay, Annexin V binds the phospholipid PS, marking apoptotic and necrotic cells, while PI bind DNA, marking only necrotic cells (Ranalli et al., 2003).

Anticancer properties of brown alga including *T.decurrens* most likely related to its fucoxanthin content. Fucoxanthin is one of the most abundant carotenoids, and contributes more than 10% of the estimated total production of carotenoids in nature, especially in the marine environment. Fucoxanthin is a xanthophyll with formula of $C_{42}H_{58}O_6$. It is an orange-colored pigment, along with chlorophylls *a* and *c* and b-carotene, present in *Chromophyta* (*Heterokontophyta* or *Ochrophyta*), including brown seaweeds (*Phaeophyceae*) and diatoms (*Bacillariophyta*). Fucoxanthin that was firstly isolated from the marine brown seaweeds *Fucus*, *Dictyota*, and *Laminaria* (Peng et al., 2011) have the allene structure and epoxide and hydroxyl groups. As a type of carotenoid, fucoxanthin has been attracting people's attention for its effect to prevent cancer. Fucoxanthin has been reported to inhibit tumor cells mediated by

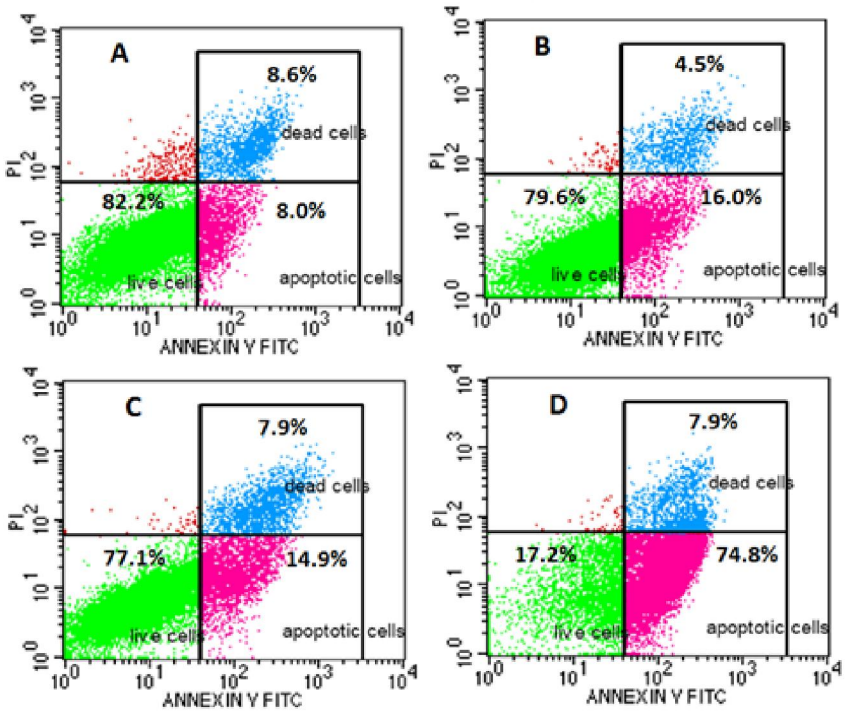

Figure 3. Flow cytometry analysis of T47D cells treated with n-hexane and ethyl acetate fractions. A, cell control (without treatment); B, cell treated with *n*-hexane fraction (40 μg ml^{-1}); C, cell treated with ethyl acetate fractions (50 μg ml^{-1}); D, cell treated with doxorubicin (5 μg ml^{-1})

apoptosis induction. Fucoxanthin reduced the viability of HepG2 cells accompanied with the induction of cell cycle arrest during G_0/G_1 phase (Das et al., 2008). The apoptosis-inducing effect of fucoxanthin on human promyelocytic leukemia HL-60 cell line has been investigated by Hosokawa et al. (1999), who found that fucoxanthin exhibited strong antiproliferative activity and could induce apoptosis of HL-60 cells. In HL-60 cells, fucoxanthin caused cleavages of procaspase-3 and poly-ADP-ribose polymerase, and apoptosis induction by fucoxanthin was mediated through mitochondrial membrane permeabilization and caspase-9 and caspase-3 activation. Kim et al. (2010) showed that fucoxanthin induced reactive oxygen species generation, inactivated the Bcl-xL signaling pathway, induced caspase-3, -7, and poly-ADP-ribose polymerase cleavage, and thus triggered the apoptosis of HL-60 cells, indicating that the generation of reactive oxygen species was a critical target in fucoxanthin-induced apoptosis in HL-60 cells.

Steroid compound, fucosterol, also have been isolated from the genus *Turbinaria*. This compound have been reported to possess cytotoxic properties against various cancer cell lines (Sheu et al., 1999) as well as showed antiviral, antibacterial and antifungal activity. Fucosterol also inhibited histamine and acetylcholine (Kumar et al., 2009; Shanmugam et al., 2010).

The MTT assay showed that extract of *T.decurrens* had a medium cytotoxic activity. In this research, the ability of *n*-hexane and ethyl acetate fractions to induce apoptosis in T47D cells was assayed. The DNA electrophoresis showed that both n-hexane and ethyl acetate fractions could not fragment DNA. In apoptosis assay using DNA fragmentations test, cleavage of DNA depends on the concentrations of the extract tested. In our experiment the concentrations of extract was might be too low so this concentrations was not enough to induce the apoptosis event. Also, it was possible that the time of exposed extract to T47D cell was not enough to induce apoptosis, may be the cell undergoing apoptosis after 48 hours incubation. In contrast, a clear DNA ladder was visible in the lyophilized apoptotic U937 cells as a positive control. The double staining by annexin V-propidium iodide analysis showed that n-hexane and ethyl acetate fractions could induce apoptosis in T47D cells (16.0% and 14.9%, respectively, Figure 3). It was suggested that killing T47D cells by *T.decurrens* extract was mediated by apoptosis. Doxorubicin as a positive control induced apoptosis in T47D cells (74.8%). Doxorubicin (14-hidroxydaunorubicin) is an antracyclic antibiotic drug widely used in the treatment of a variety of cancers. Doxorubicin has multiple mechanisms of action, including its interaction with the enzyme topoisomerase II, metal ion chelation and free radical

generation. More recently doxorubicin was found to reduce the viability of cancer cells via RNA damage (Brilhante et al. 2011).

Based on the cytotoxicity and apoptosis assay, *T.decurrens* is suspected to have a potency to induce apoptosis in T47D cells. These anticancer properties may be related to fucoxanthin content although this hypothesis need to be further investigated.

CONCLUSSION

Crude extract of *T. decurrens* had medium cytotoxic activity against T47D, HepG2, and C28 cell lines. The cytotoxic activity increased after solvent partition of the crude extract. Analysis of DNA fragmentation of T47D cell showed that both *n*-hexane and ethyl acetate fractions couldn't fragmented DNA as a features of apoptosis. However, flow cytometry analysis by using annexin and propidium iodide staining revealed that *n*-hexane and ethyl acetate fractions could induce apoptosis in T47D cell. This research indicated that *T. decurrens* had potency to induce apoptosis in T47D cells

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