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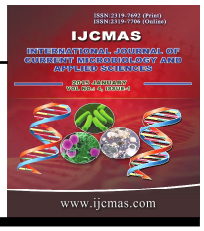

Original Research Article

Cellular mechanism of anti-cancerous activity in active marine sponge *Cinachyrella anomala* against T47D cell

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Keywords

Cinachyrella anomala, cytotoxic, apoptosis, T47D cell

A B S T R A C T

1,4,9-triazatricyclo[7,3,1,0]trideca-3,5(13),10-trien-8-ol compound was isolated from marine sponge *C.anomala*. Cellular mechanism against T47D cells by means of cytotoxic activity was IC_{50} 123.18 µg/mL; cellular proliferation inhibition was 48 hours of incubation, apoptosis induction was 11.77% and sub-G_1 phase of cell-cycle arrest was 5.87% and G_2/M phase of 50.5%.

Introduction

Cancer is a cellular malignancy characterized by loss of control function over the cell cycle regulation and homeostasis in multi-cellular organisms. As a result, the cells keep proliferating, thus leading to abnormal tissue growth. Cancer is the fourth leading causes of death after stroke, hypertension and diabetes (Ullah & Aatif, 2009).

Many efforts had been made to overcome cancer. At the beginning of the 20th century, cancer treatment was conducted using radiotherapy and surgical intervention. The success rate of the methods was no more than 30%; therefore, additional therapy or combined therapy in terms of chemotherapy and chemo-preventive intervention (DeVita & Chu, 2008). Chemotherapy and chemo-preventive intervention plays an important role in cancer treatment. In the United States and Europe, about 65% of commercially available medicine for cancer was made from natural ingredients (Wei *et al*., 2007). Anti-cancer drugs from natural ingredients such as plants, microbes, and marine organisms were more than 60%. Derivative compounds from natural ingredients had a

specific bioactive target and low side effects (Iwamaru *et al*., 2007).

Phylogenically, sponge is the most primitive multi-cellular invertebrate, living more than 635 years ago during the Precambion period (Hochmuth *et al*., 2010). Sponge is sessile animal; the filter feeder has a physiological strategy, reproduction and effective defensive mechanisms against bacterial infection, fungi, viruses, and predators (Brackman & Daloze, 1986), spatial competition with other organisms (Schupp *et al*., 2009), and defensive against marine pathogens (Muller *et al*., 2004). Sponges that produce varied types of secondary metabolite (Bell, 2008) have pharmacological potentials (Faulkner, 2001).

Cinachyrella anomala belongs to Demospongiae class, Spirophorida orde, and Tetillidae family (Vacelet *et al*., 2007). This sponge is dominant at intertidal area of Kukup beach, Gunung Kidul, DIY. The sponge *Tetilla* sp. of Tetillidae family has an antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* (Perkasa & Budiyanto, 2010). Ethanolic fraction of marine sponge *C. anomala* shows an arrest condition of G_0-G_1 phase in WiDr cell (Nurhayati *et al*., 2011). Arrest condition in G_0-G_1 phase is the first phase in the cell cycle. It is a condition similar to normal cells that undergo *senescent* (resting period). This needs further investigation since many anti-cancer drugs are cytotoxic and inhibitory against cellular growth in M (mitosis) phase. The study aims at finding out the anti-cancer cellular mechanisms of active compounds isolated from *C. anomala*.

Materials and Method

Sponge *C. Anomala* was collected from Kukup Beach, Kemadang Village, Tanjungsari Sub-district, Gunung Kidul Regency, DIY at intertidal area by means of direct collection (Wright, 1998). Sponge samples were put into plastic bag and stored in an icebox under a temperature of 5°C until the extraction day. Alkaloid compound of *C.anomala* that had been isolated and identified is a group of *cinachyramine* derivative group, with a molecular formula of $C_{10}H_{13}N_3O$ and structural name of ,4,9-*triazatricyclo*[7,3,1,0]*trideca*-3,5(13),10-*trien*-8-*ol* (Nurhayati *et al.*, 2014). The compound underwent cytotoxic assay, apoptosis and cell cycle against T47D cell.

Cytotixic Test

Cell suspension of 100 µL with a density of 2 x 10^4 cell/100 µL media was distributed into 96-well plate and incubated for 24 hours. After the incubation, 100 µL of active compound of *C.anomala* in different concentrations was put into the well. For positive control, 100 µL of doxorubicin was used, and 100 µL of culture medium was used as cell control. The cells were incubated for 24 hours in the incubator with 5% CO_2 and 95% O_2 flows. At the end of incubation, culture media were removed and added with 110 µL of MTT solution (5 mg/mL PBS) and incubated for 3-4 hours. MTT reaction was stopped by adding stopper reagent SDS (100 µL). Cell containing micro-plate was sealed for ± 5 minutes, then wrapped with aluminum foil and incubated overnight under room temperature. The results of assay were read using ELISA reader at a wavelength of 595 nm. Percent cell death was calculated based on the formula [{(A-D)-(B-C)}/(A-D)] X 100 %, in which A = control absorbance, b=extract absorbance, C=extract control absorbance, and D=media control absorbance. The value of *Inhibitor Concentration* 50 (IC_{50}) was determined

using probit analysis using SPSS 13 statistic application.

Apoptosis Test Using *Flow cytometry* Method

Apoptosis test was conducted in flow cytometry method and double staining Annexin-V-FLUOS staining kit. T47D cell was treated using active compound of *C.anomala* and doxorubicin for 24 hours. The cells were harvested using 0,25% trypsin and washed with PBS. The cells were treated using 100 µL of Annexin-V-FLUOUS staining kit, and incubated under a dark room for 10 minutes under temperatures of 25^0C-27^0C. Histogram of apoptotic and necrotic cells was analyzed using flocytometer *FACSCalybur* (Becton-Dickinson).

Cell Proliferation Kinetic Assay (*Doubling Time* test)

The method used for proliferative kinetic assay include cytotoxicity test. Three variations of dosages used included LC_{50} dosage, and 2 dosages below LC_{50}; cytotoxicity and incubation assays were conducted in time interval of 24, 48 and 72 hours.

Cell cycle analysis using *Flow Cytometry* method

Cell cycle analysis was conducted using *flow cytometry* method (Vermes *et al*., 2000); 10^6 cells/well were distributed into the 6-well plate. The cells were treated with active compound of *C.anomala* and incubated for 24 hours, then harvested using trypsin-EDTA. The harvests were centrifuged (2000 rpm, 30 seconds). The cell suspension was homogenated and transferred into *flowcyto-tube*. *Flowcytometry* data were analyzed using Modfit LT 3,0 to see the cell distribution in each phase of G1, S and G2/M.

Result and Discussion

Cytotoxic activity test showed increasing death of T47D cells, along with the increase of compound dosage (Figure 1).

One-way Duncan statistic analysis using active compound of *C. anomala* showed a significant increased at dosage range of 62.5 - 1000 µg/mL. Doxorubicin was found to be significant at a dosage of 25; 50 and 100 µg/mL (Table 1).

The results of probit analysis are presented in Table 2. Proliferative kinetic assay of active compound *C. anomala* at dosages of 125 and 31.25 µg/mL and 48 and 72 hours of incubation significantly influenced proliferation of T47D cell (Figure 2).

Doubling time data were further transformed into the profile of relationship between percent alive cells and incubation time. The equation slope shows proliferative kinetics of T47D cells (Table 3). The decreasing slope resulted in longer doubling time. In other words, there was an increasing proliferative inhibition. The lowest slope during the treatment with *C. anomala* active compound occurred at a dosage of 12.5 µg/mL (Table 3).

The result of apoptosis assay using *Annexin-PI* method for *C. anomala* active compound at a dosage of 12.25 µg/mL showed early apoptosis (1.54%) and end apoptosis (1.94%), while the dosage of 31.25 µg/mL resulted in early apoptosis (11.77%) and end apoptosis (4.84%) (Table 3).

Analysis of using flow cytometry method showed inhibitory activity against the active compound of T47D cell in different phases.

A dosage of 31.25 µg/mL was capable of inducting cell cycle inhibition (*cell-cycle arrest*) at sub-G_1 phase (5.87%) and G_2/M phase (50.50%). Combined dosage between 12.25 µg/mL of active isolate and 5 µg/mL of doxorubicin showed cell cycle inhibitory induction at sub-G_1 phase (5.75%) and G_2/M phase (36.89%) (Table 4).

Induction of T47D cell apoptosis was assumed to happen through intrinsic pathway. This s because 1,4,9-*triazatricyclo*[7,3,1,0] *trideca*-3,5(13),10-*trien*-8-*ol* compound lead to the damage of T47D cell DNA. Cells with damaged DNA could induce p53 gene transcription (King, 2000). Furthermore, transcription of p53 gene would stimulate pro-apoptosis protein expression, which is an important regulator of apoptosis in the intrinsic pathway (Van der Heiden *et al*., 1997). Damage of DNA would lead Bax to penetrate the mitochondrial membrane and induce cytocrome c expression. Cytochrome c and Apaf-1 could activate caspase-9 and further activate caspase-3, which is an important chain reaction (cascade) and able to degrade actin, lamin, and DNA and finally lead to apoptosis (Ghobriel *et al*., 2005).

Inhibition of 1,4,9-*triazatricyclo* [7,3,1,0] *trideca*-3,5(13), 10-*trien*-8-*ol* against proliferative inhibition and T47D cell cycle occurred at sub-G_1 and G_2/M phases. The compound is assumed to play a role as a cdk2 inhibitor. Cdk2 inhibitor causes phosphorilation of Thr-14 and Tyr-15 and degrades E/Cdk2 dan A/Cdk2 cyclin complex, thus interfering with G_1 to S progress (Morgan *et al*., 1995).

Cells, which were interrupted during the early phases to mid-G_1 phase, would be delayed at *checkpoint* G_1. The *Checkpoint* G_1 depended upon the increase of expression and activation of gene p53 (Kasta *et al*., 1991). Gene p53 has a huge role in maintaining genome stability, since it acts as a conductor (Nylander *et a*l., 2000). Gene p53 drives the expression of downstream effectors, such as p21, gadd45, mdm2 and Bcl-2 *associated* X protein (Bax) (Miyashita & Reed, 1995), to stop the cell cycle and to improve DNA or apoptosis (Malanga & Pleschke, 1998). The response occurred because the effector gene has a specific spot that can identify P53 in the regulator. After induction, P53 would activate transcriptions of a number of genes, including p21 (Zhao *et al*., 2000). Gene p21 is directly connected to the cyclin/cdk complex and acted as Cdk inhibitor. The gene P21 inhibited cyclin A/Cdk2 activities (Harper *et al*., 1993). Over-expression of P21 lead to cell arrest at G_1 phases (Dulic *et al*., 1998).

Table.1 Cytotoxic activity assay of active compound C. anomala against T47D cell

No	Fraction dosage (µg/mL)	Dead cells (%)	Doxo dosage (µg/mL)	Dead cell (%)
		Active isolate F3.2.1		Doxorubicin
1	15.625	1.4a	1.5625	2.1a
2	31.25	6.4a	3.125	5.1a
3	62.5	42.9b	6.25	0.5a
4	125	43.7c	12.5	97.3a
5	250	64.0c	25	99.0b
6	500	95.0d	50	98.0b
7	1000	98.4d	100	99.7b
8	IC_{50} value	123,178	Dosage of Doxo (µg/mL)	Dead cells (%)

Description: subscript a,b,c,d connotation shows significant values (one-way Duncan analysis).

Table.2 Cytotoxic activity assay of active compound C. anomala against T47D cells

No	Sample	IC_{50} (µg/mL)
1.	Isolate F3.2. 1	123.178
2.	Doxorubicin	86.660

Table.3 Slope resulting form the doubling time equation for C. anomala active compound against T47D cell

MTT Fractionation	Treatment-Fraction Dosage of *C. anomala* sp. (µg/mL)	Equation	Slope	R^2
Active isolate F3.2.1	125	-1.63 x + 94.84	-1.64	81.6
	31.25	-1.16 x + 90.44	-1.17	0.84
	12.50	-2.68 x + 194.97	-2.68	0.05
	50	7.70 x + 49.44	7.70	-0.99
Doxorubicin	12.25	-1.31 x + 88.06	-1.32	0.71
	5	1.78x + (-105.52)	1.78	0.92

Table.3 Percent apoptosis and necrosis of T47D cell after treatment with active compound of C. anomala and 24 hours of incubation

No	Treatment	% living cells	% early Apoptosis	% End Apoptosis	% dead cells
1	Control, T47D cell	95.12	2.53	1.23	1.12
2	Active isolate 12.25 µg/mL	94.81	1.54	1.94	1.70
3	Active isolate 31.25 µg/mL	76.20	11.77	4.84	7.18
4	Doxorubicin 5 µg/mL	16.50	0.72	6.34	76.44
5	Doxorubicin 12.25µg/mL	2.54	0.00	0.56	96.89
6	Combination (active isolate 12.25 µg/mL and Doxorubicin 5 µg/mL)	17.76	0.01	1.57	80.67

Table.4 Cell cycle assay for active compound of C. anomala against T47D cell

No	Treatment	Sub-G_1 (%)	G_0/G_1 (%)	S (%)	G_2/M (%)
1	T47D cell as control	3.04	43.83	25.60	30.83
2	Active isolate 12.25 µg/mL	3.99	47.51	23.20	29.44
3	Active isolate 31.25 µg/mL	5.87	30.53	18.90	50.50
4	Doxorubicin 5 µg/mL	5.35	44.07	25	30.87
5	Doxorubicin 12.25 µg/mL	6.60	42.71	26.30	30.80
6	Combination of 12.25 of active isolate and 5 µg/mL of Doxorubicin	5.75	43.14	19.90	36.89

Figure.1 Curve of dosage response of active compound C. anomala against T47D cells

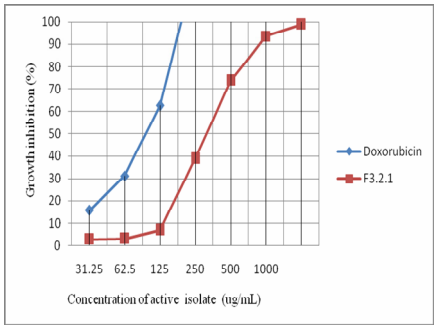
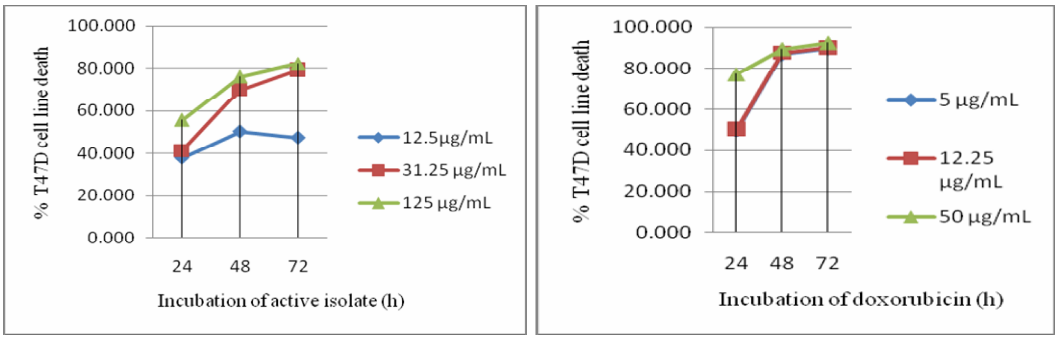


Figure.2 Proliferative kinetic inhibitory assay for the active compound of C. anomala against T47D cell and 24, 48, and 72 hours of incubation. A. Active compounds. B. Doxorubicin



a. b.

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