Article

Apoptosis and Necrosis on T47D Cells Induced by Shiga-Like Toxin from Local Isolates of *Escherichia coli* O157:H7

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Abstract: Apoptosis and cell cycle arrest induction are targeted in the strategy of cancer therapy. Furthermore, bacterial toxin such as shiga-like toxin producing *Escherichia coli* has been suggested to be used as novel therapeutic agent against tumor malignancies either as independent anti-neoplastic agents or in combination treatment with chemo or radiotherapy. The aim of study was to investigate the potency of shiga-like toxin originated from local strains of E. coli O157:H7 as a new cancer therapy. As many as 10 culture cells T47D cell line were subjected by crude extract Shiga like toxin originated from five local isolates of *E. coli* O157:H7 i.e. KL-48(2), SM-25(1), SM-7(1), DS-21(4), and one isolate ATCC 43894 as a control with IC50 doses, respectively. The treatment was observed for 24 h, with two replications. An FITC-Annexin V and PI assay was used to observe apoptosis and necrosis effect, and simultaneously with cell cycle analysis using propidium iodide (PI) staining. Results of study showed T47D cell treated with Shiga-like toxin from local strain KL-48 (2) show the lowest viable cell, followed by SM 7(1), ATCC 43894, SM-25(1), DS-21(4) and contrary with control with each percentages as 15.20, 16.36, 22.17, 22.64, 33.86, and 94.36%, respectively. The results were also confirmed by the induction of the cell cycle arrest in phase G0-G1 as inactive phase, i.e. 66.41, 63.37, 61.52, 55.36 and 47.28% for T47D cell treated with toxin of KL-48(2), ATCC 43894, SM 25(1), SM 7(1), and DS 21(4), respectively. These results show tendency deleterious effect of Shiga-like toxin from local isolates on T47D cell, so that concluded they have potency as a good anticancer drug in Gb3-expressing breast cancer

Keywords: Apoptosis; breast cancer; *E. coli* O157:H7; necrosis; Shiga-like toxin

Key Contribution: Apoptosis and necrosis induced by Shiga-like toxin.

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1. Introduction

Shiga toxin Escherichia coli (STEC) is major public health concerns in developed and developing countries due to the severity of the diseases they cause. The infection by this bacterium may results in bloody diarrhea, and the subsequently of life-threatening sequalae, including acute renal failure and neurological abnormalities [1]. On the other hands, STEC also has been exploited for medical purposes such as cancer therapy or imaging [2]. STEC has multifunctional capable to inactivate multiple cell stress signaling pathways which may result in apoptosis, autophagy or activation of the innate immune respone [3]. Apoptosis may be triggered by activation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38MAPK) [4]

Scientific studies indicate that Shiga toxin (Stx)-induced apoptosis on specific cell as an important process in the pathophysiological response of humans to this bacterial toxin. Apoptosis has been reported in several different cell types as a results of Stx1 and Stx2 action associated with infection by this bacterium [5]. Apoptosis, or programmed cell death, is a multy-step process that is important to eliminate damaged or abnormal cell [6]. Furthermore, apoptosis and cell cycle arrest induction are targeted in the strategy of cancer therapy [7].

Shiga-like toxin produced by Escherichia coli O157:H7 damages cellular nucleic acids by removing a specific adenine from 28S rRNA in ribosome [8]. All STEC contain a pentameric ring of identical B-subunit which each subunit aproximately 7.7 kDa that non-covalently associated with a single A-subunit of approximately 32 kDa [9]. The B subunit of STEC specifically binds to sugar moiety of glycosphingolipid globotriaosylceramide (Gb3) in the plasma membrane of target cells, and mediates uptake and intracellular transport of the toxin, and is then transported to the endoplasmic reticulum following the retrograde route. The A subunit is cleaved in the trans-Golgi network, and the enzymatically active A1 part is translocated from the lumen of the endoplasmic reticulum to the cytosol. The A1 fragment irreversibly modifies ribosomal 28S RNA, leading to the inhibition of biosynthesis and cell death by apoptosis [10].

Shiga-like toxins efective against specific signaling pathways could reduce treatment side-effects to normal tissue and be an approach to generate specific anti tumour agents [8]. Shiga and Shiga-like toxins producing *Escherichia coli* have been suggested to be an anti-cancer due to its low general toxicity and high specificity against tumors expressing its receptor globotriasosylceramide (Gb3) [11]. Moreover, Gb3 has been reported to be incraesed on the surface of several tumour cells lines such as breast cancer [12]. Remarkably, many types of cancer cells overexpress Gb3 on their surface, and therefore, the binding of toxins or the non-toxic pentameric Stx B-subunits coupled to anti-cancer agents [13].

The aim of this research was to evaluate the potential of Shiga-like toxin producing Escherichia coli O157:H7 local isolates as a novel agent for enhancing apoptosis and necrosis in T47D cells. We demonstrate that verotoxin has potency to be use as anticancer drug in Gb3-expressing breast cancer.

2. Results

2.1. Toxicity assay

Results of toxicity assay showed the differences in the viable or deleterious cells of T47D among each treatments and control. The percentage of deleterious cells was calculated as cytophatic effect / CPE based on the OD at 550 nm λ and it is briefly summarized in Table 1.

Table 1.

The inhibitory concentration 50% (IC 50) of Shiga-like toxin on the formation of cytophatic effect (CPE) on T47D cells after 24 h of observation

Shiga-like toxin strains	Source of strains	IC 50 Concentration (μg/μL)
ATCC 43894	Human, America	0.92
KL-48(2)	Human, Indonesia	0.94
SM 25(1)	Cattle feces, Indonesia	1.08
SM 7(1)	Cattle feces, Indonesia	1.03
DS 21(4)	Beef, Indonesia	1.03

Note: IC50 (Inhibitory Concentration 50%) is the concentration of drug that is require for 50% inhibition *in vitro*

The results of toxicity test (Table 1) appears Shiga-like toxin producing *E. coli* O157:H7 strain KL-48(2) from human origin having IC 50 values almost equally with control isolate ATCC 43894, and slight different from others. The concentration of toxin that is require for 50% inhibition *in vitro* of ATCC 43894 is 0.92 ug / mL and KL-48(2) is 0.94 μg / mL, On the other hand, the toxin of SM-25(1), SM-7(1), and DS 21(4) require higher concentration to inhibit 50% of cells i.e. 1.08; 1.03; and 1.03 ug / mL, respectively.

2.2 Cell apoptosis assay

Detection of toxicity effects (apoptosis or necrosis) caused by each isolates of *E. coli* O157:H7 on T47D cell was analyzed by using FITC-Annexin V and PI methode (Fig.1). Annexin V binding was assessed using bivariate FCM, and cell staining was evaluated with fluorescin isothiocyanate (FITC)-labelled Annexin V (green fluorescence), simultaneously with dye exclussion of propidium iodide (PI) (negative for red fluorescence). The test described, descriminates intact cell ($FITC^-/PI^-$), apoptotic cells (($FITC^+/PI^-$) and necrotic cell ($FITC^+/PI^+$) [14].

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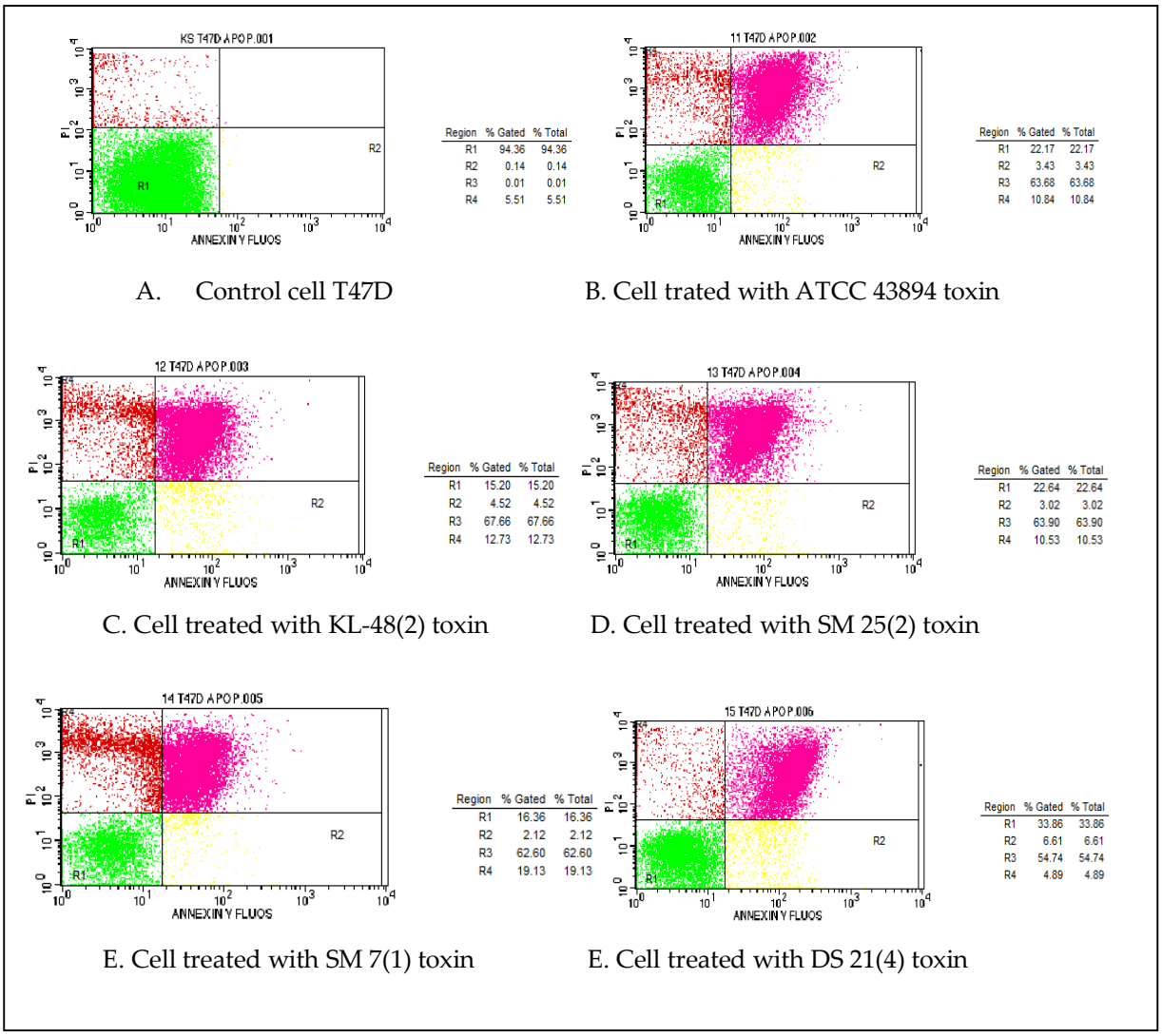

A. Control cell T47D

B. Cell trated with ATCC 43894 toxin

C. Cell treated with KL-48(2) toxin

D. Cell treated with SM 25(2) toxin

E. Cell treated with SM 7(1) toxin

E. Cell treated with DS 21(4) toxin

Figure 1. Contour diagram of FITC-Annexin V/PI flow cytometry of T47D cell line with and without treatment of Shiga-like toxin. The lower left quadrants of each panels show the viable cells, which exclude PI and are negative for FITC-Annexin V binding. The upper right quadrants (R1) contain the non-viable, necrotic cells, positive for FITC-Annexin V binding and for PI uptake. The lower right quadrants (R2) represent the apoptotic cells, FITC-Annexin V positive and PI negative, demonstrating cytoplasmic membrane integrity.

The results of T47D cells treated with Shiga-like toxin isolated from various strain (Figure 1) showed various visible cell, apoptosis, late apoptosis, and necrosis that are summary on Table 2.

Table 2.

Percentages of T47D cells with apoptosis, late apoptosis, and necrosis as well as viable cells after 24 h treated Shiga-like toxin with inhibitory concentration 50 (IC 50).

	Percentages of T47D cell after treatment			
Treatment	Viable cell	Apoptosis	Late apoptosis	Necrosis
T47D control cell (without toxin)	94.36	0.14	0.01	5.51
T47D cell + ATCC 4389 toxin	22.17	3.43	63.68	10.84
T47D cell + KL-48(2) toxin	15.20	4.52	67.66	12.73
T47D cell + SM 25(1) toxin	22.64	3.02	63.90	10.53
T47D cell + SM 7(1) toxin	16.36	2.12	62.60	19.13
T47D cell + DS 21(4) toxin	33.86	6.61	54.74	4.89

T47D cells treated with Shiga-like toxin isolated from local strain show various percentages effect on T47D cell cycle after 24 h incubation (Table 2). The result contrary with T47D cell control. T47D cell treated with Shiga-like toxin KL-48(2) show the lowest percentage of viable cells, followed by SM 7(1), ATCC 43894, SM-25(1), DS-21(4) and control with each percentages as 15.20, 16.36, 22.17, 22.64, 33.86, and 94.36%. These results show tendency deleterious effect of Shiga-like toxin treatment on T47D cell. The equal effect of Shiga-like toxin on breast cancer tissue has been report by previous study [15,16].

2.3. Cell cycle analysis

Flow cytometry as a method for differentiating of DNA content in various of cell cycle phases. The ploidy of cells in G1, S, G2 and M is 2N, 2-4N, 4N and 4N, respectively. However, G0 and G1 phase, G2 and M phase, which both have an identical DNA content, could not be discriminated based on their differences in DNA content. The result of study which analyzed of T47D cell cycle with and without treatment of Shiga-like toxin is presented in Figure 2, and the various cycle cell phase i.e. G0-G1, S, and G2-M phases with each percentages that are summary on Table 3.

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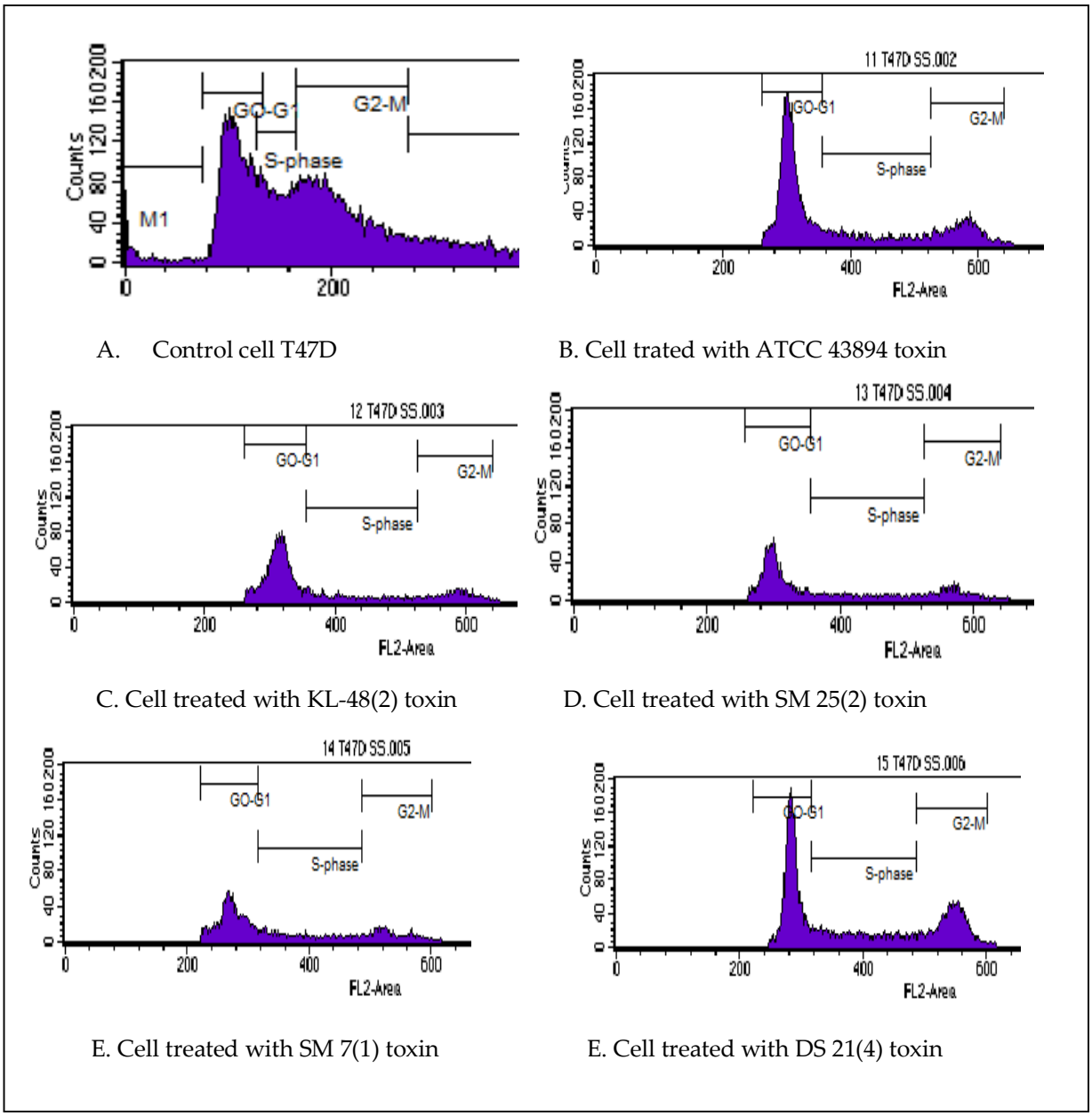

A. Control cell T47D

B. Cell trated with ATCC 43894 toxin

C. Cell treated with KL-48(2) toxin

D. Cell treated with SM 25(2) toxin

E. Cell treated with SM 7(1) toxin

E. Cell treated with DS 21(4) toxin

Figure 2 Contour diagram of T47D cell cycle after 24 h treated with and without Shiga-like toxin.

Table 3.

Percentages of T47D cells with each cell cycles arrest after 24 h treated /without Shiga-like toxin with inhibitory concentration 50 (IC 50).

	Percentages of T47D cell cycle after treatments		
Isolate	GO-G1	S-phase	G2-M
T47D control cell (without toxin)	40.69	22.56	42.30
T47D cell + ATCC 4389 toxin	63.37	17.58	19.06
T47D cell + KL-48(2) toxin	66.41	17.10	16.61
T47D cell + SM 25(1) toxin	61.52	20.14	18.35
T47D cell + SM 7(1) toxin	55.36	25.33	19.25
T47D cell + DS 21(4) toxin	47.28	22.95	29.71

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The treatment of T47D cell with different Shiga-like toxin (Table 3) shows various effect on the cell cycle arrest. The cell treated with KL-48(2) toxin show the highest effect on the cell cycle arrest in G0-G1 phase compare with othrers. The G0-G1 phase is known as a resting phase or the cell has left the cycle and has stopped deviding. The percentage of cells in G0-G1 phase are 66.41, 63.37, 61.52, 55.36 and 47.28% for T47D cell treated with toxin of KL-48(2), ATCC 43894, SM 25(1), SM 7(1), and DS 21(4), respectively. The results are different with T47D cell control without toxin treatment which show the highest percentage on G2-M phase as active phase for preparation of cell to divide or mitosis.

3. Discussion

Results of study indicated the treatment of Shiga-like toxin originated from local strain of *E. coli* O157:H7 effective to decrease of T47D viable cell compare with control. Aplication of Shiga-like toxin proven to trigger T47D cell entering apoptosis, late apoptosis, and necrosis stages. This study reinforce the statement of previous study [15] which states the bacterial toxins such as verotoxin is known to be used as a therapeutic agent against malignant tumors, either used alone or in combination with medication chemo or radiotherapy including the breast cancer cells. The study also evident apoptosis was induced rapidly (60%) in HeLa cells after exposure to Shiga toxin within 4 hours [5]. Therapy cancer with toxins are known to be very effective and can reduce the side effects on normal tissue because the mechanism of action through a very specific signaling pathways [8].

All members of the Stx family are composed of 1A and 5B subunit protein. Each B subunit (StxB) binds with high affinity to the glycosphingolipid globotriaosylceramide, Gb3 (CD77) present on select eukaryotic cell [17,18]. The A subunit is an N-glycosidase that removes adenine 4342 of 28S RNA of the 60S ribosomal subunit [8], rendering ribosomes inactive for protein synthesis [19]. The Stx1B induced apoptotic with accompanying DNA fragmentation, whereas the Stx1A were found to be necrotic and no DNA fragmentation occurred [20,21]

Generally, exposure of cancer cells to Stx activate caspase 3, 6, 8, and 9. Caspase 8 is known to active Bid, an endogenous protein known to permeabilize mitochondrial membranes. The cleavage of Bid will convert from inactive 26 kDa form to an active 15 kDa capable of the disrupting mitochondrial outer membrane. This activity will induce release of cytochrome C from mitochondria and triger activation of caspase-9 which then accelerated apoptosis by activating caspase-3 [5]. Furthermore, the previous study also showed that apoptosis involving caspase-3 activation is induced after Stx is transported to the Golgi apparatus, which is similar to the inhibition of protein synthesis caused by Stx [21].

The ability of Shiga-like toxin to trigger T47D cell entering apoptosis, late apoptosis, and necrosis stages compare with control cell also proven by the results of cell cycle analysis (Table 2). Result of study shows some treatment of T47D with Shiga-like toxin of ATCC 43894, KL-48(2), SM-25(1), and SM-7(1) was more arrest the cell cycle on G0-G1 pahse, except for DS-21(4) with the lowest effect. Propidium iodide (PI) is a fluorescent dye that binds specifically to double stranded nucleic acids [22,23]. In the flow cytometry assay employed, PI fluorescence is indicative of the DNA content of the cells. Cell in the G2/M phase are preparing to divide and they contain double

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amount of DNA (4n) compared to cells in the G1 phase that have not yet replicated their DNA (2n DNA content) [15].

Cell cycle analysis (Table 3) showed simultaneously effect with cytotoxic assay. The higher viable cell on control is correlate with the higher cell cycle on G2-M phase as a active phase, as well as the lower percentages of viable cells correlate with the higher cell cycle on S-phase or G0-M phase. The result strengthened by several articles have presented correlations between DNA ploidy classification and cell cycle variables and clinico pathologic variables [24]. The reability of flow cytometry method to detect cell cycle phase and equal phenomena also found by several researehers. The reserchers showed a decrease of leukemic cells in the S, G2 and M phase was followed by an increase of G1 phase [25]. Another result also showed in respone to genotoxic induced lymphocyte that was showed an accumulation of the cell in G2+M phase was accompanied by a decrease in the G0 + G1 population [26].

4. Conclusion

Cell treated with Shiga-like toxin show higher apoptosis and necrosis effect than cell control, and also higher cell arrest on G0-G1 phase of cell cycle. The results confirmed locally strain to be novel candidate for anticancer drug in Gb3-expressing breast cancer althouh further research was still needed in order to investigate its potency as anticancer completely.

5.Materials and Methods

5.1. Cultivation of Escherichia coli O157:H7 isolates

Cultivation of the five isolates of *E. coli* O157:H7 i.e. KL-48(2), SM-25(1), SM-7(1), DS-21(4), and control isolate ATCC 43894 was initiated by culturing on lactose broth medium (LB) at 37°C, and incubated aerobic for overnight. Presumtive *E. coli* O157 isolates were re-confirmed using *E.coli* O157 latex agglutination test (Ovoid, DR120M) according to previous method [27,28].

5.2 Isolation of Shiga-like toxin

Isolation of Shiga-like toxin was performed by culturing of isolates on *Luria Bertani* / LB broth (Sigma, L3022) and incubated on 37°C , 24 h, subsequently it was centrifugated 2000 rpm, 40 min at 4°C. Amount 15 ml of the supernatant was added 5.97 g of ammonium sulfate (Sigma, A4418) gradually in order to obtain 65% percentage of saturation. The solution was recentrifugated on 2000 rpm for 40 min. The supernatant was removed, and the precipitate was diluted with 3 ml of sterile physiological saline, and then dialyzed at 4°C overnight. Furthermore, the toxin was sterilized by Millipore filtered with 0.22 μm filters (Corning, 431 219). The concentration of the toxin was measured by calculation of optical dencity at a wavelength of 595 nm [29,30].

5.3 Preparation of T47D cancer cells

One ml of T47D cell maintained under standard cell culture condition was grown as monolayer culture in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, D6046) with supplemented by 10% Newborn Calf Serum (Sigma N4887), 100 IU penicillin/ml, 100 mg/ml Streptomycin, and 50 μg fungizon (Fisher Scientific, BW17-745H). It was incubated at 37°C, in a humidified atmosphere containing 5% CO2.

5.4.Toxicity assay

The analyzed of the toxicity effect in the form of cytophatic effect (CPE) among treatments and control was done according previous study by measuring inhibitory concentration 50% (IC 50) value of the cells. Amount 50 µL of T47D cells were implanted into 96 well micro plate (Merck) and incubated at 5% CO_2 for 24 hours to obtain confluent growth with its density of 5 x 10^4 cells/well. Then the media was replaced with the new ones and added with 50 µL of crude toxin with serial dilution. After 15 min of incubated at room temperature, the crude toxin was removed and monolayer cells were washed two times with Dulbecco's Modified Eagle Medium (DMEM). The cells then added with 100 µL complete growth medium (DMEM with 10% Newborn calf serum, 100 IU penicillin/ml, 100 mg/ml Streptomycin, and 50 µg fungizon) before they were incubated at 37°C, 5% CO_2 for 24h. Positive test was showed by amount of T47D cell lyses after incubation. At the end of incubation, the media were removed and then the cells were washed with a solution of phosphate buffer saline (PBS). Each well was added with 100 µL of culture media and 10 µL of MTT reagent (3-(4, 5 dimetiltiazol-2-yl) -2.5-diphenyl tetrazolium bromide) 0.5%. Cells were incubated again for 4-6 h in 5% CO2 incubator at 37°C to form formazan. The reaction was stopped by 100 µL of MTT reagent stopper (sodium dodecyl sulfate). The cells were incubated for overnight at room temperature, and then analyzed by ELISA reader at λ 550 nm [29,30].

5.5. Cell apoptosis and necrosis assay

Apoptosis of T47D cells was determined according to the method previously with slight modification [14,31]. An FITC-Annexin V and PI method (Invitrogen; Thermo Fisher Scientific, Inc.) was used to assess apoptosis. Briefly, 1×10^6 T47D cells were harvested, washed twice with cold PBS by centrifugation at 2000 rpm for 5 min, and resuspended in 100 µL binding buffer (Thermo Fisher Scientific, Inc.). A total of 100 µL Annexin V-fluorescein isothiocyanate and 2 µL PI were added to the solution. Following 10 min incubation in the dark at room temperture, 400 µL binding buffer was added to the solution and cells were analyzed using the Accuri™ C6 Flow Cytometer. The results were analyzed using CellQuest™ software 1.0 (BD Biosciences). A quadrant dot plot was used to identify whether cells were in the early or late phase of apoptosis and whether they were living or necrotic.

5.6.Cell cycle analysis with propidium iodide staining

The method according to the previous method with slight modification [15]. The T47D cell with density 7 x 10^5 cells upon completion of 24 h incubation with / without IC 50 of each Shiga-like toxin locally isolates and control ATCC 43894. The cell cultures were washed with PBS by centrifugation at 2000 rpm for 5 min and treated with 0.1% trypsin at 37°C. The cell suspension was collected, wash once with PBS (2000 rpm, 5 min), and re-suspended for 30 min, 4°C in PBS containing 70% cold absolute ethanol for fixation and permeabilization of the cell membrane. After that, the cells were washed twice with PBS by centrifugation 2000 rpm for 5 min, and the cell were treated with 40 µg/mL Rnase in PBS (final volume 100 ml), for 15 min at 37°C. Finally, 2 µl of PI staining solution was added to the cells, followed by 10 min incubation in the dark at room temperature. The cell cycle analysis was performed by a Fluorescence Activated Cell Sorter (FACSCalibur, Becton Dickinson, San Jose CA USA), and PI fluorescence (designated as Fl-2 Height

in the histogram plots) was measured at 488 nm. Ten thousand cells were analyzed in each experiment. The percentage of cells arrest in the G0/G1, S, and G2/M phases of the cell cycle were then determined.

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Competing interests

The authors declare that have no competing interest that might inappropriately influence the reported work.

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