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Original Article

CYTOTOXICITY OF CRUDE EXTRACT FROM SPONGE-ASSOCIATED BACTERIA AGAINST MOLT4 LEUKEMIC CELL LINES THROUGH APOPTOSIS

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ABSTRACT

Objective: The objectives of this study were to isolate bioactive compounds from sponge-associated bacteria and analyze their cytotoxicity against MOLT4 leukemic cell lines through apoptosis.

Methods: Crude extract of sponge-associated bacteria encoded SAB E 35, SAB E 38 and SAB E 40 was obtained by extraction using ethyl acetate. The cytotoxicity assay was determined by Cell Counting Kit 8. Apoptosis assay was determined by Annexin V Apoptosis Detection Kit. Further, cell cycle was determined by DNA-specific dye Propidium Iodide(PI).

Results: The ethyl acetate extract showed cytotoxicity effect on MOLT4 cell line. Based on cytotoxicity rate value, SAB E 35 has the strongest capability of inhibiting MOLT4. The estimated ethyl acetate extract concentration required to inhibit the growth of MOLT4 cells by 50% (IC_{50}) was 263.93 µg/ml. SAB E 35 showed the strongest apoptosis effect on MOLT4 cell line based on its ability to decrease viable cells, that remained22.70 % and 37.07% cells on their late apoptosis phase. Cell cycle on treated cells resulted decrease in the number of cells in S phase, G_0/G_1 and G_2/M, in addition to the accumulation of cells in sub-G_1 fraction.

Conclusion: The results of this study demonstrated that sponge-associated bacterial extracts have cytotoxicity effect on MOLT4 cell lines. Other results indicated that apoptosis occurred in MOLT4 treated by the extract, confirmed by the result of cell cycle analysis. The results suggested that the apoptotic effects of sponge-associated bacteria on MOLT4 can provide contribution to their claimed-anticancer activity.

Keywords: Sponge-associated bacteria, MOLT4, Anticancer, Cell cycle.

INTRODUCTION

Sponges were reported as the best producers of new marine natural compounds. However, they are mostly not yet ready to be developed further because obtaining continuous and large supplies of the compounds [1]. These natural products have interesting biomedical and pharmaceutical potentials regarding their antimicrobial, antiviral, antitumor, anticancer and general cytotoxic properties. Since almost 40-60% total sponge biomass are microorganisms, isolation of sponge-associated bacteria is an alternative that can be used to produce a wide range of bioactive compounds in large quantities through the production of microbial culture [2]. Some of the bioactive compounds produced by sponge are expected to be derived from sponge-associated microorganisms. Previous studies proved that sponge-associated bacteria produce diverse bioactive compounds from sponge [3]. It is hypothesized that symbiotic marine-microorganisms harbored by sponges are the original producers of these bioactive compounds [4].

Cancer was characterized as abnormal cells grown from a relatively small number of inherited or environmentally-induced genetic mutations [5]. Cancer is one of the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer-related death in 2012 [6]. In Indonesia, cancer is a major problem of public health. Based on Basic Health Research 2007, cancer ranks 7th of all death causes (5.7%). The national prevalence of cancer is 4.3 per 1000 populations [7]. Leukemia is a blood cell cancer, mostly found in children where 32% cancer patients are children under 15 y old and 74% out of them were leukemia [8]. Nowadays, cancers can be successfully cured by surgery and chemotherapy. While physically removing a tumor is an optimal option, it is still difficult to take out the entire tumor cells and thus, to increase the probability of the tumor cells spread to other organs [9]. The development of new anticancer with the lack of side effects is highly important in cancer treatment. The aims of the present study were to isolate bioactive compounds from sponge-associated bacteria and analyze its activities against MOLT4 leukemic cell line through apoptosis.

MATERIALS AND METHODS

Materials

Bacterial isolates used in this study were SAB E 35, SAB E 38 and SAB E 40 isolated from *Jaspis* sp. taken from Raja Ampat Islands, Indonesia. Based on the molecular identification, the three isolates SAB E 35, SAB E 38 and SAB E 40 were closely related to *Providencia rettgeri* YL, *Bacillus aerius* 24K, and *Bacillus amyloliquofasciens*, respectively (Fadhillah 2014, unpublished data). These bacteria have the ability to produce antimicrobial substances against *Staphylococcus aureus, Vibrio harveyii, Escherichia coli, Pseudomonas aeruginosa,* EPEC K-11, *Candida albicans* and *C. tropicalis.* The bacteria were cultured in Sea Water Complete (SWC) media. The human acute lymphoblastic leukemia MOLT4 cell line was obtained from RIKEN Cell Bank. The cell lines were cultured in Roswell Park Memorial Institute (RPMI)1640 media supplemented with 10% Fetal Bovine Serum (FBS) in addition to 1% penicillin and streptomycin. The cell lines were then maintained at a temperature of 37^{o}C in 5% CO_2 atmosphere with 95% humidity. All other ingredients used were of analytical grade.

Extraction of biactive using ethyl acetate

Sponge-associated bacteria were grown in 1000 ml SWC media and incubated for three days on a shaker (100 rpm, 30^{o}C). After incubation, the cultures were added with 1000 ml ethyl acetate and stirred for 12 h. Ethyl acetate phase on the upper part of the culture was then discarded and concentrated with rotary evaporator to obtain the crude extract. Crude extracts produced were stored at 5^{o}C for further assay.

Cytotoxicity assay

The cytotoxicity assay was determined by Cell Counting Kit 8. Approximately 1x10^4 cells/well of cell line were plated in 100 µl of

RPMI 1640 with 2% FBS and seeded into 96 well plates. The cells were loaded with 50 µl of the extract at a concentration of 50 µg/ml, 100 µg/ml, 200 µg/ml and 400 µg/ml. The plates were then incubated in CO_2 incubator at 37 °C for 48 h prior to addition of 10 µl counting reagent each well. The plate was then re-incubated at 37°C in a CO_2 incubator for four hours and the absorbance of which was read using microplate reader at 450 nm. This assay was carried out in triplicate.

The percentage of inhibition rate was calculated by formula:

$$\% \text{ cytotoxicity rate} = \frac{\text{Mean OD of control cells} - \text{Mean OD of tested cells}}{\text{Mean OD of control cells}} \times 100$$

The IC_{50} was determined using relation curve between the concentration of extract (x) and percentage of inhibition (y). The IC_{50} is provided in µg/ml.

Apoptosis assay

Apoptosis assay was determined using Annexin V Apoptosis Detection Kit following the protocol of the manufacturer. Cells were incubated with SAB E extracts for two hours before their suspension being washed with ice-cold medium and centrifuged at 4 °C and 500 x g for 5 min. The supernatant was discarded and the pellets were re-suspended to $5x10^6$-$10x10^6$ cells/ml in ice-cold 1x binding buffer and stored in ice. A total of 100 µl cell suspension was used to prepare the assay. Cell suspension was stained with PI and Annexin V and then incubated for 15 min at room temperature. The samples were analyzed by flow cytometry. This assay was carried out in triplicate.

Cell cycle analysis

Cells were incubated with SAB E extracts for two hours before being collected and washed three times with PBS prior to addition of 0.9 ml staining solutions and mixing using vortex. The cell suspension was incubated for 10 min in ice prior to addition of 0.1 ml 1.5 M NaCl. Samples were analyzed by flow cytometry. Staining solution for 10-11 samples consisted of 4.5 ml PI stock solution (55.6 mg/500 ml DDW), 1 ml Na-citrate stock solution (0.588/50 ml DDW), 0.45 ml RNase (10 mg/ml), 0.1 ml 10% Triton X-100, 3.95 ml DDW.

RESULTS AND DISCUSSION

Bioactive extraction using ethyl acetate

The three isolates of SAB produced crude extracts as yield, ranging from 0.01% to 0.02% weight per volume with SAB E 38 as the highest yield producer (table 1).

Table 1: Yield of ethyl acetate extract from sponge-associated bacteria

Bacterial isolates	Bacterial culture volume (ml)	Extract weight (g)	Yield(%(w/v))
SAB E 35	1000	0.115	0.01
SABE 38	1000	0.182	0.02
SABE 40	1000	0.113	0.01

Cytotoxicity assay

The SAB extract showed cytotoxicity effect on MOLT4 human leukemic cell lines. At concentration of 400 µg/ml, inhibitory rate of SAB E 35, SAB E 38 and SAB E 40 were 74.87%, 65.92% and 62.48%, respectively.

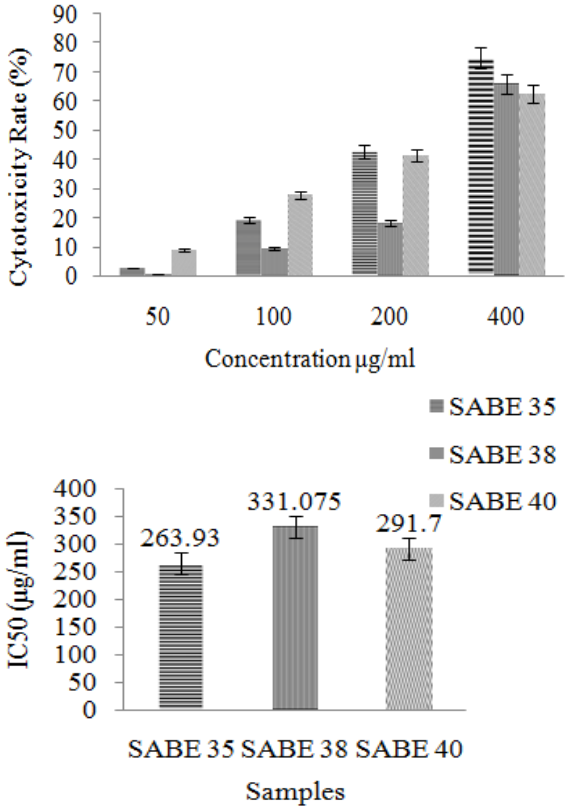

Fig. 1: Cytotoxicity effect of the extract of sponge-associated bacteria (SAB) and its IC_{50} values on MOLT4 cells line

Based on cytotoxicity rate, SAB E 35 possessed the strongest ability to inhibit MOLT4 cells. Estimated ethyl acetate extract concentration required by SAB E 35 to inhibit the growth of MOLT4 cells by 50% was 263.93 µg/ml (fig. 1). This concentration can be decreased since only crude extracts were used.

Metabolites produced by sponges and their associated microorganisms can be classified chemically into alkaloids, terpenoids, glycosides, phenols, phenazines, polyketides, fatty acid products and peptides, amino acid analogues, nucleosides, porphyrins, aliphatic cyclic peroxides and sterols [10,11]. The chemical diversity of bioactive compounds produced by sponge-microbe associations revealed that certain chemical classes, such as quinones, steroids, fatty acids, diketopiperazines, alkaloids, terpenes, terpenoids, trichoverroids and prodigiosin derivatives, diglucosyl-glycerols, polyketides, cyclopeptides, glycoglycero lipid, benzoic acid derivatives, are responsible for anticancer or antitumor activities [12]. Further purification to obtain pure extract is necessary and IC_{50} value is expected can be decreased significantly.

Apoptosis assay

Toxicity mechanisms can occur through apoptosis which is a genetically-regulated form of cell death. In the early stages of apoptosis, several changes occur on the cell surface. One of the plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer side, by which PS becomes exposed on external cell surface. PS changes can be detected using Annexin V anticoagulant which has affinity binding to PS. Cell membrane integrity is lost during the apoptotic process. Using DNA-specific viability dyes such as PI, it is possible to distinguish early apoptotic, late apoptotic, and dead cells [13].

The apoptosis effect of the extracts on MOLT4 showed several results, as reported in table 2 and one of flow cytometry outputs in Fig.2. The viability of cells treated with SAB extracts decreased and their apoptosis activity appeared as late-phase apoptosis. SAB E 35 was capable of decreasing viable cells the most, i.e. remained only 22.70 % and 37.07% cells on late apoptosis phase. However, dead cells cannot be detected whether they death or not by apoptosis

effect or any other mechanisms. The number of cells treated for this analysis has dramatically decreased, compared with the control cells. This was expected because cells were dead early while being treated and wasted away during assay process.

Table 2: Apoptosis effect on MOLT4 cells incubated in samples for 2 h

Samples	Viable cells (%)*	Early apoptosis (%)*	Late apoptosis (%)*	Dead cells (%)*
Control	92.1±0.45	5.23±0.75	2.60±1.31	0.10±0.17
SAB E 35	22.7±1.21	0.40±0.69	37.07±1.86	40.07±1.10
SAB E 38	54.03±2.62	0.47±0.72	0.37±0.40	42.70±1.51
SAB E 40	57.33±2.46	0.0±0.0	13.23±2.48	23.23±4.68

*mean±SD, n=3

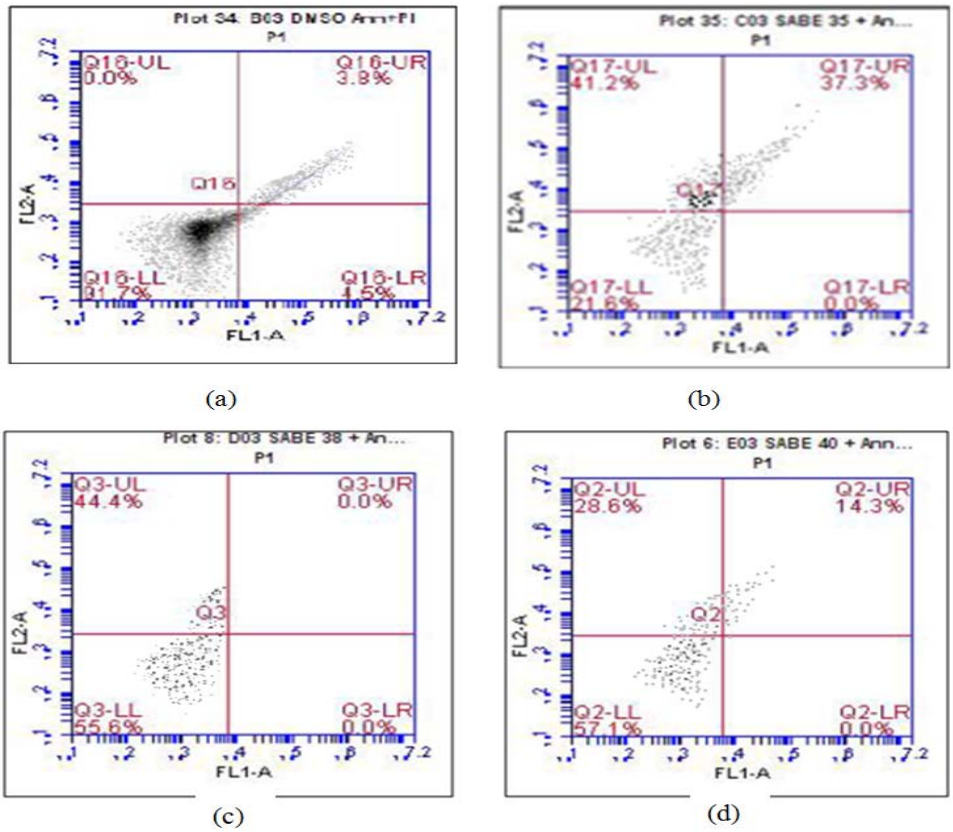

Fig. 2: Flow cytometry outputs of apoptosis effect on MOLT4. LL quadrant: viable cells; LR quadrant: early apoptosis; UR quadrant: late apoptosis; UL quadrant: dead cells. (a) control cells; (b) cells treated with SAB E 35; (c) cells treated with SAB E 38; (d) cells treated with SAB E 40

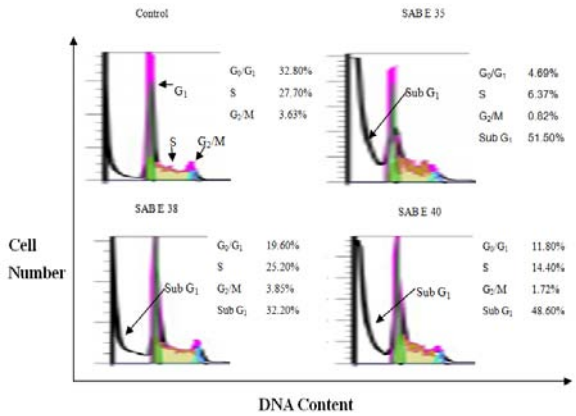

Fig. 3: DNA content frequency histogram representing cells from control cultures and cells treated with samples that affect the cell cycle distribution and induce apoptosis Cell cycle analysis

Cells treated with SAB E 35 sample at IC_{50} concentration contained different DNA compared with the control ones. A significant decrease was seen in the number of treated cells in S phase, G_0/G_1 and G_2/M fraction, in addition to the accumulation of cell populations in sub-G_1 fraction (fig. 3). The most dramatic change of DNA content occurred in cells incubated in SAB E 35. The G_0/G_1 phase, S phase and G_2/M phase of cells treated with SAB E 35 decreased into 4.69%, 6.37% and 0.82%, respectively, while for the control cells were 32.80%, 27.7% and 3.630%, respectively. However, sub-G_1 fraction increased from 2.45% to 51.50%.

In addition, the induction of apoptotic cell death in MOLT4 cells treated by sponge-associated bacterial extract was confirmed by cell cycle analysis result. Apoptotic cells frequently have fractional DNA content because the fragmented DNA, which has low molecule weight, was extracted during staining procedures. Some cells also lose DNA chromatin by shedding apoptotic bodies [14]. Thus, only a fraction of DNAs remains in the apoptotic cell. They are then represented by DNA content frequency histograms in sub-G1 peak [15]. The proportion of cells in sub-G_1 fraction was accumulated, indicating that apoptotic DNA cleavage increased significantly after two hours samples treatment.

There are several research, development of new anticancer with the lack of side effects. An example of a cancer drug that targets the cell cycle is 5-fluorouracil, which blocks the synthesis of thymine, one of the four bases in DNA. The drug taxol prevents the functioning of microtubules in the mitotic spindle. Both drugs inhibit the cell cycle, and apoptosis causes tumor shrinkage [9]. More such research ongoing is Pterostilbene, a polyphenolic compound present in grapes and other fruits. Pterostilbene at the IC_{90} concentration of 44 μM inhibited proliferation and induced apoptosis in MOLT4 human leukemia cells. Treatment with pterostilbene resulted in a transient accumulation of cells in the G_0/G_1-cell cycle phase followed by the S-phase arrest [16].

In summary, combining the cytotoxicity assay and the detection of apoptosis confirmed with cell cycle analysis could be applied to efficient screening of the potent bacterial isolates and also predicting their related compounds. These related compounds could be developed and applied in pharmaceutical industry in order to treat leukemia.

CONCLUSION

The results of this study demonstrated that sponge-associated bacterial extracts gave the cytotoxicity effect on MOLT4 cell lines. Other results indicated that apoptosis occurred in MOLT4 treated by the extract, confirmed by the result of cell cycle analysis. The results suggested that the apoptotic effects of sponge-associated bacteria on MOLT4can provide a contribution to their claimed-anticancer activity.

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CONFLICT OF INTERESTS

Declared None

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