Cytotoxic Effects of Methanol Extracts of Soursop Leaves (Annona muricata) on MCF-7 Cell Line and Its Effect on Expression of bcl-2

Eka Prasasti Nur Rachmani1, Tuti Sri Suhesti1, Retno Widiastuti2, Aditiyono2

1Department of Pharmacy, Faculty of Medicine and Health Sciences, Jenderal Soedirman University, Purwokerto Indonesia 53123
Email: ekasholehab@yahoo.com
naylifa@yahoo.com
2Department of Medicine, Faculty of Medicine and Health Sciences, Jenderal Soedirman University, Purwokerto Indonesia 53123
Email: astiss2004@yahoo.com
aditiyono@yahoo.com

Abstract — The leaves of the soursop (Annona muricata) contain annonaceous acetogenin that have cytotoxic effects on pancreatic carcinoma (PACA-2) and colon adenocarcinoma (HT-29) cell lines. The purpose of this study was to determine the effects of cytotoxic of methanol leaf extract of soursop in MCF-7 breast cancer cell line. The methanol extract of soursop leaves was obtained by maceration using methanol for 3 x 24 hours. Cytotoxic activity was examined by MTT assay. The percent of living cells analyzed using the probit in SPSS (Statistical Product and Service Solution) program to obtain the IC50 values. The expression of Bcl-2 protein was observed by immunocytochemistry method. The results showed that the methanol extract of soursop leaves has a cytotoxic activity in MCF-7 breast cancer cell line with IC50 values of 88.53 μg/ml. The methanol extract of soursop leaves decreasing Bcl-2 protein expression.

Keywords — MCF-7; soursop leaves; Annona muricata; cytotoxic; Bcl-2.

I. INTRODUCTION

Breast cancer is an abnormal multiplication of cells, continuous and uncontrolled. Propagation of these cells can be regulated through the regulatory process of apoptosis [1]. Important proteins that play a role in the regulation of apoptosis such as p53, Bcl-2 family proteins and caspase proteins [2]. Treatment of breast cancer are expected to reduce the multiplication of cells. It can by the induction of apoptosis in cancer cells. Soursop plant is one of the plants used as traditional medicine. Empirically used for antimotor soursop leaves [3]. Soursop leaf has a sedative effect, anti-spasmodic, hypotensive, antioxidant and antitumor [4]. Chemical compounds contained in soursop are alkaloids, essential oils, and acetogenin annonaceous in various parts of the plant, including the leaves. Acetogenin have cytotoxic activity against to cancer cells [5]. Other studies showed that ethanol and methanol extracts of leaves of the soursop has cytotoxic activity in T47D breast cancer cells and be able to induce apoptosis [6] [7]. One of the characteristics of MCF-7 cells was experiencing overexpression of Bcl-2 protein [8].

II. RESEARCH METHODE

A. Extraction

Methanol extract made from the leaves of Annona muricata soursop taken from Banyumas, Central Java. Extraction was done by maceration method using methanol. Maserat obtained by the evaporator and then evaporated to remove the solvent to obtain methanol extract of soursoup leaves.

B. Cytotoxic Test

MCF-7 cells with a density of 1.0 x 104 cells / wells are distributed in 96 well plate. After 24 hours, it was gave the extract with concentration in a raw 500 μg/ml, 250 μg/ml, 125 μg/ml, 62.5 μg/ml, 31.25 μg/ml and then incubated for 24 hours. At the end of incubation, the culture was added 15 mL reagent MTT 5 mg/ml in PBS. After 4 hours, was added 10% SDS stopper reagent (Sigma) in 0.01 N HCl (Merck). After overnight incubation at room temperature, absorbance reading was done by cell ELISA reader (λ, 595 nm). Absorbance results were unreadable converted in percentage of living cells.

C. Bcl-2 Protein Expression Test

Cells with a density of 5x104 cells/well were grown on cover slips in 6 wells plate. After the cells were treated with a test compound concentration of 1C50 88.53 mg/mL and incubated in a CO2 incubator at 37°C for 24 hours. At the end of the incubation period, cells were washed PBS and then added to cold methanol. After methanol was removed with a Pasteur pipette gently on the wall microplate. Furthermore, it were given with hydrogen peroxide and let stand for 10 minutes and then sprinkled with prediluted blocking serum, let stand for 10 minutes. Solution was removed by micropipette. Primary monoclonal antibody for antigen Bcl-2 dropped as much as 100 mL, let stand for 60 minutes. Biotin-labeled
secondary antibody (biotinylated universal secondary antibody) 100µL dropped, let stand for 10 minutes. Reagent containing streptavidin-peroxidase complex dropwise, let stand for 10 minutes. DAB chromogen substrate solution is dropped, let stand for 5 minutes, then washed with distilled water. Cells sprinkled with Hematoxylin solution, let sit for 30 minutes, then cells were washed with distilled water again. Coverslip removed and dipped in xylol, then dipped in alcohol. Once dried coverslip placed on the object and etched glass with glue (mounting media). Then covered with a coverslip slides were observed with a light microscope.

III. RESULT AND DISCUSSION

Extraction is done by maceration method using methanol solvent. Solvent-free extract obtained as 51.1 g (11%).

Cytotoxic test results showed the percentage of living cells is inversely proportional to the concentration increases. The higher the concentration of the test material, the lower the percent of the mean number of living cells MCF-7 cells that life is getting a little. At the smallest concentration on concentration (31.25 mg / mL) soursop leaf methanol extract produced an average percentage of cell survival by 75.32%, while the largest concentration (500 mg / mL) produced an average percentage of cell survival by 5.31%. Tamoxifen at a concentration of 50 mg / mL cause percent MCF-7 cells were alive at 3.186%, while the concentration of 3.125 mg / mL per cent cell survival by 87.990% (Figure 1).

IC50 values of methanol extract of soursop leaf and tamoxifen, respectively, 88.53 mg / mL and 15, 185 mg / mL. IC50 values of methanol extract of soursop leaves less than 1000 mg / mL, it can be concluded soursop leaf methanol extract has cytotoxic activity (Figure 2).

**Figure 1.** Graph showing the relationship between the percentage of live MCF-7 cells with various concentrations of methanol extract and tamoxifen

**Figure 2.** IC50 value of methanol extract of soursop leaf and tamoxifen

IC50 values lower tamoxifen group compared with the methanol extract of leaves of the soursop was 15, 185 mg / mL. Tamoxifen is a non-steroidal agent that are antiestrogenic. Tamoxifen competitively binds to estrogen receptors on the cancer that can block the effects of estrogen that can interfere with the growth of cancer cells [9].

Pamungkas stated that the methanol extract of soursop leaves has a cytotoxic effect on T47D breast cancer cells with IC50 value of 46.194 mg / mL and were able to induce apoptosis [6]. Soursop leaf ethanol extract showed IC50 value of 17.149 mg / mL against breast cancer cells T47D [7]. Several studies have shown that the compounds in soursop leaves and is thought to have the potential cytotoxic acetogenins annonaceous. Acetogenin compound contained in the leaves and seeds of soursop is muricin H, muricin I, and cis-annomontacin has cytotoxic effects on liver cancer cells Hep G [10]. Acetogenin compounds from Annona muricata is annomuricin E, muricapeptocin, muricoreacin and murihexocin C have cytotoxic activity against MCF-7 cells with IC50 values are respectively 1.45 mg / mL, 1.90 mg / mL, 1.30 mg / mL and 3.80 mg / mL [11].

Cytotoxic mechanism acetogenin several compounds against cancer cells is the pathway of apoptosis and inhibition of cell cycle. Bullatacin acetogenin compounds able to reduce the production of ATP through inhibition of NADH:ubiquinone oxidoreductase (Complex I) in the mitochondrial electron transport system and ubiquinone associated NADH oxidase in the plasma membrane of tumor cells [11]. Decreased ATP production which will lead to the occurrence of apoptosis [5]. Annonacin acetogenin compounds are able to induce apoptosis through inhibition of anti-apoptotic proteins Bcl-2 and Bcl-xL, squamocin able to increase the expression of pro-apoptotic proteins Bax and Bad [12], stimulate mitochondrial release of cytochrome c and activation of caspase cascade [1], activates caspase 3 [12]. Annonacin acetogenin compounds capable of inducing G0/G1 growth arrest with increased expression of p21, p27 and decreased cyclin D1 [13].

Test results of Bcl-2 protein expression by immunocytochemistry method showed that the intensity of the brown color of the cytoplasm of MCF-7 cells after methanol extract treated with IC50 concentrations of 88.53 mg / mL decrease compared with control cells (Figure 3.). This means that the expression of Bcl-2 protein decreased after treated with methanol extract of leaves of soursop. These results indicate that decreased expression of Bcl-2 protein has an important role in the induction of apoptosis of MCF-7 cells with methanol extract of leaves of the soursop.
MCF-7 cell morphology after treated with methanol extract of changes compared with control cells without treatment. This suggests that the cells have undergone apoptosis. Morphology of MCF-7 cells undergoing apoptosis is indicated by the fragmentation of the nucleus, cytoplasm is constricted, and formed apoptotic bodies [14].

Protein Bel-2 proteins both pro-apoptotic and anti-apoptotic and caspase 9 as an initiator caspase regulator intrinsic apoptotic pathway. Pro-apoptotic proteins and anti-apoptotic role in mitochondrial membrane permeability change that will affect the expenditure of cytochrome c from mitochondria. Decreased expression of Bel-2 resulted in induction of apoptosis due to release of cytochrome c by mitochondria is not inhibited which can further activate the caspase pathway [15]. Presence of CASP-3 gene deletions cause undergo apoptosis of MCF-7 through the activation of caspase sequences 6 and 7 and not through caspase 3 [16].

Research on the Annona squamosa seed extract containing compounds acetogenin, suggesting that the extract may induce apoptosis through caspase 3 and decreased the expression of Bel-2 protein and Belxl [17]. In the study by Ko et al. (2011) showed that annonacin (acetogenin annonaceous) of soursop and sugar apple crop is able to increase apoptosis of MCF-7 cells through a decreased expression of Bel-2 protein, induces G0/G1 growth arrest with increased expression of p21, p27 and decreased cyclin D1 [13]. Observations protein expression of Bel-2 gives an overview of the mechanism that mediate cytotoxic activity of methanol extract of leaves of the soursop in MCF-7 cells. Mechanism that mediates cytotoxic activity of methanol extract of leaves of the soursop in MCF-7 cells is through a decrease in Bel-2 protein expression.

IV. CONCLUSION

Methanol extract of leaves of soursop (Annona muricata) has cytotoxic activity on breast cancer cells MCF-7 with IC50 of 88.53 mg / mL and were able to decrease the expression of Bel-2 protein.

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