Apoptosis Effect of Ethanol Extract Soursop Leaves On WiDr Cancer Cell Line

Yoni Astuti[1]*, Wahyu Joko Priambodo[2], Sabtanti Harimukti[3], Agus Suharto[4]

[1]*Biochemistry Department, Medical School, Faculty of Medical And Health sciences, Universitas Muhammadiyah Yogyakarta, Jl Brawijaya, Tamantirto,Kasihan,Bantul, DI Yogyakarta, Indonesia*
[2]*Medicinal plant and Traditional Medicine Research and Development Centre, Jl Lawu no 10 Tawangmangu, Karanganyar, Solo, Indonesia.*
[3]*Pharmacy School, Faculty of Medical And Health sciences, Universitas Muhammadiyah Yogyakarta, Jl Brawijaya, Tamantirto,Kasihan,Bantul, DI Yogyakarta, Indonesia*
[4] *Patology Anatomi, Medical School, Faculty of Medical And Health sciences, Universitas Muhammadiyah Yogyakarta, Jl Brawijaya, Tamantirto,Kasihan,Bantul, DI Yogyakarta, Indonesia*
Corresponding Author email : yonia@umy.ac.id

ABSTRACT
Apoptosis is a critical physiological process responsible for the homeostatic mechanism and maintenance of cell populations in tissues. Due to the close correlation between the mechanism of apoptosis and the effect of anticancer agents, extensive research has been done on this mode of cell death. Annona muricata has a significant antiproliferative effect on some cancer cells. This research carried out to explore the apoptosis effect of ethanol extract soursop leaves from Tawangmangu on WiDr cancer cell line. Methods To gain the ethanol extract of soursop leaves use maserasi method. To calculate the death cell used colouring Tryphan blue. The result showed that the average of death cell at dose IC 50 in 24 hrs was (6.35±2.05) 104 in 48 hours were (11.1±1.27) 104. meanwhile that the average of death cell at dose ½ IC in 24 hours were (6.15±0.64)104 and (10.25±1.59) 104. The Increasing of death cell at dose IC 50 during 24 hours were 1,71 times and at dose ½ IC 50 were 1,67 times. Conclusion: Ethanol extract Annona muricata from Tawangmangu able to increase death cell around 1,71 times per hours in IC50 and 1,67 in 1/2IC50 on WiDr cell line.

Keywords: *Apoptosis effect, Annona muricata, WiDr Cancel cell*

1. INTRODUCTION

Apoptosis is a critical physiological process responsible for the homeostatic mechanism and maintenance of cell populations in tissues. Apoptosis is a mechanism for removing unwanted or no longer needed cells and is a highly regulated process [1]. Cancer cells usually have abnormalities in the mechanisms that regulate proliferation, differentiation, and survival [2].

Cancer can result from abnormal proliferation of one of the different cell types in the body, so there is more than one hundred different types of cancer, which can vary in behaviour and response to the treatment [3]. Control or cessation of uncontrolled growth of cancer cells is one way to treat cancer. So that utilizing the mechanism of cell death or targeting the mechanism of apoptosis is an effective method, for all types of cancer cells. Various anticancer drugs have targets at various stages of cancer either through intrinsic or extrinsic pathways [4]. There are two general ways of targeting therapy: increasing the activity of proapoptotic molecules and inhibiting the activity of antiapoptotic molecules.

Cell lines derived from human tumours are therefore called tumour cell lines; however, when inoculated into experimental animals these cell lines do not always produce tumours. WiDr cell line can form tumours within 1-4 weeks after inoculation in experimental animals and the results are highly effective which was confirmed histologically with four xenogenic hosts. In addition, WiDr easily proliferated in culture within 15 hours and showed an effective and high yield of 51%. This condition is more effective than other types of colon cancer cell lines. In addition, WiDr is also able to express beneficial biochemical markers even in small amounts. So, the WiDr cell line is very efficient as a model for cancer biology research especially as colon cancer model [5].

Cancer is a disease resulting from the division of a cell that is not regulated and controlled [6]. Colorectal cancer is the third form of malignancy that can affect both men and

women. The prevalence of colorectal cancer is increasing in Asia recently. The incidence of colorectal cancer is similar in many eastern and western countries. East Asian countries such as South Korea, Japan, China, and Singapore experienced a two- to four-fold increase in the incidence of this disease [7]. Current therapy of cancer still remains the worse side effect and spend a lot of money but still the cancer will rise again. Many people use the herbal formula to prevent also to cure the cancer [8].

The phytochemicals contained in A.muricata are flavonoids and acetogenins. While Annonacin is generally found in various families Annonaceae. Annonacin is the main acetogenin of A. muricata. Several studies have shown the ability of A. muricata to have anti-tumor effects against endometrial, breast and skin cancer cells. Antitumor mechanisms through cell cycle arrest and other cell signaling pathways [9]. Antitumor activity was mostly demonstrated through the induction of apoptosis in breast cancer and colon cancer cell lines. Annona muricata has been reported to exhibit antiproliferative activity in HL-60 f cells. After 24 hours of induction with A. muricata extract, characteristic apoptotic cells appeared, namely with an increase in bright blue colour on Hoechst 33258 staining. It also showed a clear DNA fragmentation at the extract content of 100 g/mL. DNA fragmentation is one of the important characteristics of apoptosis [7]. Annona muricata also showed as anti-proliferative to soma cancer cell [10,11]. This research address to explore the apoptosis effect on WiDr as a colon cancer cell model.

2. MATERIALS AND METHOD

To gain the ethanol extract of soursoup leaves is using maserasi methods. Simplicia or dried leaves of A. muricata were obtained from BPPTO Tawangmang is crushed into powder. 5 g of anoona leaf powder was soaked in 200 m 96% ethanol for 3 days at room temperature. The solvent extract suspension was then filtered through Whatman type 2 filter paper then the filtrate was dried using a rotary evaporator at low temperature [28]. The extract obtained was stored at 20 °C in a sealed tube for further use. The dose level of soursop leaf ethanol extract used for the apoptosis test was based on its IC 50 value of 450 mg/dl and its 12IC 50 value of 225 mg/dl.C50 (unpublished).

To maintain cell line is used RPMI 1640 (Rosewell Park Memorial Institute) medium enriched with antibacterial streptomycin and penicillin, also Fetal Bovine Serum (FBS). FBS or FCS 10%; Penicillin Streptomycin (Penstrep) 2%, photericin B (Fungizone) 0.5%; RPMI medium up to 100 mL. Method of manufacture: FBS is taken as much as 10%, Penstrep as 2% antibiotic material is used and Fungizone as antifungal agent 0.5% is used. Preparation of 100 mL of RPMI culture media was made with how to mix 10 mL of FBS; 2 mL Penstrep; 0.5 mL Fungizone. Sufficient with RPMI media to 100 mL, mixing was carried out in LAF and clearly identified on the RPMI culture medium vial, for then stored at a temperature of 2 – 8 °C.

Colon cancer cell subculture was carried out by taking 500 L harvested cells and put into a culture flask and added 6 mL of media RPMI culture and homogenized. Then incubate the cells in a 5% CO2 incubator. 37°C and observed cell conditions the next day. Harvesting is done when the cells are 80% confluent. Remove the culture medium from the flask with micropipette or pasteurized pipette, the cells were washed 2 times with 10 mL of phosphate buffer saline (PBS) and added 400 L Trypsin EDTA 0.25% evenly, then incubated in a 5% CO2 incubator at 37°C for 5 minutes and 4 mL of RPMI culture medium was added to inactivate EDTA Trypsin. Subsequently, the cells were resuspended using a micropipette so that the cells apart one by one (not clustered), observed the state of the cell in inverted microscope. If there are still cells observed in a clustered state immediately resuspended cell. Cell transfer into conickel tube. In 6 dish wells to treat cells, after planting 10 cells with enough WiDr cells, then waiting for 24 hours, after that they were treated with several doses IC50 (450,05ug/ml) and 1/2IC50(250,0 ug/ml), then harvested and observed after 24 and 48 hours of treatment. To calculate the death cell used coloring Tryphan blue. To find out the percentage dead cells done with trypan blue and staining counted on haemocytometry. The death cell was in blue. Calculations are carried out below inverted microscope dead cells counted with the formula [12]:

$$Number\ of\ cells = \frac{cells}{number\ of\ squares\ x\ dilution\ x\ total\ volume} \times 104\%$$

$$\boldsymbol{Viabillity} = \frac{\boldsymbol{Living\ cells}}{\boldsymbol{Total\ number\ cells}} \times \mathbf{100\%}$$

3. RESULTS

The average of percentage death cell as shown in table 1. The increasing of percentage death cell in line with the addition of time also the addition of dose. The percentage death cell after induced eesl in 24 hrs almost similar between dose 225 ug/ml and 450 ug/ml were 6.15±0.64 and 6.35±2.05 respectively. But during 48 hrs the increasing of death cell on dose 225 ug/ml and 450 ug/ml were 10.25± 1.59 and 11.1± 1.27 respectively.

Table 1. The average of percentage death cell of WiDr colon cancer cell line (10^4)

Doses (EESL)	24 hr	48 hr
450,05 ug	6.35±2.05	11.1±1.27
225,0 0ug	6.15±0.64	10.25±1.59

As shown in figure 1. there were significant different between dose 450 ug/ml in 24 hrs and 48 hrs, also on dose 225 ug/ml in 24 hrs and 48 hrs. The significant different with p< 0,05., CI :0,95.

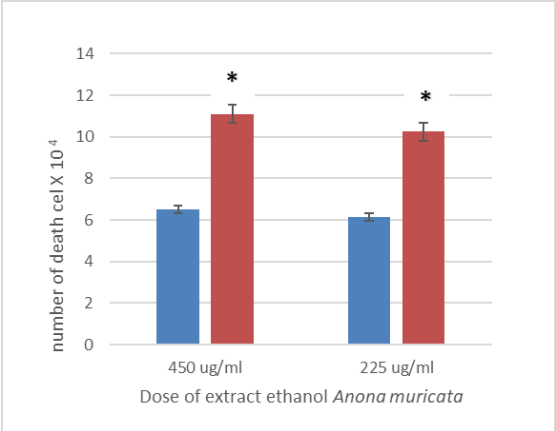

Figure 1. The significant Difference of death cell number of WiDr Colon Cancer cell after induced Extract ethanol *Annonamuricata* leaves

Table 2. Showed the folding of death cell number of widr colon cancer cell line between dose 450 ug/ml and dose 225 ug/dl in 24 hrs and 48 hrs were 10,33 and 10 ,83 respectively.

Table 2. The folding percentage of Death cell

	0 Hrs - 24 hr	24hrs - 48 hrs
Dose 225 ug/ml	-	1,67
Dose 450 ug/ml	-	1,71
Folding	10,33	10,83

Apoptosis is the death programmed of cell, as a target of cancer therapy. In this study showed the death cell of WiDr colon cancer cell line after induced extract ethanol Annona *muricata* leaves using dose 450 ug/ ml also 225 ug/ml during 24 and 48 hours. The higher dose of *Annona muricata* showed on the higher of death cell also the time during induced extract ethanol *Annona murica* any longer the time showed the increase of death cell. It assumes that extract ethanol of *Annona muricata* able to damage the WiDr colon cancer cell line.

Previous study reported that Apoptosis effect of *Annona muricata* leaf on COLO cancer cell line showed able to increase the activity of caspase 3 as proapoptotic marker. The effect of activity Caspase 3 stimulate apoptotic cell began to shrink and causes changes in the plasma membrane that indicate on macrophage response [13]. Meanwhile *Annona muricata* ethyl acetat extract (AMEAE) leaves were able to upregulated Bax and down regulated BCL2 on A549 cell line. [10]. Bax as proapoptotic protein involved in releasing of cytochrome c from mitochondria to cytosol via dimerization and translocation to the outer mitochondrial membrane [14]. Bcl-2 expression decreased significantly after treated with 20 and 40 µg/mL of AMEAE after 24 hours on A549. BCL2 is an antiapoptotic protein that is responsible for protecting cells from the apoptotic process by regulating mitochondrial membrane function. BCL2- which is associated with protein X (BAX) is able to encode a pro-apoptotic protein that plays a role in the induction of cell death [15]. *Annona muricata* induced apoptosis in HL-60 tumor cells in a dose-dependent manner. The apoptosis occurred on G0/G1 cell cycle arrest in HL-60 cells at different concentration after 24 h of treatment after induced *Annona muricata*. Therefore, suggest that the anticancer effects of A. muricata extract is associated with G0/G1 cell cycle arrest and cell differentiation [11].

Annona muricata ethanol extract due to its secondary metabolic such as acetogenins and polyphenols, among others [16,17], and can be used as reducing agents for the biosynthesis of nanoparticles [18]. It should be noted that the anticancer activity is directly attributed to acetogenins, specifically the lactone functional group. Lactones are an organic compound of the cyclic ester type. This functional group is responsible for blocking the complex I at the mitochondrial level in cancer cells, creating the accumulation of protons through the mitochondrial membrane, stopping production of ATP and, therefore, forcing selective apoptosis [19].

In this study as a preliminary study for next study showed the effect of extract ethanol *Annnona muricata* to the death cell on Widr colon cancer cell using trypan blue increase on the higher dose of Annona muricta. However, mechanism of death cell induced by extract ethanol Annona muricata on WiDr colon cancer cell line need more investigated.

4. CONCLUSION

Extract ethanol Annona muricata leaves had the apoptotic effect on Widr colon cell line. The apoptotic effect was 1,71 times /hour in dose 450ug/ml and 1,67 times in dose 225 ug/ml of extract ethanol Annona muricata.

ACKNOWLEDGMENTS

The title "ACKNOWLEDGMENTS" should be in all caps and should be placed above the references. The references should be consistent within the article and follow the same style. List all the references with full details.

REFERENCES

[1] C.M. Pfeffer, A.T.K. Singh, Apoptosis: A Target for Anticancer Therapy, Int. J. Mol. Sci. 19 (2018) 448. https://doi.org/10.3390/ijms19020448.

[2] G.M. Cooper, The cell : Molecular approach., Sinauer Associates, Sunderland, 2000.

[3] G. Runel, N. Lopez-Ramirez, J. Chlasta, I. Masse, Biomechanical Properties of Cancer Cells, Cells. 10 (2021) 887. https://doi.org/10.3390/cells10040887.

[4] A.-A.A.A. Azzwali, A.E. Azab, Mechanisms of programmed cell death, J. Appl. Biotechnol. Bioeng. 6 (2019) 156–158. https://doi.org/10.15406/jabb.2019.06.00188.

[5] P. Noguchi, R. Wallace, J. Johnson, E.M. Earley, S. O'Brien, S. Ferrone, M.A. Pellegrino, J. Milstien, C. Needy, W. Browne, J. Petricciani, Characterization of the WIDR: a human colon carcinoma cell line, In Vitro. 15 (1979) 401–408. https://doi.org/10.1007/BF02618407.

[6] T. Reya, S.J. Morrison, M.F. Clarke, I.L. Weissman, Stem cells, cancer, and cancer stem cells . Nature, Nature. 414 (2001) 105–111. https://doi.org/10.1038/35102167.

[7] M.A. Pourhoseingholi, Increased burden of colorectal cancer in Asia, World J Gastrointest Oncol. 4 (2012) 68–70. https://doi.org/10.4251/wjgo.v4.i4.68.

[8] WHO, International Agency Research for Research on Cancer.).'Latest World Cancer Statistic Global Cancer Burden Rises to 14,1 Milion New Cases in 2012 : Marked Increace in Breast Cancers Must be Addressed, Geneva, 2013.

[9] K. Foster, O. Oyenihi, S. Rademan, J. Erhabor, M. Matsabisa, J. Barker, M.K. Langat, A. Kendal-Smith, H. Asemota, R. Delgoda, Selective cytotoxic and anti-metastatic activity in DU-145 prostate cancer cells induced by Annona muricata L. bark extract and phytochemical, annonacin, BMC Complement. Med. Ther. 20 (2020). https://doi.org/https://doi.org/10.1186/s12906-020-03130-z.

[10] S.Z. Moghadamtousi, M. Fadaeinasab, S. Nikzad, G. Mohan, H.M. Ali, H.A. Kadir, Annona muricata (Annonaceae): A Review of Its Traditional Uses, Isolated Acetogenins and Biological Activities, Int J Mol Sci. 16 (2015). https://doi.org/10.3390/ijms160715625.

[11] C.A. Pieme, S.G. Kumar, M.S. Dongmo, B.M. Moukette, F.F. Boyoum, J.Y. Ngogang, A.K. Saxena, Antiproliferative activity and induction of apoptosis by Annona muricata (Annonaceae) extract on human cancer cells, BMC Complement Altern Med. 14 (2014) 516. https://doi.org/10.1186/1472-6882-14-516.

[12] R. Rinendyaputri, F. Dany, A. Polim, A. Boediono, Vitrification Method Efficacy of Mesenchymal Stem Cells (MSCs) Derived from Wharton's Jelly, J. Biotek Medisiana Indones. 6 (2017). https://doi.org/https://doi.org/10.22435/jbmi.v6i1.1681.

[13] M. Abdullah, A.F. Syam, S. Meilany, B. Laksono, O.G. Prabu, H.S. Bekti, L. Indrawati, D. Makmun, The Value of Caspase-3 after the Application of Annona muricata Leaf Extract in COLO-205 Colorectal Cancer Cell Line, Gastroenterol. Res. Pract. (2017). https://doi.org/10.1155/2017/4357165.

[14] M. Ocker, M. Höpfner, Apoptosis-modulating drugs for improved cancer therapy, Eur Surg Res. 48 (2012) 111–120. https://doi.org/10.1159/000336875.

[15] M.İ. Karagül, S. Aktaş, D. Yetkin, G. Bayrak, D. Çelikcan, Expression and Apoptosis in Perifosine and Vitamin D-Treated Endometrial Cancer Cell Line (HEC1A), in: 2nd Int. Cell Death Res. Congr., 2018. https://doi.org/https://doi.org/10.3390/proceedings2251564.

[16] J.C. Gordillo, D. Ortiz, J.E. Larrahondo, M.S. Mejía, H. Pachón, Soursop (Annona muricata L.) antioxidant activity: A literature review, Bol. Latinoam. y Del Caribe Plantas Med. y Aromat. 11 (2012) 111–126.

[17] V.C. George, D.R.N. Kumar, P.K. Suresh, R.A. Kumar, Antioxidant, DNA protective efficacy and HPLC analysis of Annona muricata (soursop) extracts, J Food Sci Technol. 52 (2015) 2328–2335. https://doi.org/10.1007/s13197-014-1289-7.

[18] A.K. Mittal, Y. Chisti, U.C. Banerjee, Synthesis of metallic nanoparticles using plant extracts, Biotechnol Adv. 31 (2013) 346–356. https://doi.org/10.1016/j.biotechadv.2013.01.003.

[19] S. Hafezi, M. Rahman, Review Targeting BCL-2 in Cancer: Advances, Challenges, and Perspectives, Cancers (Basel). 13 (2021). https://doi.org/https://doi.org/10.3390/cancers13061292.