PharmTech

International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.6, No.4, pp 1392-1396, Aug-Sept 2014

Effects of Inhibition Cell Cycle and Apoptosis of Sabrang Onion extract (*Eleutherine bulbosa* (Mill.) Urb.) on Breast Cancer Cells

Yanti Fitri[1]*, Rosidah[2], Edi Suwarso[3]

[1,2,3] Pharmacology Laboratory, Pharmacology Department, Faculty of Pharmacy, North Sumatera University, Indonesia.

***Corres.author: fitriyantiyanti94@yahoo.co.id +6285260061608**

Abstract: Sabrang onion bulbs (*Eleutherine bulbosa* (Mill.) Urb.) a typical plant in Central Kalimantan, also known as dayak onion or ghost onion, This plant has been used for generations by Dayak community as a medicinal plant for various types of diseases such as breast cancer and colon, hypertension, diabetes mellitus, hipercholesterol. strokes. Sabrang onion bulbs containing naphtoquinonens and its derivatives such as elecanacine,eleutherine, eleutherol, eleuthernone which efficacious as anticancer. This study aimed to determine the cytotoxic effect of leaf extract Sabrang onion bulbs (*Eleutherine bulbosa* (Mill.) Urb.) and cell cycle. Extraction is carried out by graded maceration using solvents based on the level of polarity. The solvent used are n-hexane, ethyl acetate and ethanol. Extracts tested against T47D by using MTT method and cell cycle by using *flowcytometry* method. Cytotoxic test results in the treatment with T47D cells giving IC_{50} values n-hexane extract of onion sabrang (NESO), ethyl acetat extract of onion sabrang (EAESO), and ethanolic extract of onion sabrang (EESO) row at 265.023 µg/ml, 147.124 µg/ml and 3782.29 µg/ml respectively. Furthermore EEABS tested for cell cycle, the result is Inhibition of cell cycle on G_0-G_1 phase with a percentage 40.88%.
Key words: MTT, Citotoxic, flowcytometri, cell cycle, T47D, sabrang onion.

Introduction

Cancer is a major health problem in the world and many patients die in this disease. Breast cancer is one type of cancer causes of death worldwide after lung cancer, stomach, liver and colon[1,2].Data from the WHO in 2008, breast cancer is the commonest cancer with 1.38 million new cases and a cause of death in the world 458 000 people per year[3]. In Indonesia, the incidence of breast cancer is 26 women per 100,000 women, followed by cervical cancer with 16 women per 100,000 women. According to data from the Hospital Information System (SIRS) In the year 2007, breast cancer is the first rank in hospitalized patients overall the hospital in Indonesia[4]. One model of breast cancer cells that is used frequently used in research is T47D (Human ductal breast epithelial tumor cell line). T47D cells is a model of breast cancer cells that has mutated p53 which is resistant to the apoptotic mechanism (physiological mechanism of reduction cells in order to repair the tissue and to disposal the damaged cells that can be dangerous for the body)[5,6]. Onions bulbs sabrang also known as dayak onion or ghost onion is a typical plant in Central Kalimantan. This plant has been used as traditional medicine by dayak people for various types of diseases such as breast cancer and colon, hypertension, diabetes mellitus, hipercholesterol and strokes. The use sabrang onions can be used as fresh onion, simplicia ad powder[7]. The study showed that the *Eleutherine bulbosa* (Mill.) Urb. contains of naphtoquinonens and derivatives such as elecanacine, eleutherine, eleutherol, eleuthernone[8]. Naphtoquinones has biological activity as antimicrobial, antiviral, anti inflammatory, antipyretic, antifungal, antiproliferative

and cytotoxic effects against colon cancer and cervical cancer[9,10]. Ethanolic extract of *Eleutherine bulbosa* (Mill.) Urb. has cytotoxic effects against HeLa uterine cervical cancer with LC50 value of 84.027 ug/ml decrease the expression levels of cyclin E and reduce the level expression of Bcl2 and induces apoptotic pathways of HeLa cells[11]. Ethanolic extract of sabrang onion has cytotoxic effects against colon carcinoma HT29 with LC50 value of 3.125 mg/ml and that mutant p53 can suppress which is caused by triterpenoid, flavonoids, anthraquinone and kaumarin[12]. The research of Li, et al., 2008 showed that sabrang onions has cytotoxic effects against colon cancer cells. Elutherine and elecanacin compounds inhibit transcription of TCF/β-catenin in SW480 colon cancer cells depending on the dose. Both of these compounds also showed selective cytotoxicity against colorectal cancer[13]. Preparation of extract was carried out by graded maceration with *n*-hexane, ethyl acetate and ethanol as solvent, to withdraw secondary metabolit of sabrang onion that is effective as anticancer. The objective of this study to determine whether the extract of n - hexane, ethyl acetate and ethanol from onion bulbs sabrang have a cytotoxic effect on T47D cells and determine whether the active extracts of onion bulbs sabrang and doxorubicin can inhibit cell cycle.

Materials and Methods

The aparatus were glasses, autoclave (Hirayama), blender (Philips), conical tube, eksikator, Elisa reader (Biorad BenMark), CO2 incubator (Heraceus), an inverted microscope (Olympus), porcelain crucible , laminar air flow (Labconco), micropipette, a rough balance (Home Line), electrical balance (Vibra AJ), oven (Memmert), water bath (Yenaco), rotary evaporator (Haake D1), sentrifugator, a set of water content determination, set of tools distillation, flatbed porcelain cup, porcelain crucible with a lid, desiccator furnace, vortex, 96 - well plate, 6 - well plate and flowcytometry.

The materials used were aged sabrang onion bulbs ± 4 months (harvest time) were taken from the village of Simalingkar B Tuntungan Medan District, North Sumatra Province. 96% ethanol, ethyl acetate and n-Hexana distilled, T47D breast cancer cells is a collection of the Laboratory of Parasitology Faculty of Medicine Yogjakarta. Media grower Roswell Park Memorial Institute (RPMI), Media M 199-serum, Fetal Bovine Serum (FBS) 10% (v/v) (Gibco), penicillin- streptomycin 2% (v/v) (Gibco), and Fungizone (amphotericin B) 0.5%. In addition to the above materials are also used 0.25% Trypsin-EDTA (Gibco), Fetal Bovine Serum(FBS), MTT [3-(4,5-dimetiltiazol-2-yl)-2,5 difeniltetrazolium bromide] (Sigma), at a concentration of 5 mg / mL and propidium iodide. Stopper used was sodium dodecyl sulfate in 0.01 N HCl.

Preparation of Extract onion Bulbs Sabrang (Eleutherine bulbosa (Mill.) Urb.)

A total of 10 parts of simplicia was inserted into a vessel, then pour with n-hxane 75 parts, closed and left for 5 days protected from light, with stirring frequently. After 5 days it was filtered diserkai juice, pulp squeezed and washed with solnvents to obtain 100 parts. Maserates moved into a closed vessel, left in a cool and protected from light for 2 days. Filtered, evaporated by rotary evaporator and freeze dryer. The pulp was dried and macerated again with ethyl acetate and ethanol[14].

Citotoxic assay

T47D cell viability were assessed using MTT colorimetric assay. The cell were cultured in 96-well plates. Each well contained 5x10 cell. The culture cells were the incubated in a humidified incubator at 37 ^oC in an atmosphere of 5% CO_2 and 95% air for 24 h. after 24 h incubation, the medium was discharge and treated by NEOS, EAEOS and EEOS with concentration was 15.625ug/ml, 31.25ug/ml, 62.5ug/ml, 125ug/ml and 250ug/ml. after incubation for 24h, the cell were incubated with 0,5 mg/mL MTT for 4h in 37 ^oC. viable cell react with MTT to produce purple formazan crystals. After 4 h, the stopper 10% SDS in 0.01 N HCl was added to dissolve the formazan crystal. The cell were then incubated for 24h in room temperature and protected from light. After incubation, the cell were shaken, and cell absorbance was measured by ELISA reader at λ 595 nm. The experimental data was absorbance of each well and then coverted to percentage of viable cell.

$$percentage\ of\ viable\ cells = \frac{B-C}{A-C} x100\%.$$

Where A, B and C are absorbances of control group, treatment group and medium respectively[15].

Flowcytometry assay

T47D cell (5x10^5 cells/well) were seeded into 6-well plate and incubated for 24h. after that, the cell were treated by EAEOS and control, and then incubated for 24 h. both floating and adherent cell were collected using 0,025% tripsin and transferred into 1,5 mL tube. The cell was washed twice cold PBS and centrifuged. The supernatant was discharge, while the pellet was collected and fixed gently in cold 70% ethanol in PBS at -20 ^{o}C for 1 h. the fixed cell were then washed twice with cold PBS and resuspenden in PBS containing PI (40 ug/ml), RNAse (100ug/mL) and triton-100 at 37 $^{o.}$ C for 30 min. the samples were then analysed using FACScan flowcytometer. Based on DNA contents, percentage of cell in each stage of cell cycle (G_1, S and G_2/M phases) were calculated using ModFit Lt.3.0.s. [15].

Result and Discussion

Test cytotoxic of n-hexane, ethyl acetate and ethanol extract of sabrang onions on T47D cells

Test cytotoxic of n-hexane, ethyl acetate and ethanol extract from sabrang onions was done on T47D cells (cell line). The principle of this test is MTT assay method. Methods MTT [3-(4,5-dimetiltiazol-2-yl)-2,5-diphenyl tetrazolium bromide] is one quantitative cytotoxicity assay. This test is based on measurement of the intensity of the color (colorimetric) that occurs as a result of metabolism of a substrate by living cells become a colored product[16]. Mitochondrial dehydrogenase activity of living cells will reduce the yellow MTT become purple. Absorbance of color can be measured quantitatively with a wavelength of 595 nm by using elisa reader. The reduction occurs only when mitochondrial reductase enzymes is activeted then the conversion can be directly related to the number of living cells. Cytotoxic effects of the IC50 value is the concentration that causes death of 50 % of the cell population. Treatment n-hexane, ethyl acetate and ethanol extract of sabrang onion was 15,625ug/ml, 31.25ug/ml, 62.5ug/ml, 125ug/ml and 250ug/ml. Cytotoxic effects on cells T47D was directly proportional to the concentration of the test compound. The greater the concentration of test compound, the smaller the percentage of living cells T47D will be. It wasshown the cytotoxic effect of sabrang extract is dose dependent.

The test results of the test solution cytotoxic T47D cells IC50 value 265.023 ug/ml for n-hexane extract of onion sabrang (NESO), 147.24 ug/ml for ethyl acetat extract of onion sabrang (EAESO), and 3782.29 ug/ml for ethanolic extract of onion sabrang (EESO). Where in the concentration of each extract was able to inhibit 50% growth of T47D cells. Each of concentration other whise, the extract was called potent if the IC_{50} value less then 500 ug/ml[17]. From the test results and calculation of IC50 extracts against T47D cells, IC50 results was obtained below 500 ug/ml for NESO and EAESO . While on EESO above 500 ug/ml was 3782.2 ug/ml. The data showed that EAESO effective on T47D cells with IC50 values in cell extracts of T47D 147.124 ug/ml, it gives great hope treatment ER-positive breast cancer (T47D cells represented)

Inhibition of cell cycle

The data were analyzed by flow cytometry cell quest program to see the distribution of cells in the phases of the cell cycle sub G1, S, G2/M and cells undergoing polyploidy. Flow cytometry was performed with 488 nm light beam and the medium speed (500 cells/sec).

Accumulation of cells in the cell cycle was one of the main targets of anticancer agents. In this observational study was of cell cycle by flowcytometry method. With this method, we can be see the distribution of cells in each of phase of the cell cycle after treatment, so it can be estimated EAESO inhibitory pathways cell cycle. Phase of the normal cell cycle have differences on the number of sets of chromosomes that G1 phase is the number of sets of chromosomes 2n. Continuesly in S phase, the number of sets of chromosomes between 2n and 4n as a process of replication, whereas the G2 and M phases, replication has been perfectly formed set of 4n chromosomes. With the fluorochrome that has the ability combinated with bases such as propidium iodide DNA strand so each cell has a different number of sets of chromosomes will provide different fluorescence intensities. The more sets of chromosomes the greater fluorescence intensity will be. The tools that were used to read the fluorescence intensity of each cell in this study were FACS (Fluorescence Activated Cell Sorting) or flowcytometer[18].

Testing of the cell cycle in T47D cells with flowcytometri method was performed by a variety of treatments. Among them there was the control which is shown in Figure 1 and EAESO on ½ IC50

concentration was 73.50 mg/mL (Figure 1). T47D cell cycle profile after treatment was shown in Table 1 and shown below.

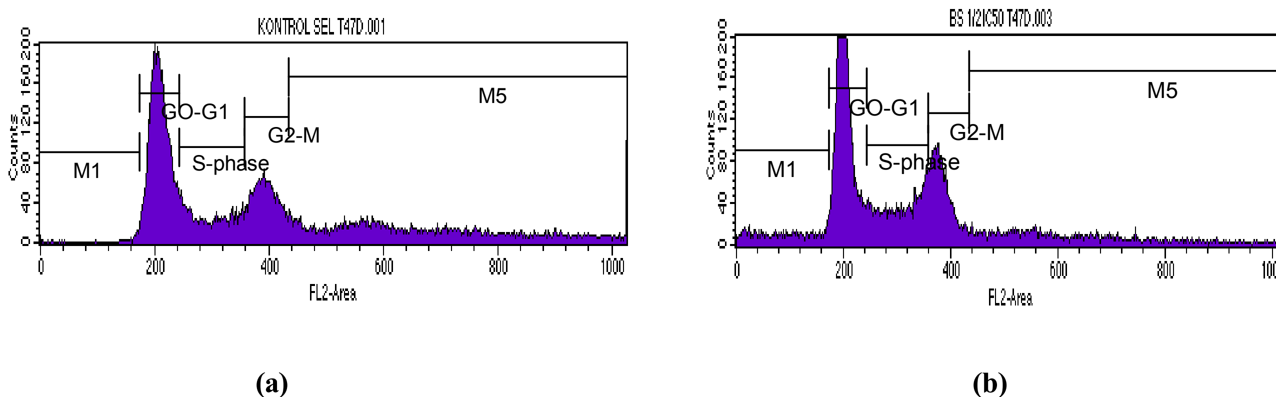
Data analysis was done descriptively, by comparing with the control treatment. Cell cycle analysis was performed on the phase of the cell cycle that the largest accumulation of the cells in each treatment was occured.

Accumulation of cells in the treatment EAEOS with concentration 73.50ug/ml (½IC50) was a G0-G1 phase with value 40.88%. When compared with controls accumulation in G0-G1 phase with value 36.18% and the effect of treatment with the extracts of G0-G1 phase is much different, so it can be said that the concentration EAEOS ½IC50 (73.50mg/ml) showed an influence on the cycle T47D cells .

Constraints on the cell cycle regulation of G0-G1 cycle by EAESO 73.50 ug/ml was occurred by the decreasing of expression level of cyclin D so it was not activated of CDK4 and CDK6 which was cause the inhibition of PRB (retinoblastoma protein), which is not phosphorylated Rb binds with E2F transcription factors bind DNA and inhibit transcription of genes whose products are required for S phase of the cell cycle so that the cells retained in the G1 phase or G1 arrest occurs[19]. Inhibition of the cell cycle could be caused by the ability of the compounds contained EAESO increase expression of p21 and p27 proteins become a complex bond with Cyclin D and Cyclin Dependent Kinase 4/6 (CDK), so that would inhibit the phosphorylation PRB (retinoblastoma protein). Thus it was inactive E2F, that led to the cessation of the cell cycle[19-20]. Cessation of the cell cycle at G0-G1 phase provides an opportunity for cells to repair damaged DNAif it could n ot be repaired further in to the apoptosis process. Accumulation of cells in the M1 phase was assumed as apoptosis ½ IC50 concentration (73.50 mg/ml) is equal to 7.22 %. This activation is likely to cause inhibition of the protein expression of Bcl2 (anti- apoptotic protein).

Table 1 Distribution of T47D cells after treatment with IC50 concentrations EAESO ½ (73.50ug/ml)

Treatment	Concentration	Cell phase (%)				
		M1	G_0-G_1	S	G_2–M	M5
Kontrol	0	0.55	36.18	12.77	17.41	33.63
EAESO	½ IC_{50}	7.22	40.88	19.71	17.77	15.13



(a) **(b)**

Figure 1 Overview of the cell cycle T47D (a) control, (b) given EAESO ½ IC50 (73.50 mg/ml)

Conclusion

IC50 values obtained NEOS, EAEOS and EEOS the treatment of T47D cells in a row at 265.023 ug/ml; 147.124 ug/ml and 3782.29 mg/ml

EAEOS at a dose of 73.50 mg/ml inhibits the cell cycle at G0-G1 phase with a percentage of 40.88 % and leads to apoptosis of 7.22 %.

References

1. Yacoob, N, K., Nurraihana, H., Nik, N., Nik, M, Kamal, Siti, A, Z., et al.. "Anticancer activity of a sub-fraction of dichloromethane extract of Strobilanthes crispus on human breast and prostate cancer cells in vitro", BMC Complementary and Alternative Medicine., 2010;10: 1.
2. Jemal, A., Rebecca, S., Jiaquan, X., dan Elizabeth, W. "Cancer Statistics 2010", CA Cancer J Clin., 2010;60: 277-300.
3. Globocan. "International Agency for Research on Cancer. World Health Organization". 2008. http://globocan.iarc.fr/.
4. Depkes R.I. "Panduan Memperingati Hari Kanker Sedunia". Depkes RI: Jakarta, 2013; pp: 3.
5. Ruddon, R.W. "Cancer Biology", Oxford University Press: New York, 2007; pp: 117-196.
6. Junedi, S., Susidarti, R.A., dan Meiyanto, E. (2010). "Naringenin Meningkatkan Efek Sitotoksik Doxorubicin pada Sel Kanker Payudara T47D Melalui Induksi Apoptosi", Jurnal Ilmu Kefarmasian Indonesia., 2010;8: 85-90.
7. Galingging, R.Y. (2009). "Bawang Dayak (Eleutherine palmifolia) Sebagai Tanaman Obat Multifungsi", Warta Penelitian dan Pengembangan., 2009; 15: 2-4.
8. Hara, H., Maruyama, N., Yamashita., S., Hayashi, Y., Lee, K.H., Bastow, K.F., Chairul, Marumoto, R., dan Imakura, Y. "Elecanacin, A Novel New Napthoquinon From The Bulb Of Eleutherine americana", Chem Pharm Bull., 1997; 45: 1714-1716.
9. Babula, P., Vojtech, A., Ladislav, H., dan Rene, K. "Noteworthy Secondary Metabolites Naphthoquinones–their Occurrence, Pharmacological Properties and Analysis", Current Pharmaceutical Analysis., 2009; 5: 47-68.
10. Alves, M.A.A., Helmut, K., dan Carlos, L.Z. "Eleutherine, a Novel Fungitoxic Naphthoquinone from Eleutherine bulbosa (Iridaceae)", Mem inst Oswaldo Cruz., 2009; 98: 709-712.
11. Budityastomo, H. (2010). Pemberian Fraksi Etanolik Ekstrak Bawang Dayak Terhadap Tingkat Ekspresi Cyclin-E Galur Sel Kanker Serviks Uteri Hela. Tesis, Surakarta, Fakultas Kedokteran Universitas Sebelas Maret., 2010; pp: 58.
12. Yusni, M.A."Perbedaan Pengaruh Pemberian Fraksi Etanolik Bawang Dayak (Eleutherine palmifolia L. Merr) Dengan 5-Fluorourasil Terhadap Penghambatan Pertumbuhan Galur Sel Karsinoma Kolon HT29 Dan Ekspresi p53 Mutan", Tesis. Surakarta, Fakultas Kedokteran Universitas Sebelas Maret., 2008; pp: 75.
13. Li, X., Ohtsuki, T., Koyano, T., Kowithayakorn, T., dan Ishibashi, M. "New Wnt/β-Catenin Signaling Inhibitors Isolated from Eleutherine palmifolia", Chemistry An Asian Journal., 2008;4: 540-547.
14. Ditjen POM. "Farmakope Indonesia". Third Edition, Depkes RI: Jakarta, 1979; pp: 33, 649, 696.
15. Nugroho, A.E., Hermawan, A., Putri, D.D.P., Novika, A., dan Meiyanto, E. "Combinational Effects of Hexane Insoluble Fraction of Ficus septica Burm. F. and Doxorubicin Chemotherapy on T47D Breast Cancer Cells", Asian Pacific Journal of Tropical Biomedicine., 2012;2: 1-6.
16. Kupcsik, L., dan Stoddart M.J. "Mammalian Cell Viability: Methods and Protocols", Humana Press: New York, 2011; pp: 13-18.
17. Machana,S., Natthida, W., Sahapat, Barusrux, Apiyada, N., Bungorn S, dan Thaweesak, T. "Cytotoxic and apoptotic effects of six herbal plants gainst the human hepatocarcinoma (HepG2) cell line",. Chinese Medicine. 2011;6: ,39.
18. Givan, A.L. "Flow Cytometry First Principles". Wiley-Liss Inc: New York, 2011; pp: 108-115.
19. Kumar, V., Abas, A.K., dan Foustro, N. "Pathology Basic of Dissease", Elsevier Inc; New York, 2005; pp: 270-336.
20. Foster, J.S., Henley, D.C., Ahamed, S., dan Wimalasena, J. "Estrogen and Cell Cycle Regulation in Breast Cancer", Trend in Endocrinology and Metabolism., 2011;12: 320-327.
21. King, R.J.B. "Cancer Biologi", Second Edition, Pearson Education Limited: London, 2000; pp: 157-165.

*****