

# Ethyl Acetate Extract Of Sabrang Onion (*E. Bulbosa* (Mill.) Urb.) Increases Apoptosis Of Widr Cells

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**Abstract** : Cancer is one of the deadly diseases. Every year there's an increase of cancer patients, one of which is colon cancer, which ranks third worldwide. Chemotherapy is a cancer treatment option with high effectiveness, but there are problems of drug resistance and side effects that limit the use of chemotherapy. Sabrang onion (*E. bulbosa* (Mill.) Urb.) One of the plants from Kalimantan, Indonesia which has a cytotoxic effect on colon cancer cells WiDr. This study aims to determine the apoptotic activity of Sabrang (*E. bulbosa* (Mill.) Urb.) Ethyl acetate extract on WiDr cells. The study was conducted in vitro on WiDr cells. The test was carried out by exposing ethyl acetate extract of sabrang onion to WiDr cells for 24 hours then apoptosis test was performed using the flowcytometry method in the Laboratory of Parasitology, Faculty of Medicine, Gadjah Mada Indonesia. The results of the study showed that sabrang onion ethyl acetate extract caused an increase in initial apoptosis and end apoptosis of WiDR cells and was concentration dependent.

**Index Terms** ; apoptosis, ethyl acetate extract, sabrang onion, WiDr cells.

## 1. INTRODUCTION

CANCER is a non-infectious disease that is responsible for the majority of deaths worldwide and is thought to be a leading cause of death as well as a obstacle to the rising life expectancy of every country in the world today. Colon cancer is the third largest based on the number of new cases and is also the second biggest cause of death worldwide in 2015 [1]. Colon cancer treatment is carried out in various method including surgery, chemotherapy, and radiotherapy, depending on the stage of the cancer patients [2]. Chemotherapy is often the first choice for colon cancer treatment by using various drugs to kill cancer cells at an advanced stage [3]. One problem that arises in the use of chemotherapy in colon cancer is resistance caused by various things such as an increase in the number of drugs released from cancer cells, decreased absorption of drugs into cancer cells, or changes in enzymes involved in metabolism [4]. Resistance problems that arise in cancer therapy trigger the development of drugs from natural materials that can be used not only as new chemotherapy drugs but as additional therapies and preventive chemotherapy in cancer patients, because natural materials have several advantages in therapeutic management including relatively small side effects [ 5], [6]. Many natural compounds in plants are known to have cytotoxic activity against cancer cells [7]. One plant that is known to have cytotoxic activity against cancer cells is a plant of the genus *Eleutherine*. Sabrang onion plant (*E. bulbosa* (Mill.) Urb.) or Dayak onions are plants from the Kalimantan region that are traditionally used by the community to treat various types of diseases such as cancer, hypertension, diabetes mellitus, hypercholesterolemia and stroke [8]. The in vitro method is a preliminary study that can be carried out to

analyze the anticancer activity of a compound [9]. WiDr cells are a model of colon cancer cells that are often used in in vitro studies, isolated from a 78-year-old female colon with adenocarcinoma types [10]. Adenocarcinoma is a type of cancer that develops in glandular cells, where almost 96% of colon cancers are adenocarcinomas [11]. Previous sabrang onion cytotoxic studies conducted on various cell models such as *E. palmifolia* L.Merr extract showed cytotoxic effects on T47D cancer cells and HT-29 colon cancer cells [12], [13]. Extracts of *E. bulbosa* (Mill.) Urb in the T47D breast cancer cycle in the G0-G1 phase and inhibits apoptosis [14]. *E. palmifolia* L. Merr. has a cytotoxic effect on cervical cancer HeLa uteri and also induces apoptotic pathways [15]. *E. palmifolia* L. Merr extract also has a cytotoxic effect on HT29 colon carcinoma [13]. An extract declared potent as a cytotoxic material if it had an IC50 value of less than 500 µg / ml [16]. The results of previous studies stated that the ethyl acetate extract of Sabrang onion (*E. bulbosa* (Mill.) Urb) has cytotoxic activity against WiDr cells with IC50 14,186 µg / mL [17].

Cancer cells are cells that lose their control over the regulation of the cell cycle and the function of cell homeostasis in multicellular organisms. Because of this failure, cells cannot proliferate normally. As a result, cells will continuously proliferate, causing abnormal tissue growth [18]. Failure to control cell proliferation normally through various mechanisms including apoptosis (cell death program). Future studies aimed to determine the ability of apoptosis from ethyl acetate extract of Sabrang onion (*E. bulbosa* (Mill) on WiDr cells.

## 2 MATERIAL

The materials used in this study were Sabrang onion layer bulbs that had been harvested after ± 4 months of age (harvest time) with a height of ± 20-40 cm and a width of ± 1.5-3 cm bright red, distillate n-hexane , distillate ethylacetate, and distillate ethanol, and chemicals used unless otherwise stated are pro-analysis quality, namely: α-naphthol, ammonium hydroxide, acetic acid anhydride, concentrated acetic acid, concentrated chloride acid, concentrated nitric acid, sulfuric acid concentrated, benzene, iron (III) chloride, bismuth (III) nitrate, ether, ethylacetate, hepes, iodine, isopropanol, potassium iodide, chloroform, methanol, sodium hydroxide, sodium sulfate anhydrous, petroleum ether, mercury (II)

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chloride, powder magnesium, zinc powder, sodium hydrogen sulfate, lead (II) acetate, toluene, distilled water, and dimethyl sulfoxide (DMSO), WiDr colon cancer cell is a collection of the Laboratory of Parasitology at the Faculty of Medicine UGM, Roswell Park Memorial Institute (RPMI), Fetal Bovine Serum (FBS) 10% (v / v), penicillin-streptomycin 2% (v / v), and Fungizone (amphotericin B) 0.5%. In addition to the above ingredients, also a 0.25% trypsin-EDTA solution, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] with a concentration of 5 mg / mL, Phosphate Buffer Saline (PBS), sodium dodecyl sulfate in 0.01 N HCl, propidium iodide (PI), Annexin V.

### 3 WORKING PROCEDURE

#### 3.1 Preparation of Test Material

Preparation of test material includes the collection of plant material carried out purposively ie without comparing with the same plant from other regions. Plant identification was carried out by Medanense Herbarium (MEDA) of the University of North Sumatra, Medan, then proceed with the manufacture of simplicia, and the manufacture of Sabrang onion layer tubers extract. The sample used was Sabrang onion layer bulbs  $\pm$  4 months old (harvest time). Plant material was taken from Simalingkar B Village, Medan Tuntungan District, North Sumatra Province. The collected Sabrang onion bulbs were wet sorted, washed clean with running water, drained and then thinly sliced in each layer and weighed as wet weight. This material is then dried in a drying cabinet to dry, then weighed as dry weight. Simplicia that has dried is blended into powder and then weighed as the weight of the simplicia powder. Simplicia is placed in a plastic container and tied, labeled and stored in a place that is protected from sunlight. A total of 1 kg (10 parts) of simplicia powder on the bulb of the Sabrang onion is put into the vessel, added 7.5 L of ethyl acetate and then the vessel is closed and left for 5 days protected from light while frequently stirring. Then filtered and the pulp is rinsed again with ethyl acetate until 10 L (100 parts) is obtained. Maserat is accommodated in a dark bottle, left in a cool place and protected from light for 2 days then filtered. The simplicia pulp residue is dried by aerating. Then the extract was concentrated using a rotary evaporator then the extract was dried with a freeze dryer [19].

#### 3.2 Cell Growth

##### 3.2.1 Colon Cancer Cell Growth (WiDr)

Prepare the equipment and condition the material at room temperature, take 10 mL of RPMI media in a 15 mL conicellular tube, take ampoules (WiDr cells) from the -80°C freezer or nitrogen tank and melt at room temperature, take the cell suspension in the ampoule, insert dropwise into prepared RPMI media, centrifuge at 600 rpm for 5 minutes, remove the supernatant and add 4 mL MK-RPMI and resuspend until homogeneous. Transfer 2 mL each into a new culture flask. Add 5 mL MK-RPMI to each culture flask, and homogenize. Observe cell conditions using an inverted microscope. Make sure the cells are homogeneous on the entire surface of the culture flask (not clustered in certain parts). Identify the culture flask, then store it in a CO<sub>2</sub> incubator [20].

##### 3.2.2 Colon Cancer Cell Subculture (WiDr)

Prepare the equipment and condition the material at room temperature, do work on LAF. The process of harvesting WiDr cells is done by taking 500  $\mu$ L of cell harvesting and inserting it into a culture flask. Add 6 mL MK-RPMI, homogeneous. Cell incubation in CO<sub>2</sub> incubator, observe the condition of the cell the next day [20].

##### 3.2.3 Colon Cancer Cell Harvest (WiDr)

Prepare the equipment and condition the material at room temperature, observe the condition of the cell. Harvesting is done according to the cells carried out in 80% confluent conditions, all work is done at LAF. Remove MK from the flask with micropipette or pasteur pipette, wash cells 2 times with 5 mL PBS (Phosphate Buffer Saline), add 400  $\mu$ L Tripsin-EDTA 0.25% in a row, then incubate in CO<sub>2</sub> incubator for  $\pm$  5 minutes, and add 4 mL MK to activate trypsin. Save cells with a micropipette so that the cells are separated one at a time (not to cluster). Observe the state of cells in an inverted microscope. Cell resuspension returns if there are still cells that are clustered together. Transfer cells into the cone tube [20].

##### 3.2.4 Calculation of WiDr Cells

Prepare the equipment and condition the material at room temperature, take 10  $\mu$ L of the harvest and drop it into the hemocytometer. Count the number of cells under a microscope using a counter. Hemocytometer consists of 4 counting rooms (A, B, C, and D), each counting room consists of 16 boxes. Count cells in 4 hemocytometer chambers, cells that are dark (dead) and cells that are on the outer boundary on the left and top do not count. Cells on the right and bottom boundary are counted. Calculate the number of cells per mL using the formula:

$$\sum \text{cell/ml} = \frac{\sum \text{cell A} + \sum \text{cell B} + \sum \text{cell C} + \sum \text{cell D}}{4} \times 10^4 \quad (1)$$

Count the total number of cells needed. For example to plant cells in each 96-well plate, then the total number of cells needed is  $1 \times 10^4 / \text{wells} \times 100 \text{ wells (made more)} = 1 \times 10^6 \text{ cells}$ . Calculate the required harvest volume (in mL) with the formula:

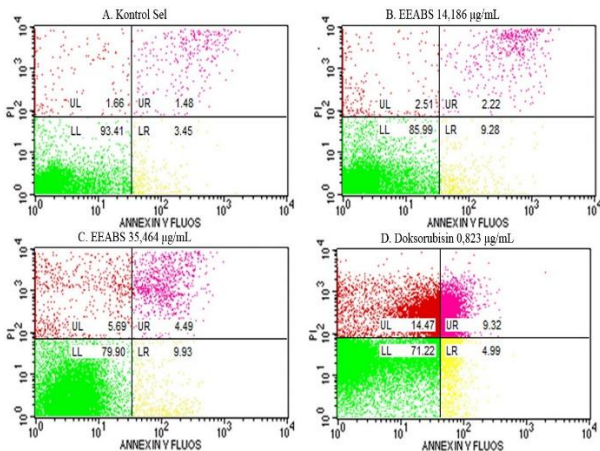
$$\text{cell harvest volume} = \frac{\text{total number of cells needed}}{\text{cell counts/ml}} \quad (2)$$

Take the cell harvest volume, transfer to a new tube then get the MK to the total required volume [21].

### 4 APOPTOSIS TESTING OF SABRANG ONION ACTIVE EXTRACTS ON WIDR CELLS

Apoptosis testing is done by the flow cytometry method. This method is a method to count live cells, apoptosis, and necrosis quickly. In this test, a protein called Annexin V can be used which can bind specifically to the phosphatidylserine present in the cell plasma membrane during apoptosis. DNA in damaged cells, both necrosis and apoptosis, will be colored by propidium iodide (PI), which produces orange to red fluorescence. When passing through a laser beam, the cell will

be excited and scatter its light producing fluorescent light [22]. Analysis of the percentage of living cells, apoptosis, and necrosis using the Cell Quest program can be seen in Fig. 1.



**Fig 1.** WiDr cell apoptosis test at (A) = Control, WiDr cells without EEABS exposure. (B) = exposure to EEABS 14,186 µg / mL for 24 hours. (C) = EEABS exposure of 35,464 µg / mL for 24 hours. (D) = exposure to doxorubicin 0.823 µg / mL for 24 hours.

The results of the apoptotic test analysis using the Cell Quest program can be seen in Table 1.

**Table 1** Percentage of Living WiDr Cells, Apoptosis, and Necrosis

Treatment	LL (%)	LR (%)	UR (%)	UL (%)
Control	93,41	3,45	1,48	1,66
EEABS 14,186 µg/mL	85,99	9,28	2,22	2,51
EEABS 35,464 µg/mL	79,90	9,93	4,49	5,69
Doxorubicin 0,823 µg/mL	71,22	4,99	9,32	14,47

Note: LL = living cell; LR = cells undergo initial apoptosis; UR = cells undergo final apoptosis; UL = cells undergoing necrosis

EEABS test results on WiDr cells above indicate an increase in the number of cells undergoing initial apoptosis and final apoptosis that depend on concentration. At an EEABS concentration of 14.186 µg / mL an increase in the number of apoptotic cells from 4.93% (control) to 11.5% and at a concentration of 35.464 µg / mL an increase in the number of cells undergoing apoptosis from 4.93% (control) to 14.42%. The increase in the number of WiDr cells undergoing apoptosis in EEABS exposure with a concentration of 35,464 µg / mL was higher when compared to the doxorubicin concentration of 0.823 µg / mL, namely 14.42% and 14.31% of 4.93% (control). Doxorubicin can be used as a positive control because it is a chemotherapy agent that can stimulate apoptosis in colon cancer cell models [23].

## 5 DISCUSSION

This study uses ethyl acetate extract of Sabrang onion (*E. bulbosa* (Mill.) Urb.) As a test material with IC<sub>50</sub> value of 14.186 µg / mL [17]. An extract can be said to have moderate cytotoxic activity if it has an IC<sub>50</sub> value of 100-500 µg / mL,

and has a strong cytotoxic activity if it has an IC<sub>50</sub> value of 10-100 µg / mL [16]. The cytotoxic ability of the ethyl acetate extract of Sabrang onion (*E. bulbosa* (Mill.) Urb.) has been demonstrated from the increase in the initial and final apoptosis of WiDr cells exposed to the extract for 24 hours and increases in increasing concentration. Apoptosis is a process of programmed cell death as an important mechanism of normal cell replacement [24]. Cells that have DNA damage will undergo apoptosis (cells die themselves without undergoing an inflammatory process) [18]. This is different from cancer cells that are not familiar with cell death programs (apoptosis) so that cells proliferate uncontrollably [18]. Ethyl acetate extract of Sabrang onions contains flavonoid derivatives. Flavonoids have been used as disease prevention and therapeutic agents in traditional medicine in Asia, one of which is as an anticancer [25]. Flavonoid compounds work by inhibiting cell proliferation through inhibition of oxidative processes that can cause cancer initiation. Flavonoids inhibit the expression of enzymes topoisomerase I and topoisomerase II which play a role in catalysis of DNA playback and relaxation. In some previous studies flavonoids have been shown to be able to inhibit proliferation in various cancer cell models. The topoisomerase enzyme inhibitor will stabilize the topoisomerase complex and cause DNA to be cut off and be damaged so that it continues into the apoptotic process [26]. Ethyl acetate extract of Sabrang onions has anthraquinone compounds. Other studies have shown that anthraquinone compounds have a cytotoxic activity against the COLO 320 cancer cell model through the mechanism of activation of the P53 protein in the mitochondria (intrinsic pathway). P53 protein will increase the expression of proapoptotic protein families such as Bax and suppress the expression of antiapoptotic proteins such as Bcl-2, changes in expression in the Bcl-2 family will change the permeability of the mitochondrial membrane which will regulate the release of cytochrome c from mitochondria. Cytochrome c in cytosol binds to and activates Apaf-1 and procaspase-9, forming apoptosomes which will activate the caspase cascade and ultimately cause apoptosis [27] [28]. In addition, suppression of mutant p53 expression can also stimulate cells to undergo apoptosis [29]. Anthraquinone also works by increasing the production of intracellular reactive oxidative species in the HCT-116 colon cancer cell model, an increase in ROS will trigger an increase in P53 expression due to oxidative stress response [30].

## 6 CONCLUSION

Ethyl acetate extract of Sabrang onion (*E. bulbosa* (Mill.) Urb.) increases apoptosis of WiDr cells and is dependent on concentration.

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