

# The Effect of Grape Seed Extract and 5-Fluorouracil toward Apoptosis Induction and Cell Cycle Modulation of WiDr Cells

Ibrahim Arifin\*, Farhana\*, Dwi Setyorini\*, Anis Fauzia\*, Dwi Nilawati\*

\*Faculty of Pharmacy, Universitas Wahid Hasyim, Semarang

Email : [drs.ibrahimarifin@gmail.com](mailto:drs.ibrahimarifin@gmail.com)

## Abstract

5-Fluorouracil is a commonly used chemotherapeutic agent in patients with colon cancer. The side effect using of 5-fluorouracil such as cardiotoxicity and immunosuppression can lead to death. One of the approach to overcome overloaded use of 5-fluorouracil is the combination with a chemopreventive agent, including the grape seeds extract (*Vitis vinifera* L.). This research aims to reviewing the effect of grape seeds extract on the cytotoxic activity of 5-fluorouracil in modulating cell cycle and apoptosis induction of colon cancer cells WiDr. Determination of the cytotoxic activity of grape seeds extract and 5-fluorouracil as well as a combination of both conducted by MTT assay. Modulation surveillance of cell cycle and apoptosis induction is done by using flow cytometry and analyzed by FACS Calibur program. Cytotoxicity assay single treatment of grape seeds extract ( $IC_{50}$ ) is  $403,957 \mu\text{g/ml}$ , whereas  $IC_{50}$  values 5-fluorouracil is  $848 \mu\text{M}$ . Observations modulation of cell cycle and apoptosis induction combination of grape seeds extract and 5-fluorouracil at concentrations of  $403,957 \mu\text{g/ml}$  -  $212 \mu\text{M}$ , showed that a combination of grape seeds extract and 5-fluorouracil to inhibit the proliferation of cells in S phase and able to induce apoptosis of colon cancer cells WiDr.

**Keywords:** Grape seeds extract, 5-Fluorouracil, Cell cycle, Apoptosis, WiDr cells

## INTRODUCTION

Colon cancer is the third leading cause of death in the world caused by cancer (Dehkordi and Safaee, 2012). Nowadays, the colon cancer treated using chemochemical agent. The first line chemotherapeutic agent for colon cancer was 5-fluorouracil (Longley and Johnston, 2007). Longterm use of 5-fluorouracil could decreased cancer cell sensitivity toward chemotherapeutic agent and cause side effects of cardiotoxicity and immunosuppression (Thomas, et al., 2004).

To overcome the side effect of 5-fluorouracil, one strategy developed is by combining 5-fluorouracil and natural material that has cytotoxic effect, of it is grape seeds (*Vitis vinifera* L.) (Parhi, et al., 2012). In this study, we observed the ability of a combination of grape seeds extract (GSE) and 5-fluorouracil in modulate cell cycle and improve induction of cell apoptosis on WiDr colon cancer cells.

The molecular search of the mechanism is going to be beneficial as the basic development of grapes seeds as a cochemotherapy agent that can be minimize the therapeutic dose of 5-fluorouracil so that it will minimize the side effect of the use of 5-fluorouracil an also to convince the health practitioners to use natural material in the practice of treating colon cancer cell.

## EXPERIMENTAL SECTION

### Materials:

Grape seeds (*Vitis vinifera* L.), methanol (Berlico).

**Cytotoxic assay, cell cycle and apoptosis materials** : methanol extract of grapes seeds, DMSO (Merck), 5-fluorouracil (Kalbe Farma), WiDr colon cancer cell lines (collection of Parasitology Laboratory of Gadjah Mada University), RPMI media (Gibco), grower media containing FBS 10% and penicillin-streptomycin 1% PBS (Gibco), MTT reagent (Sigma), Stopper reagent in the form of SDS 10% in HCL 0,01 N, Flowcytometry reagent (*Propidium Iodide* (PI) (Sigma), RNase and 0,1% (v/v) (Laboratory of Animal Science, NAIIST, Japan) Triton-X 100 in PBS (Gibco), alcohol 70% (Merck), annexin V-FLOUS Apoptosis *Detection* Kit, Tripsin-EDTA 0,25% (Gibco).

### Instruments:

Soxhletation instrument (Iwaki), rotary evaporator (Heidolph), beakerglass (Iwaki), measuring glass, funnel, boiling stone (Merck), heater, CO<sub>2</sub>5% incubator (Heraeus), treated tissue culture dish 10 cm (Iwaki), inverted microscope (Zeiss MC 80), LAF hood (Labconco), micropipet Gilson), yellow-tip, blue-tip, reaction tube, conical tube (Iwaki), 96 well-plate (Iwaki), 6 well-plate (Iwaki), sentrifuge tube, sentrifuge (Sorvall), object glass, flowcytometer (BD Accuri), digital camera (Canon, Japan), ELISA reader (SLT 240 ATC).

### Methods :

#### Cytotoxicity and Combinational Assay

Test Solution of GSE dissolved in DMSO and made the series a concentration of 1000, 500, 250, 125, 62,5; and 15,625 µg/ml. WiDr cells in a nitrogen tank subcultured in a culture medium until ready to test. A number of  $8 \times 10^3$  cell/well distributed in wells on a 96 well plate and incubated in wells on a 96 well plate and incubated in 5% CO<sub>2</sub> incubator at 37<sup>o</sup> C for overnight. Step in single testing or in combination, namely the test solution was added to the wells and incubated for 24hours. Media is removed, then each of the wells are washed with PBS, then added to the culture medium of 100 mL and 10 mL MTT with a concentration of 5 mg/ml. cells were incubated for 2-4 hours to form formazan. MTT reaction was stopped by the stopper reagent in the form of 10% SDS in 0.01 N HCl and incubated overnight at room temperature in dark place. Uptake is read by ELISA reader at a wavelength of 595 nm (Mossman, 1983).

### Observation of Cell Cycle using Flowcytometry

A number of  $5 \times 10^5$  Cells/ well transferred into a 96-well plate incubated in 5 % CO<sub>2</sub> incubator at 37 °C for 24 hours. Test solution is added to the wells and incubated for 24 hours. The cell media transferred to conical, 500 mL of PBS was added to the wells and transferred in the same conical. Cells were harvested by adding 200 mL of 0.25% trypsin-EDTA and incubate 2 minutes, added 1 mL of culture medium/well and resuspended. The harvested cells are transferred into the same conical and pitting.

Rinsed with PBS than transferred to the same conical. Centrifuged by 600rpm for 5 minutes and the supernatant was discarded. The precipitated cells were washed with cold PBS 500 ml and centrifuged by 600 rpm for 5 minutes and then PBS discarded. Added 500 ml of 70% alcohol and stored conical at temperature of 37°C for 30 minutes and then centrifuged by 600 rpm for 5 minutes and the alcohol is removed, add 500 µl PBS and centrifuged by 2000 rpm for 3 minutes. Flowcytometry reagent is added and allowed to stand 30 minutes in conical wrapped in aluminium foil. The cell suspension is transferred to flowcyto-tube and analyzed by FACS Calibur flowcytometer program to determine the profile of the cell cycle (Ayers, et al.2011)

### Observation of Apoptosis Using Flowcytometry

A number of  $5 \times 10^5$  cell well transferred into a 96 -well plate, incubated in a 5% CO<sub>2</sub> incubator at 37°C for overnight. Test solution is added to the wells and incubated for 24 hours. Conical transferred to the cell media and the wells are washed with 500 ml of PBS, then transferred to the same conical and harvesting with the addition of 0,25% trypsin-EDTA as much as 150 mL/well and incubated 3 minutes. Media culture is added 1000 mL/well and cells resuspended. Cell centrifuged by 600 rpm for 5 minutes, the media removed. The cell sediment was dissolved in buffer kit annexin V-FLOUS. The cell suspension was homogenized and incubated for 15 min at room temperature in conical wrapped in aluminium foil. Cells are then transferred to flowcyto-tube and analyzed (Gattenlohner, et al., 2009).

### Analysis of Cytotoxicity Assay Data

Data absorbance or OD (Optical Density) of ELISA reader is converted to percent of cell viability using Equation 1:

$$\% \text{ Cell viability} = \frac{\text{OD treatment cell} - \text{OD media control}}{\text{OD cell control} - \text{OD media control}} \times 100\% \quad (1)$$

The result of calculation were analyzed using Microsoft Excel 2007 to obtain single treatment IC<sub>50</sub> of GSE and 5-fluorouracil. IC<sub>50</sub> obtained from a single treatment is used to determine the concentration of the GSE and 5-fluorouracil. Potential cytotoxic 5-

fluorouracil-GSE combination determined by calculating the Combination Index using equation 2 (Reynolds and Maurer, 2005) :

$$CI = (D)_1 / (Dx)_1 + (D)_2 / (Dx)_2 \quad (2)$$

The concentration of each test compound is used to test the combination started in  $D_1$  and  $D_2$  whereas the concentration of a single compound derived from the interpolated percent cell viability caused by combination treatment (X) in a single regression equation expressed in  $D_{x1}$  and  $D_{x2}$ .

### **Analysis of Cell Cycle Observation Data**

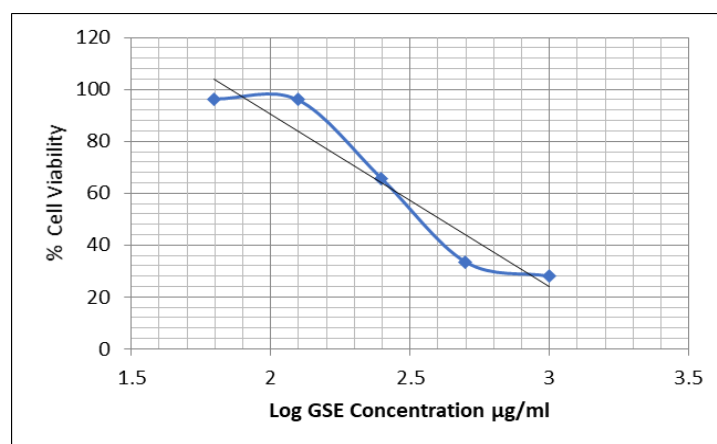
Data were analyzed by flowcytometry flowing program to see the distribution of the percentage of cell in G1 phase S, and G2/M. The inhibition of the cell cycle can be determined by comparing the treatment effect of the test solution with control cell.

### **Analysis of Apoptosis Observation Data**

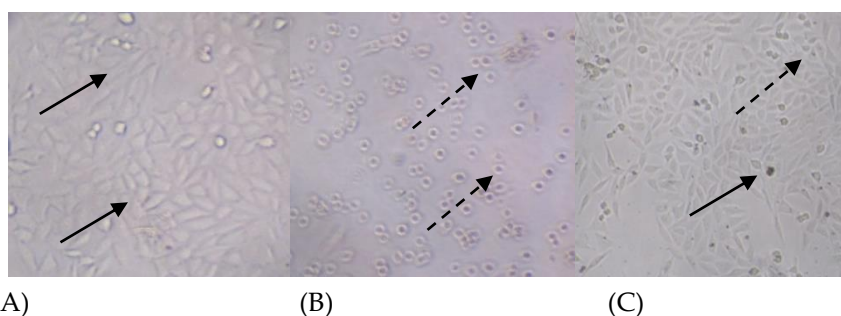
Flowcytometry data showed the percentage of cells contained in four quadrants, namely LL (lower left), LR (lower right), UL (upper left), and UR (upper right). LL quadrant showed the percentage of cells, LR quadrant showed the percentage of cells undergoing early apoptosis, UL quadrant showed percent cell necrosis, UR quadrant showed the percentage of cells undergoing late apoptosis. Induction of apoptosis known to compare the effects of single compounds and combination treatment with control cells.

## **RESULTS AND DISCUSSION**

The single cytotoxicity assay give the GSE  $IC_{50}$  values of 403,957  $\mu\text{g/ml}$ , whereas 5-fluorouracil cytotoxicity give  $IC_{50}$  value of 848  $\mu\text{M}$  (Nurulita et al., 2011). Cytotoxicity profile of GSE which shows its ability in lowering WiDr cells viability is presented in figure 1, while the morphology of colon cancer cell after treatment with 5-fluorouracil and GSE is shown in Figure 2.



**Figure 1.** The ability of GSE to decrease the viability WiDr cell. GSE inhibits the growth of WiDr colon cancer cell gives  $IC_{50}$  value of 403,957 µg/ml



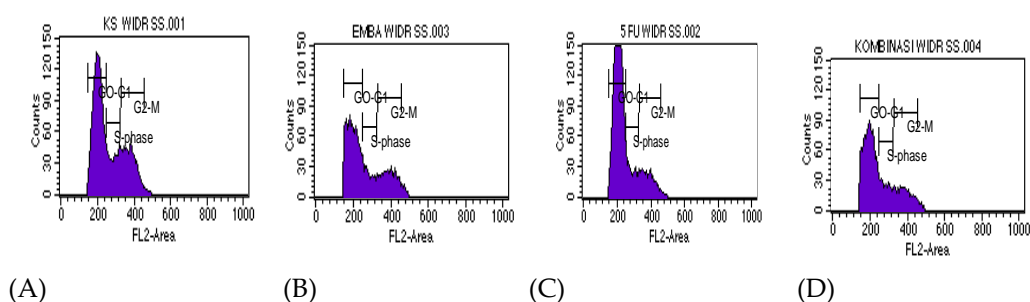
**Figure 2.** The Morphology of WiDr Cell after incubation for 24 hour. Control of WiDr Cells (A); 5-fu 212 µM (B); GSE 403,957 µg/ml. The living cells is indicated by ( —>), while the dead cells is marked by (- ->)

Cytotoxicity combination assay was conducted to know the effectivity of the combination GSE and 5-fluorouracil in inhibiting the growth of WiDr cells. In the combination assay, the concentration of GSE and 5-fluorouracil was lowered to under its  $IC_{50}$  respectively so can suppress the side effects of 5-fluorouracil. The results show the Combination Index of GSE and 5-fluorouracil as show in Table I.

**Table I. Combination Index (CI) of GSE and 5-fluorouracil in WiDrColonCancerCells.**

GSE Concentration (µg/ml)	5-Fluouracil Concentration (µM)			
	212	282,67	424	848
100,99	0,6	1,0	0,6	0,7
134,65	1,1	1,1	1,0	0,9
201,98	1,4	0,8	1,3	0,6
403,957	0,5	0,6	1,0	0,5

Combination Index in table I shows that the combination GSE of 403,957  $\mu\text{g/ml}$  with 5-fluorouracil of 212  $\mu\text{M}$ ; 282,67  $\mu\text{M}$  and 848  $\mu\text{M}$ ; GSE of 201,98  $\mu\text{g/ml}$  with 5-fluorouracil of 282,67  $\mu\text{M}$  and 848  $\mu\text{M}$  provide a synergistic effect which means it has greater cytotoxic effect than the sole effect of each compound. The best synergistic effect is shown by the combination GSE of 403,957  $\mu\text{g/ml}$  and 5-Fluorouracil 212  $\mu\text{M}$  and 848  $\mu\text{M}$  with CI value of 0,5. The combination that recommended is combination GSE of 403,957  $\mu\text{g/ml}$  and 5-fluorouracil 212  $\mu\text{M}$ .



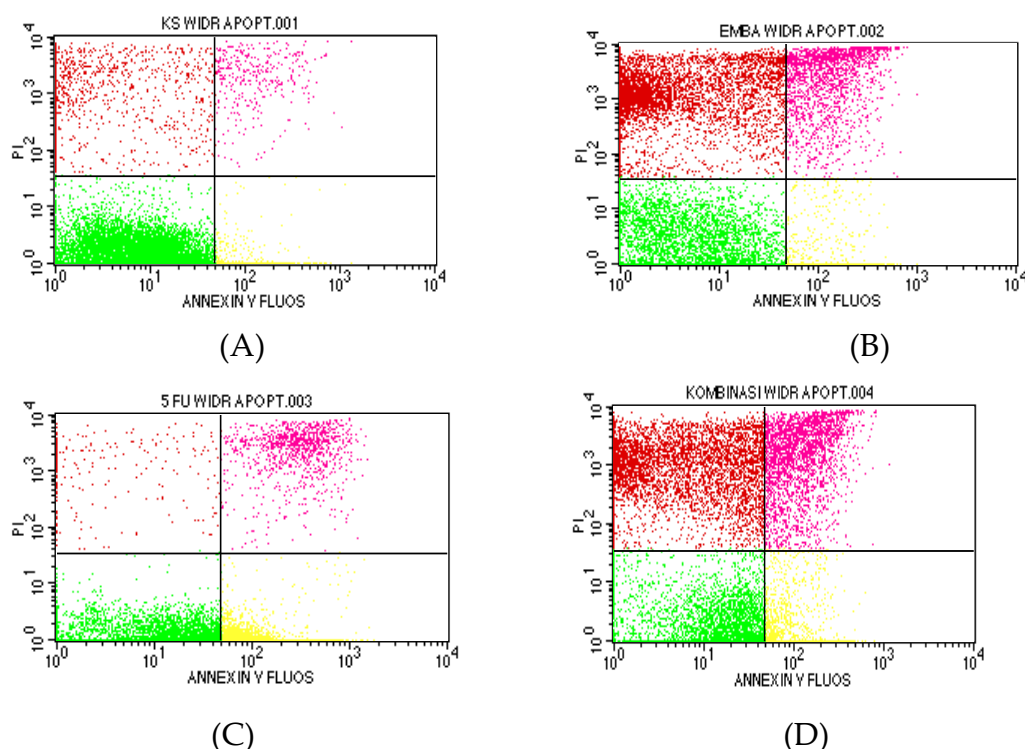
**Figure 3. Analysis of WiDr Cell Cycle Distribution using Flowcytometry. Cell control (A); GSE 403,957  $\mu\text{g/ml}$  (B); 5-fu 212  $\mu\text{M}$  (C); Combination GSE 403,957  $\mu\text{g/ml}$  + 5-fu 212  $\mu\text{M}$  (D).**

Flowcytometry analysis using FACS Calibur program is presented in figure 3, while the percentage of cell cycle distribution in table 2. The result shows that the control cells undergo cell distribution in phase G<sup>1</sup>, S and G<sup>2</sup> / M. Single treatment of GSE causes an accumulation of cells in G<sup>2</sup> / M phase, while single treatment of 5-fluorouracil causes an accumulation of cells in G<sup>1</sup> phase. The different mechanism of action may occur for a synergistic effect in combination GSE and 5-fluorouracil. Combination Index calculation results as shown in table 1 and figure 3 confirmed the synergism GSE and 5-fluorouracil. 5-Fluorouracil and GSE combination treatment is lowering the number of cells that accumulate in the G<sup>1</sup> phase which was originally raised by 5-fluorouracil, while increasing accumulation of cells in S phase. It shows the influence GSE in increasing cell accumulation in S phase, which means giving an inhibitory effect on S phase.

**Table 2. The Distribution of WiDr Cells in Cell Cycle phases after Treatment**

Sample	G1 phase (%)	S phase (%)	G2/M phase (%)
Cell Control	57,71	18,55	23,44
GSE 403,957 $\mu\text{g/ml}$	61,38	11,32	12,54
5-fu 212 $\mu\text{M}$	75,98	15,63	22,19
GSE 403,957 $\mu\text{g/ml}$ + 5-fu 212 $\mu\text{M}$	62,80	17,61	18,79

The results of apoptosis induction is presented in figure 4, while the percentage of cell death are show in table 3



**Figure 4. Analysis of WiDr apoptosis using flowcytometry. Cell Control (A); GSE 403,957 µg/ml (B); 5-fu 212 µM (C); Combination GSE 403,957 µg/ml + 5-fu 212 µM (D)**

**Table 3. Death Percentage Cells after Treatment in Colon Cancer Cells WiDr**

	Cell Control	GSE 403,957 µg/ml	5-fu 212 µM	GSE 403,957 µg/ml + 5-fu 212 µM
Early Apoptosis (%)	4,74	4,87	46,51	13,58
Late Apoptosis (%)	1,68	9,65	5,07	12,68
Necrosis (%)	9,10	57,37	1,31	40,73
Apoptosis total (%)	6,42	14,52	51,61	26,26
Death Total (%)	15,52	71,89	52,92	66,99

As show in table 3, combination of GSE and 5-fluorouracil provides apoptosis percentage of 43.92% which is presenced the induction of apoptosis in WiDr colon cancer cells. Treatment of 5-fluorouracil sole give the apoptosis percentage of 26,24% indicates the lower induction of apoptosis than its combination, which means that the addition GSE is able to increase the apoptosis effects of 5-fluorouracil involves its bond with DNA

through intercalation between the base pairs and inhibit the synthesis of DNA and RNA through rioting tempelate and steric hindrance. Another mechanism of action involves binding to the lipid membrane of cells that will turn various cellular functions and interacts with topoisomerase II to form the complex of DNA cutter. GSE contains flavonoids and Quercetin glycosides. Quercetin is Able to induce apoptosis through the mitochondrial pathway, which disrupt MMP thereby triggering the release of cytochrome c into the cytoplasm and the activation of caspase-3 and caspase-7 (Gibellini, et al.,2011).

## CONCLUSION

Based on observations of modulation of cell cycle apoptosis induction, GSE and 5-fluorouracil combination with a concentration of 403,957  $\mu\text{g/ml}$  – 212  $\mu\text{M}$  is able to inhibit cell proliferation in S phase and increase the induction of apoptosis of colon cancer cells WiDr.

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