

The ethyl acetate fraction of *Gynura procumbens* sensitizes widr colon cancer cell line against 5-fluorouracil but shows antagonism with cisplatin

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Abstract

Our recent study has evaluated the ethyl acetate fraction of *Gynura procumbens* (FEG) as co-chemotherapeutic agent in combination with 5-fluorouracil (5-FU) and cisplatin (CISP) against WiDr colon cancer cells. This study aimed to assess whether FEG produced synergistic effect with 5-FU and CISP and to evaluate its regulation on proliferation, cell cycle, and cell death induction on WiDr colon cancer cells.

(3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT assay was performed to determine the growth inhibitory effect of both single (FEG, 5-FU, or CISP) and combination treatments. FEG (25-500 µg/mL), 5-FU (25-1000 µM) and CISP (5-100 µM) inhibited cells growth in a dose dependent manner and exhibited an IC₅₀ value of 125 µg/mL, 848 µM and 43 µM, respectively. FEG sensitized WiDr cells that was treated by 5-FU, boosting its therapeutic potential. Conversely when FEG was combined with CISP, it caused antagonism. The antiproliferative effect of single and combination treatment was determined by studying the cell proliferation kinetics using MTT assay. Flowcytometry and (4',6-diamidino-2-phenylindole) DAPI staining was used to disclose the mechanism of cell cycle arrest and apoptosis. FEG inhibited cell proliferation, induced G₁ and S phase arrest and apoptosis. The inhibitory effect was enhanced when FEG was combined with 5-FU, differing from CISP.

According to the datas obtained, FEG possess sensitizing properties causes cell cycle arrest and cell death suppose to be apoptosis on WiDr cells. FEG demonstrates a possibility of additive to synergism properties when combined with 5-FU but shows antagonis with CISP.

Keywords: *Gynura procumbens*; WiDr; G₁ and S phase arrest; apoptosis.

INTRODUCTION

G. procumbens is a medicinal plant found throughtout South-East Asia, especially in Indonesia, Malaysia and Thailand. The plant has been traditonally used for treatment of several

diseases such as fever, rash, kidney disease, migrains, constipation, hypertension, diabetes mellitus and cancer [1]. Previous studies show several pharmacological properties of this plant such as anti-inflammatory and reduced blood pressure [2-3]. *G. procumbens* extract and its fractions exhibit high antioxidant properties

shown by xantine oxidase inhibition and radical scavenging activity [4]. The uncontrolled production of radical oxygen species (ROS) and unbalance antioxidant protective system have an important part on cancer initiation and development. These natural antioxidants may become a beneficial agent to be developed as chemoprevention substance for cancer disease.

Recent studies on cancer prevention and therapy show *G. procumbens*'s chemo-preventive properties to inhibit tumor development on benzo(a) pyren-treated mice and breast cancer on DMBA-treated rat [5-6]. *G. procumbens* ethanolic extract was reported for its antimutagenic property against lung cancer on benzo(a)pyren-treated mice. Its phenolic fraction suppresses cell proliferation and induces apoptosis on HeLa cells [7]. It also has an antiangiogenesis potency [8]. *G. procumbens* decreased COX-2 expression and increased p53 and Bax expression on breast cancer cells. Co-chemotherapeutic study of ethyl acetate fraction of *G. procumbens* (FEG) showed the synergism effect of its combination with doxorubicin on T47D and MCF-7 breast cancer cell lines [9]. Aqueous extract of *G. procumbens* inhibited cell proliferation and DNA synthesis on human mesangial cells [10]. Sugiyanto *et al.* (2003) found flavon/flavonol as active compounds of *G. procumbens* ethanolic extract. *G. procumbens* may contain β -sitosterol and stigmasterol, kaempferol-3-O-Rutinoside, and quercetin [11,4]. Proposed mechanism of *G. procumbens* should be through several way, such as block on carcinogenesis and suppress cell proliferation through cell cycle arrest and apoptosis induction. Both of mechanism are suggested by its phenolic and flavonoid mostly contained in FEG.

Cancer disease develops into serious problem of health because of its prevalency increasing year by year. World Health Organisation (WHO), reported more than 10 millions cancer cases every year in the world. At Amerika, colorectal cancer is the third most common cancer in both men and women and is estimated 106,100 cases of colon and 40,870 cases of rectal cancer are expected to occur in 2009. An estimated 49,920

deaths from colorectal cancer are expected to occur in 2009, accounting for almost 9% of all cancer deaths [12]. Despite advances in the management of this condition, including improved surgical techniques, the use of chemo- or radiotherapy, have not been decreased the mortality value for decades. These treatment methods are only useful for early detection cancer but not for metastasis stage. Cancer treatment failure, especially with chemotherapy, because of low selectivity of the anticancer and uncertain for the molecular targeted. The major contributor of the limited effectiveness of treatment is the resistance of cancer cell to current chemotherapeutic agents. The numerous mechanism which contributes to chemo-resistance include the amplification and mutation of drug target lead to the inhibition or avoidance of drug-induced apoptosis, drug transport defect or over expression of multi drug resistance protein (MRP) [13-14]. In line with this problem, side effect rising to cancer patient inconvenience and further may reduce the life quality and productivity of patient.

Several study report that plant constituents have high potency as oncogene negative regulator and tumor suppressor gene positive regulator that refer to their anticancer potency [15]. Anticancer studies on medicinal herbs tend to focus on developing their potency as co-chemotherapy agent. These studies aim to increase cell sensitivity, suppress cancer cell resistance, and to control side effect of chemotherapy agent.

Recent study of *G. procumbens* on several cancer cell showed high potency to be developed as cancer chemoprevention and co-chemotherapy. Study of co-chemotherapy potency of *G. Procumbens* was limited and no report found about the anticancer properties of this plant on colon cancer. Therefore it is a chance to conduct the advanced study of it and examined of its molecular mechanisms. Our study was carried out to elucidate the anticancer of *G. procumbens* on colon cancer cell and to observe its co-chemotherapy effectiveness.

Material and Methods

Chemicals and Reagents

5-Fluorouracil (5-FU) (Ebewe) was purchased from PT. Ferron Par Pharmaceutical Cikarang, Indonesia. Material were used in this study: Cisplatin (CISP) (Kimia Farma), DMEM (Nacalay, Japan), Fetal Bovine Serum (FBS) (PAA), Dimethyl sulfoxide (DMSO) (Sigma, Aldrich, Germany), penicillin and streptomycin (Gibco), trypsin (Sigma), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *sodium dodecyl sulphate* (SDS). Tris-HCl, triton X-100, propidium iodide (PI), RNaseA (Sigma) petroleum ether, ethanol, and ethyl acetate, and fraction of ethyl acetate *G. procumbens* (FEG). The fractions was dissolved in DMSO with 0,1 % as maximum concentration.

Human colon cancer cell lines

Human colon cancer WiDr was kindly provided by Prof. Masashi Kawaichi, Nara Institute Science and Technology (NAIST). The cells were routinely grown in DMEM containing 10% FBS, 1 % Penicillin-streptomycin (v/v), and L-glutamine (1 mM) at 37 °C and 5% CO₂.

Plant material

The leaves of *G. Procumbens* were collected by Balai Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (BP2TO2T) Indonesia, and was determined at Laboratorium of Pharmacognocny, Faculty of Pharmacy Gadjah Mada University, Indonesia. Dried powdered leaves were first extracted with ethanol 96 %, and concentrated by evaporation under reduced pressure and the temperature was kept not more than 40°C. The extract was diluted in hot water and then fractionated with n-hexane. The aqueous fraction than was fractionated again with ethyl acetate. The ethyl acetate fraction was concentrated by evaporation under reduced pressure and the temperature was kept not more than 40°C. The extract was stored at 4°C prior to use.

Cytotoxicity and co-chemotherapy assessments

WiDr cells (10⁴ cells/well) were seeded to 96-well plate (100 uL/well). After 24 hours growth, cells culture medium was discarded and replaced with 5-FU, CISP, and/or FEG-contained medium, as single and combination treatment. After incubated for 24 hours, the medium was discarded and replaced with MTT-containing medium (0,5 mg/mL) and incubated for 4 hours at 37°C, 5 % CO₂. The reaction was stopped with 10% SDS in 0,1 N HCl solution and was incubated for overnight in light protected chamber, to dissolve formasan salt. The absorbance of each well was measured with ELISA reader at 595 nm. The ratio between treated and control cells absorbance refer to percentage (%) of viable cells.

Calculation of combination effects

The cell response from treatment with chemotherapy agent as a single agent was compared with its response treated with *G. procumbens* combination to obtain the combination index (CI).

$$CI = D_1/Dx_1 + D_2/Dx_2$$

Where,

D₁, D₂ = concentration of each agent that used in combination

Dx₁, Dx₁ = the concentration of single compound single compound producing the same effect with combination treatment (Reynolds *and* Maurer, 2005). CI value interpretation: < 0.1 very strong synergism, 0.1–0.3 strong synergism, 0.3–0.7 synergism, 0.7–0.9 mild to moderate synergism, 0.9–1.1 almost additive, 1.1–1.45 mild to moderate antagonism, 1.45–3.3 antagonism, >3.3 strong to very strong antagonism

Cell proliferation assay

The WiDr cells (3.10³ cells/well) were seeded in 96-well plate and treated with the indicated concentrations of FEG or combined treatment with chemotherapy agent (5-FU and CISP). Control cells were supplemented with complete media containing maximum 0.5% dimethyl

sulfoxide (DMSO) (vehicle control) for 12, 24, 48, and 72 h. Following treatment, cell number and viability were determined by MTT assays.

Cell cycle analysis

The WiDr cells ($5 \cdot 10^5$ cells/well) were seeded in 6-well plate and were treated as indicated concentration after 24 hours growth. After 24 hours treatment, all cells were recovered (both attached and detached cells) using 0.025% trypsin. The cell were washed with cold PBS and fixed in cold 70% ethanol (-20°C) carefully. The fixed cells were washed twice with PBS and incubated with PBS containing PI (50 $\mu\text{g}/\text{mL}$), RNaseA (100 $\mu\text{g}/\text{mL}$), and tritonX-100 for 30 minutes at 37°C . The mixture solution was analyzed using flowcytometer. The percentage of cells was determined based on DNA contents using ModFit Lt. 3.0 to quantified cell distribution on each stage of cell cycle.

Results

Inhibitory effect of 5-FU, Cisp, and FEG on growth of WiDr cells.

To determine the potency of FEG as co-chemotherapeutic agent, first the cytotoxicity properties were examined in WiDr colon cancer cell line. Cell viability was examined using MTT reagent after 24 hours incubation. 5-FU (50-1000 μM), Cisp (5-100 μM), and FEG (25-500 μM) inhibited the growth of WiDr cells in dose dependent manner with IC_{50} value of 848 μM , 43 μM , and 125 $\mu\text{g}/\text{mL}$, respectively. (Figure 1A and 1B)). FEG had high potency as cytotoxic agent on WiDr cell. This cell was more sensitive to CISP rather than 5-FU shown by IC_{50} value (Table 1). The combination treatment of FEG and 5-FU or CISP was intended to improve the chemotherapeutic agent potency.

Combined treatment with 5-FU+FEG resulted additive to synergism effect on WiDr cells growth inhibition but produced antagonism effect with Cisp+FEG combination.

We used sub-toxic concentration both of chemotherapy agent (5-FU or CISP) and FEG to determine the combination effect on WiDr cells viability. FEG enhanced the inhibitory effect of 5-FU on WiDr cells viability within 24 hours, but gave no enhancement effect when combined with CISP. The calculation of Combination Index (CI) showed that the combination of 5-FU and FEG exhibited additive to slight synergism effect on WiDr cells (CI value 0,2–1,1), but seemed antagonism after combination treatment of Cisp and FEG (CI value $> 1,1$). The cells morphology was changed after 24 hours treatment by sample as single or combination. The combination treatment of 5-FU and FEG show more extensive morphologically changes compared with single treatment, but this phenomenon did not show after combination treatment of CISP and FEG. FEG with sub-toxic concentration (100 $\mu\text{g}/\text{mL}$) suppressed cell proliferation after 24 hours incubation and enhanced the inhibition of cell proliferation of 5-FU. But when FEG combined with CISP failed to produce higher on the suppression of cell proliferation and resulted antagonistic effect (Figure 2A and B). This result proposed that FEG enhanced the cytotoxic effect of 5-FU and cell proliferation inhibition. The suppression of cell growth could be occurred through cell death induction and cell cycle modulation.

Table 1. The cytotoxicity effect on WiDr cells after treatment of FEG, 5-FU, and Cisp for 24 hours

Sample/compounds	IC_{50} *
FEG	125 $\mu\text{g}/\text{mL}$
5-FU	848 μM
Cisp	43 μM

* IC_{50} value as average from two independent experiment with the consistent result

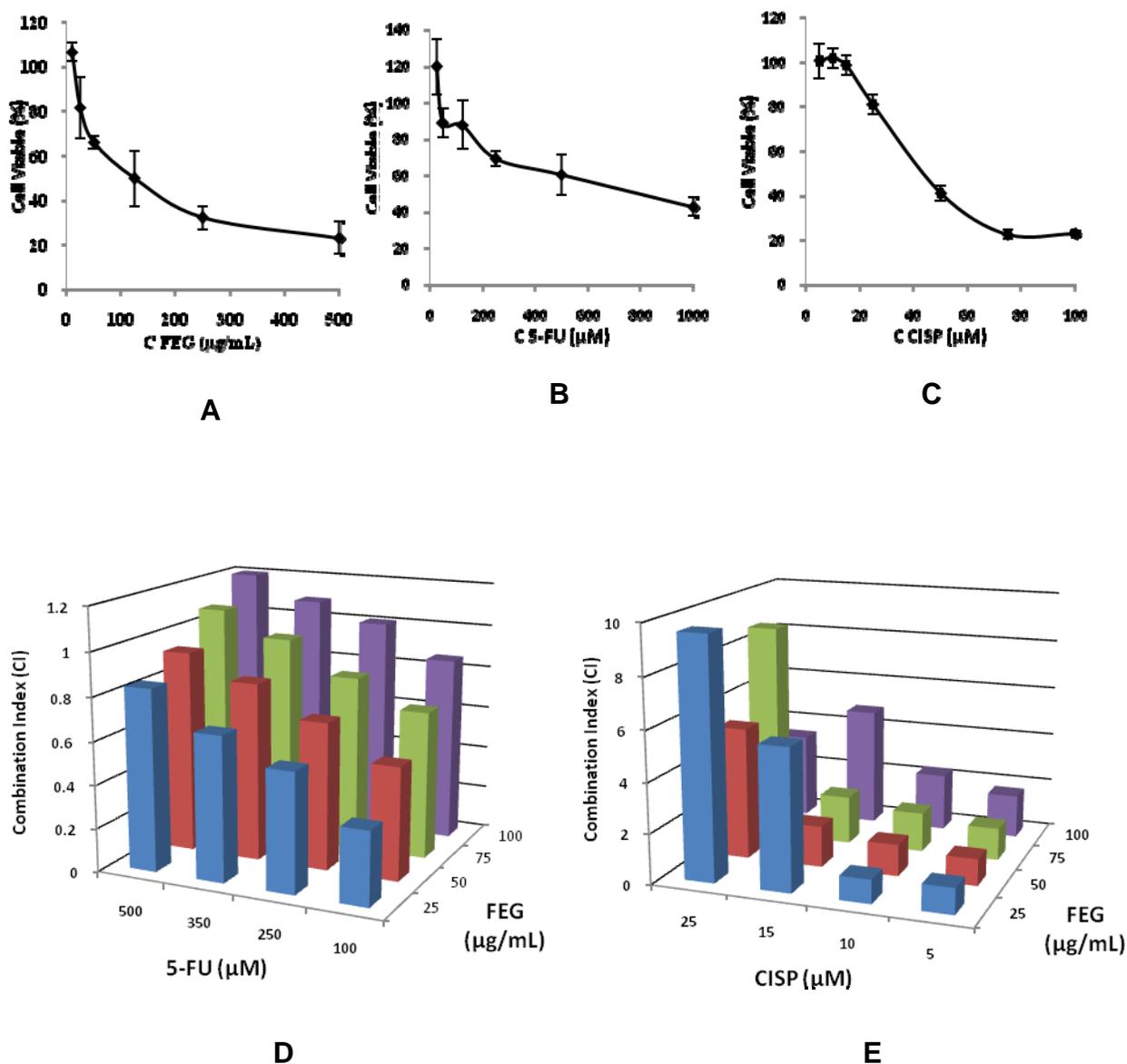


Figure 1. The FEG, Dox and Cisp inhibit WiDr cell growth in dose dependent manner and FEG produced additive to synergistic effect when combined with 5-FU but not with Cisp on WiDr cells. (A, B, and C) WiDr cell viability was determined by MTT assay after treated with the various concentration of FEG, Dox, or Cisp for 24 hours incubation. (D and E) The cell viability was determined by MTT assay treated with the indicated concentration of 5-FU+FEG or Cisp+FEG for 24 hours incubation. The combination index (CI) were calculated to determine the combination effect. The results are from one representative data of three experiment that showed similar results. Each point represents the mean \pm SD of three experiments.

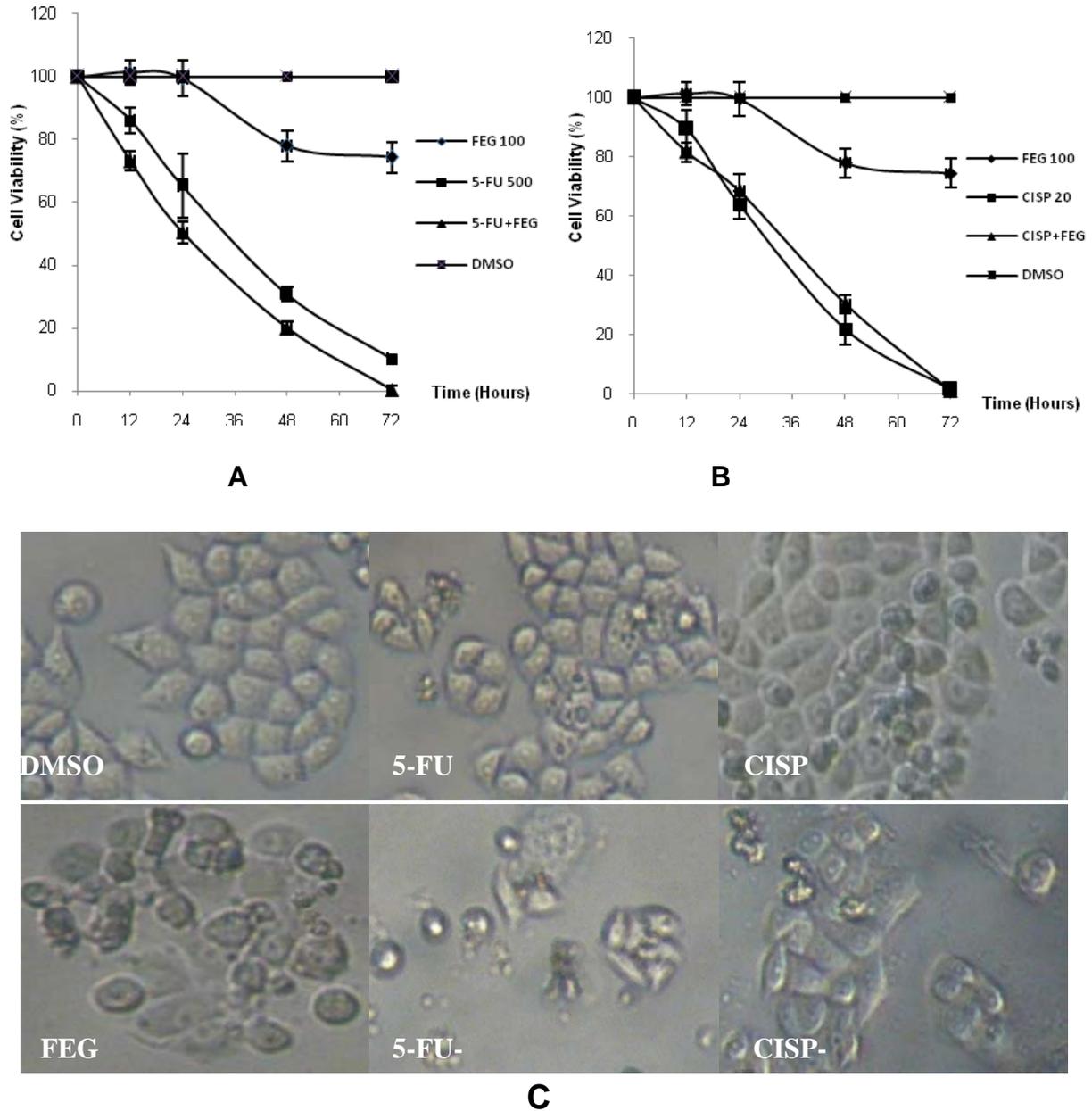


Figure 2. Combined treatment between 5-FU+FEG suppressed cell proliferation on WiDr and caused morphological changing (A and B) The cell proliferation was examined with cell viability measurement using MTT assay after treated with indicated concentration of FEG, 5-FU, Cisp, and combination between 5-FU+FEG or Cisp+FEG for 0, 12, 24, 48, 72 hours. The combination of 5-FU+FEG suppressed the cell growth but not with Cisp+FEG. (C) The cellular morphology of the cells was examined under inverted microscope (x 400). The results are from one repretative data of three experiment that showed similar results. Each point represents the mean \pm SD of three experiments.

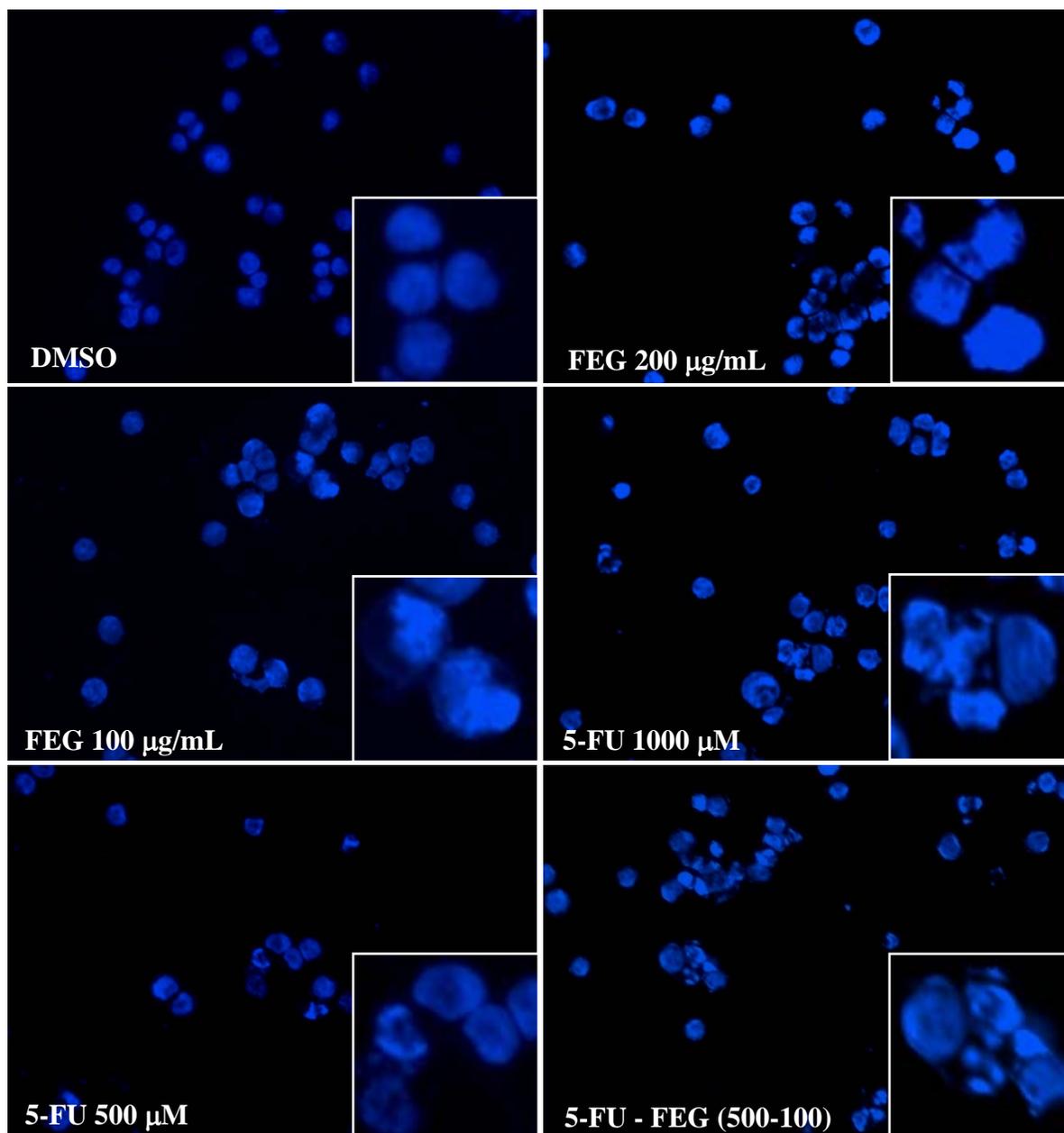


Figure 3. The combined treatment with Dox/5-FU and FEG induced nuclear shrinkage and chromatin condensation on WiDr cells. A. The cells were treated with the indicated concentration of 5-FU, FEG, or 5-FU+FEG for 24 hs. The cells fixed with cold methanol and stained with DAPI and quantified for percentage of condensed nuclei. B. To determine whether all treatment caused the morphological changing, the cells morphology was examined under fluorescence microscope with 400x in magnification.

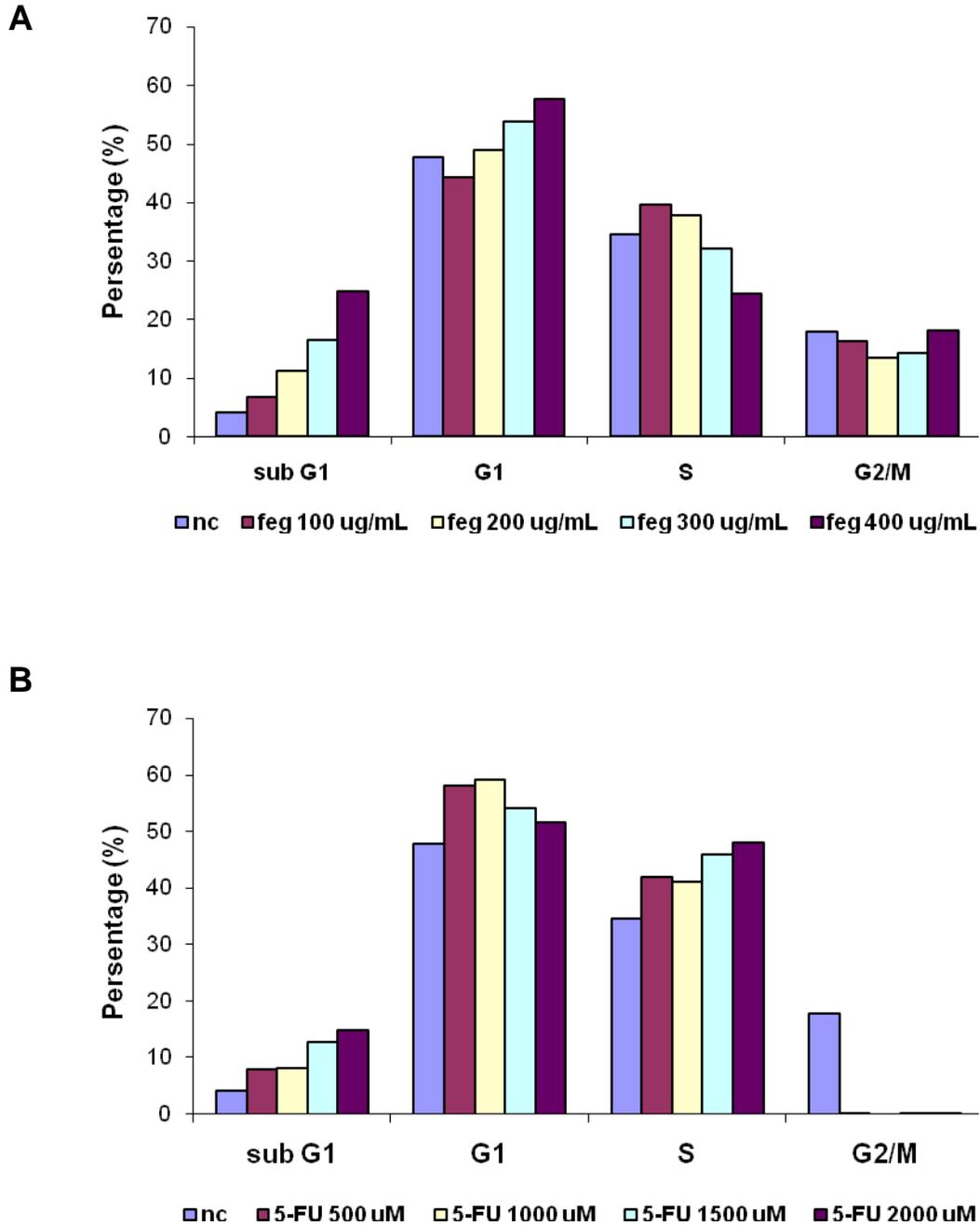
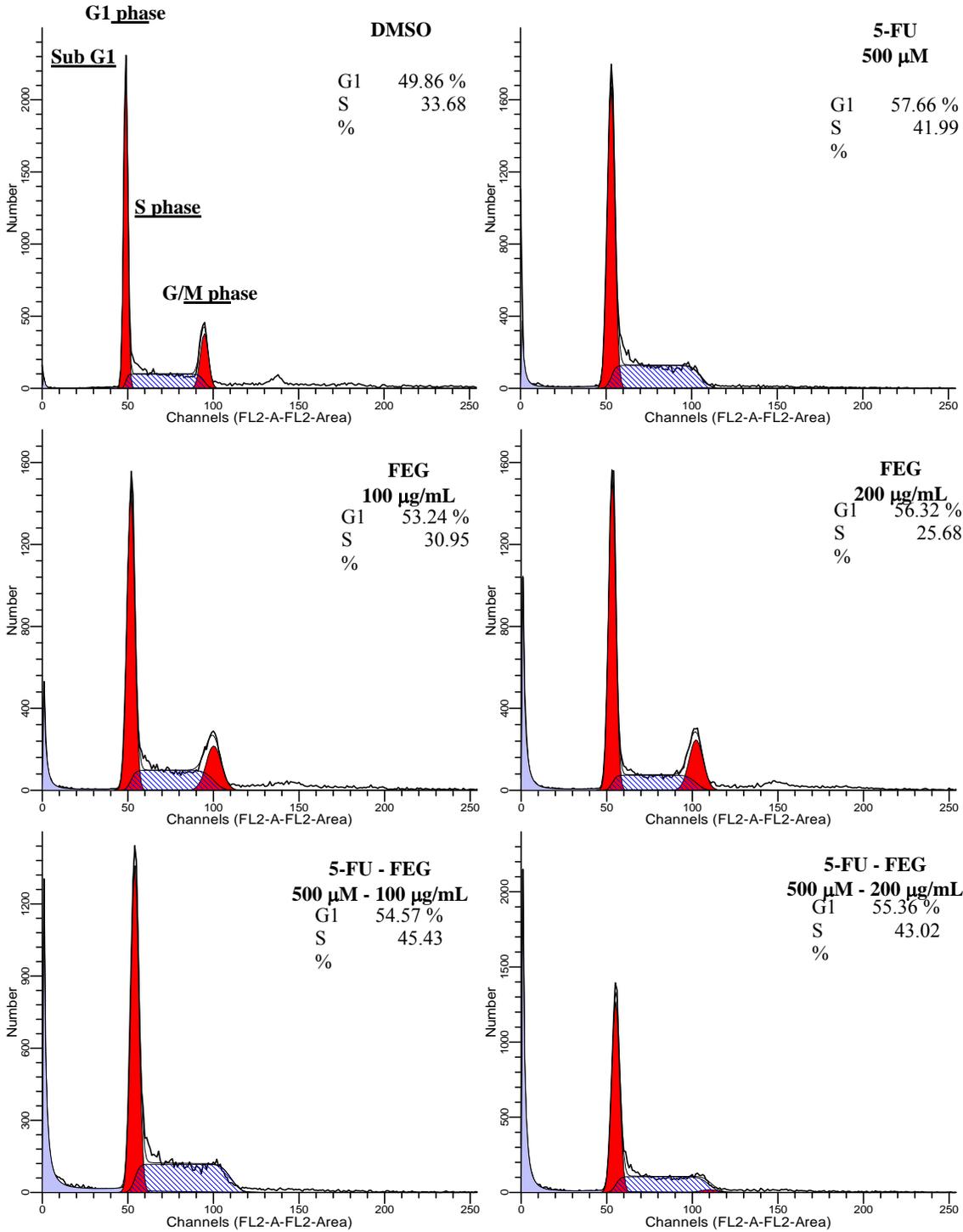


Figure 4. The effect of 5-FU and FEG on cell cycle progression. (A and B) The cells were treated with the indicated concentration 5-FU and FEG, then analyzed by flowcytometry. 5-FU caused S phase arrest after 24 hs incubation in dose dependent manner, while FEG produced G1 arrest. The results are from one representative data of two experiment that showed similar results.

A



B

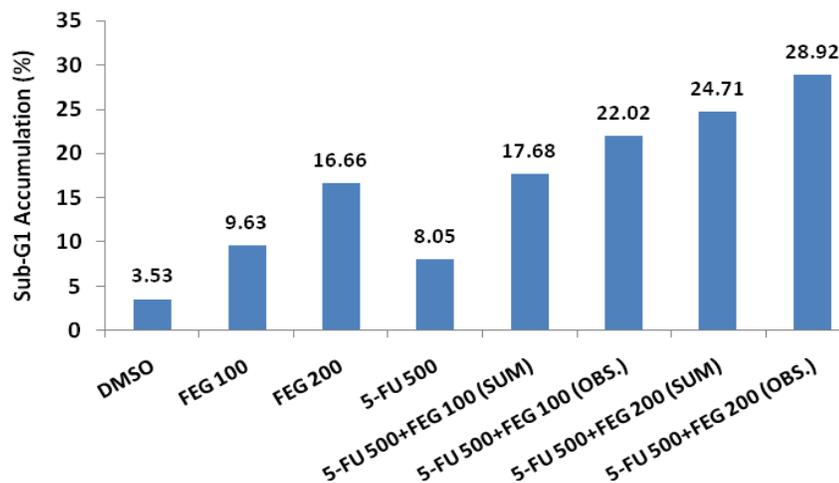


Figure 5. The 5-FU+FEG resulted G1 and S phase arrest and produced synergistic effect on sub-G1 accumulation. The cells were treated with the indicated concentration of Dox, 5-FU, FEG, or 5-FU+FEG for 24 hs. (A and B) The DNA content was analyzed by flowcytometry. The observed effect of sub-G1 induction shows higher when compared with the sum of single one and to the effect of 5-FU alone. The results are from one representative data of two experiment that showed similar results.

Table 2. The cell cycle distribution on WiDr cells after treatment of 5-FU, FEG and their combination for 24 hours

Treatment	Percentage (%)		
	G1-phase	S-phase	G2/M-phase
DMSO	49.86	33.68	16.47
FEG 100 $\mu\text{g/mL}$	53.24	30.95	15.81
FEG 200 $\mu\text{g/mL}$	56.32	25.68	18.01
5-FU 500 μM	57.66	41.99	0.35
5-FU -FEG (500 μM -100 $\mu\text{g/mL}$)	54.57	45.43	0.00
5-FU-FEG (500 μM -200 $\mu\text{g/mL}$)	55.36	43.02	1.62

The combined treatment with 5-FU+FEG induced nuclear shrinkage and chromatin condensation on WiDr cells

For further investigation on cell death induction, we predominantly focused on the combined treatment of 5-FU and FEG compare with single treatment. The morphological examinations of WiDr cells showed the difference between the treated groups and the control. after the single treatment of 5-FU, CISP, or FEG, cells were detached from the surface and contained some debris, whereas the control group was well spreaded with a normal morphology. The chromatin condensation and membrane shrinkage were showed at WiDr cells after treated by single treatment of 5-FU and FEG while at control cell (DMSO) no condensed nuclei found. The chromatin condensation, membrane shrinkage, and also nuclear fragmented were showed extensively after cell treated by combined treatment of 5-FU and FEG. These phenomenon especially nuclear fragmented indicated the DNA fragmentation and nuclear membrane damage as one of main indicators for late apoptosis (Figure 3). All the result suggested that FEG increased 5-FU-induce cell death. Chromatin condensation, membrane shrinkage and nuclear fragmented addressed to apoptosis cell death.

The FEG resulted additive to synergism effect on sub-G1 accumulation when combined with 5-FU and also resulted G1 and S phase arrest.

To determine whether the anti-proliferative activity of FEG as a single or combination treatment with 5-FU would lead to cell cycle arrest and cell death, we analyzed the cell cycle distribution of WiDr cells using fluorescence-activated cell sorting (FACS). FEG treatment alone caused on sub-G1 accumulation referred to cell death induction trough apoptosis or necrosis. FEG modulated cell cycle progression resulting G1 phase arrest in dose dependent manner (Figure 4A). 5-FU treatment exhibited very significance cell cycle arrest both on G1 and S phase, but just produced a slight sub-G1

accumulation (Figure 4B). Combined treatment of 5-FU and FEG exhibited higher accumulation on sub-G1 measurement if compared with single one (both FEG and 5-FU). This combination also caused both G1 and S phase arrest. The combination treatment mainly produced strong inhibition on S phase (Figure 5A). As we know that 5-FU already well-known as a DNA synthesis inhibitor could lead both of G1 or S phase arrest at cell cycle progression. Co-treatment with FEG enhanced this effect shown by the increasing of S phase measurement (Table 2). Combined treatment also caused increasing of cell death induction shown by sub-G1 measurement (Figure 5B). Thus, FEG augmented 5-FU-modulated cell cycle shown by G1 and S phase arrest. The increasing of sub G1 accumulation refered to cell death induction.

Discussion

WiDr colon cancer cell line, has molecular characteristic with mutated p53. Tumor cells with p53 mutant have less respond to apoptosis-induced agent and cell become resistant to DNA damage and apoptosis as target. For long-term usage chemotherapy agent, cancer cell become resistance to several chemotherapy agent. Development of drug resistance in human tumor is primary cause of the failure of chemotherapy. The extract and fraction of *G. procumbens* represented many flavonoid compound as identified as flavon/flavonol [5]. Previous study of *G. procumbens* has many pharmacological effects supporting its anticancer properties. *G. procumbens* extract and fraction exhibited high antioxidant shown by xantine oxidase inhibition and radical scavenging activity [4]. Since uncontrolled cellular ROS production and unbalanced antioxidant protective have important part on cancer development, chemoprevention and co-chemotherapy using *G. procumbens* may support colon cancer treatment with its resistance problem without higher side effect risk. The 5-FU, Cisp, or FEG treatment on WiDr cells caused cell growth inhibition and morphological changing in dose dependent manner. The result of

cytotoxic assay exhibited more sensitive to CISP compared to 5-FU on WiDr cell, as shown by IC_{50} value ($1/10 IC_{50}$ 5-FU). The long expose of 5-FU as establish chemotherapy for colon cancer was known to lead colon cancer resistance [16]. 5-FU and CISP are chemotherapy agent for colon cancer as single and combination usage with other chemotherapy agent. 5-FU has spesifik target on DNA synthesis while CISP could be intercalated with DNA and lead to DNA damage. Combined treatment of 5-FU and FEG produced additive to slight synergistic effect on cell growth inhibition at sub-toxic concentration on WiDr cells. The usage of *G. procumbens* as co-chemotherapy agent sensitized cancer cells therefore at sub-toxic concentration 5-FU resulted higher effect than that of single one at toxic concentration. Meanwhile combined treatment Cisp+FEG resulted to antagonistic effect as shown by high value as CI ($CI > 1,1$). Combination treatment of 5-FU and FEG caused morphological changes more extensive compare to single treatment. Opposited with that phenomenon, combination treatment of CISP and FEG gave no effect on cell morphology compared with control. Combination treatment of 5-FU and FEG also produced inhibition effect on cell proliferation higher than single one. Conversely, CISP and FEG treatment exhibited lower effect than 5-FU as single one. We determined the antiproliferative potential of 5-FU, CISP, and FEG as single and combination treatment on WiDr cells. In parallel with previous cytotoxic effect as combination treatment, just 5-FU and FEG combined treatment produced stronger inhibition on cell growth and proliferation.

WiDr cells were proved more resistance to 5-FU than that to CISP, shown by higher IC_{50} value. The long usage of 5-FU lead to colon cancer resistance with the increasing BCl-2 and BCl-x1 and also suppressing the Bax level was determined as the marker of cells resistance development [16]. The ethyl acetate fraction of *G. procumbens* previously resulted the increasing of p53 and Bax on breast cancer cells [17]. Ethyl acetate fraction of *G. procumbens* may contain β -

sitosterol and stigmasterol [11]. kaempferol-3-O-Rutinoside, astragalinalin and quercetin [4]. Kaempferol resulted cell growth and proliferation inhibition, and also increase the Bax and decrease BCl-2 protein level, leading to apoptotic induction trough mitochondria dependent [18].

For further analysis, the nuclear cells stained with DAPI and PI to assessed nuclear changes that might be correlated with cell death phenomenon. 5-FU and FEG as single and combination treatment caused morphologically changes as identified as chromatin condensation. Combined treatment with 5-FU+FEG was not only generated more extensive chromatin condensation, but also lead to nucleus fragmentation. Cell death induction effect also was shown by the increasing sub-G1 accumulation on cell cycle analysis. The 5-FU+FEG treatment resulted additive to slight synergistic effect on cell death induction. Chromatin condensation was one of marker for apoptosis. Because of WiDr cells have p53 mutant, so apoptosis induction on WiDr cells may be through p53 independent.

The cell cycle analysis was conducted to determine the modulation effect of 5-FU and FEG as single and combined treatment on WiDr's cell cycle. As reported before that kaempferol as one of compound may contain in FEG, caused significance increased in S-phase in cell population on MDA-MB453 breast cancer cell line [19]. Combination between quercetin and several chemotherapy agent for colon cancer, such as oxaliplatin, leucovorin, and 5-FU, increased superoxide dismutase and decreased BCl-2 expression through inhibition of NF κ B activity. This combination suppressed HT29 cell growth and prolong survival on *in vivo* experiment [20]. 5-FU and FEG as single treatment could modulate cell cycle. 5-FU is already well known as antimetabolite compound at DNA and RNA synthesis process. WiDr cells arrested at G1 and S phase after 5-FU treatment. Single treatment of FEG lead to G1 phase arrest, might cause protein synthesis inhibition at early phase of cell cycle. Therefore the cell will be accumulated at restriction point before entering S

phase. Combined treatment 5-FU+FEG resulted to accumulation G1 and S phase with dominant on S phase arrest. Combination treatment of 5-FU treatment with sub-toxic concentration produced higher inhibition of DNA and RNA synthesis than that of single one. It might be caused by sensitized effect of FEG to WiDr cells

Conclusion

The FEG as co-chemotherapy treatment with 5-FU on WiDr cell exhibited additive to slight synergistic effect on proliferation inhibition and apoptosis induction, but Cisp and FEG treatment produced antagonism effect. Combined treatment of 5-FU and FEG arrested cell on G1 and S phase. This combination has potency to develop as combination therapy that mainly aim to reduce side effect and suppress resistance of chemotherapy agent. The extended research need to explore the mechanism and interaction of combined treatment for colon cancer.

Author's Contribution

NAN have made conception and design of this study, acquisition of data, analysis and interpretation and statistical of data, and drafted the manuscript. EM, S, EisM, and MK analyzed and interpreted data and reviewed the manuscript. All authors have already read and approved the final manuscript.

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