

Cytotoxic effects and anti-proliferative cancer activity of coelomic fluid from *Lumbricus rubellus* promotes apoptosis and reduces G2/M phase progression in HT-29 cells

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ABSTRACT

This study has investigated the effect of chemotherapy drugs combined Coelomic Fluid (CF) derived from *Lumbricus rubellus* in HT-29 cells. HT-29 cells were treated with a combination of 5-fluorouracil (5-FU) (5 µg/ml) and Coelomic Fluid Lumbricus (CFL) (5 µg/ml, 10 µg/ml, or 20 µg/ml), respectively. The cells were incubated for 24 hours and then analyzed by flow cytometry to assess apoptosis, cell cycle progression, and proliferation. The cytotoxicity effect was measured by MTT assay that causes 50% decrease in cell viability (IC₅₀ value). Results showed that the percentage of apoptosis in combined-therapy groups significantly higher than 5-FU single therapy group ($p < 0.001$). Additionally, the percentage of proliferation and cell growth significantly inhibited of 5-FU and CFL combination groups ($p < 0.001$). However, cell cycle slightly reduced the percentage of G2/M phase. Our results showed that CFL inhibited cell proliferation that is strictly dependent on colon cancer. These findings suggest that CFL have cytotoxicity effect to inhibit cell growth, anti-proliferative activity and increased apoptosis against human colon cancer cells.

INTRODUCTION

Colorectal cancer is the third most common cancer in the world, with 1.4 million cases diagnosed and 694,000 deaths per year. Colorectal cancer accounts for 10% of the total cancer cases in men (746,000) and 9.2% of the total cases in women (614,000) (Ferlay *et al.*, 2015). In Indonesia, the rate of incidence of colorectal cancer is 19.1% for men and 15.6% for women (Ferlay *et al.*, 2010). Colon cancer is a colonic epithelium disease identified by the loss of control of malignant cell growth, resulting in the invasion of surrounding tissue and other organs (Niederhuber *et al.*, 2014).

The common treatment for colon cancer is surgery, with a high recovery rate at early stages and utilization of radiotherapy

and chemotherapy drugs at advanced stages (Mishra *et al.*, 2013). One therapeutic agent, adjuvant 5-fluorouracil (5-FU), is consumed by patients at high risk in the second or third stage (Watanebe *et al.*, 2015). The active metabolites of 5-FU, fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FTUTP), can inhibit the activity of thymidylate synthase (Chua *et al.*, 2011). However, the therapy presents side-effects such as neutropenia, stomatitis, diarrhea, and cardiotoxicity (Ciccolini *et al.*, 2010). Increasing the activity of 5-FU by combination treatment with natural materials is an important avenue to explore for the treatment of colon cancer. A combination treatment of 5-FU and a natural material could increase the sensitivity of cancer cells by capturing cell cycle mechanisms, inhibiting proliferation, and promoting apoptosis (Redondo-Blanco *et al.*, 2017). Recent epidemiological studies show that herbal treatment improves prognosis in patients with colon cancer; therefore, it is possible to further explore for new

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target therapy in the future (Au-Yeung and Ko *et al.*, 2010).

Earthworms are reported to possess bioactive molecules that can be used as remedies for various diseases, especially cancer (Cooper *et al.*, 2012). *Eisenia foetida* has coelomic fluid (CF) macromolecules capable of significantly inhibiting the proliferation of HeLa cells and human lung adenocarcinoma cell line LTP-A2 (Zhang *et al.*, 2011). Administration of CF from *Eudrilus eugeniae* has shown increased apoptotic activity in SiHa cells, and 85% cell death in lung cancer cell line A549 and colorectal tumor cell line HTC 116 (Jaabir *et al.*, 2011; Vidya *et al.*, 2016).

Proliferation, cell cycle regulation, and apoptosis are targets for cancer therapy. This study reviews the role of these targets in colon cancer and treatment strategies using various inhibitors. The distinct mechanisms between 5-FU and CFL by combination therapy are expected to enhance the performance of 5-FU in colorectal cancer therapy and improve its therapeutic efficacy.

MATERIALS AND METHODS

Lumbricus rubellus

Earthworm was collected from a commercial vermiculture unit CV Rumah Alam Jaya Organik, Malang and maintained in a container containing decomposed organic matter. All experimental procedures were approved by the Ethical Committee Brawijaya University, Malang, Indonesia (No. 299/EC/KEPK/08/2017).

Preparation of coelomic fluid *Lumbricus rubellus*

Earthworm was weighed for 15 grams and washed in distilled water. They were placed in boxes which have filter paper and were transferred to a new box in order to be given heat and cold treatment (Dinesh *et al.*, 2013). Briefly, water in a glass beaker (10 ml) was prepared by heating in an electric water heater with an inserted inside it to measure the temperature between 45-50°C was used to give the heat shock. The ice pack prepared and put in plastic was used to give the cold shock. The heat and cold shock treatment were done at 3 minutes and repeated until the earthworm collapse they were given stimulus from beaker glass filled hot water and ice pack in the plastic shifting on the surface of the earthworms' body CFL were released through the back pores of the body due to "heat and cold shock". CFL was collected in a sterile Eppendorf tube using a pipette. The filtrate was stored in aliquots at -20°C for further use. The supernatant measured on nanodrop to see protein levels.

Cell culture and treatment

HT-29 cell line was obtained from American Type Culture Collection (ATCC® HTB38™) was cultured in McCoy's 5A (Sigma, USA) culture medium supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 1% penicillin-streptomycin and 1% amphotericin and maintained at 37°C in a humid atmosphere containing 5% CO₂. Passage of the cells was achieved every 3 days through trypsinization (0.25% trypsin (GIBCO)) and then the cells were treatment with or without or different concentration of CFL (5, 10, 0 µg/ml), respectively, for 24 hours.

Apoptosis analysis

HT-29 cells were cultured in a 24-well plate at a density of 2×10^5 /plate. The cells were treated with several concentrations of 5-fluorouracil (5-FU) 5 µg and CFL (5, 10 and 20 µg/ml) for 24 h and collected by trypsinization. The cells were washed with phosphate buffer saline (PBS). For each sample, 2.5 µg/ml Annexin-V-FITC and 50 µg/ml propidium iodide (PI) (Biolegend) were added to the cell suspension and incubated for 5 min at room temperature (25°C) in the dark. Cell death by apoptosis was scored by quantifying the population of Annexin V-FITC-positive cells. Flow cytometry data were plotted and analyzed by Cell-Quest software (Becton Dickinson FACS Calibur).

Cell cycle analysis

HT-29 cells were cultured in a 24-well plate at a density of 2×10^5 /plate. The cells were treated with several concentrations of 5-FU 5 µg and CFL (5, 10 and 20 µg/ml) for 24 hours and collected by trypsinization. The cells were washed with PBS and then, suspended in 25 µg/ml propidium iodide (PI) (Biolegend) solution and incubated at 4°C in the dark for 25-30 minutes. The cell cycle percentage was measured by Flow cytometry and then analyzed by Cell-Quest software (Becton Dickinson FACS Calibur).

Cell proliferation analysis

HT-29 cells were cultured in a 24-well plate at a density of 2×10^5 /plate. The cells were treated with several concentrations of 5 µg 5-FU and CFL (5, 10, and 20 µg/ml) for 24 hours and collected by trypsinization. The cells were washed with PBS and then, labeled with bromodeoxyuridine (BrdU) conjugated fluorescein (FITC) for 30 minutes at 4°C. The cell proliferation was measured by flow cytometry analyzed by Cell-Quest software (Becton Dickinson FACS Calibur).

Cytotoxic assay

HT-29 cells were cultured in a 96-well plate at a density of 1×10^3 /well. The cells were treated with 5 µg of 5-FU and different concentrations of CFL (5, 10 and 20 µg/ml) for 24 hours. The cells were treated with 20 µl MTT (Sigma, USA) solution and followed with incubation at 37°C for 4 hours. Subsequently, 150 µl of solvent MTT was added and shaken the plate for 15 minutes. The quantity of formazan (presumably directly proportional to the number of viable cells) was measured by recording changes in absorbance at 630 nm using spectrophotometer. The inhibition concentration was calculated following the formula:

$$\text{Inhibitory \%} = \frac{(\text{Control absorbance} - \text{control medium absorbance}) - (\text{Treatment absorbance} - \text{control medium absorbance})}{(\text{Control absorbance} - \text{control medium absorbance})} \times 100\%$$

Statistical analysis

The results were analyzed by one-way ANOVA followed by Post Hoc Tukey test. Pearson correlation and regression test to determine the relationship between each group. Data were described as a mean \pm standard deviation. Statistical significance of a difference between groups, with $P < 0.05$ being considered

significant. Statistical analysis was performed with SPSS Version 11.0 statistic software package (SPSS Inc, Chicago IL, USA).

RESULT AND DISCUSSION

Results

Synergistically effect of 5FU and coelomic fluid induced apoptosis

In our research, we evaluated the response of CFL and 5-FU combinations. We investigated that the apoptosis effect of cervical cancer HeLa cells in the combination of 5-FU and coelomic fluid on HT-29 cells using double staining method

FITC-conjugated annexin V and PI. The results of flow cytometry showed the percentage of apoptosis significantly increase on the combination of 5-FU and CFL ($P < 0.05$) followed by increasing concentrations of CF (Figure 1A and B). The administration of CFL at dose in group IV (10 $\mu\text{g/ml}$), $P < 0.001$; group V (20 $\mu\text{g/ml}$), $P < 0.001$ increased the levels of apoptotic cell presentation compared single therapy group. These results suggested that combination of 5-FU and CFL had synergistically increased apoptosis in HT-29 cells. In this study, a combination of 5-FU with 10 and 20 $\mu\text{g/ml}$ CFL able to suppress carcinogenesis by increasing the apoptosis of HT-29 cells. Therapy combination of 5-FU and different concentration of CFL (5, 10, 20 $\mu\text{g/ml}$) significantly enhances cell apoptosis ($P < 0.05$).

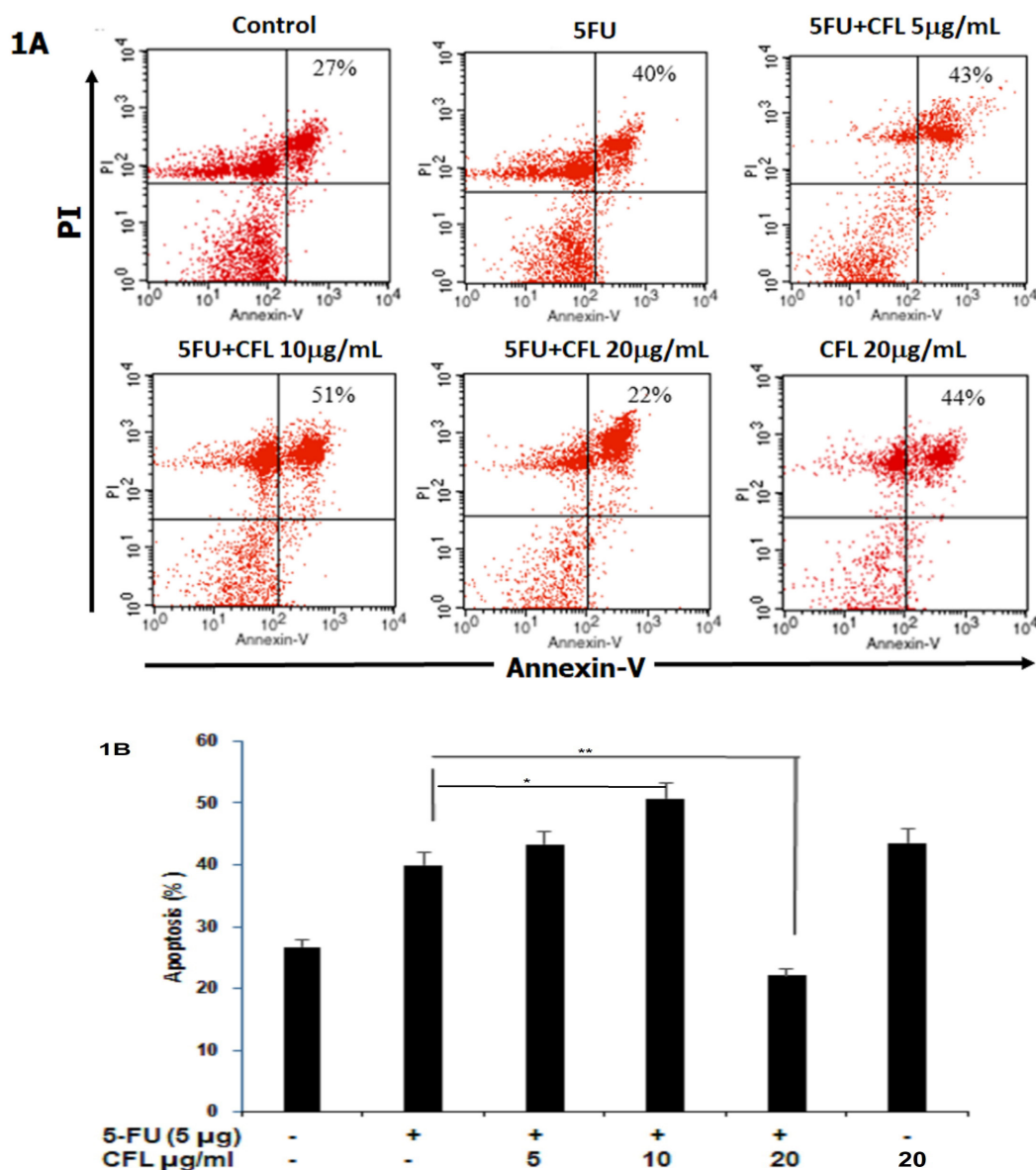


Fig. 1: The effect of 5-FU with or without coelomic fluid on the percentage of apoptosis. The effect of the combined 5-Fu and CFL to the apoptosis on HT-29 cells were treated with 5-Fu with or without 5, 10, and 20 $\mu\text{g/ml}$ CFL were added for another 24 hours. (A) The percentage of apoptosis cells were analyzed using a FACS Calibur flow cytometer. Representative results of four replicates in each group are shown. (B) The combination of 5-Fu and CFL increased the percentage of apoptosis in HT-29 cells. Results shown are mean \pm SD, with $n = 4$ replicates in each group. * $P < 0.05$, ** $P < 0.001$ versus 5-FU only group.

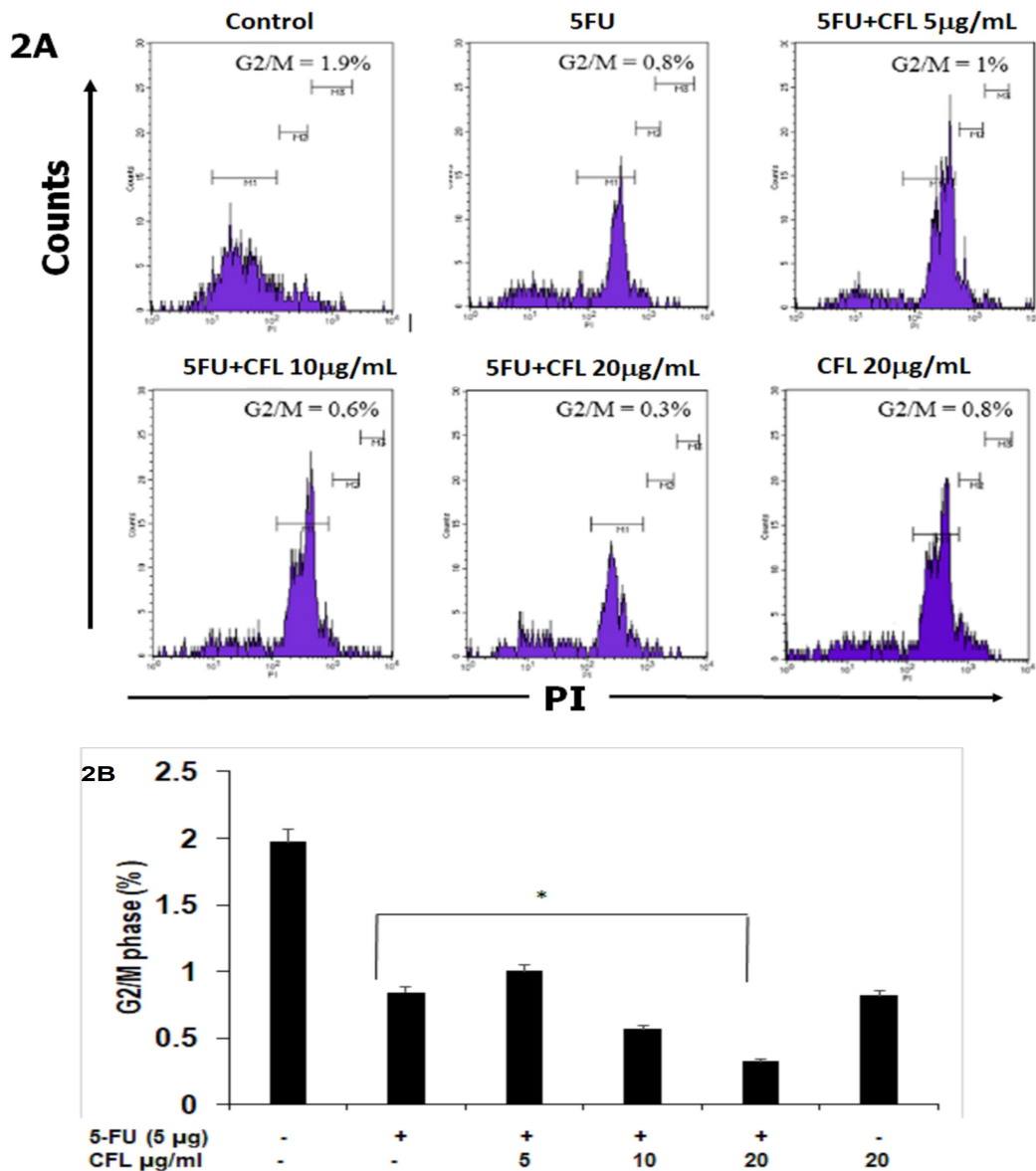


Fig. 2: Effects of 5-FU with or without coelomic fluid on the G2/M phase in HT-29 cells. HT-29 cells were treated with 5 µg 5-FU with or without CFL (5, 10, and 20 µg/ml) were added for another 24 hours. (A) Percentages of G2/M phase in each histogram were shown inside the panels. (B). Numbers represent the percentages of G2/M phase in each histogram were shown. The percentage of G2/M phase decreases in all treatment groups compared with 5-FU only. Results shown are mean + SD, with $n = 4$ replicates in each group. * $P < 0.05$, ** $P < 0.001$ versus 5-FU group.

The combined effect of 5-FU and coelomic fluid inhibits the G2 phase of the cell cycle

To know the combination effect of 5-FU and CFL on cell cycle progression at G2/M phase involved in this study, we determined the percentage of G2/M phase. The histograms showed the percentage of the cell cycle in HT-29 cells was treated with 5-FU 5 µg and CFL (5, 10, and 20 µg/ml) for 24 hours. The G2/M phase of the cell cycle was represented on the histogram as M3 (Figure 2A). The effect of combination therapy shown slightly reduce the percentage of G2/M phase in the cell cycle (Figure 2B). The G2/M phase in combination

groups decreased 66.5% compared with single therapy group. These results indicated the percentage of G2/M phase slightly increased in CFL combinations groups compared to the single therapy group.

The combination therapy of 5-FU and CFL inhibit proliferation in HT-29 cells

HT-29 cells were treated using the single 5-FU with or without CFL in various concentrations (5, 10, and 20 µg/ml), proliferation was observed by anti-BrdU staining using flow cytometry. The proliferation was represented on the histogram

as M1 (Figure 3A). HT-29 cells have undergone proliferation as induced by the combination of 5-FU and 20 µg/ml CFL. Our data presented the percentage of cell proliferation significantly

decreased ($P < 0.05$) in 20 µg/ml concentration (Figure 3B). In this study, a combination of 5-FU and CFL suppressed carcinogenesis by reducing the proliferation of HT-29 cells.

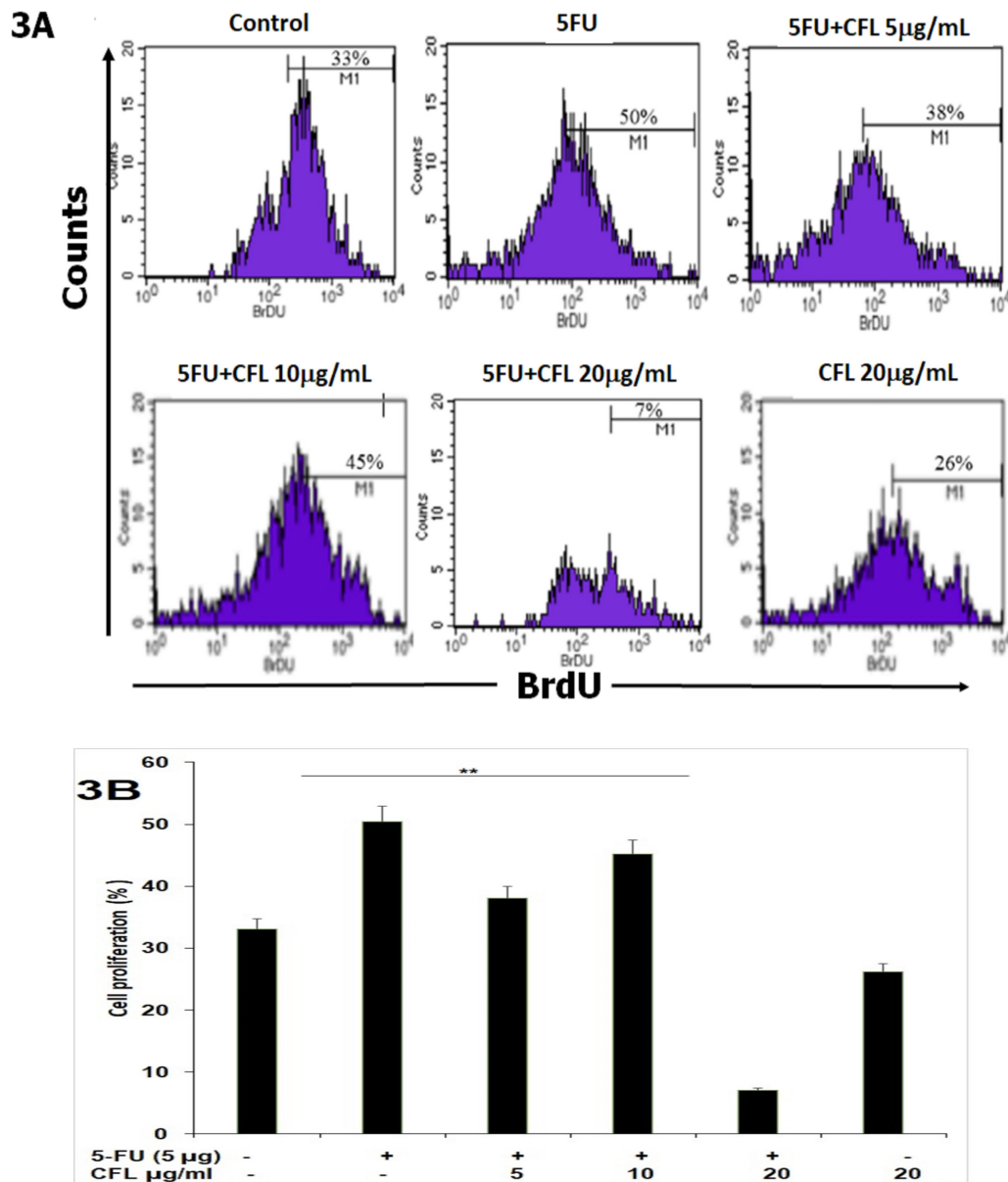


Fig. 3: The inhibitory proliferation of 5-FU with or without coelomic fluid on the growth of HT-29 cells. (A). The proliferation of HT-29 cells following 24 hours treatment of 5-FU with or without CFL (5, 10, and 20 µg/ml). The percentage of BrdU cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences). Representative results of four replicates in each group are shown (B). Numbers represent the percentages of BrdU in each histogram are shown. Results shown are mean \pm SD, with $n = 4$ replicates in each group. * $P < 0.05$, ** $P < 0.001$ versus 5-FU only group.

The combination effect of 5-FU and CFL on cytotoxic effect

Combination therapy of 5-FU and CFL able to kill HT-29 cells effectively. The cytotoxic effect was represented on the histograms. The combination of 5-FU and CFL with various concentrations (5, 10, and 20 µg/ml) could kill HT-29 cells by 13%, 29%, and 87%, respectively (Figure 4). CFL enhances the cytotoxicity effect of 5-FU in inhibiting the growth of HT-29 cells. ($p < 0.05$). These results suggested that combination of 5-FU and

CFL inhibited cell growth.

Discussion

Colon cancer is caused by many factors, including chronic inflammatory disease, environmental effects, and lifestyle (Endharti *et al.*, 2016, Endharti *et al.*, 2017a, Endharti *et al.*, 2017b). Currently, the most widely used chemotherapy drug is 5-Fluorouracil (5-FU) (Chua *et al.*, 2011). 5-FU is frequently

used in *in vitro* research using colon cancer cell line HT-29 for developing antiretroviral drugs against anti-apoptotic activity (Qiu *et al.*, 2015).

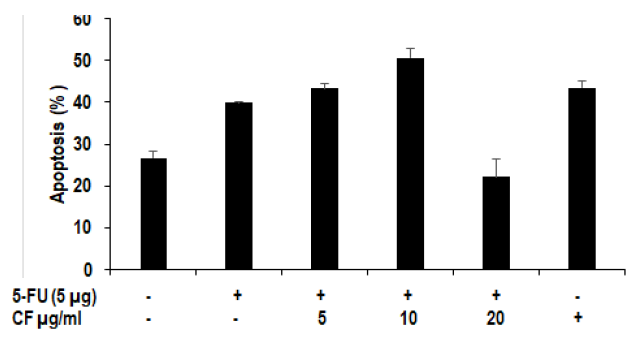


Fig. 4: The cytotoxic effects of the combination of 5-FU and coelomic fluid against HT-29 cells. The cytotoxic effect of 5-FU with or without different concentration of coelomic fluid (5, 10, and 20 µg/ml) on HT-29 cells determined by MTT assay. Values represent mean \pm SD with triplicate determinations for 50% inhibitory concentration (IC₅₀). Data values represent (mean \pm SD) that obtained in triplicate assays from four independent experiments (* p < 0.05 or ** p < 0.05 compared with 5-FU as control).

CFL of earthworms reported have the function of cell proliferation and apoptosis in HeLa cells. (Zhang *et al.*, 2011). CFL contains coelomic cytolytic factor-1 (CCF-1) and lysenin, which have known cytotoxic effects on *in vitro* cancer cells (Chen *et al.*, 2007; Kobayashi *et al.*, 2004). CCF-1 acts similar to tumor necrosis factor alpha (TNF- α), which binds the tumor necrosis factor receptor 1 (TNFR1) to activate the proliferative pathway via mitogen-activated protein kinase (MAPK) (Mancikova, 2011). It has been suggested that CFL containing CCF-1, analogous to TNF- α , binds TNFR1 and thereby inhibits the cell proliferative mechanism (Horsen *et al.*, 2006). Lysenin, through ceramide-sphingomyelin pathways, is essential for cell apoptosis, proliferation, and cell cycle inhibition (De Colibus *et al.*, 2012).

Our study has confirmed that CFL treatment with or without combination drug chemotherapy has an effect on the colon cancer cell line HT-29. CFL was used to propose as a way to minimize doses of 5-FU as a chemotherapy agent and thus minimize resistance to chemotherapy, which contributes to most cancer-related deaths. In addition, with increasing of CFL concentration, cell cycle changed markedly including the percentage of G2/M phase decreased, but not a significant difference between groups. Our study found that the combination therapy able to enhance the percentage of apoptosis. The apoptosis was associated with DNA damage that stops DNA replication (Kracikova *et al.*, 2013) and G2/M checkpoint prevents cells from undergoing mitosis before they have a chance to repair damaged DNA after replication (Medema and Macůrek, 2011).

Lumbricus rubellus has a cytotoxic effect through CFL containing lysenin protein (Kobayashi *et al.*, 2004; Endharti *et al.*, 2018). Lysenin activates caspase 9 to signal the mitochondrial pathway to promote apoptosis (Chalfant *et al.*, 2002; Ghosh *et al.*, 2007).

The combination of 5-FU and CFL in a dosage-dependent manner is simultaneously capable of increasing

apoptosis with inhibiting cell proliferation, capable of increasing cytotoxic effects but does not alter the cell cycle. The maximum dose of coelomic fluid that increased cytotoxic effects was 5 µg/ml, that increased cell apoptosis was 10 µg/ml and that decreased cell proliferation was 20 µg/ml.

CONCLUSION

In conclusion, the study showed that the combination of 5-FU and coelomic fluid able to enhance the percentage of apoptosis cells, cytotoxic effect and inhibit cell proliferation of HT-29 cells. Our results showed that CFL offers a promising anticancer candidate. These findings highlight the new therapeutic concept which combines 5-FU chemotherapy agents and coelomic in the treatment of colon cancer.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this paper.

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