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by Joko Setyono

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Zingiber officinale Var. Rubrum Extract Increases the Cytotoxic Activity of 5-Fluorouracil in Colon Adenocarcinoma Widr Cells

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ABSTRACT

One of the main chemotherapy agents used to treat colon cancer is 5-fluorouracil (5-FU). However, over the years, the effectiveness of this medication has significantly decreased, therefore, co-chemotherapy with another agent is needed to enhance the activity. This study evaluates the effect of *Zingiber officinale* var Rubrum (red ginger) extract as a co-chemotherapy agent with 5-FU in the WiDr human colorectal cancer cell line. The MTT assay used to determine the cytotoxic effect for 24 and 48 hours. A 48 hours treatment of 5-FU in WiDr cells showed an IC_{50} value of 130 µg/mL, with none obtained during the 24 hours treatment. Evaluation of the extract for 24 and 48 hours exhibited a cytotoxic effect of 68 µg/mL and 65 µg/mL, respectively. Furthermore, the addition of extract with a concentration less than half of their IC_{50} caused a significant decrease in cell viability than a single application of 5-FU after 48 hours incubation. In conclusion, red ginger extract increases the cytotoxic activity of 5-FU in colon adenocarcinoma WiDr cells.

Keywords: *Zingiber officinale* var Rubrum, 5-Fluorouracil, WiDr cell lines, chemotherapy, cytotoxic effect

INTRODUCTION

In 2018, colorectal cancer was the third most common cancer type in men and the second in women worldwide. According to Ferlay *et al.* (2019), the prevalence of colorectal cancer in Indonesia ranks second after breast cancer. One of the main chemotherapy agents for colorectal cancer is 5-fluorouracil (5-FU), with high selectivity on the thymidylate synthetase (Longley *et al.*, 2003). However, the effectiveness of 5-FU as a single chemotherapy agent has only reached 15%. Therefore, the development of additional agents is needed to increase efficacy. Several chemotherapy agents, such as leucovorin, oxaliplatin, and irinotecan, have been combined with 5-FU to enhance its efficiency (Meyerhardt *et al.*, 2005). Unfortunately, these combinations had adverse effects, such as drug resistance and toxicity at high doses (Saif *et al.*, 2009).

A more recent approach in overcoming the above problems is the combination of chemotherapy agents with natural product secondary metabolites. This combination increases metabolic activities in low-dose drugs. Over the past few years, numerous studies have been conducted using natural ingredients combined with 5-FU (Hakim *et al.*, 2014; Nurulita *et al.*, 2011; Nur *et al.*, 2011). The natural ingredients studied and known to have anti-cancer activity are red ginger (*Zingiber officinale* cv. Rubrum).

Ginger is one of the herbs widely used in traditional medicine. According to Wahyuni *et al.* (2003), there are three varieties of ginger in Indonesia, namely big ("elephant") ginger (*Z. officinale* var. Roscoe), small ("emprit") ginger (*Z. officinale* var. Amarum), and red ginger (*Z. officinale* var. Rubrum). Red ginger has smaller rhizomes and a spicier taste due to differences in the content of chemical compounds compared to the other two varieties. It contains 6-gingerol and 6-shogaol compounds known to possess anti-cancer effects (Li *et al.*, 2015; Wu *et al.*, 2015;

Hwang *et al*., 2015; Kim *et al*., 2015; Prasad *et al*., 2015; Hsu *et al*., 2015; Han *et al*., 2015; Fan *et al*., 2015; Radhakrishnan *et al*., 2014; Pan *et al*., 2008). Studies have shown that combining chemotherapy agents with natural ingredients is one of the promising approaches to overcoming cancer treatment problems. Natural ingredients have been used in traditional medicine with anti-cancer activity. In this study, a combination of 5-FU and red ginger was tested on WiDr colon cancer cells to evaluate the potency of red ginger as co-chemoterapeutic agent.

MATERIAL AND METHODS

Z. Officinale var Rubrum was obtained in powdery form from Merapi Farma Herbal (Hargobinangun, Pakem, Sleman, Yogyakarta). The plant authentication was carried out at the Plant Taxonomy Laboratory, Faculty of Biology, Jenderal Soedirman University confirmed that the plant is *Zingiber officinale* var. Rubrum, and belongs to the family Zingiberaceae.

Extracts preparation

A total of 1380 grams of the powder was extracted using 4 liters of 96% ethanol for 4×24 hours. The filtrate was filtered once a day and evaporated at 80rpm, 80°C to obtain a thick solvent-free extract with a yield percentage of 7.7%, which is less than 6.6% according to the provisions of the Indonesian Herbal Pharmacopoeia.

Cell culture

WiDr colon cancer cells were obtained from the Laboratory of Parasitology, Gadjah Mada University, Yogyakarta, Indonesia. They were grown in RPMI 1640 media (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco).

Determination of cytotoxicity

Cytotoxicity of the extract and 5-FU was evaluated by MTT assay. The stock solution (5mg/mL) of the extract was prepared in DMSO (Sigma), while the concentration of 5-FU used in the combination test ranged from 30-1000µg/mL. WiDr cells were harvested using 0.25% v/v trypsin-EDTA (Gibco) and were grown to 96-bottom wells (Iwaki) with a density of 10,000 cells per 100µL culture medium, then incubated at 37°C, using 5% CO_2. Cells were treated with extract concentrations of 15-500µg/mL for 24 and 48h. After incubation, 100µL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) (5 mg/mL in PBS) was added to each well and further incubated for 3hours. The formed formazan crystals were dissolved in 100µL stopper sodium dodecyl sulfate (SDS) 10% in 0.01N HCl (Sigma). Absorbance was measured by spectrophotometer at a wavelength of 595 nm (Biorad) with the cell viability calculated using the following formula % viability = (absorbance of treatment - absorbance of media)/(absorbance of control - absorbance of media) x 100%.

The combined treatment of red ginger extract and 5-FU

The IC_{50} value of the extract and 5-FU were first examined, followed by the combination of 5-FU and the extract at a concentration of less than half of their IC_{50} value in 24 and 48 h. The absorbance was obtained from the co-chemotherapy test by MTT assay, then the cell viability was calculated.

Data analysis

Cell viability data were presented as means ± standard deviation (SD). GraphPad Prism 8.0 (San Diego, CA, USA) was used to generate graphs. Data were analyzed using ANOVA, with the Tukey's range test used to determine the statistical significance. Furthermore, a value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Extract of *Z. officinale* var Rubrum inhibits the growth of WiDr colon cancer cells

Cytotoxicity was evaluated to determine *Z. officinale* var Rubrum extract's potential to inhibit the growth of WiDr colon cancer cells. The results indicated that the treatment of WiDr colon cancer cells with the extract inhibiting the growth (Figure 1). Furthermore, cell viability obtained from 24 and 48h incubation of WiDr cells decreased with the increase in the extract's concentration (Figure 2). Treatment of WiDr cells with the extract at a concentration of 125µg/mL for 24 and 48h incubation inhibited cell growth close to 100%. The cytotoxicity evaluation of the extract showed that the IC_{50} value at 24 and 48h incubation was 68 and 65µg/mL, respectively.

5-FU decreases cell viability of WiDr colon cancer cell

Figure 3 shows that the cytotoxicity test of 5-FU in WiDr cells at 24 and 48 incubation hours decreased in cell viability with an increase in 5-FU

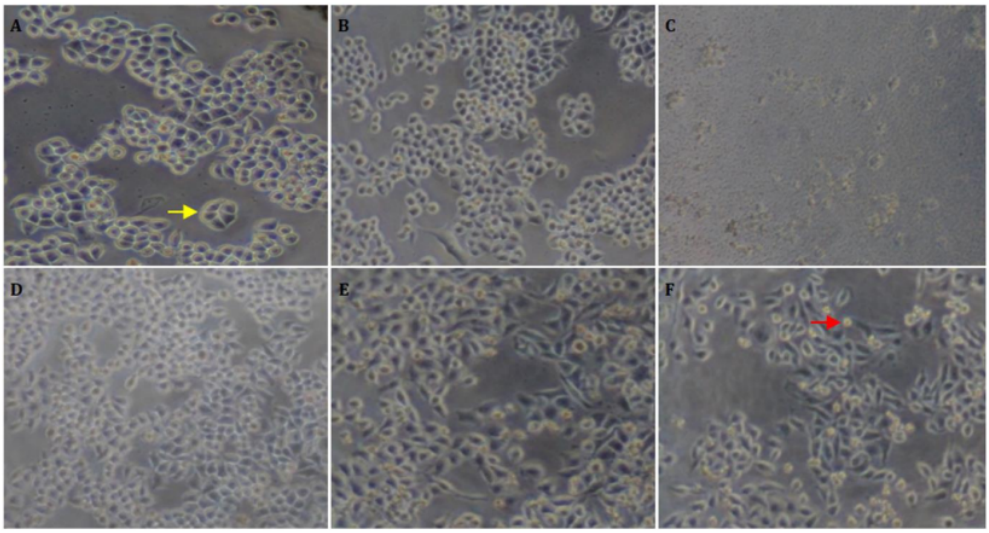

Figure 1. Morphology of WiDr cells after treated with extracts. (A) Control WiDr cells in 24h incubation. (B) Treatment of red ginger extract at 15µg/mL for 24h. (C) Treatment of red ginger extract at 500 µg/mL for 24h. (D) Control WiDr cells in 48h incubation. (E) Treatment of red ginger extract at 15 µg/mL for 48h. (F) Treatment of red ginger extract at 500 µg/mL for 48h. Photographs were taken at 10x magnification. Yellow arrow showed viable cells, while red arrow showed death cells.

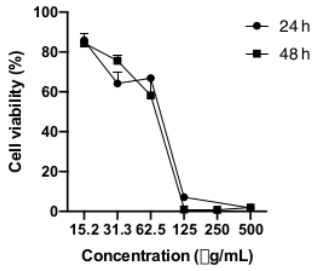

Figure 2. Profile of red ginger extract cytotoxicity on WiDr cells by MTT method. WiDr cells were plated on 96 well-plates then treated with red ginger extract at concentration 15.2, 31.3, 62.5, 125, 250, 500µg/mL for incubation 24 and 48h. Cytotoxicity was expressed by % cell viability shown as mean ± SD.

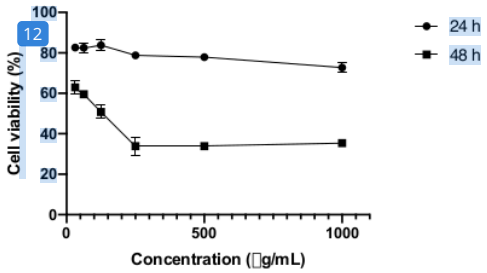

Figure 3. Profile of 5-FU cytotoxicity on WiDr cells by MTT method. WiDr cells were plated on 96 well-plates then treated with 5-FU at concentration 31.3, 62.5, 125, 250, 500, 1000µg/mL for incubation 24 and 48h. Cytotoxicity was expressed by % cell viability shown as mean ± SD.

concentration. Treatment of WiDr cells with 5-FU at a concentration of 1000µg/mL for 24h incubation caused cell viability of 70%, which implies that the highest concentration was unable to inhibit cell growth by 50%. Furthermore, the incubation of WiDr cells with 5-FU at a concentration of 1000µg/mL for 48h produced a cell viability of 35%. The IC_{50} of 5-FU at 24h incubation was undeterminable, while the value at 48h incubation was 130µg/mL.

Combination of *Z. officinale* var Rubrum extract and 5-FU inhibits WiDr colon cancer cell growth.

The combination treatment of 5-FU with the extract was carried out to determine whether the extract was able to increase the cell growth inhibition compared to the single 5-FU treatment. A 24h treatment was carried out to determine the cell viability, with the evaluation carried out using 5-FU at a 1000µg/mL concentration with varying extract concentration of 8.5, 17, 25, and 35 µg/mL. The results shown in Figure 4 indicated that the combination of 5-FU 1000µg/mL and red ginger extract at 17 and 35µg/mL was able to suppress cell viability compared to a single treatment. Similarly, the 48h combination treatment of 5-FU 60µg/mL and red ginger extract at 6.3, 18.7, and 25µg/mL were able to suppress cell viability compared to a single treatment.

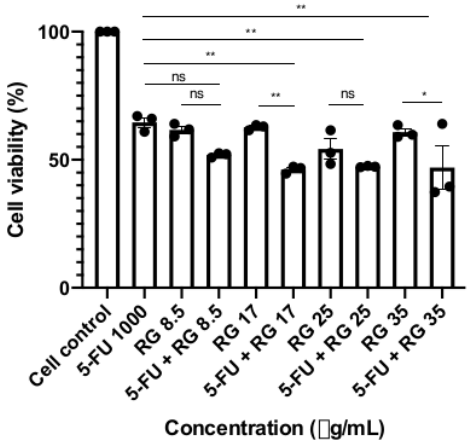

Figure 4. Effect combination of 5-FU and red ginger (RG) extract in WiDr cells at 24 hours incubation. Cells were treated 5-FU at concentrations of 1000 µg/mL, and in combination with various values of red ginger extract, such as 8.5, 17, 25, and 35 µg/mL. Percentage of cell viability was expressed as mean ± SD. Statistical analysis used One-way Anova and the Tuckey's range test. ns = not significance, * p<0.05, ** p< 0,01.

Red ginger is a potential agent used to increase the cytotoxic activity of 5-FU in WiDr colon cancer cells. This study evaluated the cytotoxic of red ginger extract, 5-FU, and their combination of WiDr colon cancer cells. The results showed that red ginger extract increases the cytotoxic activity of 5-FU in WiDr colon cancer cells.

A single treatment of WiDr cells with the extract led to IC_{50} values of 68 and 65µg/mL after 24 and 48h incubation. This value was not significantly different for both treatment and shows that the extract sufficiently inhibits 50% growth of WiDr cells at a 65µg/mL concentration. These data are consistent with the previous research carried out by Ekowati *et al.* (2012), which indicated that the IC_{50} value of the same extract was 74µg/mL after 24h treatment on WiDr colon cancer cells.

The use of *Z. Officinale* var. Roscoe extract on the cytotoxicity evaluation of several colon cancer cells has been reported in numerous studies. Elkady and co-workers (2014) stated that treatment of 116 HCT colon cells with this extract for 24 and 48h produced IC_{50} values of 20 and 25µg/mL, respectively. Hakim *et al.* (2014) also used the same extract for the treatment of HCT-116, which produced IC_{50} value of 3mg/mL after 72h. The combination of this extract and Gelam honey modulated the Ras/ERK and PI3K/Akt pathway genes in HT29 colon cancer cells (Tahir *et al.*, 2005).

The treatment of WiDr cells with only 5-FU for 24 and 48h created different cell viability values. A 24h treatment of 5-FU on WiDr cells did not produce IC_{50} values because the viability was above 50%, even at the highest concentration. Therefore, the IC_{50} of 5-FU for 24h incubation time was not determined in this study may due to the resistance of WiDr cells. Zhang *et al.* (2008) stated that it is widely accepted that the overexpression of thymidylate synthetase is recognized as the main mechanism responsible for 5-FU resistance. Several possibilities can induce thymidylate synthases, such as decreased accumulation of active metabolites, target-associated resistance, and pharmacokinetic. Decreased accumulation of active metabolites due to reduced activation, increased inactivation of 5-FU nucleotides. In addition, WiDr colon cancer cells have molecular characteristics with p53 mutations (Rodrigues *et al.*, 1990). Tumor cells with mutant p53 lack response to apoptotic-inducing agents. Therefore, the combination of 5-FU chemotherapy with red ginger extract is expected to increase the cytotoxic effects of 5-FU.

A combination test was performed to determine whether the extract of *Z. Officinale* var. Rubrum increases the cytotoxicity of the 5-FU.

The test results showed that the addition of the extract increased cytotoxic activity compared to a single 5-FU treatment. For instance, the treatment of WiDr cells with only 5-FU at a concentration of 1000µg/mL produced viability of 65%, while a combination of 35µg/mL of the extract with 1000µg/mL 5-FU produced a viability value of 40%. This combination created a smaller cell viability value compared to every single treatment, and similar to the use of 5-FU at 48-hour incubation. Figure 5 shows that the combination of 5-FU at a concentration of 60 µg/mL with extract at 25 µg/mL caused a more significant decrease in cell viability compared to a single treatment. This further reinforces that the extract can increase the cytotoxic effect of 5-FU compared to a single administration.

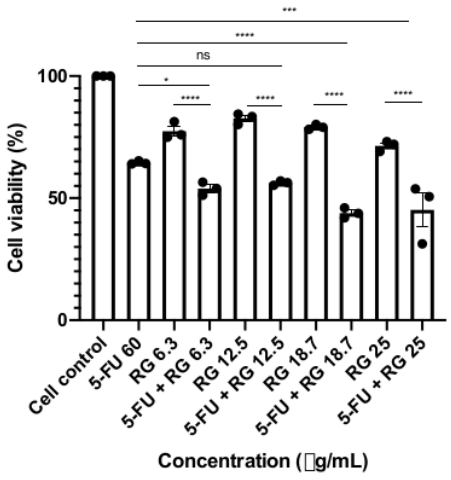

Figure 5. Effect combination of 5-FU and red ginger (RG) extract in WiDr cells at 48h incubation. Cells were treated 5-FU at concentrations of 60µg/mL, and in combination with red ginger extract with various values of red ginger extract, such as 6.3, 12.5, 18.7, and 25µg/mL. Percentage of cell viability was expressed as mean ± SD. Statistical analysis used One-way Anova and the Tuckey's range test. * p value <0.05.

The combination of 5-FU and red ginger extract led to a decrease in viability at all incubation times with a significant difference compared to a single 5-FU treatment. Furthermore, the decrease in cell viability due to the two agents' combined treatment in this study obtained differences in the mechanism of action between red ginger extract and 5-FU on cancer cells. The active ingredients in red ginger are 6-gingerol, 6-shogaol, and zingeron (Ali *et al.*, 2008). 6-shogaol is a dehydrated form of 6-gingerol, and is more pharmacologically attractive because of its higher cytotoxic activity in colon and lung cancer cells (Sang *et al.*, 2009). 6-gingerol induces caspase-dependent apoptosis and prevents PMA-induced proliferation in colon cancer by inhibiting MAPK/AP-1 signaling (Radhakrishnan *et al.*, 2014). Pan *et al.* (2008) stated that 6-shogaol induces apoptosis in colorectal cancer cells through ROS production, caspase activation, and GADD 153 expression. Meanwhile, Qi *et al.* (2015) stated that 6-shogaol triggers cell cycle arrest in G2/M and can overcome TRAIL resistance in colon cancer through survivin inhibition (Hwang *et al.*, 2015).

5-FU, as a pyrimidine analog acts antagonistically with dUMP to determine the activity of the thymidylate synthetase. Furthermore, 5-FU induces apoptosis through inhibition of DNA synthesis caused by cell deficiency of deoxythymidine triphosphate (dTTP) (Giovannetti *et al.*, 2007). When two test materials with different mechanisms are combined, the obtained results used to reduce cell viability become higher due to increased cancer cell cytotoxicity. Therefore, the combination treatment led to a better decrease in cell viability compared to a single 5-FU treatment.

This is the first study that reveals the potential of red ginger extract in increasing the cytotoxic activity of 5-FU in WiDr colon cancer cells. Previous studies combined 5-FU in WiDr colon cancer cells using the ethyl acetate fraction of *Gynura procumbens* (Nurulita *et al.*, 2011) and *Moringa oleifera* leaf extract (Nur *et al.*, 2011). The molecular mechanism by which red ginger extracts increase the cytotoxic activity of 5-FU on colon cancer cells has not be revealed yet, therefore, further research is needed, such as apoptosis assay examination.

In conclusion, red ginger extract increases the cytotoxic activity of 5-FU, therefore it acts as a nutraceutical agent in the treatment of colon cancer.

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