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Calotropis gigantea Leaf Extract Increases the Efficacy of 5-Fluorouracil and Decreases the Efficacy of Doxorubicin in Widr Colon Cancer Cell Culture

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ABSTRACT

Colon cancer is a malignant neoplasm with high incidence and causes the death of more than 30% of the patients. Although there have been efforts to increase the life of the patient using chemotherapy agents, unspecified targets of drugs cause serious side effects and lead to multiple drug resistance (MDR). This is a major problem in cancer therapy in general. Efforts to use substances from plants that have low toxicity give new hope as a co-chemotherapy agent that can increase the efficacy of chemotherapy agents and reduce their toxicity to normal cells. This study aimed to determine the synergistic effects of therapy combination of *Calotropis gigantea* (EDCG) leaf extract with the chemotherapy drug 5-Fluorouracil (5-FU) and doxorubicin against WiDR colon cancer cells. The analysis results of MTT showed that the combination of EDCG and 5-Fluorouracil gives synergism effect at the dose combination of EDCG+5FU (3 µg/ml + 62.5 nM; 3 µg/ml + 125 nM; 3 µg/ml + 250 nM). While the combination of doxorubicin and EDCG creates antagonistic effects of mild to the strong antagonist, EDCG may enhance the efficacy of 5 Fluorouracil but decrease the efficacy of doxorubicin. Therefore, a combination of EDCG and 5-Fluorouracil may be recommended for further study.

INTRODUCTION

Colon cancer is included as five leading causes of death in the world other than breast, lung, stomach and liver cancer. Colon cancer causes 693,900 deaths per year (American Cancer Society, 2015). In developed countries like America, it has been reported in 2013 that there were new cases of 102,480 thousand colon cancers and 40,340 rectal cancers. These cause 50,830 million deaths (NCI, 2013). Some attempts for colon cancer treatment have been done intensively like surgery, chemotherapy, and radiotherapy. Until now, cancer prevention efforts, mainly through chemotherapy, are still a failure. This is caused by the fact that chemotherapy drugs are not selective and specific in killing cancer cells causing serious side effects in patients. Cancer cell resistance to chemotherapy drugs is also the second cause of cancer treatment failure, so it is necessary to increase the therapeutic dose to keep giving anticancer activity (Tacar *et al.*, 2013).

In general, almost all cancer chemotherapy drugs are a combination of several drugs. It is hoped that a combination of several chemotherapeutic agents may provide a synergistic effect and suppress the growth of cancer cells but with a toxicity profile that can still be tolerated. The use of such combinations is more clinically effective than single agents. However, until now, there has been no effective combination of cancer chemotherapy, especially when it comes to the metastasis phase. The use of cytotoxic agents for the treatment of this disease is confronted with the problem of cancer cell's resistance to the drug. Chemotherapy agents often give high toxicity to normal organs (Putri *et al.*, 2016). Hence, the development of therapies for cancer is very necessary. One approach that can be done is co-chemotherapy approaches

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which combine a chemopreventive agent from natural materials with a chemotherapeutic agent, thereby increasing efficacy and decreasing the toxicity of chemotherapy on the normal tissue (Laksmiani *et al.*, 2015; Putri *et al.*, 2016).

Calotropis gigantea (*C. gigantea*) is a plant that has been investigated as potential anticancer properties. As reported by the researchers in a previous study, *C. gigantea* leaf ethanol extract can inhibit the growth of *in vivo* fibrosarcoma at doses of 100 and 150 mg/kg with the mechanism of increased expression of caspase-3 (Mutiah *et al.*, 2016). Ethanol extract of the roots and *C. gigantea* leaves have an anticancer activity which is higher than the flower (Mutiah *et al.*, 2016). Ethyl acetate fraction from the leaves (IC_{50} 41, 79 µg/mL) and dichloromethane fraction (IC_{50} 40.57 µg/mL) have cytotoxic activity which is higher than butanol fraction (IC_{50} 737.74 ug/mL) and water (IC_{50} 8493 ug/mL) (Mutiah *et al.*, 2017). This study tested the effectiveness of a combination of *C. gigantea* leaf extract with 5 fluorouracil and doxorubicin drug chemotherapy on WiDrcolon cancer cells. The use of such combinations is expected to produce synergistic effects of both. The effect is much larger than the cumulative effect of the use of the single effect. Meanwhile, the toxic effects caused can be minimized since the concentration used is also relatively low, below the IC_{50} (Putri *et al.*, 2016).

MATERIAL AND METHODS

Test material

The plant materials used in this study are the leaves of *C. gigantea* taken from Malang, East Java. Plant determination was done at Indonesian Institute of Sciences (LIPI) in Purwodadi, East Java. The plant specimens were stored in the laboratory of Pharmacognosy at UIN Maulana Malik Ibrahim Malang No. 16030232.

Materials for extraction

The solvent used for the extraction step maceration is 70% ethanol (Merck).

Cell culture material

Cancer cell used in this study is a WiDr colon cancer cell line. Cells were obtained from the Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy in Gadjah Mada University and Prof. Masasi Kawaichi, Laboratory of Gene Function in Animal, Graduate School of Biological Sciences, Nara Institute of Science and Technology. Cells were cultured in Rosewell Park Memorial Institute (RPMI) in medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (PAA Laboratories), 1% v/v penicillin-Streptomycin (Nacalay Tesque), and 1.0 mM L-glutamine (Nacalai Tesque). Then the cells were incubated in an incubator with 5% CO_2 and 95% O_2 at 37°C.

Cytotoxic test material

Dimethyl sulfoxide (DMSO) was used to dissolve the extract of *C. gigantea* (EDCG) leaf. The concentration used in this study was a maximum of 1% in the culture medium. 0.025% trypsin was used to harvest cells. Phosphate buffer saline (PBS) was used as a wash buffer solution. 3-(4,5-dimetiltiazole-2-yl)-2,5-diphenyltetrazolium (MTT) was used as a reagent that reacts with the succinate dehydrogenase enzyme in the cell. 5 Fluorouracil, Doxorubicin was used as chemotherapy agents combined with EDCG.

Tools

The main tools needed in this research are macerator, evaporator, centrifugation, liquid nitrogen tank, CO_2 - Jacketed Incubator, contrast phase microscopy, Laminar Air Flow cabinet (Nuaire), and Elisa reader.

Extraction

A total of 500 mg of *C. gigantea* leaf powder was macerated using 3 liters of 70% ethanol for 48 hours. Then, it was filtered and the filtrated before being separated. The residue was then dried and re-macerated with 3 liters of 70% ethanol for 48 hours. This process was repeated for four times. After that, the filtrate was collected and evaporated in a vacuum rotary evaporator to obtain a thick extract. The condensed extract was then dried in an oven at 40°C. (BPOM, 2010).

Anticancer combination test with MTT method

100 µl of WiDr colon cancer cell suspension at a density of 3×10^4 cells/100 µl media was distributed into wells in 96-well plate and incubated for 24 hours. After incubation, 100 µl of test solution at different concentrations was added to wells. The test solution used was EDCG with a combination of 5 fluorouracil and doxorubicin. As a positive control, 100 µl of culture medium, then 100 µl of doxorubicin and 5 fluorouracil at various concentrations were added into wells containing 100 µl of WiDr cancer cells. As a cell control, 100 µl of culture medium was added to the well containing 100 µl of cell suspension and as a solvent control, 100 µl of DMSO was added to the well containing 100 µl of culture medium and 100 µl of cell suspension with dilutions corresponding to the dilutions of test solution concentration, then it was incubated for 24 hours in an incubator with a flow of 5% CO_2 and 95% O_2. At the end of the incubation, the culture medium was discarded and then 10 µl solution of MTT (5 mg/µlPBS) was added, and the medium was replaced with 190 µl of complete 1640 RPMI medium. Then, cells were incubated for 3-4 hours. MTT reaction was stopped by adding SDS stopper reagent (100 µl). The microplate was then wrapped with tissue paper and incubated for overnight at room temperature in dark. Living cells react with MTT forming purple color. The test result is read by ELISA reader at a wavelength of 595 nm (Mutiah, 2014).

Data analysis

The data obtained are in the form of absorbance of each well converted to a percentage of living cells:

$$\text{Percentage (\%) living cell} = \frac{(\text{abs. treatment} - \text{abs. media control})}{(\text{abs. cell control} - \text{abs. media control})} \times 100\%$$

Description: Abs: absorbance

The percentage of living cells is calculated to obtain the IC_{50} value, which is the concentration that causes growth inhibition of 50% of cells population where the cytotoxic potential can be known. IC_{50} values were determined by probit analysis (Statistical Product and Service Solution (SPSS) 16.0 for Windows). Combined cytotoxicity was determined by calculating

the interaction index between chemotherapy agents with EDCG, using the equation:

Combination Index CI = (D) 1/(Dx) 1 + (D) 2/(Dx) 2

Where D1 and D2 are the sample concentrations used in the combination treatment. (Dx) 1 and (Dx) 2 are single concentration which can produce the effect of a given treatment combination (Reynolds and Maurer, 2005). CI or *Combination Index* gained is interpreted as follows: < 0.1 means the synergy is very strong, 0.1–0.3 is a very strong synergy, 0.3–0.7 is synergist, 0.7–0.9 is mild-moderate synergy, 0.9–1.1 is the mild-moderate antagonist, 1.45–3.3 is the antagonist, 3.3 > strong-powerful antagonist.

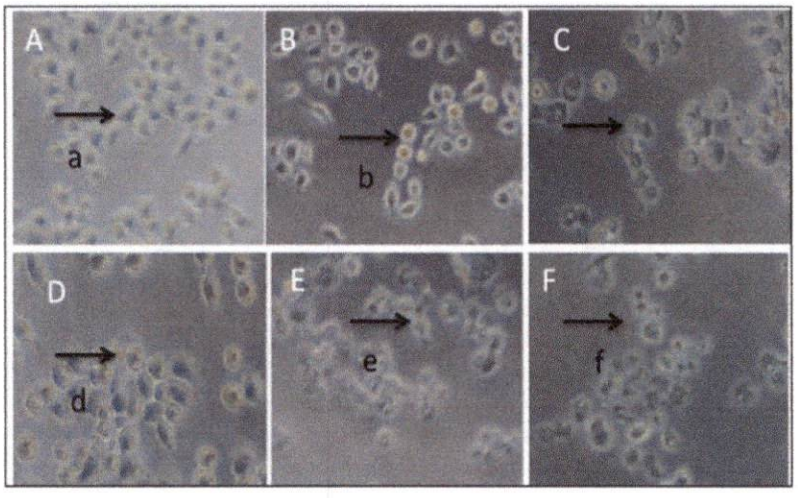

Fig. 1: Comparison of cell growth inhibitory effects (anticancer activity) for the treatment of test material on WiDrcolon cancer cells with MTT reduction method.

RESULT AND DISCUSSION

Results

Analysis of combination results of EDCG+5-fluorouracil (5 FU) and EDCG+Doxorubicin (DOX) on morphology and viability of cells

The concentration used in the test is a combination of 16 combination doses with a concentration below IC_{50}. The percentage of viable cells was determined using the MTT method. The result of the effectiveness of combination forms is compared with the single therapy, so they can compare the efficacy of the combination preparation on the single stocks.

In EDCG treatment, 5-FU and DOX either singly or in combination lead to morphological changes in WiDrcells which were linear with the increasing concentrations of the test. WiDr cells in the control look oval with clear cytosol and attached to the basic Tissue Culture Dish (TCD). After the test material treatment, some cells appear rounded and detached from TCD. The cell looks dull and compact, it looks like it is condensed and undergoes core and granulation shrinkage in the cytosol. The morphological changes are more pronounced along with the increase in test concentration. In line with the morphological changes, single and combination material test decrease the viability of the WiDr cells which are linear with the increasing concentration of the test. In the control treatment, there is no change in cell morphology, in which the cells' shape is oval, looks like clear cytosol and is attached to the basic TCD (Figure 1).

10^4 cells per well were put in 96 well plates, incubated for 24 hours in complete RPMI media, which were then treated with single and combination test preparation. Cell morphology was observed under a contrast phase microscope using 200x magnification. A) Control of WiDrcolon cancer cell DMSO treatment; B) *C. gigantea* Leaf Extract monotherapy (EDCG) Treatment; C) Treatment of Doxorubicin monotherapy; D) 5-Fluorouracil monotherapy treatment; E) Treatment of combinations of EDCG and doxorubicin; F) Treatment of EDCG and 5-Fluorouracil combination. The changes that occurred can be seen at test material treatments indicated by the cells that undergo cell death as the cells are detached from TCD and blebbing happened (b, c), are dull (e), are enlarged (d, f). The living cell is shown by the arrow (a). The experiment is performed with 3 × replications.

The treatment of EDCG+5-FU combination for WiDr colon cancer cell causes deeper cell viability decrease compare to the treatment of EDCG+DOX combination. The increase of EDCG dose for the combination with 5 FU causes the decrease of WiDr colon cancer cell viability. The graphic picture of isobologram is presented below (Figure 2):

A) The treatment of EDCG+5-fluorouracil (5-FU) therapy combination gives synergist effect.

B) The treatment of EDCG+doxorubicin (DOX) therapy combination gives antagonist effect.

10^4 cells/same age is plated in the well plate, incubated for 24 hours in the RPMI media with or without EDCG+5-FU combination treatment and EDCG+DOX combination with determined concentration. Cell viability is determined by MTT method. The analysis result is a representation from two different experiments; each of them is done with three replications.

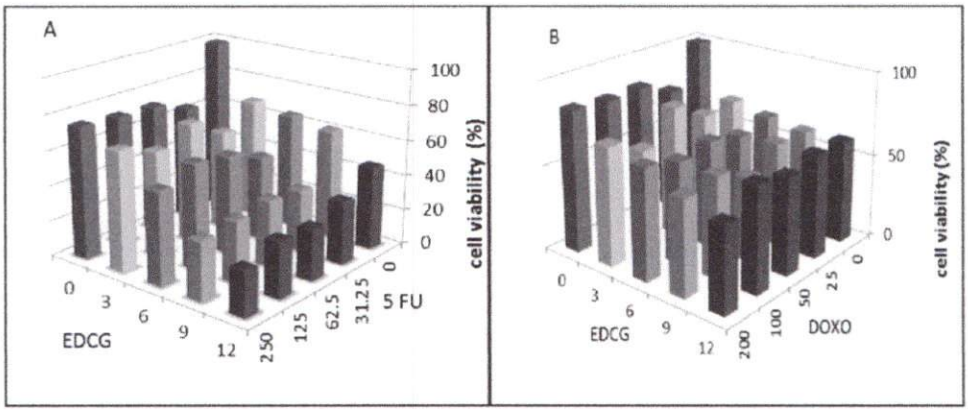

Fig. 2: The influence of treatment of EDCG+5-FU combination and EDCG+DOX combination for the growth of WiDr colon cancer cell.

The analysis of index combination result

The methods used in the evaluation of EDCG+5-FU and EDCG+DOX combinations are Isobologram and Index Combination (IC). IC analysis produces a score of the quantitative parameter that describes the efficacy of combination by using the equation of IC = (D) 1/(Dx) 1 + (D) 2/(Dx) 2. IC is used to determine additive effect from two compound combinations, whether in form of synergist, additive, or antagonist effects.

The result of EDCG+5-FU Index Combination analysis shows that from 16 combination doses, there are three doses combinations that give synergist effect, they are EDCG+5-FU (3 µg/ml + 62.5 nM; 3 µg/ml + 125 nM; 3 µg/ml + 250 nM). Other doses give light synergist, antagonist, strong antagonist, and powerful antagonist effects. The EDCG+5-FU Index Combination analysis result is presented in Table 1 and Figure 3A. The result of EDCG+DOX Index Combination analysis shows that from 16 combination doses, the combination which shows no synergism effect but that combination gives light antagonist up to powerful antagonist effect. The table of the analysis result of EDCG+DOX Index Combination is presented in Table 2 and Figure 3B.

A) EDCG+5 FU combination treatment for WiDr colon cancer cell gives synergist up to strong antagonist effects. B) EDCG+DOX combination treatment for WiDr colon cancer cell gives antagonist up to strong antagonist effect. The combination test is done with each four concentration series under IC_5 concentration Index Combination, < 0.1 powerful synergist, 0.1–0.3 strong synergist, 0.3–0.7 synergist, 0.7–0.9 light-medium synergist, 0.9–1.1 light-medium antagonist, 1.45–3.3 antagonist, 3.3 > strong-powerful antagonist.

Discussion

The objective of this research is to know the efficacy of *C. gigantea* (EDCG) leaf extract in combination with 5-Fluorouracil (5 FU) and doxorubicin (DOX) as chemotherapy medicines. The cancer cells used in this test was WiDr colon cancer cells. WiDr cell is human colon cancer cell that was successfully isolated from a 78 years old female patient. These cells were successfully derived from HT-29 cells (Nurulita *et al.*, 2011). The specifications of WiDr cells are, able to produce carcinoembryonic antigen and can finish one cell cycle in 15 hours (Dai *et al.*, 2012).

Table 1: The analysis result of Index Combination of EDCG+5-FU combination treatment for WiDr colon cancer cell.

Doses combination		Cell viability (mean ± SD)*	CI	Therapy effects
EDCG (µg/ml)	5 FU (nM)	means ± SD		
3	31.25	57.08 ± 1.18	1.24	Antagonist
6	31.25	48.04 ± 0.33	22.06	Powerful Antagonist
9	31.25	35.39 ± 2.16	702.55	Powerful Antagonist
12	31.25	35.76 ± 1.03	857.49	Powerful Antagonist
3	62.5	68.90 ± 0.86	0.3	Synergist
6	62.5	56.25 ± 2.40	0.87	Light Synergist
9	62.5	37.69 ± 5.17	2.46	Antagonist
12	62.5	29.17 ± 4.26	4.39	Powerful Antagonist
3	125	60.06 ± 3.15	0.4	Synergist
6	125	59.59 ± 16.99	0.79	Antagonist
9	125	34.92 ± 2.54	2.7	Strong Antagonist
12	125	31.42 ± 2.09	4.07	Strong Antagonist
3	250	68.06 ± 3.09	0.35	Synergist
6	250	52.43 ± 2.79	1	Antagonist
9	250	33.30 ± 2.82	2.86	Antagonist
12	250	25.30 ± 3.64	5.02	Powerful Antagonist

*Means of cell viability test ± Deviation, n = 3

Doxorubicin and Fluorouracil 5 are selected chemotherapy agents that have been used more than three recent decades. However, the use of those chemotherapy agents is still limited because of many side effects (Putri *et al.*, 2016). The side effects caused by doxorubicin chemotherapy are immunosuppression, nauseous, and reversible arrhythmia. Long-term use of doxorubicin causes

cardiomyopathy effect followed by heart failure. The side effect caused by doxorubicin depends on used doses. Therapy strategy in the form of combination with a natural chemopreventive agent in the lower doses combination under IC_{50} can improve the efficacy of chemotherapy medicine and reduce the side effect.

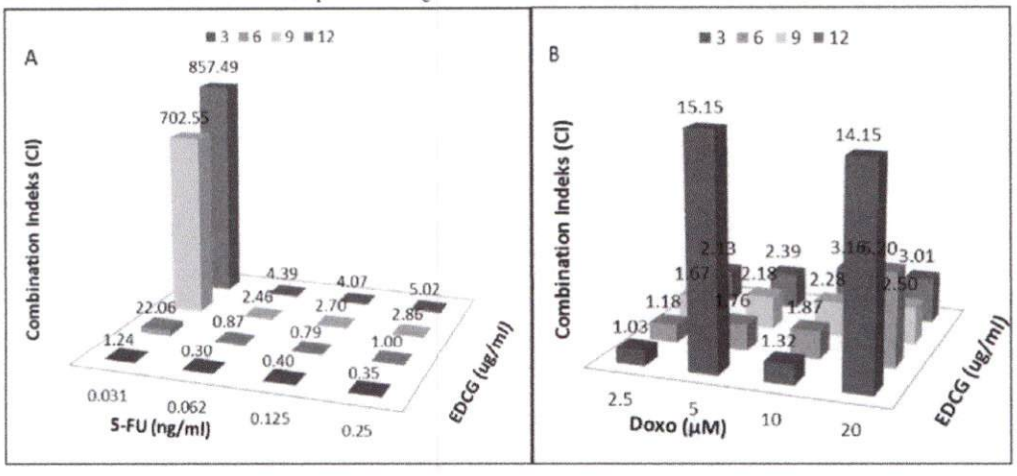

Fig. 3: Index Combination in the EDCG+5FU and EDCG+DOX combination treatments.

Table 2: The analysis result of Index Combination of EDCG+DOX combination treatment for WiDr colon cancer cell.

Doses combination		Cell viability (means ± SD)*	IC	Therapy effect
EDCG (µg/ml)	Dox (nM)	means ± SD		
3	25	65.41 ± 2.99	1.03	Light antagonist
6	25	58.91 ± 0.57	1.18	Light antagonist
9	25	61.15 ± 1.19	1.67	Antagonist
12	25	61.53 ± 0.71	2.13	Antagonist
3	50	77.76 ± 10.16	15.5	Powerful Antagonist
6	50	63.77 ± 3.05	1.76	Antagonist
9	50	49.67 ± 0.38	2.18	Antagonist
12	50	57.54 ± 4.37	2.39	Antagonist
3	100	59.84 ± 3.18	1.32	Light Antagonist
6	100	60.27 ± 1.09	1.87	Antagonist
9	100	59.67 ± 4.12	2.28	Antagonist
12	100	62.35 ± 2.48	3.16	Antagonist
3	200	70.44 ± 4.28	14.5	Powerful Antagonist
6	200	65.74 ± 1.23	6.2	Powerful Antagonist
9	200	56.39 ± 3.36	2.5	Antagonist
12	200	50.33 ± 2.70	3.01	Antagonist

*Means of cell viability test ± Deviation, n = 3.

5 FU is one of the drugs of choice in the cancer therapy. The use of 5 FU is suggested in the form of combination with another chemotherapy agent. It is caused by the decrease of 5 FU's sensitivity for a cancer cell. Several side effects caused by cancer therapy with 5 FU chemotherapy agent are stomatitis, neutropenia, diarrhea, and hand-foot syndrome (Hess *et al.*, 2010). The use of 5 FU for the long term may possibly cause cardiotoxicity, even though it is rare (Rodgers *et al.*, 2010). The excessive of 5 FU, compared to other chemotherapy medicine, is having higher selectivity and lower side effect. Nevertheless, 5 FU's selectivity for cancer cell is still reaching 15% so that until now the chemotherapy agent is still needed to be developed to improve the efficacy of 5 FU (Parhi *et al.*, 2012).

In the analysis of Index Combination (IC) EDCG+5-FU, it is obtained synergism effect in the three doses combinations; they are 3 µg/ml + 62.5 nM, 3 µg/ml + 125 nM and 3 µg/ml +250 nM. The synergism effect of EDCG+5-FU combinations is a greater effect compared to every effect summation in its sole form. The synergism effect of those combinations is caused by activity mechanism that supports each other among terpenoids compounds contents in the EDCG and 5 FU chemotherapy medicine. Similar results were reported by Alwi *et al.* (2017) where a combination of 5FU-Leucovorin with *Phaleria macrocarpa* has greater synergist effect when compared to the individual effect on mouse strain of adenocarcinoma.

Leaf extract of *Calotropis gigantea* (EDCG) has been reported of its ability in impeding the growth of cancer cell through apoptosis induction by raising the three caspase expression (Mutiah, 2016). While the 5 Fluorouracil is a chemotherapy agent with a working mechanism through improving the expressions of p21 and pRb without involving the role of p53. Protein P21 has a very important role in the checkpoint system of G1 phase. P21 expression causes the inhibition of cell cycle in the G1 and S phases caused by the E/CDK2 and A/CDK2 cycling activity inhibitions. The impeding of the cell cycle in the G1 and S phases causes cancer cell apoptosis induction (Meiyanto, 2011). The synergism effect of EDCG+5 FU combination is presumed that EDCG can be inducted and it can improve 5 FU efficacy through cancer cell apoptosis intrinsic path. For the synergism

effect of molecular mechanism, EDCG+5 FU must be proven further.

CONCLUSION

The combination of EDCG and 5-Fluorouracil gives synergism effect at the dose combination of EDCG+5FU (3 µg/ml + 62.5 nM; 3 µg/ml + 125 nM; 3 µg/ml + 250 nM). The combination of doxorubicin and EDCG creates antagonistic effects of mild to the strong antagonist. EDCG may enhance the efficacy of 5 Fluorouracil but decrease the efficacy of doxorubicin. Therefore, a combination of EDCG and 5-Fluorouracil may be recommended for further study.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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