

Red Betel Leaves Methanolic Extract (*Piper crocatum* Ruiz & Pav.) Increases Cytotoxic Effect of Doxorubicin on WiDr Colon Cancer Cells through Apoptosis Induction

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Abstract

Doxorubicin is a chemotherapeutic agent that causes a lot of side effects in high doses. Thus, combination with a co-chemotherapeutic agent which can increase its toxicity on cancer cells, is needed to reduce its therapeutic dose. Red betel leaves (*Piper crocatum* Ruiz & Pav.) have been known to contain flavonoids and alkaloids that have anticancer activity. This study was conducted to determine the cytotoxic effect and apoptosis induction of red betel leaves methanolic extract (RBM), doxorubicin (dox) and the combination of them on WiDr cells as model of colon cancer. RBM was extracted by soxhlet method using methanol. Cytotoxicity assay was performed using MTT assay for both single and combination treatments for 24 hours to determine IC₅₀ and CI as their parameters. Apoptosis induction was analyzed by double staining method using ethidium bromide-acridine orange staining. Treatment of RBM and dox on WiDr cells for 24 hours showed cytotoxic activity with IC₅₀ 100 µg/mL and 1.6 µM respectively. Combination of RBM and dox performed synergism effect with CI<0.9 ($p<0.05$). Combination of RBM (12.5 µg/mL) and dox (0.4 µM) increased the number of apoptosis cells compared to each single treatment. Based on this study, it can be concluded that red betel leaves methanolic extract is potential to be developed as a co-chemotherapeutic agent of doxorubicin on colon cancer but still need further study to disclose the underlying molecular mechanisms.

Keywords : Red betel leaves (*Piper crocatum* Ruiz & Pav.), doxorubicin, WiDr cells, co-chemotherapeutic agent

INTRODUCTION

The incidence rate of colon cancer is predicted to increase as the population grows in both developing and developed countries (Siegel, *et al.*, 2014). Based on the data from Indonesian Ministry of Health (2015), colon and rectal cancer is ranked fourth of cancer deaths after lung, liver, and stomach cancer every year. Therefore, the best therapy was needed to improve the quality of life and prevent mortality of colon cancer patients.

Doxorubicin is a chemotherapeutic agent that has broad-spectrum antitumor activity. This drug is often used in cancer therapies, one of them is in colon cancer treatment. However, the use of doxorubicin is limited because it can cause toxicity on normal cells, cardiotoxicity leading to heart failure (Ferreira, *et al.*, 2008), hepatotoxicity (El-Sayyad, *et al.*, 2009), and chemoresistance (Riganti,

et al., 2009) so the treatment becomes less effective. To overcome this problem, a co-chemotherapeutic agent is required to improve the effectiveness of doxorubicin.

Based on previous research study, it is known that red betel extract (*Piper crocatum* Ruiz & Pav.) contains a number of active compounds including flavonoids, alkaloids, essential oils and tannins (Farida, *et al.*, 2010). Piperine, the main alkaloid in Piper genus showed cytotoxic activity on several cancer cells. Piperine was reported to have cytotoxic activity in blood cancer (CEM and HL-60), melanoma cancer (B16), and colon cancer (HCT-8) (Bezerra, *et al.*, 2005) and breast cancer cells

Submitted: Feb 15, 2018

Revised: Feb 20, 2018

Accepted: Feb 20, 2018

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(T47D) (Widowati, *et al.*, 2011). Piperine was also reported to inhibit the growth of HT-29 colon cancer cells by induction of apoptosis (Kim, *et al.*, 2009).

This study was conducted to develop an agent which could enhance the effectiveness of doxorubicin. Cytotoxic effect of doxorubicin, red betel leaves methanolic extract (RBM) and its combination was performed *in vitro* using WiDr cells. Observation of apoptosis was done by double staining method using ethidium bromide-acridine orange. The results of this study are expected to provide scientific evidence of RBM in improving the cytotoxicity effect of doxorubicin so that it can become the basis of utilization of red betel leaves as the a co-chemotherapeutic agent of doxorubicin in the treatment of colon cancer.

MATERIALS AND METHODS

Sample Preparation

Simplicia of *Piper crocatum* Ruiz & Pav leaves were collected from Bina Agro Mandiri, Inc., Bantul, Yogyakarta, Indonesia, and were determined at Biological Pharmacy Laboratory, UGM. An amount of simplicia were powdered and extracted by soxhlet with 400 mL methanol in 3 cycles soxhlet. Then, filtrate was dried using rotary evaporator on 40°C. Identification of chemical compounds in RBM was determined by thin layer chromatography (TLC) using naringenin as positive control for flavonoid and piperine as positive control for alkaloid.

Cell Culture

WiDr colon cancer cells were obtained from Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada. Cells were grown in Roswell Park Memorial Institute (RPMI) medium (Sigma) with 10% FBS (Sigma), sodium bicarbonate, 1.5% Penicilin-Streptomycin and 0.5% Fungizone (Gibco).

Cell Viability Assay

Cell viability assays were performed using MTT assay. WiDr cells were seeded in 96-well plate at a density of 13×10^3 cells/well and were incubated for 24 hours. Cells were treated with increasing concentrations of doxorubicin (0.25, 0.50, 0.75,

1.00, 1.25, 1.50, 1.75, 2.00 μ M) and RBM (10, 30, 50, 70, 90, 110, 130, and 150 μ g/mL). After 24 hours of incubation, culture medium was removed and cells were washed with PBS (Sigma). Then cells were added 100 μ L MTT (Sigma) 5 mg/mL diluted with culture medium in each well for 4 hours. The absorbance of each well was measured using ELISA reader at 595 nm (Bio-Rad). The single absorbance data converted into percentage of cell viability and will be used to determine of the IC₅₀ value.

Apoptosis Detection

Apoptosis was detected using ethidium bromide (50 mg) and orange acridine (15 mg) reagents (EtBr-AO) (Sigma, Sigma-Aldrich Corp., St. Louis, MO, USA) dissolved in 1 mL of ethanol 95%. WiDr cells (5×10^4 cells/well) were seeded on coverslips in 24-well plates until 50-60% confluent. Cells were then incubated with RBM alone, doxorubicin alone and their combination for 24 hours at a temperature of 37°C. At the end of incubation, culture medium was removed and cells were washed with PBS. Coverslips were placed into object-glass and added with 10 μ L 1x working solution acridine orange-ethidium bromide (Sigma). Morphological features of apoptosis were identified using fluorescence microscope (Zeiss MC 80) at 100x magnification.

Data Analysis

Absorbance data were obtained from viability assays then converted into a percentage of cell viability and log values. Inhibitory concentration (IC₅₀) value calculation is performed by the logit method. Synergistic cytotoxicity was determined by calculating the interaction index (CI = Combination Index) between doxorubicin and RBM. The observation of apoptotic induction was conducted with qualitative approach by observing the specific morphological criteria, including condensation and fragmentation of chromatin, also formation of apoptotic bodies. Live cells show a normal green nucleus, early apoptotic cells a bright green nucleus with condensed or fragmented chromatin, late apoptotic cells show condensed and fragmented orange chromatin, and cells that have died from direct necrosis show a structurally normal orange nucleus (McGahon, *et al.*, 1995).

RESULTS

Cell Viability Assay

Cell viability assay was done to determine the IC_{50} of RBM alone, doxorubicin alone and their combination on WiDr cells. A lower IC_{50} means a more potent cytotoxic activity against cancer cells. Doxorubicin (dox) shows cytotoxic effect on WiDr cells after 24 hour incubation with the IC_{50} value of 1.6 μ M, while RBM has the IC_{50} value of 100 μ g/mL (Fig. 1). The curves show that RBM and dox decrease the cell viability with dose dependent manner. In higher doses, more cells appear to

morphological changes and the quantity of cells decreasing. Cell shrinkage may occur because the cytoskeleton of cells is cut into pieces so that the cell shape becomes irregular. It is a sign of cells heading for death.

The administration of doxorubicin at concentrations of 0.25-2.00 μ M (Fig. 1a) and RBM at concentrations of 10-150 μ g/mL (Fig. 1b) are able to decrease the viability of WiDr cells. The IC_{50} value of single treatment of doxorubicin and RBM was then used as a basis for determining the concentration for combination treatment.

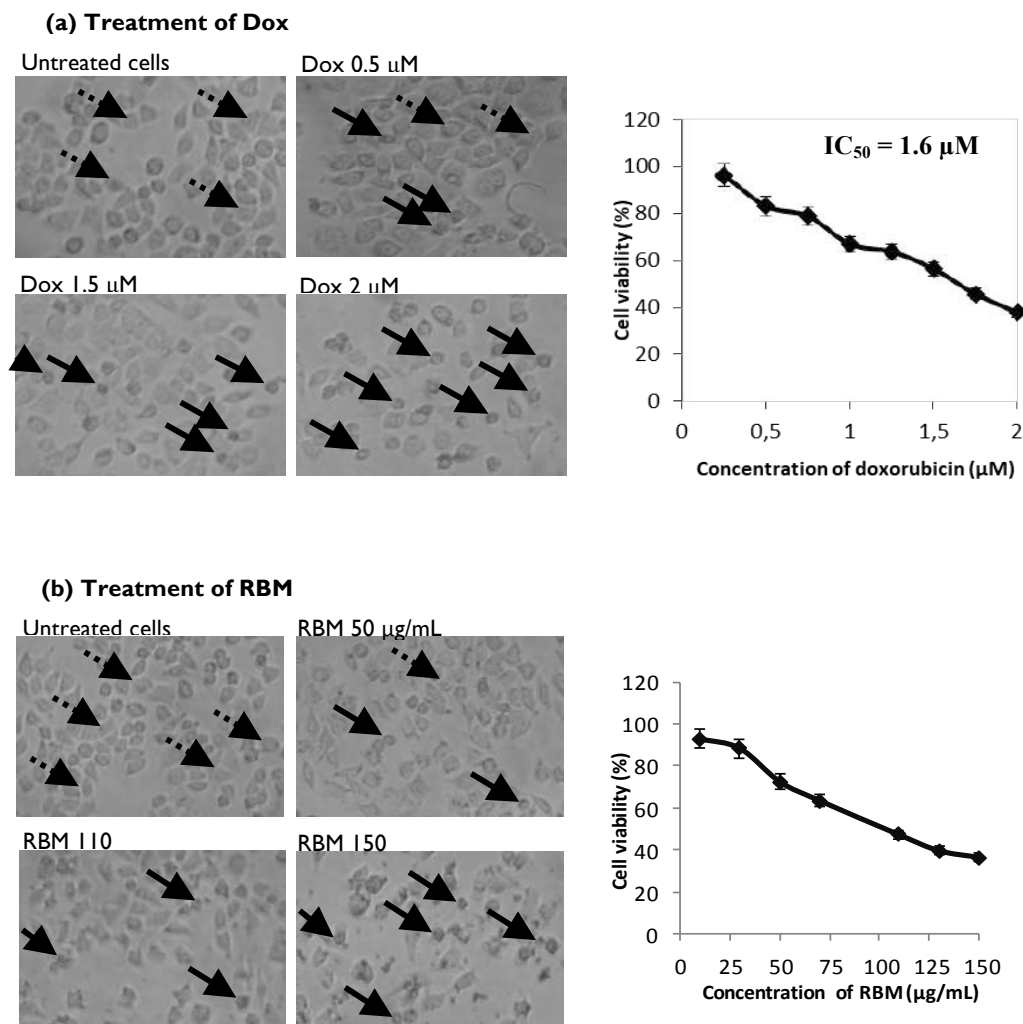


Figure 1. Effect single treatment of Dox and RBM on WiDr Cell's Viability. WiDr cells with density (13×10^3 cells/well) were treated by Dox and RBM in the concentration as indicated in the picture. Observations were made after treatment under an inverted microscope with magnification of 400x. (a) Morphology of WiDr cells after treatment of Dox (left side) and the cell viability profile at various concentrations of Dox (right side). (b) Morphology of WiDr cells after treatment of RBM (left side) and the cell viability profile at various concentrations of RBM (right side). The IC_{50} value performed triplicate and was calculated by using linear regression in three independent experiments ($p < 0.05$). Description: Signs show normal WiDr cells, cells undergoing morphological changes.

Effects of combination of RBM and Dox on WiDr cell viability

Cell viability assay in combination was also done to see the effectiveness of doxorubicin and RBM combination in inhibiting the growth of WiDr colon cancer cells. A synergistic combination demonstrates the ability of RBM to increase the sensitivity doxorubicin on WiDr cells. Increased sensitivity indicates increased efficacy of doxorubicin which can reduce treatment dose to obtain the same cytotoxic effect.

The concentrations for combination treatment were dose 1/16, 1/8, 1/4, and 1/2 of IC₅₀. Doxorubicin was administered on WiDr cells at concentrations of 0.1 μM; 0.2 μM; 0.4 μM; and 0.8 μM, while RBM was given at a concentration of 6.25 μg/mL; 12.5 μg/mL; 25 μg/mL; and 50 μg/mL. Results show that the *combination index* (CI) values of all combinations are less than 1 (Table 1), which means that all combinations show a significant synergism (Reynolds and Maurer, 2005). Some concentration combinations can also reduce cell viability to lower than 50% (Table 2).

The single treatment of RBM and doxorubicin proved to be capable of decreasing cell viability when compared to untreated/ control cells, meanwhile in the combined treatment of dox and RBM seen that the number of live cells is less than the single treatment at the same doses (Fig. 2a). The observation of cell viability also showed that in some combination treatments decreased significantly the number of viable cells (Fig. 2b).

From the combined treatment, RBM shows to increase the cytotoxicity of doxorubicin on WiDr colon cancer cells. The increase of cytotoxicity effect of doxorubicin and RBM combination need to be further investigated by apoptosis detection. RBM concentration 12.5 μg/mL (1/8 IC₅₀) and dox 0.4 μM (1/4 IC₅₀) were then used for the observation of apoptosis detection by double staining method. These concentrations were chosen because the cell viability in this combination was close to 50%. Therefore, the cell count would be optimal for the observation of apoptosis detection.

Table 1. Combination Index (CI) value of RBM and Dox combination

Concentration of RBM (μg/mL)	Concentration of Dox (μM)			
	0.1	0.2	0.4	0.8
	1/16 IC ₅₀	1/8 IC ₅₀	1/4 IC ₅₀	1/2 IC ₅₀
6,25 (1/16 IC ₅₀)	0.21	0.27	0.48	0.67
12,5 (1/8 IC ₅₀)	0.23	0.27	0.44	0.60
25 (1/4 IC ₅₀)	0.37	0.40	0.51	0.68
50 (1/2 IC ₅₀)	0.54	0.57	0.61	0.74

Table 2. The cell viability percentages in some dose combinations

Concentration of RBM (μg/mL)	Concentration of Dox (μM)				
	0	0.1	0.2	0.4	0.8
	0	1/16 IC ₅₀	1/8 IC ₅₀	1/4 IC ₅₀	1/2 IC ₅₀
0	100	73.9	69	64.3	57.3
6,25 (1/16 IC ₅₀)	84.3	73.5	68.6	69.5	59.4
12,5 (1/8 IC ₅₀)	73.3	62.3	55.8	59.2	49.2
25 (1/4 IC ₅₀)	71.8	60.7	55.5	53.1	46.4
50 (1/2 IC ₅₀)	63.7	50.4	46.9	39.3	32.2

(a) The Morphological Observation

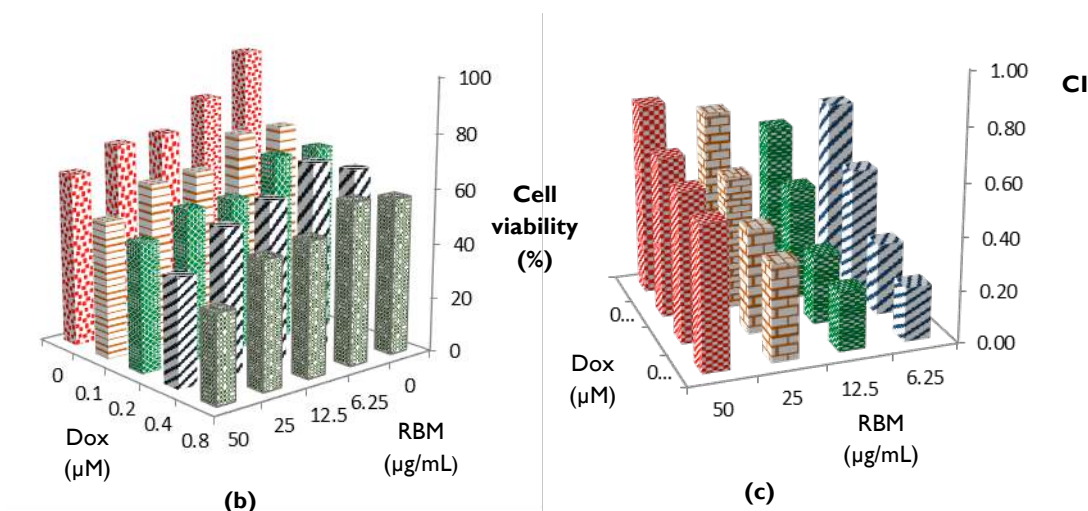
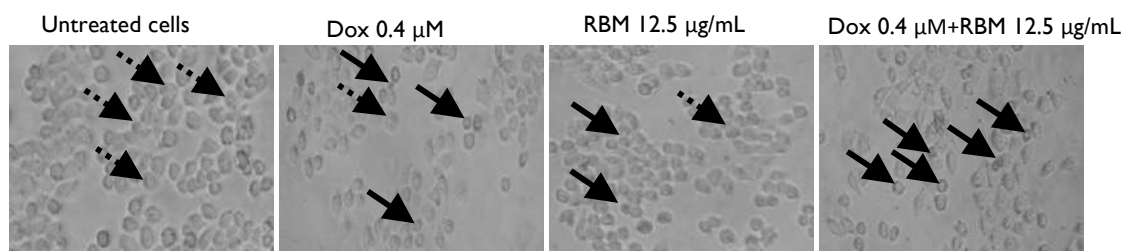


Figure 2. Effects of combination of RBM and Dox on WiDr cell viability. WiDr cells with density (13×10^3 cells/well) were treated by Dox and RBM in the concentration as indicated in the picture, then incubated for 24 hours. Cells viability were obtained from the conversion of absorbance values of formazan that formed by MTT treatment as described in the research procedure. (a) The morphological observation was using inverted microscope at magnification 400x. (b) The effect of RBM and Dox combination treatment on viability of WiDr cells ($n = 3$, $p < 0.05$) (left side). The value of inhibition was expressed as percentages of cell viability. (c) The concentration combination of test compounds vs CI. Description: Signs $--\blacktriangleright$ show normal cells, \blacktriangleright cells undergoing morphological changes.

Apoptosis Test using Double Staining

Observation of apoptosis detection was performed on WiDr cells after single treatment of RBM 12.5 μg/mL, dox 0.4 μM, and its combination at the same concentration. From apoptosis detection, combination of RBM and dox are able to increase the number of apoptosis cells than their single treatments (Fig. 3). The green fluorescence indicates the viable cells while the orange-red fluorescence indicates the death cells. Apoptosis cells were indicated by occurrence of chromatin condensation and the orange-red apoptotic bodies.

In single treatment of doxorubicin and RBM, some cells experienced early apoptosis that the membrane still has green fluorescence but the nucleus showed chromatin condensation. The highest incidence of apoptosis was in the combination treatment of doxorubicin and RBM which was characterized by a bright green nucleus with condensed or fragmented chromatin (early apoptosis) and condensed or fragmented orange chromatin (late apoptosis) (Fig. 3). From apoptosis detection, it shows that the combination of RBM and doxorubicin increase the number of apoptosis cells compared with their single treatments.

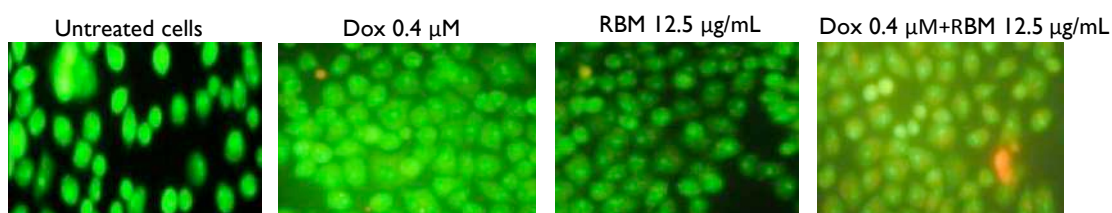


Figure 3. Apoptosis detection of RBM and dox on WiDr cells. WiDr cells (5×10^4 cells/well) were seeded on coverslips. The treatment was administered after 24 hours after the cell planting process, then cells were stained with acridine orange-ethidium bromide and observed under fluorescence microscope with 100x magnification. The apoptotic cells are indicated by dashed arrows colored white. From the figure it can be seen that in the combination treatment of dox $0.4 \mu\text{M}$ and RBM $12.5 \mu\text{g/mL}$ shows the number of cells experiencing apoptosis more than the single treatment.

DISCUSSION

The results show that red betel leaves methanolic extract (RBM) treatment for 24 hours incubation shows cytotoxic activity and apoptosis induction against WiDr cells. RBM has potential to be developed as a co-chemotherapeutic agent of doxorubicin because it contains some secondary metabolites including alkaloids and flavonoids that are known to have anticancer activities. Arishandy (2010) reported that flavonoids found in red betel leaves were flavonols, flavanones, isoflavones, and aurones. Fitriyani (2011) also reported that the result of flavonoid screening with Wilstater test showed an orange change which means that in methanolic extract of red betel leaves also contains flavon compound.

A chemotherapeutic agent is expected to induce apoptosis on cancer cells. Single treatment of doxorubicin (dox) for 24 hours is able to demonstrate cytotoxic effect. The results of cytotoxic activity of each single treatment performed that doxorubicin shows IC_{50} values of $1.6 \mu\text{M}$, while RBM shows IC_{50} values of $100 \mu\text{g/mL}$. Increased concentration of RBM and dox followed by decreased cell viability which was indicating a dose dependent manner. The IC_{50} value of RBM (methanolic) on WiDr colon cancer cells in this study is smaller than the IC_{50} value of red betel leaves ethanolic extract on HeLa cervical cancer cells with the IC_{50} value of $266.01 \mu\text{g/mL}$ which was conducted by Suci, *et al.* (2013). RBM also shows more potent on WiDr colon cancer cells than on 4T1 breast cancer cells which has the IC_{50} value of $120 \mu\text{g/mL}$ (Zulharini, 2015). However, the IC_{50} value of RBM on WiDr colon cancer cells is greater than the IC_{50} value of red betel leaves methanolic extract on

T47D breast cancer cells with $44.25 \mu\text{g/mL}$ (Wicaksono, *et al.*, 2009).

RBM has better potential cytotoxic activity when it is compared with other methanolic extracts on WiDr colon cancer cells. The methanolic extract of the *Ficus septica* Burm rhizome has an IC_{50} value of $423.62 \mu\text{g/mL}$ (Istiqomah, 2015), and methanolic extract of Tegari (*Dianella nemorosa* Lam.) which has a larger IC_{50} value of $1871 \mu\text{g/mL}$ on WiDr colon cancer cells (Karim, 2014). Potent cytotoxic activity indicates that RBM is potential to be developed as a co-chemotherapeutic agent of doxorubicin in the treatment of colon cancer.

The result of cell viability assay the RBM and dox combination with ratio 1/16, 1/8, 1/4, and 1/2 of IC_{50} for 24 hours incubation shows a stronger cytotoxic effect as well as induce apoptosis than their single treatments. All combinations also show a significant synergism with CI value of less than 1. Observation of apoptosis induction is done further to know the activity of RBM and doxorubicin combination.

Apoptosis detection on WiDr colon cancer cells in combination dose RBM at $12.5 \mu\text{g/mL}$ (1/8 IC_{50}) and dox at $0.4 \mu\text{M}$ (1/4 IC_{50}) shows able to increase the number of cells undergoing apoptosis compared to their single treatments. This is characterized by cell morphology with a bright green-orange due to chromatin condensation and the cells begin to experience blebbing membranes, so that ethidium bromide can enter the cells. The observation of apoptosis induction has similar pattern with the result of cell viability test using MTT assay that shows combination in both concentrations have cell viability of 59.2% (Table 2). This may be happen because cells which experienced early apoptosis and towards death still

have metabolic activities so these cells still able to reduce the salt of tetrazolium into formazan crystals. Therefore, it causes cells that have experienced early apoptosis still remaining visible as viable cells in the MTT assay.

Doxorubicin can induce apoptosis either p53-dependent or p53-independent. However, WiDr cells were used in this study characterized with overexpression of COX-2 and TS which had mutant p53 protein (Palozza, *et al.*, 2005; Noguchi, *et al.*, 1979). Therefore, increased the cytotoxic effect of RBM and doxorubicin combination on WiDr colon cancer cells is possible through p53-independent apoptotic pathway. In p53-independent, doxorubicin triggers extrinsic apoptosis pathway by stimulating caspase-8 activation and PARP cleavage (Lee, *et al.*, 2002).

Apoptosis induced by doxorubicin on WiDr cells is also possible through increased reactive oxygen species (ROS). The ROS pathway may activate apoptosis through p53-independent which increases Bax expression and decreases Bcl-2 expression in cells without involving p53 protein (Tsang, *et al.*, 2003). Besides that, the apoptotic induction ability of RBM on WiDr colon cancer cells is also possible because RBM contains flavonoids and alkaloids. In previous studies, flavonoid compound was reported to stimulate apoptosis through several mechanisms such as inhibition activity of DNA topoisomerase I/ II, modulation of signaling pathways, decreased expression of Bcl-2 and Bcl-XL genes, enhanced expression of Bax and Bak genes and activation endonuclease (Ren, *et al.*, 2003). While alkaloid was known to affect expression of Bcl-2 protein, increase caspase activation, and induce apoptosis p53-independent through inhibition of survivin (Kim, *et al.*, 2009). The presence of alkaloid in RBM may activate p53-independent apoptotic pathway through inhibition of survivin that can maximize activation of caspase effectors. Therefore, RBM may lead to direct the death of WiDr colon cancer cells through apoptotic mechanism.

All data obtained in this study indicate that RBM has cytotoxic activity against WiDr cells and its combination with doxorubicin shows a significant synergism and able induce apoptosis. However, further research is needed to disclose the underlying molecular mechanism and determine the active compound in RBM which is responsible for the

mechanism of cytotoxic effect and apoptosis induction on WiDr colon cancer cells.

CONCLUSION

Based on these results, the cytotoxic effect of doxorubicin on WiDr colon cancer cells could be enhanced with the use of red betel leaves methanolic extract (RBM) as a co-chemotherapeutic agent. RBM also increase the apoptosis induction together with the use of doxorubicin. The use of RBM as a combination agent may also reduce the dose of doxorubicin and expected to reduce the risk of side effects of doxorubicin in colon cancer chemotherapy.

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