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Ethanolic Extract of *Hedyotis corymbosa* L. Increases Cytotoxic Activity of Doxorubicin on MCF-7 Breast Cancer Cell

Sari Haryanti[1], Sendy Junedi[2] and Edy Meiyanto[2*)]

1. Medical Plant and Traditional Medicine Research and Development Office, Tawangmangu, Central Java, Indonesia
2. Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract

Hedyotis corymbosa L. with ursolic acid as the main compound is one of the plants that has been used for traditional medicine including to cure breast cancer disease. The aim of this research is to examine the cytotoxic activity of rumput mutiara herb ethanolic extract (ERM) and its effect in combination with doxorubicin against MCF-7 breast cancer cell line as cell model of doxorubicin resistance. *Hedyotis corymbosa* L. herb powder extraction was done by maceration using ethanol 96% then the extract is detected for ursolic acid content. Cell viability assay of ERM, doxorubicin and the combination of ERM and doxorubicin treatments were carried out by MTT assay to determine IC_{50} and CI (Combination Index). Cell cycle distribution was determined by flowcytometry. Apoptosis assay was performed by ethidum bromide-acridine orange DNA staining method. Investigation on Bcl-2 expression was determined by immunocytochemistry method.

Thin Layer Chromatography of ERM had similar Rf with ursolic acid standard: 0,6. ERM and doxorubicin inhibited cell growth against MCF-7 with IC_{50} of 77 µg / mL and 349 nM (0,19 µg / mL) respectively. Combination of ERM and doxorubicin showed synergistic effect (CI 0.66-0.99). Combination of 25 ìg / mL ERM- 200 nM doxorubicin induced apoptosis and decreased Bcl-2 expression but showed no cell accumulation on cell cycle. Doxorubicin induced high cell accumulation in G2 / M phase, but ERM at the concentration of 25 ìg / mL had a low effect in G1 phase, and ERM IC_{50} did not induce cell accumulation otherwise apoptosis. These results concluded that the apoptosis mechanism of combination doxorubicin-ERM is mediated by cell cycle arrest and non cell cycle arrest. Therefore ERM has a potential activity to be developed as co-chemotherapeutic agent.

Keywords: *Hedyotis corymbosa* L., doxorubicin, co-chemotherapy, apoptosis , MCF-7

Introduction

Resistance to the chemotherapy is common problem in cancer therapy. MCF-7 cell line characterized by p53 wildtype is one of the breast cancer cell lines which is resistance to doxorubicin due to over expression of P-gp and Bcl-2 (Mealey *et al.*, 2001; Davis *et al.*, 2003; Simstein, 2003). Co-chemotherapy of doxorubicin with chemopreventive agent is one alternative to resolve the resistance in order to increase efficacy and reduce toxicity of doxorubicin.

Rumput mutiara (*Hedyotis corymbosa* (L.) Lamk, member of Rubiaceae family, at the beginning was used for treating appendicitis, peritonitis in China, then nowadays it is developed as anticancer (Dharmananda, 2007). The common compound which is play apart in cytotoxic activity of rumput mutiara is ursolic acid, a pentacyclic triterpenoid. Ursolic acid was known have anticancer activity through inhibition of carcinogenesis, cancer

*Corresponding author : Dr. Edy Meiyanto, M.Si., Apt. Faculty of Pharmacy, Gadjah Mada University, Sekip Utara, Yogyakarta 55281, Telp / Fax: (0274) 543120, E-mail : meiyan_e@yahoo.com http://www.ccrc.farmasi.ugm.ac.id

promotion and angiogenesis. Those activities are mediated by the abilities of ursolic acid to inhibit activation of nuclear factor-kappaB (NF-eB). NF-eB is a protein that regulating expression of some genes which play a role in cancer formation process; including antiapoptosis genes, genes regulating molecule adhesion, and genes regulating cell cycle (Shishodia *et al.*, 2003). Moreover, ursolic acid can induce apoptosis by inhibiting cell cycle of MCF-7 breast cáncer cells at G0/G1 phase and increasing p53 expression (Zhang *et al.*, 2005). Therefore, a compound which is able to inhibit NF-eB activation, increase p53 expression and inhibit cell cycle has the therapeutic potency as anticancer agent.

This research is aimed to examine the cytotoxic effect of rumput mutiara herb ethanolic extract (ERM) and its combination with doxorubicin on MCF-7 breast cancer cell line. Moreover, analysis of apoptosis, Bcl-2 expression and cell cycle are conducted to understand the mechanism of those cytotoxic effects. Finally, the result is going to be the scientific evidence of Indonesian herb usage in the effort to develop Indonesian herb as combination agent which can resolve resistance of doxorubicin toward breast cancer cell.

Materials and Methods

Materials

Doxorubicin (Dox) was purchased from Ebewe, PT. Ferron Par Pharmaceutical. *Hedyotis corymbosa* L. herb was collected in Solo, Central Java, and determined in Balai Besar Litbang Tanaman Obat dan Obat Tradisional, Tawangmangu, Central Java. Materials for extraction and detection are ethanol 96%, silika gel 60 F254 (Merck), chloroform-aceton (9:1) (Merck), 10% sulfuric acid and ursolic acid (Sigma). ERM and Dox were dissolved in Dimethyl Sulfoxide (DMSO) (Sigma). MCF-7 breast cancer cell was kindly accepted from Prof. Tatsuo Takeya (Nara Institute of Science and Technology, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing Fetal Bovine Serum (FBS) 10.% (v/v) (Gibco), penicillin-streptomycin 1 % (v/v) (Gibco) and 0.25.% trypsin-EDTA (Gibco) was used to detach cells from flask and plate. For MTT assay, use MTT [3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma), isopropanol acid (HCl 4N (Merck)-isopropanol (1:100) (Merck). For apoptosis assay, uses acridine orange (Sigma)-ethidium bromide (Sigma). For immunocytochemistry, antibody against Bcl-2 (Cell signaling), universal detection kit (streptavidin-HRP, biotinylated secondary antibody, blocking serum, 3,3'-diaminobenzidin (DAB)) (Ultravision plus detection system, Ref TP 125-HLX, Runcorn, Cheshire, WA71PR, UK; Novostain Universal Detection kit NCL-RTU, Novocastra Lab Ltd., Newcastle NE12 8EW, UK), mayer's hematoxylin (Dako). For cell cycle analysis usesTriton-X (Merck), RNAse, propidium iodide (Sigma) in PBS.

Methods

Extraction of rumput mutiara herb (Hedyotis corymbosa L.).

ERM was prepared by extracting the dried herb with ethanol 96%. Then, the menstrum was evaporated by rotary evaporator and dried by freeze drying to get the viscous extract.

Detection of ursolic acid

1% ERM and 0.1% ursolic acid in methanol spotted on silica gel plate, then eluted in chloroform-aceton (9:1). The plate was dried and spried by 10% sulfuric acid then heated at 110°C for 5 min and observed under visible light.

Assessment of cell viability

Cells were seeded at density of 5x10

cells / well in 96 well plate and incubated with various concentration of ERM (50-200 mg / mL), Dox (115-1150 nM) and combination of ERM (8, 20, 25, 40 mg / mL)-Dox (4, 100, 135, 200 nM) for 48 h in incubator 37°C, 5% CO_2. Thereafter the medium was changed and 100 µL MTT 0.5 mg / mL was added to incubate for 4 h in incubator 37°C. The viable cells were directly proportional to the production of formazan. Following dissolution in 100 µL isopropanol-HCl, the absorbations were read at 595 nm with ELISA reader (Bio-Rad microplate reader Benchmark). IC_{50} value of ERM and Dox were determined based on the equation of linier regression of log concentration vs % cell viability. Combination Index (CI) was determined according to the equation developed by Chou (Reynolds and Maurer, 2005). CI>1 shown antagonist, CI=1 shown additive, and CI<1 shown synergy (Zhao *et al.*, 2004; Reynolds and Maurer, 2005).

Apoptosis assay

Cells were seeded on cover slip at a density of $3x10^4$ cells / well in 12 well plate and incubated with ERM (25 mg / mL), Dox (200 nM) and combination of ERM-Dox for 24 h at 37°C, 5% CO_2. Thereafter, the medium was removed and rinse with PBS. 10 mL acridin orange-ethidium bromide was added to cover slip. Observation was conducted by fluorescence microscope (Zeiss MC 80). The color of apoptotic cell was orange and there were nuclear fragmentation and apoptotic body. Otherwise, the color of normal cell was green.

Immunocytochemistry

Cells were seeded on cover slip at density of $5x10^5$ cells / well and incubated with ERM (25 mg / mL), Dox (200 nM) and combination of ERM-Dox for 18 hours in incubator 37°C, 5% CO_2. The medium was removed and rinsed with PBS. Then, cells in cover slip were fixed with methanol and rinse with PBS. Hidrogen peroxidase was added to incubate for 10 minutes followed with addition of prediluted blocking serum for 10 minutes in room temperature. Monoclonal antibody anti Bcl-2 was added to incubate for 24 h in temperature 4°C. Biotinylated secondary antibody was added fro 10 min followed with addition of streptavidin-peroxidase complex for 10 min. Cells added with DAB for 10 minutes were submerged in Mayer's Haematoxylin for 3 min. The expression of Bcl-2 was observed under light microscope (Nikon YS100). Bcl-2 expression was shown by brown color in cytoplasm.

Cell cycle analysis

Cell cycle analysis was performed with a flowcytometer (FACS Calibur). 10^4 cells / well were cultured in 6 wells plate and treated with ERM (8, 25, 40 mg / mL), Dox (40, 200 nM) and combination of ERM (8, 25mg / mL)-Dox (40, 200 nM) for 24 h. Thereafter, the cells rinsed with PBS and centrifuged at 2000 rpm for 3 min. The attached cell was detached with 100 µl tripsin-EDTA for 3 min then added 1 mL of DMEM and centrifuged at 2000 rpm for 30 sec. After that, cells rinsed with cold PBS and centrifuged at 2000 rpm for 30 sec. Prior to the samples being analyzed by the flowytometry, propidium iodide (PI) stain solution (7% Triton-X, 0.2% RNAse, 5% propidium iodide) was added to the mixture and it was incubated for 10 min in darkness at temperature of 37°C. Data acquisition and analysis were performed in the flowcytometer with accompanying software (Cell Quest). The percentage of hypodiploid cells (sub G1 phase) over total cells was calculated and represented as percent of apoptosis.

Results and Discussion

Thin Layer Chromatography detection of

ERM

TLC profile of ERM showed a pink spot in visible light and the Rf (0.6) was similar to the ursolic acid standard (Figure1). Rumput mutiara contains pentacyclic triterpenoid of ursolic acid and oleanolat acid which the difference was only take place in the location of one methyl group of E ring. Therefore the separation of both compounds was difficult in TLC even using isocratic system. Based on that, the pink spot in TLC profile of ERM was probably made up from the combination of ursolic acid and oleanolat acid.

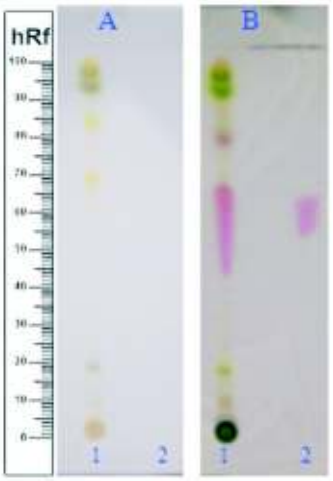

Figure 1. TLC profile of ERM. Elution was used mobile phase chloroform-aceton (9:1). Rumput mutiara herb ethanolic extract is 1 and ursolic acid standard is 2. TLC plate before (A) dan after (B) spraying with sulfuric acid 10% in ethanol which was then heated in 110°C for 5 minutes was seen pink spot in visible light.

Assessment of cell viability of rumput mutiara herb ethanolic extract (ERM), doxorubicin and its combination on MCF-7 Cell

ERM and doxorubicin inhibited cell viability of MCF-7 cell with IC_{50} value 77 µg / ml and 349 nM respectively (Figure 2).

According to the cell viability and cell morphology, ERM and Dox treatment showed linier correlation toward its concentration. Morphological change was probably caused by death cell but the mechanism neither apoptosis nor necrosis was not revealed yet.

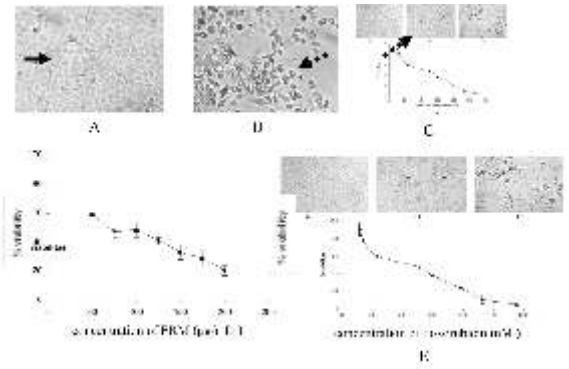

Figure 2. Cell viability after ERM and doxorubicin treatment on MCF-7 cell. The assessment were conducted by incubating 5x10⁵ cell with ERM (50-200 µg / mL), Dox (115-1150 nM) for 48 hours. Treatment ERM 125 µg / mL (B) and Dox (506 nM) shown morphological change compare with control cell (A). Graphics of cell viability percentage vs ERM concentration (D) and cell viability percentage vs Dox concentration (E) shown linier correlation.
Magnification 100x. A.normal cell, B.morphologically changed cell

Table 1. CI value of ERM and Dox combination on MCF-7

Concentration of Dox (nM)	Concentration of ERM (µg/mL)			
	8	**20**	**25**	**40**
40	0.92	1.70	1.81	2.72
100	1.61	2.17	2.34	3.41
135	1.13	1.48	1.36	1.97
200	0.66	0.90	0.85	0.99

Combination treatment of ERM and Dox gave synergistic effect at Dox concentration 200 nM and all ERM concentrations (8, 20, 25 and 40 µg / mL) with CI values 0.66-0.99 (Table 1). These results revealed that ERM has the ability to increase the sensitivity of MCF-7 cell toward Dox. Dox concentration below to ½ IC_{50} did not show synergistic effect instead it gave antagonistic effect. It might be caused by dox and ERM concentrations which were too low (1 / 10 – 1 / 3 IC_{50}) therefore the combination effects were not optimal.

The effect of ERM, dox and its combination on apoptosis

All cells in control cell gave green fluorescence showing there was no death cell. ERM and dox treatment caused some cells gave orange and red fluorescence which signed the increasing of cell membrane permeability as the indicator of apoptosis. The nuclear of several cells were fragmented and formed apoptotic bodies. The number of cells which were occurred apoptosis was just few because the concentration of ERM and dox were under IC_{50}: ERM 25 µg / mL (1 / 3 IC_{50}) and dox 200nM (1 / 2 IC_{50}). Furthermore the incubation period was 24 hours, shorter than the period of viability assessment. Combination treatment of ERM and dox shown increasing of apoptotic cells number compared with single treatment of ERM and dox (Figure 3).

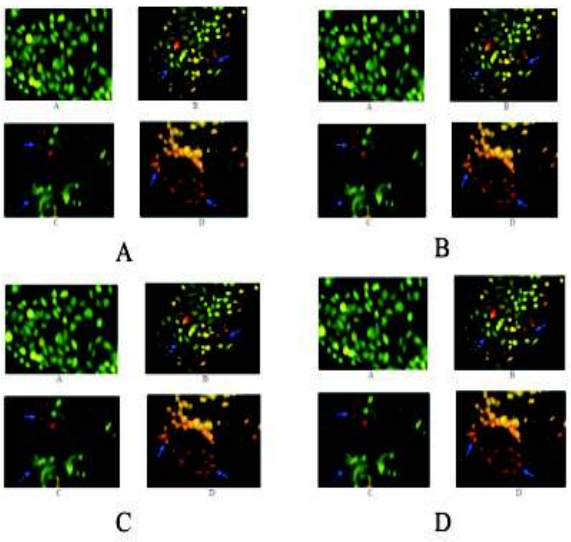

Figure 3. Apoptotic induction after treatment of ERM, dox and its combination on MCF-7 cell. 5x10^4 cells on coverslips in 24 well plate were incubated for 24 hours with ERM 25µg / mL (B), dox 200 nM (C) and its combination (D) then stained with acridine orange-ethidium bromide and observed under fluorescence microscope. Cell control was shown in A. Magnification 100x, live MCF-7 cell, apoptotic cell

The effect of ERM, dox and its combination toward Bcl-2 expression on MCF-7 cell

Antiapoptotic protein, Bcl-2, is expressed highly in MCF-7 cell and it is correlated with low effect of chemotherapy agent. Based on that, the exploration of ERM and dox apoptotic mechanism either single or combination were directed to inhibit Bcl-2 expression. The qualitative observation shown the decreasing of Bcl-2 expression after single treatment of ERM 25 ìg / mL, dox 200nM and both combination compared with cell control (Figure 4). The decreasing of Bcl-2 expression in combination treatment was more than single treatment of ERM and dox which was signed by low intensity of brown color in cytoplasm compare with single treatment. Therefore, each ERM and dox was contributed to Bcl-2 expression in combination treatment.

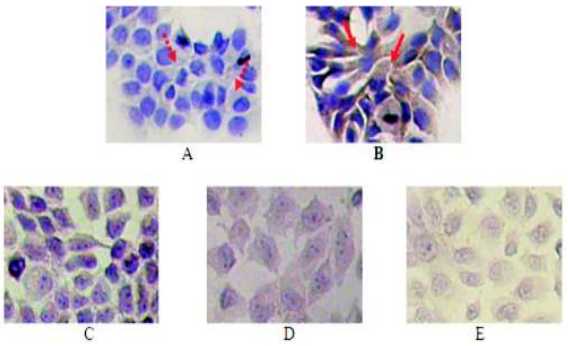

Figure 4. The effect of ERM, dox and its combination toward Bcl-2 expression on MCF-7 cell. 5x10^4 cells were seeded on coverslips in 24 well plate. Two coverslips were as cell control and remains were treated with ERM 25µg / mL, dox 200 nM and its combination for 18 hours. Thereafter it was stained by using immunocytochemistry with primer antibody anti-Bcl-2 as mentioned in the method. (A) Cell control without primery antibody Bcl-2, (B) cell control with with vehicle treatment, (C) treatment of dox 200 nM, (D) treatment of ERM 25 µg / mL, (E) combination treatment of dox and ERM (200 nM-25 µg / mL). Observation was done under light microscope, with magnification 400x.
Cell expresses Bcl-2, cell does not express Bcl-2.

The effect of ERM, dox and its combination toward cell cycle modulation on MCF-7 cell

Distributions of MCF-7 cells in cell cycle phases after single and combination treatment were described in Table 2.

Table 2. Distribution of MCF-7 cell in cell phases after treatment of ERM, dox and its combination.

treatment	concentration	Percentage of cell number (%)				
		Sub G1	G1	S	G2/M	Polyploidy
control	-	1.31	41.83	21.46	22.78	13.26
ERM	8 µg/mL	1.85	42.36	18.52	22.32	15.08
	25 µg/mL	3.05	48.89	20.96	19.88	7.12
	80 µg/mL	7.30	42.29	18.30	18.86	13.65
dox	40 nM	2.59	18.97	12.96	49.27	16.73
	200 nM	6.67	37.95	9.86	35.76	10.21
combination	25 µg/mL ERM 200 nM dox	13.61	40.02	11.26	23.68	10.96
	8 µg/mL ERM 40 nM dox	5.21	32.18	5.78	47.19	9.85

Dox 40 nM dan 200 nM shown high cell accumulation in G2/M phase but the cell accumulation of dox 200 nM was less than dox 40 nM because cells in sub G1 of dox 200 nM was more than dox 40 nM. The treatment of ERM 25 µg/mL caused low cell accumulation in G1 phase but at concentration IC_{50} (80 µg/mL) there was no accumulation in G1 phase. ERM 80 µg/mL shown increasing of sub G1 more than ERM 25 µg/mL. Increasing of sub G1 cells was showing the increasing of apoptotic cell and this result was correlated with the result of cytotoxic effect of dox and ERM.

The combination treatment of ERM-dox 8 µg/mL-40 nM increased G1 and sub G1 accumulation and decreased G2/M accumulation of dox single treatment (40 nM).This result showed the synergistic effect of ERM and dox in cell cycle which is dominated by dox. Therefore, G2/M phase was still high. The cell cycle profile of ERM-dox 25 µg/mL-200 nM was similar to cell control except sub G1 (Figure 5.H) so the synergistic effect of its combination was mediated by apoptosis. The result above revealed that the synergistic effect was not only mediated by cell cycle accumulation but also apoptosis

ERM inhibit the viability of MCF-7 cell with low IC_{50} (77 mg/mL, less than 100

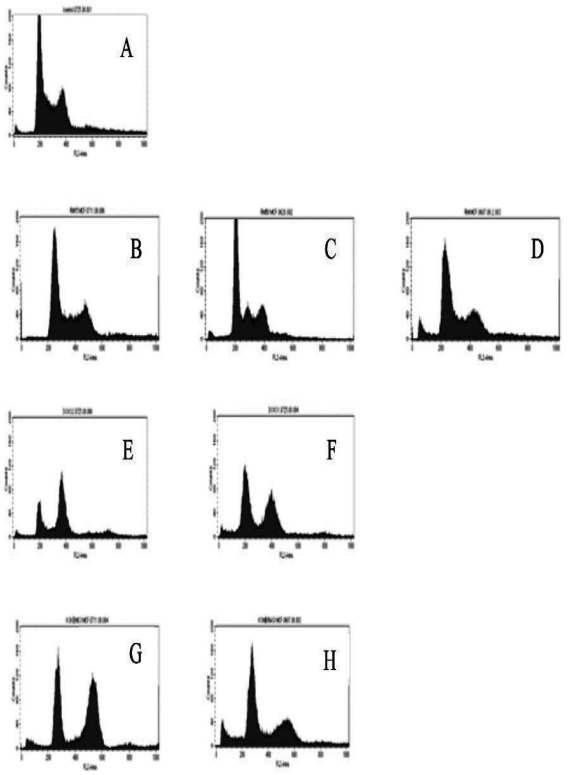

Figure 5. Cell cycle analysis of MCF-7 after treatment of ERM, dox and its combination. 10^5 cells were seeded in 6 well plate and incubated with ERM (8, 25, 80 µg/mL), dox (40, 200 nM) and combination ERM-dox (8 µg/mL-40nM; 25 µg/mL-200 nM) for 24 hours. Cells were harvested then added with PI reagent and analyzed with *flowcytometer*. (A) cell control, (B) ERM 8 µg/mL, (C) ERM 25 µg/mL, (D) ERM 80 µg/mL, (E) dox 40 nM, (F) dox 200 nM, (G) ERM-dox 8 µg/mL-40nM, ERM-dox 25 µg/mL-200 nM.

mg/mL) so it is potential to develop as alternative chemotherapy agent. TLC profile shown pink spot which Rf was similar with Rf of standard ursolic acid. The spot contained of ursolic acid and oleanolat acid that difficult to separate because of similar structure. In MCF-7 cell, ursolic acid has IC_{50} 4,7 µg/mL while oleanolat acid has lower activity than it. Until concentration 20 µg/mL, oleanolat acid only inhibited 30% of MCF-7 cell viability (Chen *et al.*, 2005). Based on that research, ursolic acid was more contributed to cytotoxic activity than oleanolat acid in MCF-7 cell.

MCF-7 cell has lowsensitivity to dox showed by the high IC_{50}, that was 349 nM. That value was much bigger than IC_{50} dox on T47D breast cancer cell, that was 43 nM (Jenie and Meiyanto, 2007). Viability assessment of combination treatment by using high concentration of dox (200 nM) and various concentration of ERM (8, 20, 25 dan 40 µg / mL) gave low CI categorized in synergistic effect and the result was correlated to apoptosis assay. Combination treatment of dox 200 nM and ERM 25 µg / mL increased apoptosis incident of dox and ERM single treatment. That synergistic effect can be used as the basic of co-chemotherapeutic development.

The exploration of apoptosis mechanism was correlated with expression of Bcl-2 regulator protein. The expression of Bcl-2 was decreasing after treatment of dox, ERM and its combination. One protein controlling Bcl-2 expression is NF-eB, a transcription factor (Aggarwal *et al*, 2006). NF-eB is expressed highly in malignancy process of breast cancer (Shehata, 2005). Ursolic acid has the ability to decrease activation of NF-eB through inhibit IeBa kinase and phosphorilation of p65 which was correlated with the decreasing of cyclin D, COX-2, and MMP 9 expression. Therefore, the decreasing of Bcl-2expression which then inducing apoptosis in ERM treatment was probably mediated by ursolic acid. ERM which inhibited Bcl-2 expression can also increase FasL expression (Srivastava, 1999) while dox can induce Fas receptor expression (Yamaoka *et al*., 2000). Both mechanism probably produce synergistic effect in combination treatment of ERM and dox, mediated by intrinsic and extrinsic pathway of apoptosis.

Analysis of cell cycle can explain further about synergistic mechanism of its combination treatment. Dox induce cell accumulation in G2 / M phase at all range of concentrations while ERM induce cell accumulation in G1 phase at concentration 25 mg / mL (1 / 3 IC_{50}). In higher concentration of ERM, 80 mg / mL (IC_{50}) percentage of apoptotic cell increased and it was probably happened because p53 was expressed highly by ERM induction. Whereas, in low concentration of ERM (25 mg / mL), p53 expression was probably relative low so cell cycle arrest was more than apoptosis. Cell accumulation in G2 / M phase after treated with dox was probably caused by increasing of p53 dan $p21^{WAF1}$ expression through Ras pathway. Then it was followed by upregulation of cyclin D3 and E, and downregulation of p16. Therefore, cell passed G1 checkpoint yet detained in G2 / M phase, because there was no upregulation of cyclin B. Different with dox, the increasing of p53 expression induced by ERM probably did not cause upregulation of $p21^{WAF1}$ so there were downregulation of cyclin D3 and E, and upregulation of p16. Thereafter cell was detained in G1 checkpoint (Mansilla *et al*, 2003; Zhang *et al*, 2005).

Combination treatment of dox-ERM 40 nM- 8 mg / mL shown synergistic effect with cell accumulation in G2 / M phase. The G2 / M cell accumulation which was like dox single treatment was happened because the effect of ERM low concentration (8 mg / mL) was lower than dox. The synergistic effect was also happened in dox-ERM 200 nM- 25 mg / mL with increasing of apoptosis but no inhibition of cell cycle. In combination of dox-ERM 200 nM- 25 mg / mL, either ERM or dox gave effect to cell cycle by increasing p53 expression. Therefore p53 expression of dox-ERM 200 nM- 25 mg / mL was much higher than single treatment and it made increasing of apoptosis incident.

Apoptotic mechanism of combination treatment dox-ERM in MCF-7 cell was mediated by cell cycle arrest and non cell cycle arrest. The mechanism of non cell cycle

arrest was probably mediated by decreasing of NF-eB activation and increasing of FasL, Fas and p53 expression. The ability of ERM to increase cytotoxic activity of doxorubicin mediated by various mechanisms of apoptosis make ERM has the potency to develop as co-chemotherapeutic agent with doxorubicin in breast cancer. In addition, rumput mutiara (*Hedyotis corymbosa* L.) grow easily and not used widely yet so it is more potential to develop further.

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