The Synergistic Effect of Doxorubicin and Ethanolic Extracts of *Caesalpinia sappan* L. Wood and *Ficus septica* Burm. f. Leaves on Viability, Cell Cycle Progression, and Apoptosis Induction of MCF-7 Cells

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Abstract

Caesalpinia sappan L. and *Ficus septica* Burm. f known as a potential plant with wide variety of medicinal properties, including anticancer. Present study was aimed to explore cytotoxic effect of sappan wood (ECS) and awar-awar leaves (EFS), and its combination with doxorubicin (dox) on MCF-7 cells focusing on cell cycle progression and apoptosis induction. The result of MTT assay showed that single treatment of ECS and dox performed cytotoxic effect with the IC_{50} value of 32 µg/mL and 6 µM respectively, while EFS performed low cytotoxic effect with the IC_{50} value of 282 µg/mL. The combination of ECS with EFS and doxorubicin showed synergistic cytotoxic effect. Flow cytometry analysis revealed that combination of ECS (16 µg/mL) with EFS (8 µg/mL) and doxorubicin (2 µM) induced apoptosis, and cell accumulation at sub-G1 and G2/M phases. Immunoblotting assay confirmed the apoptosis induction of this combination through increasing of cleavage of PARP-1. Based on these results, the synergistic cytotoxic effect of this combination was through G2/M phase accumulation and apoptosis induction and potentially to be developed as co-chemotherapeutic agent.

Keywords: Sappan wood, *Ficus septica* leaves, doxorubicin, MCF-7, cell cycle, apoptosis

Introduction

Cancer is a malignant disease, marked by uncontrolled cell proliferation and spreading due to the aberration of many regulatory growth system (Witsch *et al*., 2010). Continuing proliferation and evading apoptosis are the main factors of the malignancy and mainly driven by cell cycle progression with regardless of check point control (Barnum and O'Connell, 2014). There are many molecular event in these physiological processes involving growth signaling through MAP Kinases, cell cycle regulatory proteins, and apoptosis regulation (Santarpia *et al*., 2012). Most of the signal regulatory system are the potential pivotal targets of anti cancer agents. Therefore, the development of combinatorial anti cancer agents should consider to the main predominantly molecular event in particular cancers (Kummar *et al*., 2010).

To date, many molecules have been identified to perform cytotoxic activity targeted on (some) cancer markers such as RTK (receptor tyrosine kinase) inhibitors (Takeuchi and Ito, 2011), CDK (cyclin-dependent kinase) inhibitors (Asghar *et al*., 2015), NF-κB inhibitors (Hoesel and Schmid, 2013) and MMPs inhibitors (Cathcart *et al*., 2015). Some of these active molecules are natural plant origin. Meiyanto *et al*., 2012 noted that some citrus flavonoids perform cytotoxic activities to some cancer cells through inhibition of MAPK and NF-κB activation (Meiyanto *et al*., 2012). Curcumin and other compounds in turmeric are also reported to

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have anti cancer activity with some molecular targets in cell cycle, apoptosis and cell migration (Tuorkey, 2014). Some alkaloids component also known to have cytotoxic activity to some cancer cells, such as taxane alkaloids and phenanthroindolizidine (Chemler, 2009; Dumontet and Jordan, 2010). Interestingly, the compounds composed in the potential anticancer plants are believed to contribute in the cytotoxic activity trough different targets and may perform synergistic effect.

Caesalpinia sappan L., commonly known as sappan wood or secang in Indonesia, is a plant of Leguminosae family, which has been used widely as traditional medicine in Asia (Nirmal *et al*., 2015). Chemical investigation resulted that brazilin/brazilein is the major compound of sappan wood (Mekala and Radha, 2015). Brazilin is easily oxidized to produce brazilein by air and light. Both brazilin and brazilein responsible for various biological activities of sappan wood, including anticancer (Nirmal *et al*., 2015). *Ficus septica* Burm. f. (awar-awar) ethanolic leaves extract induced apoptosis and downregulated Bcl2 expression on MCF-7 breast cancer cell lines (Sekti *et al*., 2010). The n-hexane insoluble fraction and ethyl acetate soluble fraction from leaves ethanolic extract exhibited cytotoxic activity on T47D breast cancer cell lines (Nugroho *et al*., 2011). The hexane fraction combined with doxorubicin showed synergistic activity on T47D (Nugroho *et al*., 2013). In our previous study (unpublished data) ethanolic extract of *F. septica* leaves showed strong antimigration activity on 4T1 cells by wound healing assay, while sappan wood did not show the effect. Therefore, we suggest the combination of both extract might support each other with different mechanism and resulting a synergistic effect with lower dose of doxorubicin. Thus, the combination might be able to combat cancer through inhibition of proliferation and also metastasis. This present study was aimed to explore cytotoxic effect of extract in MCF-7, the human breast cancer cell line, and its combination effect with doxorubicin. Furthermore, we also investigated the possible combinatorial effect of doxorubicin and the extract in cell cycle progression and apoptosis induction.

Materials and Methods

Materials and extraction

Sappan wood (*Caesalpinia sappan* L.) and awar (*Ficus septica* Burm. f.) leaves obtained from Medicinal Plant and Traditional Medicine Research and Development Center, Tawangmangu, Central Java, Indonesia. They were sliced, dried in 40°C, pulverized, and extracted with ethanol 96% by maceration method. The dried extract of sappan wood (ECS) and ficus leaves (EFS), and also doxorubicin (dox) were dissolved in DMSO (Sigma), and freshly diluted in culture medium in several concentration before used.

Cell culture

MCF-7 cell lines were obtained from ATCC HTB-22 and maintained in laboratory of Molecular Biology, Medicinal Plant and Traditional Medicine Research and Development Centre, Ministry of Health, Tawangmangu, Jawa Tengah, Indonesia. Cells were cultured in Minimum Essential Medium (MEM Gibco) containing 10% fetal bovine serum/FBS (Gibco), 1% penicillin-streptomycin (Gibco), and incubated in CO_2 incubator 5% at 37°C.

Cytotoxic MTT assay

Approximately $1x10^4$ MCF-7 cells/well were seeded in 96-well plates and incubated for 48 hours. Cells were treated with increasing concentration of the extract or doxorubicin either alone and in combination for 24 hours. Cultured medium was removed and cells were washed with PBS (Sigma). MTT 0.5 mg/ml in medium were added into each well and incubated for 3–4 hours. MTT reaction was stopped by the addition of 10% SDS in 0.01 N HCl, and incubated overnight in the dark room. The absorbance was measured using ELISA reader at λ 595 nm (Biorad). Each treatment were carried out in triplicate, and the absorbance data are provided as percent viability compared to control cells (untreated).

Cell cycle and apoptosis induction by flow cytometry assay

Approximately 5x10^5 MCF-7 cells/well were cultured in 6-well plate and incubated for 48 hours. Cells were then treated with the extract and doxorubicin, either alone or combination for 24 hours. Cells were harvested with trypsin EDTA, washed with phosphate-buffered saline (PBS), and centrifuged 500 rpm for 5 minutes. For apoptosis induction, cells then incubated with annexin-V-FITC and propidium iodide (BD Pharmingen) for 15 min in the dark, and analyzed by BD Accuri C6 Flow cytometer. To determine cell cycle distribution, cells were fixed with cold ethanol 70% for 30 minutes, washed with PBS, and centrifuged 500 rpm for 5 minutes. Cells were then resuspended in PBS containing 40 µg/ml propidium iodide (Sigma), 20 µg/mL RNAse (Roche) and 0.1% TritonX-114 (Sigma) for 15 minutes in the dark, and then subjected to BD Accuri C6 flow cytometer.

Western Blot

Approximately 10^6 MCF-7 cells were seeded in 10 cm tissue culture dish, and incubated for 24 hours. Cell were treated with the extract and doxorubicin, either alone or combination for 24 hours. Protein was extracted using Pro-prep (Intron Biotechnology), then separated in 14% acrylamide gel by SDS-PAGE electrophoresis. After transferring to polyvinylidene fluoride (PVDF) membrane, the membrane was incubated overnight at 4°C with either the rabbit monoclonal antibody of PARP-1 (Cell Signaling D64E10) or mouse monoclonal antibody of β-actin (Santa Cruz sc-47778). After incubation with secondary antibody anti-rabbit (Santa Cruz sc-2357) and anti-mouse (Santa Cruz sc-516102) for 1 hour, the protein bands were visualized using ECL (Amersham) and detected by Luminograph. The relative protein levels were calculated in reference to the amount of β-actin protein.

Data analysis

To calculate the IC_{50} value from single cytotoxicity assay, we plotted linear regression of concentration and % cells viability using Excel MS Office 2013. Combination treatment was evaluated by calculating the Combination Index (CI) value with Compusyn software. The data obtained from flow cytometer was analyzed using BD Accuri C6 software.

Results and Discussion

Results

Cell growth inhibitory effect by single treatment of ECS, EFS, and dox

Our previous report noted that ethanolic extract of ficus leaves and sappan wood performed cytotoxic effect on T47D breast cancer cells through cell cycle arrest and apoptosis induction (Nugroho *et al*., 2015; Nurzijah *et al*., 2012). In this report, we use MCF7 breast cancer cells, a type of cancer cell with caspase-3 mutation (Ghavami *et al*., 2009), to explore the response of the cells to the agents in single or in combination. The MCF-7 cells treated with single ECS, EFS, and dox showed cytotoxic effect in dose dependent manner with the IC_{50} value of 32 µg/mL, 282 µg/mL, and 6 µM respectively (Figure 1). Based on these results, we were then assessed the combination effect of the extract and doxorubicin in some series concentration on MCF-7 cells.

Combinatorial effect of ECS, EFS, and dox on MCF-7 cells viability

To evaluate the combinatorial effect of these agents, we set the treatment with the series concentration of each agent under IC_{50} values. The result showed that the increasing concentration of ECS 4, 5, 8, and 16 µg/ml and EFS 2, 3, 4, and 8 µg/ml in combination was not followed by decreasing cell viability, and resulted combination index (CI) value 1.0–4.1. These CI values indicated that combinational treatment of this extract exhibited an additive and antagonistic inhibitory effect on MCF-7 cells. Additive effect was only achieved at the highest leaves concentration used in this experiment. However, when those extract combined with doxorubicin 1.0, 1.5, and 2 µM, it resulted synergistic effect. The only antagonistic effect

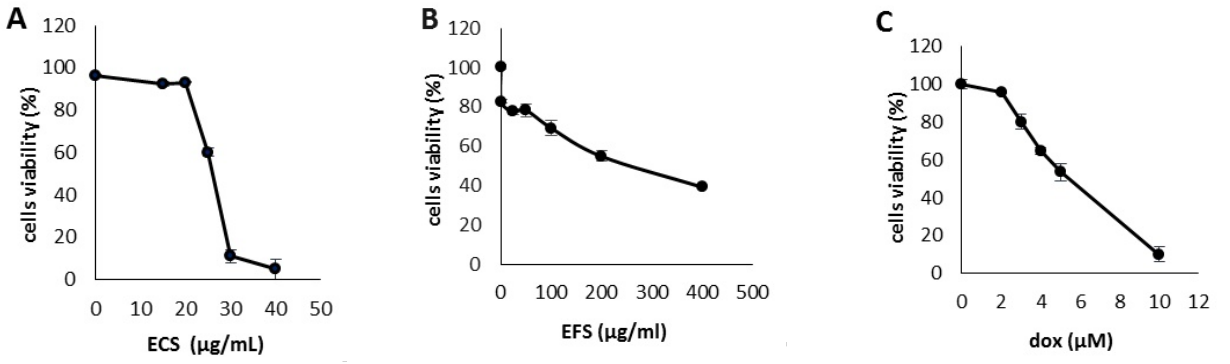

Figure 1. Cytotoxic effect of ECS, EFS, and dox on MCF-7 cells. Cells were seeded in 96-well plate and treated with ECS (A), EFS (B) or dox (C) for 24 hours with the series of concentration as indicated. Cells viability were determined by MTT assay as described in methods, performed in triplicate, and represented as mean ± SD of % cell viability. IC_{50} value was calculated using linier regression.

was showed at the lowest concentration of doxorubicin (0.5 µM) (Figure 2 and Table 1).

Cell cycle progression and apoptosis induction

The combination effect of dox and the extract in cell growth inhibition could be as the result of the modulation in physiological process of the cells. To investigate the effect of the combinatorial treatment in particular physiological process of the cells we further explored MCF-7 cell cycle progression and cell death using flow cytometry. We were selected one combination, ECS-EFS-doxorubicin with the concentration of 16:8:2. Cell cycle histogram of the treatment was presented in Figure 3. Single treatment of dox 2 µM slightly induced G2/M arrested (27.4%), while 16 µg/ml of ECS strongly induced G2/M (43.1%) compared to cell control (21.2%). Whereas, EFS did not affected cell cycle profile. Interestingly, the combination of ECS and EFS induced G2/M arrested but lower than sappan alone (41.5%). Moreover, the treatment of ECS, EFS and dox combination increased cell population in subG1 (7.1%) compared to untreated cells (2.3%) and any single treatment. The G2/M was still arrested with higher population (32.8%) than that of doxorubicin treatment, but lower than sappan alone (Figure 3).

To understand the cell death mechanism in subG1 population, whether synergistic combination was mediated through apoptosis, we stained treated cells with propidium iodide – annexin V and subjected to flow cytometry (Figure 4). The results showed the apoptotic population was significantly increased in MCF-7 cells treated by combination of ECS-EFS-dox (37.0%) compared to untreated cells (2.7%) and single

Table 1. Combination index (CI) and dose reduction index (DRI) values of doxorubicin in combination. CI < 1 indicates synergistic effect, CI = 1 indicates additive effects and CI > 1 indicates antagonistic effect (Chou, 2010).

Treatment	Concentration			% viability	CI value	DRI (doxo)
	ECS (µg/ml)	EFS (µg/ml)	Dox (µM)			
Combo 2-1	4	2	-	84.4 + 9.2	1.10	-
Combo 2-2	5	3	-	88.1 + 6.5	4.10	-
Combo 2-3	8	4	-	83.9 + 5.7	1.90	-
Combo 2-4	16	8	-	73.4 + 6.5	1.00	-
Combo 3-1	4	2	0.5	88.4 + 1.8	3.20	5.2
Combo 3-2	5	3	1	78.4 + 1.9	0.88	3.3
Combo 3-3	8	4	1.5	56.1 + 2.2	0.62	3.0
Combo 3-4	16	8	2	37.7 + 1.9	0.83	2.9

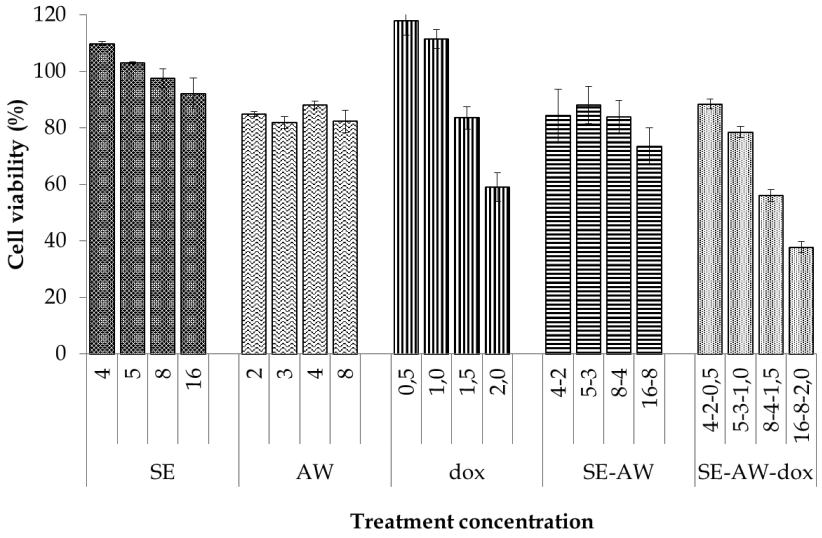

Figure 2. Combinational cytotoxic effect of ECS, EFS and dox on MCF-7 cells. Cells were seeded in 96-well plate and treated with ECS, EFS, or doxorubicin, either in single and combination. The concentration of ECS was 4, 5, 8, 16 µg/mL, EFS was 2, 3, 4, 8 µg/mL, and dox 0.5, 1.0, 1.5, 2.0 µM. The combination concentration of ECS and EFS was 4:2, 5:3, 8:4, and 16:8 µg/mL, while combination of ECS, EFS, and dox was 4:2:0.5, 5:3:1.0, 8:4:1.5, and 16:8:2.0. Cells viability were performed in triplicate, and represented as mean ± SD.

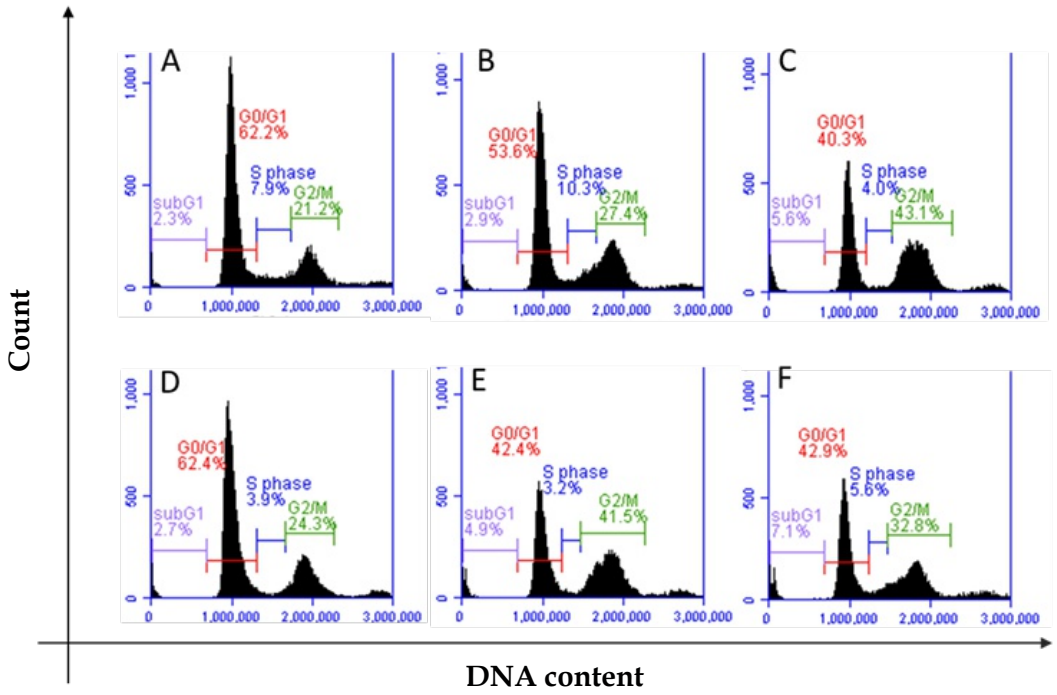

Figure 3. The effect of ECS, EFS, dox and their combination on cell cycle distribution. Cells were treated for 24 hours and stained with PI reagent, each sample was subjected to flow cytometer. (A) Cell control, (B) dox 2 µM, (C) ECS 16 µg/ml, (D) EFS 8 µg/mL, (E) combination of ECS and EFS (F) combination of ECS, EFS, and dox.

treatment of dox (7.7%), ECS (17%), and EFS (4.2%). The combination of ECS and EFS had lower apoptotic cells (14.2%), but showed the highest population on necrotic cells (4.6%). To confirm apoptosis induction of the single and combination treatment, we analyzed the expression level of protein poly (ADP-ribose) polymerase (PARP) by Western Blot. Cleavage PARP-1, as indicator of apoptosis, were shown in cells treated with single doxorubicin and its combination with ECS and EFS (Figure 4-H).

Discussion

Doxorubicin is one of the most active chemotherapeutic agent and widely used for

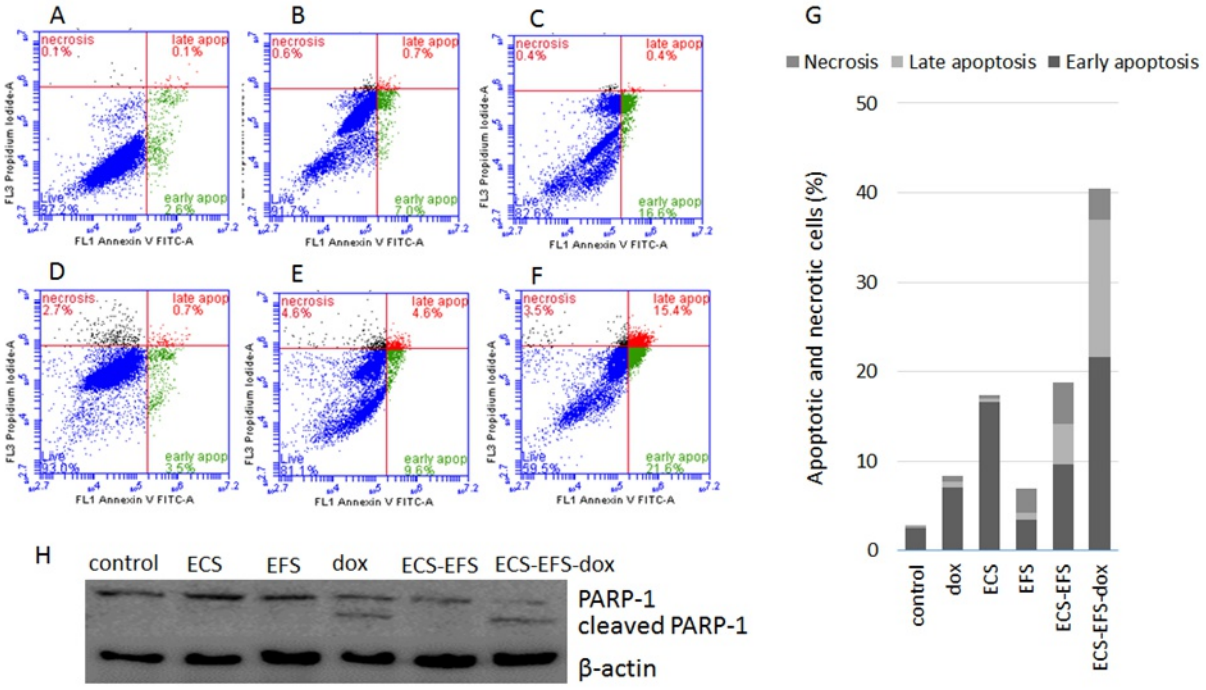

Figure 4. The effect of ECS, EFS, dox and their combination on cell apoptosis. Cells were treated for 24 hours and stained with PI reagent, each sample was subjected to flow cytometer. (A) Cell control, (B) dox 2 µM, (C) ECS 16 µg/ml, (D) EFS 8 µg/mL, (E) combination of ECS-EFS, (F) combination of ECS-EFS-dox. The diagram A-F divided into 4 area, which showed distribution profiles of living cells (bottom-left), early apoptotic (bottom-right), late apoptosis (upper-right), necrosis (upper-left) in various indicated treatment. Graphic (G) showed analysis results for apoptosis induction for each treatment. (H) The effect of ECS, EFS, doxorubicin and their combination on cellular expression levels of PARP-1 by Western blot. Protein β-actin was used as loading control.

breast cancer treatment (Anampa *et al*., 2015). Its application in chemotherapy is often limited due to cardio toxicity risk and resistance progression (Vejpongsa and Yeh, 2014). The development of doxorubicin resistance in breast cancer is multifactorial processes, mainly associated with wide and diverse expression of drug-resistance genes, and many other changes in genes responsible for cell cycle, apoptosis and DNA repair (AbuHammad and Zihlif, 2013). The combination with a natural chemopreventive agents is one of the promising strategy to improve doxorubicin anticancer effectiveness and reduce its toxicity (Ko and Moon, 2015). Therefore, we investigated the modulatory effect of ECS and EFS combined with doxorubicin on cytotoxicity, cell cycle progression and apoptosis induction in MCF-7 human breast cancer cell line.

In this study, the MTT assay revealed that MCF-7 cells viability was strongly inhibited by single ECS, and also doxorubicin, while the EFS exhibited low activity. We further demonstrated MTT assay combination of the extract and doxorubicin to obtain CI value. The CI is widely accepted as the simplest possible way to express pharmacologic drug interaction for quantifying synergism or antagonism. Synergism interaction will be very useful to treat the dreadful diseases, such as cancer. The main gains are the achievement of synergistic therapeutic effect, reduction of dose and toxicity, and also minimize or delay drug resistance (Chou, 2010).

The synergistic inhibitory effect achieved with the combination of doxorubicin 1.0, 1.5, and 2.0 µM (equal to 1/6, ¼ and 1/3 of IC_{50} respectively), with ECS (5, 8, 16 µg/mL) and EFS (3, 4, 8 µg/mL). The biggest concentration of EFS was approximately 1/35 of IC_{50}, based on its antimigration activity on 4T1 cells by wound healing method (data not shown). Therefore, the combined extract may lead to reduce of doxorubicin dose therapy and furthermore minimizing its cardio toxicity risk.

Cell cycle regulation and apoptosis induction play a critical role in cytotoxic activity. The cell cycle is a tidy and tightly regulated mechanism by which cell divide, involving four phases namely G1, S (synthesis), G2 and M (mitosis) (Deep and Agarwal, 2008). The abrogation of cell cycle checkpoints at critical time is expected to target the errors of cell cycle regulation to attain cancer cells specific cytotoxicity and to make the tumor cell susceptible to apoptosis. Currently, these agents are combined with conventional chemotherapeutic agent to overcome cell cycle mediated drug resistance and to enhance cytotoxic efficacy (Kelley *et al*., 2014). Doxorubicin modulates cell cycle through G1 and G2 phases arrest, as the result of its interaction with topoisomerase II mediated DNA damage (Lal *et al*., 2010). Our result confirmed that single treatment of dox 2 µM and ECS 16 µg/ml induced cell accumulation at G2/M phase. However, the EFS 6 µg/ml alone did not show significant changes in cell cycle profile. The combination of dox with both ECS and EFS enhanced cell accumulation at subG1, compared to the untreated cells and each single treatment. These results suggested that EFS contributed to enhance the cytotoxic effect of dox and ECS leading to cell death.

The cell cycle arrest depicted a survival mechanism for the cancer cell to repair its own damaged DNA. The disruption of cell cycle checkpoints by specific agent before completing DNA repair, can activate the apoptotic pathway leading to cell death (Carrassa, 2013). In our study, the combination of doxorubicin with both of the extract increased apoptotic and necrotic cell induction, compared to untreated cell and each single treatment. Apoptosis induction was confirmed by Western Blot analysis that showed PARP-1 cleavage in the treatment of single doxorubicin, and its combination with ECS and EFS.

PARP-1 can be cleaved by activated caspases. The cleavage of PARP-1 will inhibit uncleaved PARP-1 activity, thus promote DNA damage response in multiple pathway (Weaver and Yang, 2013). The effect of PARP-1 inhibitors in apoptosis induction, providing it as a promising target in cancer therapy (Wang *et al*., 2012). Apoptosis is one of cell death design, showed an ordered and orchestrated cellular process that occurs in physiological condition and pathological of many diseases. Apoptosis evasion plays a crucial role in carcinogenesis, therefore it is becoming as a popular target for cancer treatment strategy (Wong, 2011). Also, the aberrant cell cycle progression plays a crucial role in cancer cell growth, thus targeting the cell cycle have been regarded as an ideal cancer treatment (Deep and Agarwal, 2008).

Conclusions

Based on these findings, progressive cytotoxicity effect of doxorubicin combined with ECS and EFS extract inhibited MCF-7 cells growth through apoptosis induction and cell cycle perturbation (G_2/M arrest). Therefore, the extract combination performs potential natural sources to be developed further as co-chemotherapeutic agent.

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