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

Sari Haryanti¹, Suwijyio Pramono², Retno Murwanti³, Edy Meiyanto⁴,*

¹Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional, Kementerian Kesehatan Republik Indonesia, Tawangmangu
²Departement of Pharmaceutical Biology, Faculty of Pharmacy, UGM, Yogyakarta
³Departement of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, UGM, Yogyakarta
⁴Departement of Pharmaceutical Chemistry, and Cancer Chemoprevention Research Center, Faculty of Pharmacy, UGM, Yogyakarta

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 IC₅₀ value of 32 µg/mL and 6 µM respectively, while EFS performed low cytotoxic effect with the IC₅₀ 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-kB 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-kB activation (Meiyanto et al., 2012). Curcumin and other compounds in turmeric are also reported to

*Corresponding author:
Edy Meiyanto
Departement of Pharmaceutical Chemistry, and Cancer Chemoprevention Research Center, Faculty of Pharmacy, UGM, Yogyakarta
E-mail: meiyan_e@ugm.ac.id
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/braziline is the major compound of sappan wood (Mekala and Radha, 2015). Brazilin is easily oxidized to produce brazilin by air and light. Both brazilin and braziline 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 CO2 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 5x10^3 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 Lumigraph. 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
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>
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<th>Treatment</th>
<th>ECS (μg/ml)</th>
<th>EFS (μg/ml)</th>
<th>Dox (μM)</th>
<th>% viability</th>
<th>CI value</th>
<th>DRI (doxo)</th>
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<tr>
<td>Combo 2-1</td>
<td>4</td>
<td>2</td>
<td>-</td>
<td>84.4 + 9.2</td>
<td>1.10</td>
<td>-</td>
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<td>5</td>
<td>3</td>
<td>-</td>
<td>88.1 + 6.5</td>
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<td>-</td>
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<tr>
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<td>8</td>
<td>4</td>
<td>-</td>
<td>83.9 + 5.7</td>
<td>1.90</td>
<td>-</td>
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<tr>
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<td>16</td>
<td>8</td>
<td>-</td>
<td>73.4 + 6.5</td>
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<td>3.20</td>
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<td>0.88</td>
<td>3.3</td>
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<td>1.5</td>
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<td>2</td>
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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
breast cancer treatment (Anampa et al., 2015). Its application in chemotherapy is often limited due to cardio toxicity risk and resistance progression (Veipongsa 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 Z hilj, 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 cytoxicity, 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, 1/4 and 1/3 of IC₅₀ 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 IC₅₀ 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 cells 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 (G2/M arrest). Therefore, the extract combination performs potential natural sources to be developed further as co-chemotherapeutic agent.

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References


