



## Original Research Article

**Nobiletin Increased Cytotoxic Activity Of Doxorubicin On Mcf-7 Cells  
But Not On T47d Cells**

Edy Meiyanto\*, Adam Hermawan, Sedy Junedi, Aditya Fitriarsi, Ratna Asmah Susidarti

**\*Corresponding author:****Edy Meiyanto**

Faculty of Pharmacy  
Universitas Gadjah Mada  
Sekip Utara II, Yogyakarta  
55281, Indonesia  
Tel./Fax.:+62-274-543120  
Email : [meiyan\\_e@ugm.ac.id](mailto:meiyan_e@ugm.ac.id)  
<http://ccrc.farmasi.ugm.ac.id>

**Abstract**

Nobiletin, a citrus flavonoid, shows strong cytotoxic effect in several cancer cell lines. The aim of this research was to investigate cytotoxic activities of nobiletin alone and in combination with doxorubicin. Cell viability assay of nobiletin, doxorubicin, and combination treatments were carried out by using MTT assay. Apoptosis assay was done using double staining method using Ethidium Bromide-Acridine Orange. Cell cycle distribution was determined by flowcytometry FACS-Calibur and data was analyzed by using ModFit LT 3.0 program. Nobiletin (5, 10 and 15  $\mu\text{M}$ ) increased cytotoxic effect of doxorubicin compared with doxorubicin alone in MCF-7 cells but not in T47D cells. The strongest cytotoxic activity was showed by the combination of 200 nM doxorubicin and 15  $\mu\text{M}$  nobiletin in MCF-7 cells. Single treatment of doxorubicin 200 nM, nobiletin 15  $\mu\text{M}$  and their combination induced G2/M accumulation in MCF-7 cells. Combination of 15  $\mu\text{M}$  and nobiletin 200 nM doxorubicin showed strong synergism on apoptosis induction of MCF-7 cells. Single treatment of nobiletin 15  $\mu\text{M}$  and doxorubicin 7.5 nM induced T47D cell accumulation in G1 phase and G2/M phase respectively, while their combination induced accumulation of T47D cells in G2/M phase. Nobiletin is potentially to be developed as co-chemotherapeutic agent for breast cancer, while molecular mechanism need to be explored.

**Key words:** Nobiletin, doxorubicin, breast cancer, cell cycle, apoptosis.

**Introduction**

The incidence of breast cancer is increasing in several decades and become major as death caused cancer in women [1]. Several methods for breast cancer therapy such as surgery, chemotherapy, hormonal therapy and radiotherapy had been established. Doxorubicin is the common chemotherapeutic agent used in breast cancer therapy, but its effectivity is limited because of drug resistance problem and its toxicity in normal cells. To improve the antitumor efficacy of chemotherapy, a combination with chemopreventive agent (co-

chemotherapy) would be interesting to evaluate [2]

Nobiletin (5,6,7,8,3',4'-hexamethoxy flavone) has been proved to inhibit some kinds of tumor cells proliferation. Nobiletin blocked carcinogenesis on mice induced DMBA skin [3]. Nobiletin inhibited cell proliferation, and also induced apoptosis on HepG2 cells [4]. Nobiletin showed cytotoxic effect, induced apoptosis and modulated cell cycle on TMK-1, MKN-45, MKN-74 and KATO-III cells [5]. Nobiletin induced G1 arrest on MDA-MB-435, MCF-7 and HT-29 [6], and the newest study mentioned that

nobiletin has chemopreventive effects against colon carcinogenesis [7]. However, knowledge of the effect of nobiletin in combination with doxorubicin on breast cancer cells is unknown. Therefore, the present study was carried out to address the issue if nobiletin has potency as co-chemotherapeutic agent of doxorubicin.

Those researches showed the potency of nobiletin as chemopreventive agent and became a basic for the development of nobiletin as co-chemotherapeutic agent to increase the cytotoxic activity and reduce the side effects of doxorubicin. Therefore, the purpose of this research is to examine the effect of nobiletin alone and combination with doxorubicin on cytotoxicity, cell cycle and apoptosis induction of MCF-7 and T47D breast cancer cells.

## Materials and Methods

### Materials

Nobiletin was obtained from Sigma Aldrich Chemie GmbH, Steinheim, Germany (Cat No. N1538) while doxorubicin was obtained from Ebewe. A DMSO (Sigma Aldrich Chemie GmbH, Steinheim, Germany) solution of nobiletin was used for in vitro experiment by diluting appropriate concentration.

Doxorubicin Ebewe ((vial 10mg/5ml) obtained from P.T. Ferron Par Pharmaceutical (Cikarang, Indonesia)) was diluted directly in culture medium. The final DMSO concentration was set at less than 0.1 %.

### Cell Lines

MCF-7 and T47D cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing Fetal Bovine Serum (FBS) 10% (v/v) (FBS qualified, Gibco, Invitrogen™ USA) and penicillin-streptomycin 1 % (v/v) (Gibco, Invitrogen Corporation, Grand Island, NY, 14072, USA). Those cell lines were kindly provided by Prof. Tatsuo Takeya (Nara Institute of Science and Technology, Nara, Japan).

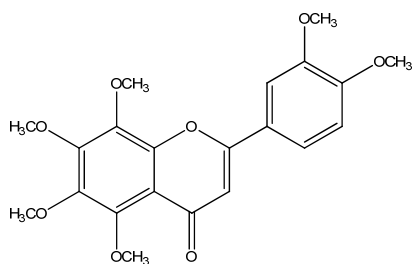
### Cytotoxic Assay (MTT Method)

The method was modified from Mosmann (1983). Cells ( $5 \times 10^3$  cells/well) were transferred to 96-well plate (Iwaki) and incubated for 24 hours (70-80% confluent). Cultures were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO<sub>2</sub> and 95% air. Cells were treated by nobiletin, doxorubicin, and their combination, and incubated for 24 hours. At the end of the incubation, 0,5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma, Sigma-Aldrich Corp, St. Louis, MO, USA) were added to each wells and the cells were incubated for 4 hours in 37°C. Viable cells reacts with MTT to form purple formazan crystal. After 4 hours, stopper sodium dodesil sulphate 10% in 0,1 N HCl solution were added to dissolve formazan crystal. Cells were incubated over night and protected from light. Cells were shaken for 10 minutes before read by ELISA reader at  $\lambda$  595 nm. The absorbance of each well converted to percentage of viable cells :

$$\% \text{ Viable cells} = \frac{\text{Treated cells abs} - \text{Medium control abs}}{\text{Cells control abs} - \text{Medium control abs}} \times 100\%$$

### Apoptotic Assay (Double Staining Method)

Cells ( $5 \times 10^4$  cells/well) were transferred to coverslips (Nunc) in 24-well plate (Iwaki) and incubated for 24 hours (50-60% confluent). Cells were treated by nobiletin, doxorubicin, and their combination, and then incubated for 15 hours. At the end of the incubation, coverslips containing cells were moved to object glass. Mixture solution of etidium bromide-acridine orange (Sigma, Sigma-Aldrich Corp, St. Louis, MO, USA) were added to the cells to form fluorescence cells. The fluorescence cells were examined by fluorescence microscope (Zeiss MC80) immediately. Green fluorescence cells showed viable cells, while red fluorescence cells showed dead cells.



**Figure 1. Chemical structure of nobiletin.**

### Cell cycle Analysis

Cells ( $5 \times 10^5$  cells/well) were transferred into 6-well plate (Iwaki) and incubated until the cells return to normal condition. Cells were treated by nobiletin, doxorubicin, and their combination, and incubated for 24 hours. Cultures were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO<sub>2</sub> and 95% air. At the end of the incubation, the media containing free cells suspension were taken and transferred into 1.5 ml eppendorfs, then it were centrifugated (2000 rpm, 3 minutes) and the supernatant were removed. The cells in 6-well plate were added by PBS, and the PBS was transferred into previous eppendorfs. The eppendorfs were centrifugated and the supernatant were removed again. This steps were repeated before the cells harvested by trypsin-EDTA 0.25% (Gibco, Invitrogen, Canada).

Harvested cells were transferred into the eppendorfs and centrifugated (2000 rpm, 30 seconds). The remaining cells in the 6-well plate were rinsed with PBS and transferred into the eppendorfs. The eppendorfs were centrifugated and the supernatant were removed. Pellet cells in eppendorfs washed by cold PBS and added by propidium iodide (PI) reagents. The eppendorfs were wrapped in aluminum foil and incubated in 37°C for 10 minutes. After 10 minutes, cell suspension was homogenated and transferred into the flowcyto-tube to be analyzed by FACS flowcytometer.

### Statistical Analysis

Statistical analysis was performed using Student's t-test and results considered significant at the  $p < 0.05$  level.

## Result and Discussion

### Cytotoxic Assay

In this study we explored the effect of nobiletin alone and combination with doxorubicin on cytotoxicity, cell cycle and apoptosis induction of MCF-7 and T47D breast cancer cells. Nobiletin have been proved to have cytotoxic and antiproliferative effect against several cancer cell lines such as HepG2, TMK-1, MKN-45, MKN-74 and KATO-III cells. Our study showed that single treatment of nobiletin did not show cytotoxic effect on MCF-7 and T47D cells because nobiletin is slightly soluble in culture medium. The maximum soluble concentration of nobiletin is 15  $\mu$ M. Our previous study showed that doxorubicin showed strong cytotoxic effect on MCF-7 and T47D breast cancer cell lines with IC<sub>50</sub> value 400 and 15 nM, respectively (Hermawan *et al.*, 2010; Junedi *et al.*, 2010). This is due to the characteristic of MCF-7 which is resistant to doxorubicin by overexpressing anti-apoptotic protein Bcl-2 and P-glycoprotein efflux pump [8]. While T47D cells resistance to apoptosis because of mutation of p53 gene. To increase the sensitivity of MCF-7 and T47D cells towards doxorubicin, we combined doxorubicin with nobiletin.

Combination of doxorubicin with nobiletin 5, 10, and 15  $\mu$ M showed higher inhibition of proliferation than single treatment of doxorubicin in MCF-7 breast cancer cell lines (Figure 2), but not increase doxorubicin cytotoxicity on T47D cells (Figure 3). This result suggested that this combination give synergistic effect on MCF-7 cells. This synergistic effect is occurred due to apoptotic induction and cell cycle modulation.

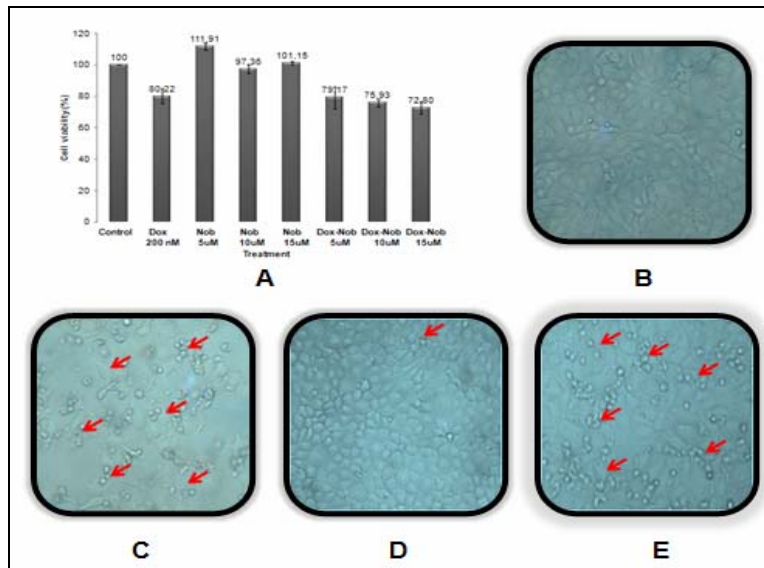


Figure 2. The effect of nobiletin and doxorubicin to the viability of MCF-7 breast cancer cells.

The combination effect of nobiletin and doxorubicin to the proliferation of MCF-7 (A), control (B), doxorubicin 200 nM (C), nobiletin 15 μM (D), combination doxorubicin 200 nM-nobiletin 15 μM (E). The assay performed by incubating  $5 \times 10^3$  cells/well with nobiletin (5-15 μM) and doxorubicin (200 nM) on MCF-7 for 24 hours. After 24 hours, cells were added by MTT reagent to calculate the absorbance which represent viable cells. Cell viability profile was shown from average  $\pm$  standard of error (SE) of 3 experiment. Death cell was pointed by red arrow. Cell morphology was examined by using inverted microscope with magnification 400 x. Nobiletin increased cytotoxicity of doxorubicin 200 nM.

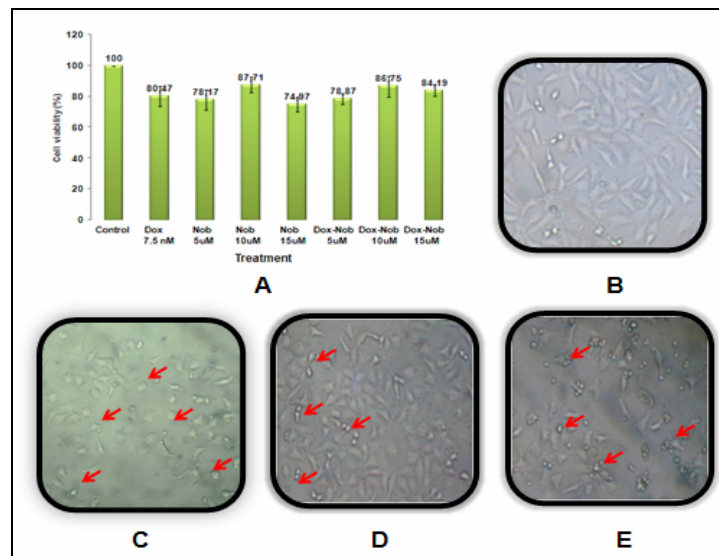


Figure 3. The effect of nobiletin and doxorubicin to the viability of T47D breast cancer cells.

The combination effect of nobiletin and doxorubicin to the proliferation of T47D (A), control (B), doxorubicin 7.5 nM (C), nobiletin 15 μM (D), combination doxorubicin 7.5 nM-nobiletin 15 μM (E). The assay performed by incubating  $5 \times 10^3$  cells/well with nobiletin (5-15 μM) and doxorubicin (7.5 nM) on T47D for 24 hours. After 24 hours, cells were added by MTT reagent to calculate the absorbance which represent viable cells. Cell viability profile was shown from average  $\pm$  standard of error (SE) of 3 experiment. Death cell was pointed by red arrow. Cell morphology was examined by using inverted microscope with magnification 400 x. Nobiletin did not increase cytotoxicity of doxorubicin 7.5 nM.

### Apoptosis Assay

We next investigated whether the nobiletin increased apoptosis induction of doxorubicin in MCF-7 and T47D cells. The results from cytotoxic assay of doxorubicin and nobiletin were parallel with apoptotic assay using double staining method. All cells in control cell showed green fluorescence means there was no death cell. Orange fluorescence cells represent apoptotic cells that loss cell membrane permeability and form apoptotic bodies. The nuclear of several cells were fragmented and formed apoptotic bodies. On MCF-7 (Figure 4) cells single treatment of doxorubicin 200 nM induced apoptosis, but there were several viable cells, while single treatment of nobiletin 15  $\mu$ M did not

show apoptosis induction. Combination of doxorubicin 200 nM and nobiletin 15  $\mu$ M increased the incidence of apoptosis, compared with single treatment of doxorubicin 200 nM on MCF-7 cells.

On T47D cells (Figure 5) cells single treatment of doxorubicin 7.5 nM induced apoptosis in almost of cells, while single treatment of nobiletin 15  $\mu$ M did not show apoptosis induction. Combination of doxorubicin 7.5 nM and nobiletin 15  $\mu$ M did not increase the incidence of apoptosis, compared with single treatment of doxorubicin on T47D cells. Probably the apoptosis incidence in combinational treatment is dominantly because of doxorubicin 7.5 nM.

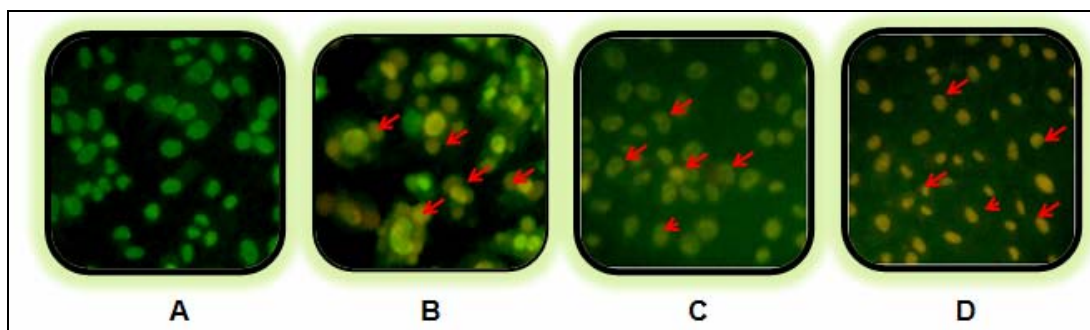


Figure 4. Apoptotic effect of doxorubicin, nobiletin and their combination on MCF-7 cells.

MCF-7 cell were treated by doxorubicin, nobiletin and their combination for 15 hours and stained by etidium bromide-acridine orange. (A) Cell control, (B) Doxorubicin 200 nM, (C) Nobiletin 15  $\mu$ M, (D) Combination of nobiletin 15  $\mu$ M and doxorubicin 200 nM. Viable cells give green fluorescence, apoptotic cells give orange fluorescence (pointed by red arrow). Examination of apoptosis was done by using fluorescent microscope with magnification 400 x. Nobiletin increased apoptosis induction of doxorubicin on MCF-7 cells.

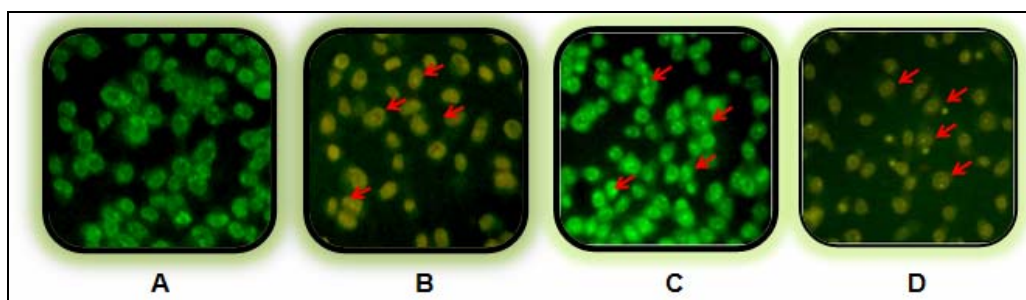


Figure 5. Apoptotic effect of doxorubicin, nobiletin and their combination on T47D cells.

T47D cell were treated by doxorubicin, nobiletin and their combination for 15 hours and stained by etidium bromide-acridine orange. (A) Cell control, (B) Doxorubicin 7.5 nM, (C) Nobiletin 15  $\mu$ M, (D) Combination of nobiletin 15  $\mu$ M and doxorubicin 7.5 nM. Viable cells give green fluorescence, apoptotic cells give orange fluorescence (pointed by red arrow). Examination of apoptosis was done by using fluorescent microscope with magnification 400 x. Nobiletin did not increase apoptosis induction of doxorubicin on T47D cells.

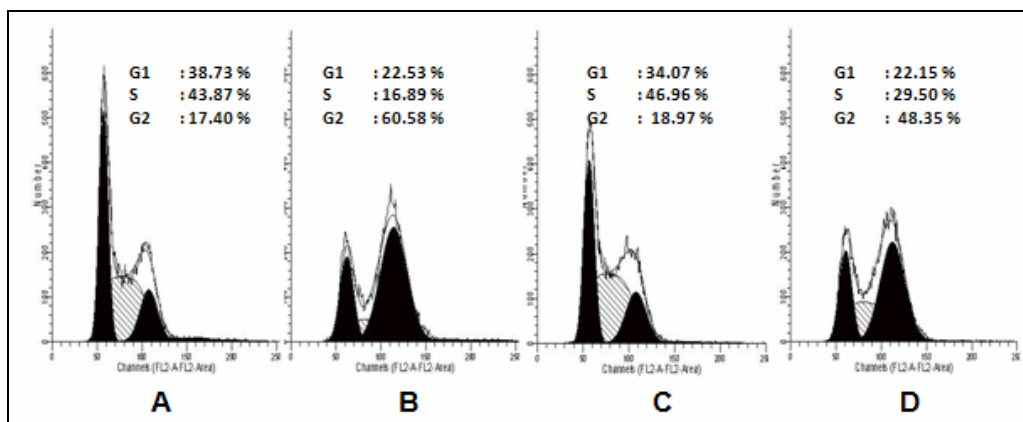


Figure 7. T47D cell cycle analysis after treatment of doxorubicin, nobiletin and their combination.

T47D cell were treated by doxorubicin, nobiletin and their combination for 24 hours and stained by PI reagent before analyzed by flowcytometer. (A) Cell control, (B) doxorubicin 7.5 nM, (C) nobiletin 15 μM, (D) Combination of doxorubicin 7.5 nM-nobiletin 15 μM.

**Cell Cycle Analysis**

In the next series of experiments, we explored the possibility of cell cycle perturbation by nobiletin, doxorubicin and their combination in MCF-7 and T47D cells. Synergistic effect of combination between nobiletin and doxorubicin could be occurred via MCF-7 cell cycle modulation (Table 1 and Figure 6). Cell cycle analysis of MCF-7 breast cancer cell lines showed that single treatment of Doxorubicin 200 nM induced G2/M

arrest. Single treatment of nobiletin 15 μM induced G1 arrest, and combination of doxorubicin 200 nM-nobiletin 15 μM induced G2/M arrest on MCF-7 cells. T47D cell cycle profile (Table 2 and Figure 7) after treatment of doxorubicin 7.5 nM induced G2/M arrest. Single treatment of nobiletin 15 μM induced S arrest, and combination of doxorubicin 7.5 nM-nobiletin 15 μM induced G2/M arrest on T47D cells.

**Table 1. MCF-7 Cell distribution after treatment of doxorubicin, nobiletin and their combination for 24 hours.**

Treatment	Concentration	G1 (%)	S (%)	G2/M (%)
control	-	47,94	40,60	11,47
doxorubicin	200 nM	34,82	45,56	<b>19,62</b>
nobiletin	15 μM	47,73	36,63	<b>15,63</b>
doxorubicin and nobiletin	200 nM -15 μM	35,92	29,48	<b>34,60</b>

**Table 2. T47D Cell distribution after treatment of doxorubicin, nobiletin and their combination for 24 hours**

Treatment	Concentration	G1 (%)	S (%)	G2/M (%)
control	-	38,73	43,87	17,40
doxorubicin	7.5 nM	22,53	16,89	<b>60,58</b>
nobiletin	15 μM	34,07	46,96	18,97
doxorubicin and nobiletin	7.5 nM-15 μM	22,15	29,50	<b>48,35</b>

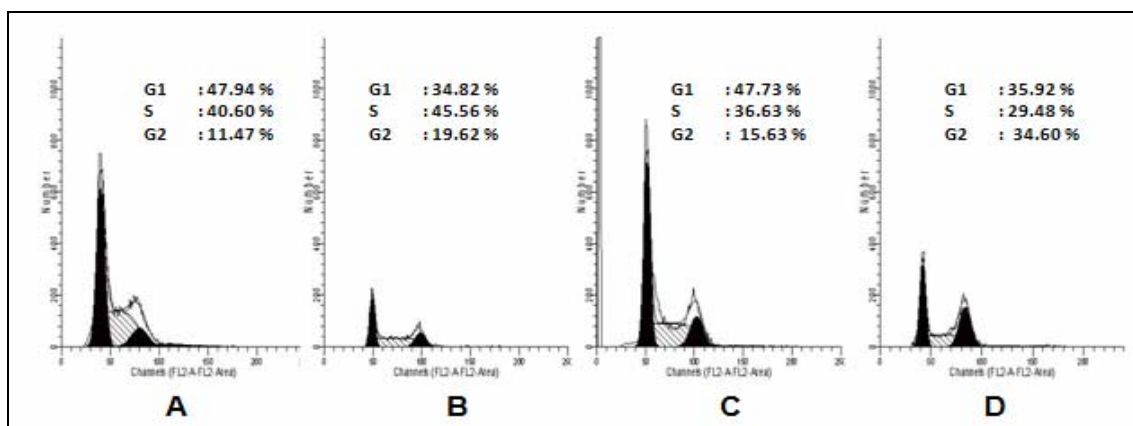


Figure 6. MCF-7 cell cycle analysis after treatment of doxorubicin, nobiletin and their combination.

MCF-7 cell were treated by doxorubicin, nobiletin and their combination for 24 hours and stained by PI reagent before analyzed by flowcytometer. (A) Cell control, (B) doxorubicin 200 nM, (C) nobiletin 15 μM, (D) Combination of doxorubicin 200 nM-nobiletin 15 μM.

MCF-7 cells showed lower sensitivity to doxorubicin than T47D cells [9]. Over expression of Bcl-2 and P-glyco Protein (PgP) in MCF-7 cells caused cells resistance to apoptosis triggered by doxorubicin. Over expression of Bcl-2 caused cells evading the apoptosis mechanism [10] while PgP transport the drug outside the cells so that concentration of chemotherapeutic agent inside the cells will be low and failed to kill the cancer cells. T47D is one one cancer cell model with mutant p53, and doxorubicin treatment in increasing dose in this cells induced drug resistance phemonema via upregulation of Akt and PgP [11,12]. Drug resistant in cancer cells could be occurred by cell cycle arrest that will repair the DNA damage caused by chemotherapeutic agent, so combination of chemotherapeutics with an agent which modulate cancer cell cycle is interesting to be studied.

Single treatment of nobiletin did not show cytotoxic effect, but the combination with doxorubicin increased cytotoxicity of doxorubicin in MCF-7 cells. Single treatment of nobiletin did not induced apoptosis, but the combination with doxorubicin increased apoptosis induction of doxorubicin in MCF-7 cells. The combination also increased G2/M arrest of MCF-7 cells. The mechanism of increasing of doxorubicin cytotoxicity by

nobiletin probably because of up-regulation of p53 and its related pathway. Doxorubicin induced DNA damage and trigger the apoptosis pathway or DNA repair depend on the degree of DNA damage [13]. In the cells with wild type p53 e.g. MCF-7 cells, upregulation of p53 could increase apoptosis induction or G2/M arrest. [14] mentioned nobiletin induced apoptosis via p53 up-regulation and those phenomena was correlated with G2/M arrest in A549 lung cancer cells. Our reults is similar to the previous study, but the mechanism need to be explored more details in MCF-7 cells.

In T47D cells, single treatment of nobiletin and its combination with doxorubicin did not increase cytotoxicity and apoptosis induction of doxorubicin. While single treatment of nobiletin increased S arrest, and its combination with doxorubicin decreased G2/M arrest of doxorubicin single treatment, so probably this mechanism triggered the cell resistance to doxorubicin. Apoptosis pathway in T47D cells did not occurred via p53 because of mutant p53 in this cells. Probably nobiletin increased p53 expression in MCF-7 cells, but did not in T47D cells so that nobiletin did not increase cytotoxicity of doxorubicin, but the mechanism need to be explored more details.

This result showed the potency of nobiletin to be developed as co-chemotherapeutic agent for doxorubicin by inducing apoptosis and cell cycle arrest. The use of doxorubicin together with nobiletin is expected to increase the activity and reduce the side effects of doxorubicin. However, the molecular mechanism of apoptotic induction and cell cycle arrest by this combination need to be explored further.

### Conclusion

This research showed that combination of nobiletin and doxorubicin synergically increase the effect of doxorubicin through apoptotic induction and cell cycle modulation. Based on this result, nobiletin is potential to be developed as co-chemotherapeutic agent for doxorubicin in breast cancer therapy.

### Author's Contribution

EM have made conception and design of this study, acquisition of data, data collection, analysis and interpretation and statistical data, drafted, and corresponding author the manuscript. All author have already read and approved the final revision of this manuscript.

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