

Ursolic Acid Enhances Doxorubicin Cytotoxicity on MCF-7 Cells Mediated by G2/M Arrest

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Abstract

Ursolic acid has been widely known to possess biological activity against numerous tumor cell lines. Previous studies revealed its cytotoxicity on several cancer cells *in vitro* by either inducing apoptosis or cell cycle modulation. This study was conducted to investigate ursolic acid's cytotoxicity solely and in combination with a chemotherapeutic agent, doxorubicin, on MCF-7 breast cancer cells, followed by observation on its mechanism. Cytotoxicity of single and combinational treatment of ursolic acid and doxorubicin on MCF-7 breast cancer cells were conducted by using MTT assay. Single treatment was then evaluated by determining IC₅₀ value, while combinational treatment was evaluated by analyzing cell viability and evaluating combination index (CI). To explore the mechanism underlying cytotoxic effect on respected cells, further analysis on cell cycle profile of single and combinational treatment was conducted by flow cytometry. Twenty four hours treatment of ursolic acid inhibited MCF-7 cells' growth with IC₅₀ value of 37 μ M, while combinational treatment showed that several concentration combinations of ursolic acid and doxorubicin exhibited synergism of cytotoxic activity on MCF-7 cells, giving optimum CI value of 0.54. Flow cytometric analysis showed that combinational treatment induced G2/M arrest in MCF-7 cells. These results show that ursolic acid is promising to be developed as either single chemopreventive agent, or as doxorubicin's co-chemotherapeutic agent in breast cancer treatment. Observation on the selectivity as part of safety aspect together with *in silico*, *in vitro*, and *in vivo* study on its molecular mechanism should be conducted.

Keywords: ursolic acid, doxorubicin, co-chemotherapeutic agent, breast cancer, cell cycle

INTRODUCTION

Ursolic acid, a pentacyclic triterpenoid found in plants, has been widely known to possess numerous biological activities, one of which is its activity against tumor cell lines. Previous researches observed that ursolic acid was able to inhibit SK-OV-3 and A2780 ovarian cancer cells growth by inducing apoptosis (Song *et al.*, 2012) and HepG2 cells proliferation by inducing apoptosis and through cell cycle modulation (Tian *et al.*, 2006). Another research reported that MCF-7 breast cancer cells proliferation was inhibited by ursolic acid through cell cycle modulation, indicating its possible potential as a medical component for breast neoplasm (Zhang *et al.*, 2005).

Breast cancer has been widely known to be the cancer suffered most among women. To date, chemotherapy using cytotoxic chemotherapeutic agents are still the major choice of treatment in cancer, especially metastatic cancer (Drummond, 2007). Doxorubicin is one of cancer chemotherapeutic agent widely used in breast cancer treatment (Childs *et al.*, 2002). Doxorubicin's cytotoxic effect occurs via p53 pathway. One of the major problems faced in doxorubicin therapy is the occurrence of resistance (Mechetner *et al.*, 1998).

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Cancer's resistance to doxorubicin occurs through various mechanisms, example drug inactivation, drug-pumping-out by efflux pumps on cell membrane, mutation of the target, and failure of apoptosis initiation (Davis *et al.*, 2003; Notarbartolo *et al.*, 2005). To overcome cancer's resistance towards existing chemotherapy agent, the application of co-chemotherapeutic agent in cancer therapy could be done.

Previous research reported that ursolic acid inhibited MCF-7 cells growth by inducing apoptosis (Zhang *et al.*, 2005). In this study, we observed ursolic acid's ability in increasing doxorubicin cytotoxicity on breast cancer cells, allowing the use of lower dose of the chemotherapeutic agent giving less toxicity on normal tissues. The study of its molecular mechanism is also necessary. This study aimed to examine the cytotoxicity of ursolic acid on MCF-7 breast cancer cells, continued with its effect in combination with doxorubicin.

MATERIALS AND METHODS

Materials

Ursolic acid (Sigma) was dissolved in Dimethyl Sulfoxide (DMSO) (Sigma). Both 5 mg/ml doxorubicin (Ebewe) and ursolic acid solution were then diluted in DMEM cell culture medium before being applied. DMSO was used as the co-solvent in dissolving ursolic acid in DMEM culture medium.

For cytotoxicity assay, 10% sodium dodecyl sulphate (SDS) (Merck) dissolved in 0.01 N HCl (Merck) as stopper reagent, phosphate buffer saline (PBS) pH 7.4 containing KCl (HPLC grade, Sigma), NaCl (HPLC grade, Sigma), Na₂HPO₄ (HPLC grade), and KH₂PO₄ (HPLC grade, Sigma) dissolved in aquadest as washing reagent, and 3-[4,5-dimethyl thiazole-2-yl(-2,5-diphenyltetrazoliumbromide)] (MTT) dissolved in PBS as MTT reagent were used.

For cell cycle analysis using flowcytometry, Phosphate Buffer Saline (PBS) pH 7.4, Propidium Iodide Solution (50 µg/ml in PBS containing 1% Triton X-100) and RNase DNase-free (20 µg/ml) reagents were used.

MCF-7 Cells Culture

MCF-7 cells were obtained from the collection of Cancer Chemoprevention Research Center (CCRC), Universitas Gadjah Mada. The cell line was a gift from Prof.

Masashi Kawaichi, Nara Institute of Science and Technology (NAIST), Japan.

Instruments

Treated tissue culture dish \approx 10 cm (Iwaki), 96-well plate (Iwaki), 6-well plate (Iwaki), glassware, LAF hood (Labconco), CO₂ incubator (Heraeus), inverted microscope (Zeiss MC80), cell counter, water bath, analytical balance (Sartorius), micropipette (Gilson), optical microscope, centrifuge (Sorvall), ELISA reader (SLT 240 ATC), yellow tip, blue tip, haemocytometer, conical tube (Nunc), shaker (MRK-RETAC), vortex, sterile eppendorf (Plasti Brand), digital camera (Canon, Japan), FACS Calibur (BD).

Cytotoxicity and Combinational Assay

MCF-7 cells were cultured in DMEM (Gibco) Culture Medium containing 10%^{v/v} Fetal Bovine Serum (FBS) (Gibco) and 1% Penicillin-Streptomycin (Gibco). Trypsin-EDTA 0.25% (Gibco) was used in to de-attached cells from TCD. The effect of ursolic acid alone and in combination with doxorubicin treatment on MCF-7 cells were measured based on MTT assay. MCF-7 cells were distributed to 96-well plate with the density of 5 x 10³ cells/well and incubated in 37°C with 5% CO₂ for 24 hours. After 24 hours exposure of sample treatment, MTT reagent was applied, followed by 4 hours incubation. Stopper reagent was then applied. Plate was then kept with protection from light overnight, continued with absorbance determination (λ 595 nm) using ELISA reader (Bio-Rad).

Flow Cytometric Analysis

MCF-7 cells were distributed in to 6-well plate with the density of 10⁶ cells/well. After 24 hours incubation, cells were treated with ursolic acid alone and in combination with doxorubicin. Following 24 hours treatment, cells were trypsinized and centrifuged at 2000 rpm for 3 minutes. Collected cell pellets were then washed twice with cold PBS. Cells were resuspended in propidium iodide solution and treated with RNase DNase-free for 10 minutes at 37°C. Treated cells were then subjected to FACS flow cytometry.

Data Analysis

Single Cytotoxicity assay. Linear regression between ursolic acid concentration and % cell viability giving the equation $y = Bx$

+ A were used to calculate IC₅₀ value, that is the concentration inhibiting 50% cell proliferation.

Combinational Cytotoxicity Assay. Cell viability resulted in combinational treatment between each treatment were analyzed statistically by one-way ANOVA by using SPSS 17.0. Combinational treatment was also evaluated by calculating

Combination Index (CI) value (Reynolds and Maurer, 2005), using the formula as follows.

$$CI = \frac{D_1}{D_{x1}} + \frac{D_2}{D_{x2}}$$

D₁ and D₂ represent concentrations used in combinational treatment, while D_{x1} and D_{x2} are single treatment concentration giving the same response as D₁ and D₂, respectively. CI value acquired will allow the evaluation of ursolic acid's potency in combinational treatment with doxorubicin on MCF-7 cells. Interpretation was done based on the classification listed in Table I.

Flow Cytometry (Cell Cycle Profile).

Cell cycle distribution was acquired by using ModFit LT 3.0 program. Analysis was done on the proportion of S-phase, G1-phase, and G2/M-phase expressed as percentage.

Table I. Interpretation of CI value representing potency of combinational application

CI value	Interpretation	CI	Interpretation
< 0.1	Very strongly synergist	0.9-1.1	Closely additive
0.1-0.3	Strongly synergist	1.1-1.45	Middle antagonist
0.3-0.7	Synergist	1.45-3.3	Antagonist
0.7-0.9	Middle synergist	> 3.3	Strongly antagonist

RESULTS AND DISCUSSION

This study was conducted to explore ursolic acid's potency as doxorubicin's co-chemotherapeutic agent against breast cancer, since MCF-7 breast cancer cells have been known to possess low sensitivity against doxorubicin (Zampieri *et al.*, 2002).

Ursolic Acid Inhibited MCF-7 Cells' Growth

Single cytotoxicity assay was conducted to evaluate ursolic acid's cytotoxicity towards MCF-7 cells. Linear regression of ursolic acid concentration against % cell viability (Fig. 1) gave value of 37 μM. Treatment of ursolic acid on MCF-7 cells showed cell growth inhibition in a concentration-dependent manner. Therefore, ursolic acid exhibited potent cytotoxic activity on MCF-7 cells according to Teng *et al.* (2005). Ursolic acid possesses

higher cytotoxicity to MCF-7 cells compared to naringenin (IC₅₀ 520 μM), a flavonoid found in the genus *citrus* (Fitriasari *et al.*, 2010). However, compared to solamargine (IC₅₀ 2.1 μM), a glycoalkaloid occurs in *solanum* species (Wei *et al.*, 2011), ursolic acid possess relatively lower cytotoxicity on MCF-7 cells. According to Meiyanto *et al.* (2012), despite of *Citrus* flavonoids' low cytotoxicity, they worked synergistically with doxorubicin to inhibit MCF-7 cells growth, revealing their potency as co-chemotherapeutic agents. We further observe ursolic acid's ability to enhance doxorubicin's cytotoxic effect on MCF-7 cells. Combinational assay was then conducted to observe whether ursolic acid would work synergistically with doxorubicin, a chemotherapeutic agent commonly used in breast cancer therapy, on MCF-7 breast cancer cells.

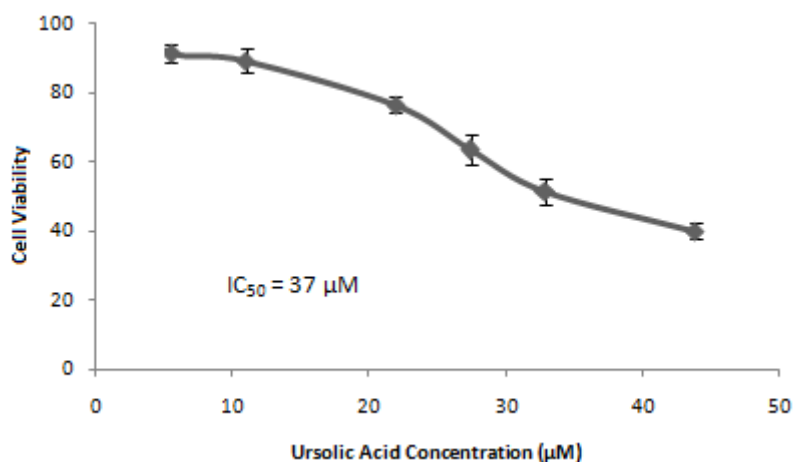


Figure 1. Ursolic acid decreased MCF-7 cells' viability. Cell viability resulted from 24-hours treatment of ursolic acid on MCF-7 cells acquired by MTT assay. Five thousand cells per well were incubated and exposed with a series concentration of ursolic acid for 24 hours each. MTT reagent was then applied, followed by addition of stopper reagent and overnight incubation. Absorbance was determined at λ 595 nm using ELISA reader. Ursolic acid inhibited cell growth in a dose-dependent manner, giving IC₅₀ value of 37 μ M. Ursolic acid's cytotoxicity was represented as percentage of MCF-7 cells' viability as the mean \pm SE of three values.

Ursolic Acid Increased Doxorubicin's Cytotoxicity on MCF-7 Cells

Combinational treatment of doxorubicin and ursolic acid on MCF-7 cells was conducted to observe the ability of ursolic acid to increase doxorubicin's cytotoxicity on MCF-7 cells. We set the experimental design of combinational treatment based on single compound cytotoxicity, represented by IC₅₀ values. Doxorubicin's IC₅₀ value on MCF-7 cells was 350 nM (CCRC, unpublished data). Combinational treatment of ursolic acid and doxorubicin on MCF-7 cells resulted in higher cell growth inhibition compared to single treatment ($p < 0.05$) (Table II, Fig. 2). Several

concentration combinations showed synergism on MCF-7 cells, with CI values less than 0.9 (Table III). Cell morphology after treatment was also observed (Fig. 3). Treatment of ursolic acid and doxorubicin alone led to cells' morphological changes, (Fig. 3(B) and 3(C)). Combination of them caused more changes compared to single treated cells, and less viable cells (Fig. 3(D)), while control cells showed only slight changes in cells' morphology (Fig. 3(A)). Flow cytometric analysis was then conducted to observe cell cycle modulation of combination of ursolic acid and doxorubicin on MCF-7 cells.

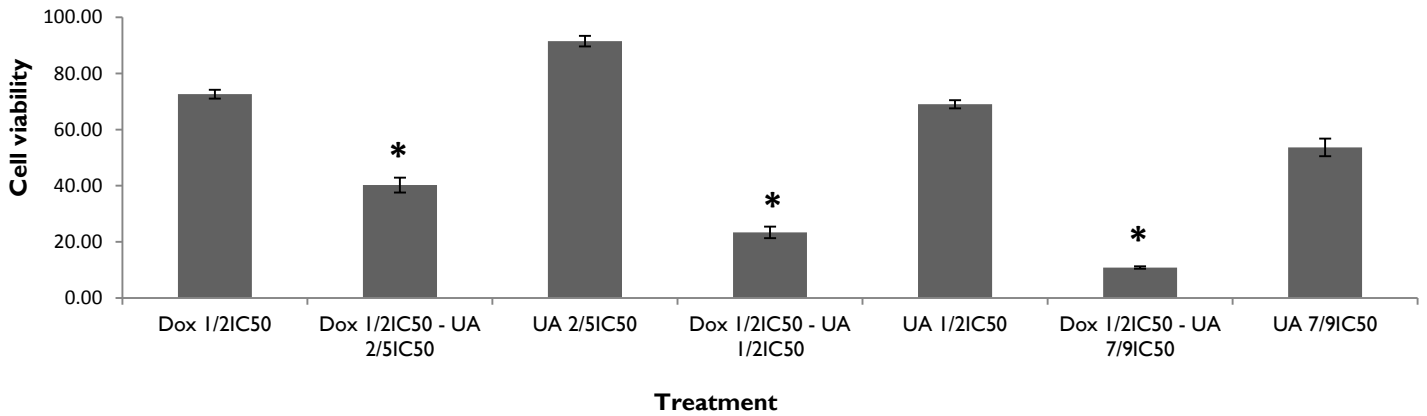


Figure 2. Combination of ursolic acid and doxorubicin inhibited T47D cells growth. Twenty four-hours treatment of ursolic acid (UA) and doxorubicin (Dox) on MCF-7 cells examined by MTT assay. Five thousand cells per well were incubated for 24 hours and were exposed with various concentrations of ursolic acid and doxorubicin solely and in combination. MTT reagent was then applied, followed by addition of stopper reagent and overnight incubation. Absorbance was determined at λ 595 nm using ELISA reader. One-way ANOVA statistical analysis was conducted to determine significant difference of cell viability yielded between treatments. Note (*) shows significant difference of combinational treatment compared to single treatments (p 0.05). Combinational treatment of ursolic acid and doxorubicin yielded less cell viability compared to single treatment. Cytotoxicity was represented as percentage of MCF-7 cells' viability as the mean \pm SE of three values.

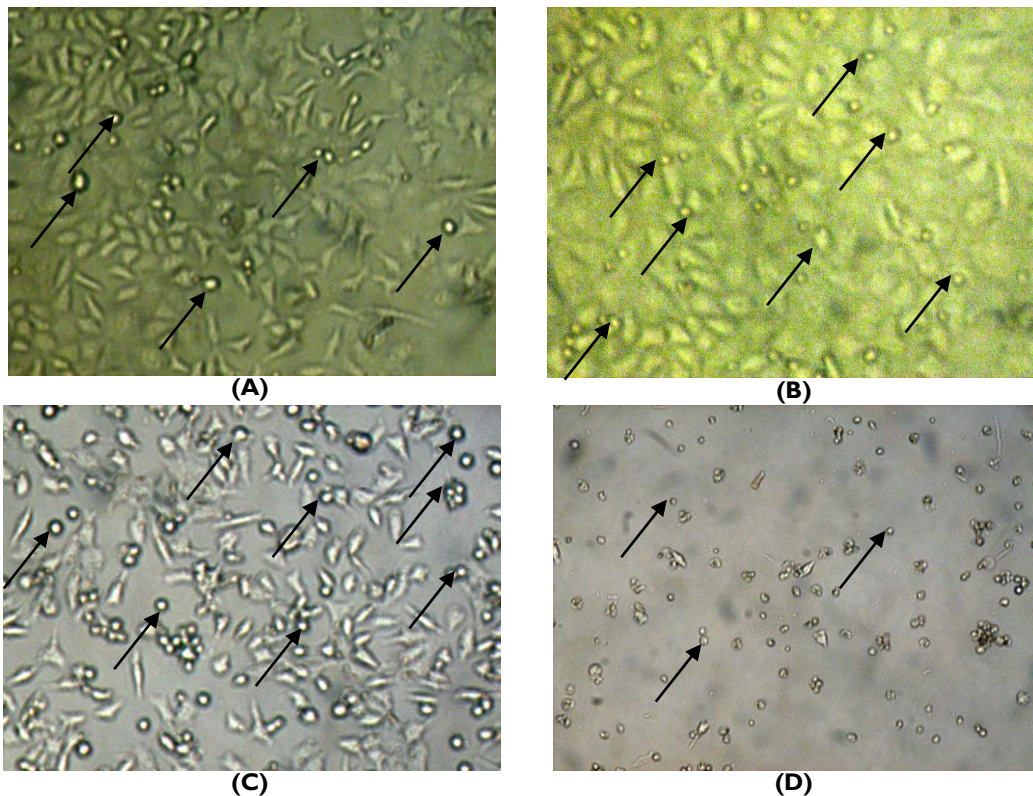


Figure 3. Combination of doxorubicin and ursolic acid showed synergism. Twenty four-hours treatment of ursolic acid and doxorubicin on MCF-7 cells. Cells were exposed with samples for 24 hours, followed by observation of 24-hours treatment of (A) vehicle only; (B) $1/4$ IC_{50} doxorubicin; (C) $2/5$ IC_{50} ursolic acid; (D) $1/4$ IC_{50} doxorubicin in combination with $2/5$ IC_{50} ursolic acid. Change in cell morphology is pointed with black arrows. Observation was done by using inverted microscope, 100x magnification.

Table II. Effect of single and combinational treatment of ursolic acid and doxorubicin on MCF-7 cells viability.

Ursolic acid concentration (IC ₅₀) IC ₅₀ = 37 μM	MCF-7 Cells Viability (% viability ± SE)				
	Doxorubicin (IC ₅₀) IC ₅₀ = 350 nM				
	0	1/10	1/4	1/3	1/2
0	-	124.20 ± 4.11*	115.37 ± 1.95*	93.54 ± 1.56*	73.61 ± 1.57*
2/5	91.50 ± 1.87	87.49 ± 3.68*	61.00 ± 2.68*	46.61 ± 1.14*	40.23 ± 2.63*
1/2	69.01 ± 1.47	63.45 ± 2.34*	28.95 ± 2.66*	28.78 ± 1.61*	23.39 ± 2.06*
7/9	53.64 ± 3.13*	40.31 ± 2.44*	13.49 ± 2.05*	13.49 ± 1.02*	10.87 ± 0.43*

* sign shows significant difference of cell viability resulted in combinational treatments compared to those yielded by both single treatments.

Table III. Combination Index of combinational treatment of ursolic acid and doxorubicin on MCF-7 cells.

Ursolic acid concentration (IC ₅₀)	Combination Index			
	Doxorubicin (IC ₅₀)			
	1/10	1/4	1/3	1/2
2/5	2.65	3.26	0.69	0.59
1/2	1.47	1.02	0.60	0.54
7/9	1.17	0.60	0.65	0.63

* Bold numbers shows combination resulting in CI value below 0.9, which are synergist.

Ursolic Acid Solely and in Combination with Doxorubicin Altered Cell Cycle Profile of MCF-7 Cells

In this research, treatment of ursolic acid alone led to S phase arrest, while doxorubicin induced accumulation in G2/M phase (Fig. 4). Combinational treatment of both compounds tends to increase cell population in G2/M phase (Fig. 4). G2/M arrest showed occurrence of disruption in cell growth process that may lead to apoptosis.

Possible Underlying Mechanism and Proposed Future Studies

Combinational treatment of ursolic acid and doxorubicin was conducted to explore ursolic acid's potency to be developed as co-chemotherapeutic agent. The use of co-chemotherapeutic agent having synergistic effect may allow the use of lower dosage of chemotherapeutic agent, resulting in the decrease of cytotoxicity on normal cells (Bastl *et al.*, 2007). Combinational treatment of ursolic acid and doxorubicin on MCF-7 cells resulted in higher cell growth inhibition compared to single treatment exhibited synergism in several combinations of concentration. Cell growth inhibition may occur via either apoptosis induction or cell cycle modulation or both occurring consecutively. Zhang *et al.* (2005) and Kassi *et al.* (2009)

reported that ursolic acid inhibited MCF-7 cells growth by downregulating Bcl-2 that leads to apoptosis induction. Our study by flow cytometric analysis showed that ursolic acid alone led to S phase arrest, while in combinational treatment it tend to cause G2/M arrest. Both S and G2/M arrest showed disruption in cell growth process, which could be in either DNA synthesis or cytokinesis. That phenomenon may lead to apoptosis.

Another study revealed that ursolic acid-induced apoptosis was followed by a decrease in CDK4/cyclin D1 expression through suppression of FoxM1 expression (Wang *et al.*, 2012). CDK4/cyclin D complex plays a role in G1 phase activation (Shah and Schwartz, 2006). Inhibition of G1 activation may lead to disruption of protein synthesis (S phase), that may responsible for the S phase arrest. A study revealing ursolic acid's effect to CDK2/cyclin, a complex that is responsible for the activation of S phase is suggested (Hsu *et al.*, 2004). It has also been observed that ursolic acid increased p53 expression (Zhang *et al.*, 2005). p53 protein will be expressed and activated due to DNA damage. The p53 will then induce the expression of cyclin dependent kinase inhibitors (CKIs), such as p21, p27, and p57 that play a role in inhibiting CDK/cyclin complex activity, resulting in the disruption of cell cycle (Foster, 2008). The effect of ursolic

acid in combination with doxorubicin on proteins involved in G2/M activation in MCF-7 cells, such as MPF, are also proposed to be further studied, to understand the mechanism of ursolic acid's ability to increase doxorubicin's cytotoxicity on MCF-7 cells. One protein playing a role in cell growth and apoptosis is

NF- κ B, commonly found as heterodimer (Moynagh, 2005). Observation on the effect of ursolic acid treatment solely and in combination with doxorubicin to NF- κ B activation in MCF-7 cells is also suggested. Besides, study on its selectivity of cytotoxicity is also needed.

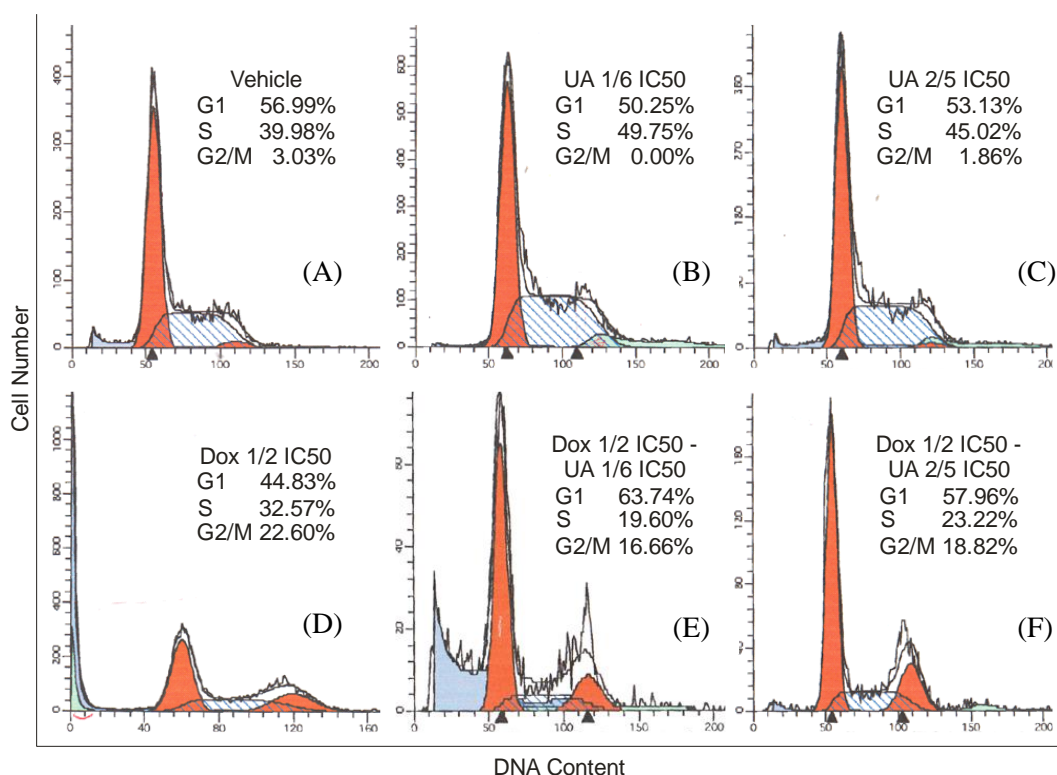


Figure 4. Ursolic acid solely and in combination with doxorubicin modulates cell cycle of MCF-7 cells. One million cells/well were incubated, followed by exposure of samples for 24 hours. Cells were then trypsinized and collected. Following addition on 50 μ g/ml propidium iodide solution and 20 μ g/ml RNase DNase-free, cells were subjected to FACS flow cytometer. Data acquired were analyzed by using ModFit LT 3.0 program. Flow cytometric analysis of (A) vehicle-treated cells; 24-hours treatment of (B) 1/10 IC₅₀ ursolic acid; (C) 1/4 IC₅₀ ursolic acid; (D) 1/2 IC₅₀ doxorubicin; (E) 1/2 IC₅₀ doxorubicin in combination with 1/10 IC₅₀ ursolic acid; (F) 1/2 IC₅₀ doxorubicin in combination with 1/4 IC₅₀ ursolic acid on MCF-7 cells.

According to the data above, we conclude that ursolic acid is promising to be developed as single chemopreventive agent, and also as doxorubicin's co-chemotherapeutic agent in breast cancer treatment. Observation on its selectivity as part of safety aspect is also needed. Further *in silico*, *in vitro*, and *in vivo* study on its molecular mechanism also should be conducted.

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REFERENCES

Bastl, A., Kaseirov, H., Den Hartog, G.J.M., Haenen, G.R.M.M., and Van Der Vijgh,

- W.J.F., 2007, Protectors Against Doxorubicin-Induced Cardiotoxicity: Flavonoids, *Cell Biol Toxicol*, **23**, 39-47.
- Childs, A.C., Phaneuf, S.L., Dirks, A.J., Phillips, T. and Leeuwenburgh, 2002, Doxorubicin Treatment *In Vivo* Causes Cytochrome c Release and Cardiomyocyte Apoptosis, as well as Increased Mitochondrial Efficiency, Superoxide Dismutase Activity, and Bcl-2:Bax Ratio, *Cancer Research*, **62**, 4592-4598.
- Davis, J.M., Navolonic, P.M., Weinstein, C.R., Steelman, L.S., Hu, Konovlepa, M., Blagosklonny, M.V. and McCubrey, J.A., 2003, Raf-1 and Bcl-2 Induce Distinct dan Commn Pathway That Contribute to Cancer Drug Resistance, *Clin. Cancer Res.*, **9**, 1161-1170.
- Drummond, C., 2007, The Mechanism of Antitumour Activity of the DNA Binding Agent SN 28049, Thesis, University of Auckland, New Zealand.
- Fitriasari, A., Susidarti, R.A. and Meiyanto, E., 2010, Peningkatan Efek Sitotoksik Doxorubicin oleh Naringenin Melalui Pemacuan Apoptosis Sel Kanker Payudara MCF-7, *Jurnal Bahan Alam Indonesia*, **7**(3), 165-170.
- Foster, I., 2008, Cancer: A Cell Cycle Defect, *Radiography*, **14**, 144-149.
- Hsu, Y. L., Kuo, P.K., Lin, C.C., 2004, Proliferative inhibition, cell-cycle dysregulation, and induction of apoptosis by ursolic acid in human non-small cell lung cancer A549 cells, *Life Sciences*, **75**(19), 2303-2316.
- Kassi, E., Sourlingas, T.G., Spiliotaki, M., Papoutsis, Z., Pratsinis, H., Aligiannis, N. and Moutsatsou, P., 2009, Ursolic Acid Triggers Apoptosis and Bcl-2 Downregulation in MCF-7 Breast Cancer Cells, *Cancer Invest.*, **27**(7), 723-733.
- Mechetner, E., Kyshtoobayeva A., Zonis S., Kim H., Stroup R., Garcia R., Parker R.J. and Fruehauf, J.P., 1998, Levels of Multidrug Resistance (MDR1) P-Glycoprotein Expression by Human Breast Cancer Correlate with *in vitro* Resistance to Taxol and Doxorubicin, *Clin. Cancer Res.*, **4**, 389-398.
- Meiyanto, E., Hermawan, A. and Anindyajati, 2012, Natural Products for Cancer-Targeted Therapy: Citrus Flavonoids as Potent Chemopreventive Agents, *Asian Pac. J. Cancer Prev.*, **13**, 427-436.
- Moynagh, P.N., 2005, The NF- κ B Pathway, *J. Cell Sci.*, **118**, 4589-4592.
- Notarbartolo, M., Poma, P., Perri, D., Dusonchet, L., Cervello, M., and Alessandro, N., 2005, Antitumor Effects of Curcumin, Alone or in Combination with Cisplatin or Doxorubicin, on Human Hepatic Cancer Cells: Analysis of Their Possible Relationship to Changes in NF- κ B Activation Levels and in IAP Gene Expression, *Cancer Letters*, **224**, 53-65.
- Reynolds, C.P. and Maurer B.J., 2005, Evaluating Response to Antineoplastic Drug Combinations in Tissue Culture Models, *Methods in Molecular Medicine*, **110**, 173-183.
- Shah, M.A. and Schwart, G.K., 2006, Cyclin Dependent Kinases as Targets for Cancer Therapy, *Update on Cancer Therapeutics*, **1**, 311-332.
- Song, Y.H., Jeong, S.J., Kwon, H.Y., Kim, B., Kim, S.H. and Yoo, D.Y., 2012, Ursolic Acid from *Oldenlandia diffusa* Induces Apoptosis via Activation of Caspases and Phosphorylation of Glycogen Synthase Kinase 3 Beta in SK-OV-3 Ovarian Cancer Cells, *Biol. Pharm. Bull.*, **35**(7), 1022-1028.
- Teng, W.Y., Huang, Y.L., Shen, C.C., Huang, R.L., Chung, R.S. and Chen, C.C., 2005, Cytotoxic Acridone Alkaloids from the Stem Bark of *Citrus Maxima*, *J. of the Chinese Chem. Soc.*, **52**, 1253-1255.
- Tian, Z., Lin, G., Zheng, R.X., Huang, F., Yang, M.S., and Xiao, P.G., 2006, Anti-hepatoma Activity and Mechanism of Ursolic Acid and Its Derivatives Isolated from *Aralia decaisneana*, *World J. Gastroenterol.*, **12**(6), 874-879.
- Wang, J.S., Ren, T.N., and Xi, T., 2012, Ursolic Acid Induces Apoptosis by Suppressing the Expression of FoxM1 in MCF-7 Human Breast Cancer Cells, *Med. Oncol.*, **29**(1), 10-15.
- Wei, G., Wang, J., and Du, Y., 2011, Total Synthesis of Solamargine, *Bioorganic*

- and Medicinal Chem. Letters*, **21**, 2930-2933.
- Zampieri, L., Bianchi, P., Ruff, P., and Arbuthnot, P., 2002, Differential Modulation by Estradiol of P-glycoprotein Drug Resistance Protein Expression in Cultured MCF7 and T47D Breast Cancer Cells, *Anticancer Res.*, **22**, 2253-2259.
- Zhang, W., Li, Y., Zhang, G., Lu, J., and Ou, H., 2005, Experimental Study on MCF-7 Cell Apoptosis Induced by Ursolic Acid, *Zhong Yao Cai*, **28**(4), 297-301.