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***Gynura procumbens* Prevents Chemoresistance through Inhibition MDR1 Expression on MCF-7 Breast Cancer Cell Line and Sensitizes the Cells to Doxorubicin**

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Abstract

The long-term exposure of doxorubicin (Dox) causes enhancement in MDR1 expression that leads to breast cancer cell resistance. This protein become a serious problem in cancer treatment and also well-known as negative prognostic factor in breast cancer malignancies. The new approach using natural chemopreventive substance was developed to inhibit this resistance progress. This study was aimed to investigate whether ethyl acetate fraction of *Gynura procumbens* (FEG) can prevent chemoresistance through suppressing the MDR1 protein expression. MCF-7 cell was used as chemoresistance cell model. The MCF-7 cells were maintained with 100 nM Dox-contained medium for five weeks. The chemoprevention effect of FEG was investigated by treated MCF-7/Dox with sub-toxic concentration of FEG. The cytotoxic properties of MCF-7 cells were determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay. Immunofluorescence and western blotting analysis was performed to detect the MDR1 expression. MCF-7/Dox cells need higher concentration for inhibiting cell growth, were compared with MCF-7, shown by IC₅₀ value. The MDR1 protein level elevated after Dox exposure in time dependent manner. The FEG treatment decreased MDR-1 protein level with dose dependent manner. FEG in combination with DOX potentiates the DOX effect on breast cancer cell growth inhibition. The FEG prevents the chemoresistance development in breast cancer cell line, MCF-7 induced by Dox through inhibiting *MDR1* expression. The additional of FEG enhances Dox effect on cell death induction. Thus, FEG could be developed as co-chemotherapy agent for reverse multidrug resistance.

Keywords : *Gynura procumbens*, Chemoresistance, MDR1, MCF-7, MCF-7/Dox

Introduction

Drug resistance becomes a major obstacle to the successful of cancer chemotherapy. There are several mechanisms mediate the drug resistance mechanism including

drug inactivation, drug efflux, drug target mutation, and the failure of apoptosis initiation (Davis *et al.*, 2003; Notarbartolo *et al.*, 2005). Over expression of drug transporter such as P-gp plays a major role in causing multi drug resistance. P-gp is an adenosine triphosphatase (ATPase), a member of ATP-binding cassette (ABC) transporter encoded by *MDR1* gene. In normal cells, P-gp localized at epithel cell and has a function to eliminate xenobiotic and metabolit endogen. However, in some human cancers P-gp overexpression has high corelation with the decreasing

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survival and become poor prognosis on cancer development (Leonessa and Clarke, 2003). There is positive correlation between overall *MDR1* expression and grade of breast cancer tumor (Surowiak *et al.*, 2005). This protein become a serious problem in cancer treatment and also well-known as negative prognostic factor in breast cancer malignancies.

P-gp substrates include substances in variable structure, chemical properties and drug mechanism. Several cancer chemotherapy drugs such as anthracyclines, taxanes, and epipodophylotoxins already well-known as the substrate of this protein. Long time exposure of these agents on cancer cells triggers the transcriptional activity of *MDR1* lead the increasing of P-gp (Germann, 1996; Nielsen *et al.*, 1996). The elevation of P-gp protein leads to chemotherapy failure due to cell resistance at several type of cancer. Doxorubicin which belongs to anthracycline group could induce breast cancer multi drug resistance after long time exposure. DOX treatment increase P-gp expression level through Ras/Raf1 activation (Davis *et al.*, 2003). P-gp-mediated drug efflux decreases intracellular Dox uptake and decrease its efficacy.

The development of P-gp inhibitors led to the third generation. Unfortunately, most of P-gp inhibitors that have been developed giving disappointing results. New approach using natural substances with moderate or low cytotoxic properties become a promising hope for reversing multi drug resistance due to *MDR1* over expression. Natural flavonoids have antitumor properties and others pharmacological activities related with its antitumor such as anti-inflammatory and antioxidant. Flavonoid, especially flavones and flavonol show modulation effect on cancer cell growth through cell cycle arrest and apoptosis induction (Limtrakul *et al.*, 2007). Quercetin and kaempferol down regulate multidrug resistance gene on human erythroleukemic K562/A cells. It causes decreasing of cells sensitivity to adriamycin

(Yanqiu *et al.*, 2011). Quercetin sensitizes cell resistance to daunorubicin through suppression on *MDR1* expression and P-gp activity at human pancreatic carcinoma (Borska *et al.*, 2010).

Gynura procumbens is commonly used as herbal medicines in South-East Asia, especially Indonesia, Malaysia, and Thailand. The extract and fraction of this plant have high anti-inflammatory and antioxidant properties (Iskander *et al.*, 2002; Rosidah *et al.*, 2008). The ethyl acetate fraction of *G. procumbens* (FEG) may contain quercetin and kaempferol. FEG sensitizes MCF-7 breast cancer cells to Doxorubicin treatment led to cell death. FEG modulates the microtubule integrity followed by cell cycle arrest and cell growth inhibition (Nurulita *et al.*, 2012). In this study, we subject FEG to doxorubicin-resistance cell. We use MCF-7 cells treated by 100 nM doxorubicin for five week as a model of cell resistance. We investigate the modulation on their sensitivity to doxorubicin, P-gp level, and cell growth.

Materials and Methods

Plant material

The leaves of *G. Procumbens* were obtained from Balai Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (BP2TO2T) Indonesia. Dried powdered leaves were first extracted with ethanol 96%, and concentrated by evaporation under reduced pressure and the temperature was kept below 40°C. The extract was diluted in hot water and then fractionated with n-hexane. The aqueous fraction then was fractionated again with ethyl acetate. The ethyl acetate fraction was concentrated by evaporation under reduced pressure and the temperature was kept below 40°C. The extract was stored at 4°C until used.

Cells and chemicals

MCF-7 cells were obtained from Laboratorium of Gene Function in Animal, Nara Institute of Science and Technology (NAIST), Japan. MCF-7/DOX cells were

developed from MCF-7 cell that induced with 100 nM DOX for five weeks. The resistance development was checked by P-gp levels. The MCF-7 cells were routinely grown in DMEM (Nacalay Tesque) supplemented with 10% FBS (PAA Laboratories), 1% v/v Penicillin-streptomycin (Sigma), and 1 mM L-glutamine (Nacalay Tesque) at 37°C in 5% CO₂. MCF-7/DOX cells were maintained in 25 nM DOX-contained medium. Doxorubicin (DOX) and 5-Fluorouracil (5-FU) (Ebewe) was purchased from PT. Ferron Par Pharmaceutical Cikarang, Indonesia, Dimethyl sulfoxide (DMSO) (Sigma, Aldrich, Germany), penicillin and streptomycin (Gibco), Tripsin (Sigma), 3-(4,5-dimethylthiazol-2-il)-2,5-diphenyltetrazolium bromide (MTT) (Sigma), triton X-100, 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) are at analytical degree.

Antibodies

Primary antibodies that used in western blotting and immunostaining were anti P-Glycoprotein (JSB-1) (GeneTex, Inc.), anti α -Tubulin (Santa Cruz), and secondary antibodies were diluted in PBS containing 5% skim milk, 0,05% Tween (blocking buffer). For P-gp detection, the anti-P-gp and its secondary antibody were diluted in Can Get Signal (CGS) solution (Cosmo Bio).

Cells growth assay

Cells (10⁴ cells/well) were seeded in 96-well plate (100 μ l/well). After 24 h of incubation, the medium was replaced with DOX, FEG, and its combination-containing medium. The medium was discarded and replaced with MTT-containing medium (0.5 mg/ml) after 24 h incubation and incubated again for further 4 h at 37°C, 5% CO₂. The reaction was stopped using 10%SDS in 0.1 N HCl solution and was incubated for overnight in light protected chamber, to dissolve formasan salt. The absorbance of each well was measured by ELISA reader at 595 nm. The ratio between treated and control cells absorbance referred to percentage (%) of cells viability.

Gel electrophoresis and immunoblotting

Cells were recovered, washed in PBS, and lysed for 30 min on ice using lysis buffer (20 mM Tris-HCl, pH 8, 0.5 mM EDTA, 1% NP40, 25 mM NaCl, and complete inhibitors of protease. Cells extract were centrifuged at 15.000 rpm for 20 min at 4°C to separate insoluble material. The protein concentration was determined using Bradford assay. Equal amount of each sample were mixed with SDS loading buffer, boiled for 3 min and subjected to SDS-PAGE at 120 V followed by electro blotting to Polyvinylidene fluoride (PVDF) membranes for 2 h at 100 V. Membranes were blocked using 5% skim milk in PBS at room temperature for 1 hand subsequently probed with the primary antibody of interest. Blots were exposed by Chemilumi-oneSuper (Nacalay Tesque).

Results

Long treatment of sub toxic concentration of DOX induced resistance cells development.

Breast cancer cells become less sensitive to chemotherapy agen during the cells resistance development. After five weeks induced by DOX, MCF-7 cells decreased its sensitivity to DOX treatment, as shown by IC₅₀ value (Table 1).

Table 1. IC₅₀ value of DOX and FEG on MCF-7 and MCF-7/DOX cells

Sample	Cell Line	IC ₅₀ value *
DOX	MCF-7	410 \pm 44 nM
	MCF-7/Dox	921 \pm 40 nM
FEG	MCF-7	259 \pm 29 μ g/ml
	MCF-7/Dox	161 \pm 40 μ g/ml

*average from two independent experiments

DOX resulted cell growth inhibition more than 2-fold higher on MCF-7/DOX sub line than that of MCF-7 cell line (Figure 1A). FEG treatment showed the opposite results. FEG caused cell growth inhibition stronger on MCF-7/DOX sub line compared with MCF-7 cell (Figure 1B).

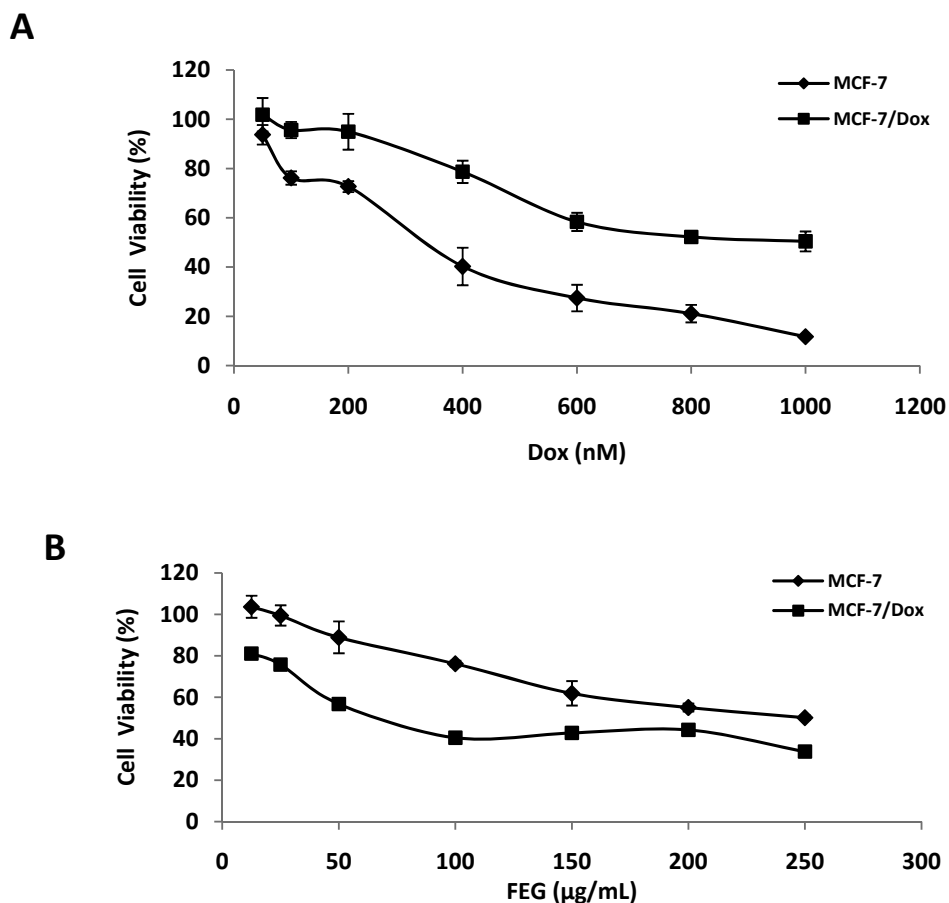


Figure 1. MCF-7/DOX cells are less sensitive to DOX (A) but more sensitive to FEG (B). Cells (10^4 cells/well) were cultured at 96-well plate, then treated with indicated concentration of DOX or FEG for 24 h incubation. Cells Viability was determined with MTT assay. This result represents from 3 independent experiments. The data was statistically analyzed by two tailed-T-test analysis with $p < 0,05$.

DOX induced the elevation of P-gp level

DOX-induced MCF-7 cells showed the elevation of P-gp expression level in time dependent manner (Figure 2A). expression level of this protein was correlated with less sensitivity of MCF-7/DOX cells to DOX. FEG treatment decreased P-gp level in dose dependent pattern (Figure 2B). To make confirmation of this phenomenon, both immunoblotting and immunofluorescence were conducted. FEG decreased the green fluorescence intensity that refer P-gp protein decreasing (Figure 3). DOX induces breast cancer cell resistance due to increasing of

P-gp expression level. The P-gp protein has 170 kDa molecule size. This protein is hyperglycosilated as such appears as a diffuse band on Western blot results (Patel *et al.*, 2002). We also found the additional band below P-gp protein size (Figure 2). The lower molecules weight bands was detected using the JSB-1 antibody for P-gp represent degraded product of P-gp protein. JSB-1 antibody recognizes a cytoplasmic epitope of P-gp and does not cross-react with other protein such as MDR3 or muscle myosin, as does the C219 antibody (Leonessa and Clarke, 2003).

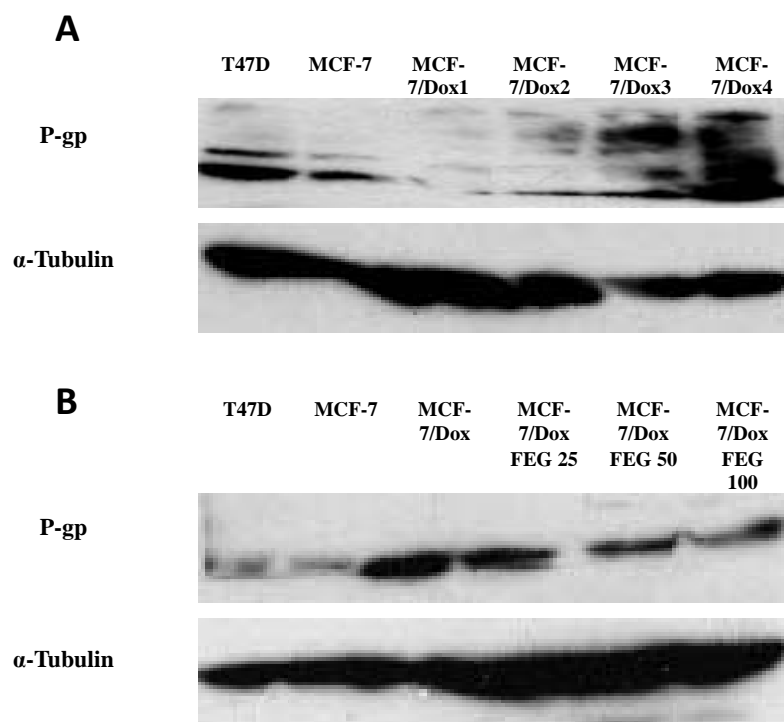


Figure 2. DOX induced P-gp expression on pada MCF-7 cells (A) and FEG prevents the elevation of this protein expression (B). Cells (10^6 cells/well) were seeded at 6-well plate, then treated with DOX, FEG or its combination for 24 h. Cell lysate then analyzed with Western blotting. Anti- α -tubulin was used for loading control. Dox1, Dox2, Dox3, and Dox4 refer to induction time for 2, 3, 4, and 5 weeks, respectively.

FEG reversed cell resistance and increased the sensitivity of MCF-7/DOX cells to DOX treatment

To investigate whether FEG could reverse the cell resistance development on MCF-7/DOX, MCF-7 cells were treated with DOX, FEG, or its combination and observed FEG reversal effect and its modulation on cell growth. Combination treatment of DOX+FEG suppressed cell growth o both of MCF-7 and MCF-7/DOX cells. The inhibition effect of combination treatment on MCF-7/DOX cells more significance compared to MCF-7 cells (Figure 4).

Discussion

P-glycoprotein (P-gp), a protein encoded by *MDR-1* gene, has important function as ATP-dependent pump of drug efflux. P-gp has specific substrate, such as vinca alkaloids,

anthrasiclin, epipodophylotoxins, and rhodamine. The exposure of These substrate to cancer cells could trigger transcriptional activity of MDR-1 (Germann, 1996; Lampidis *et al.*, 1989; Nielsen *et al.*, 1996).

Previous study have found the long time exposure of DOX induced cancer cells resistance through the increasing level of P-gp (Abolhoda *et al.*, 1999). In this study MCF-7 cells were used as resistance model of breast cancer. This cells were developed by long term sub toxic concentration of DOX induction. MCF-7 cells are cell line that express *MDR1* and *Breast cancer resistance protein* (BCRP) that will be triggered its expression after several chemotherapy agent induction (Faneyte *et al.*, 2002). MDR-1 expression elevates at patient after long time chemotherapy agent administration and had strong correlation with cancer chemotherapy

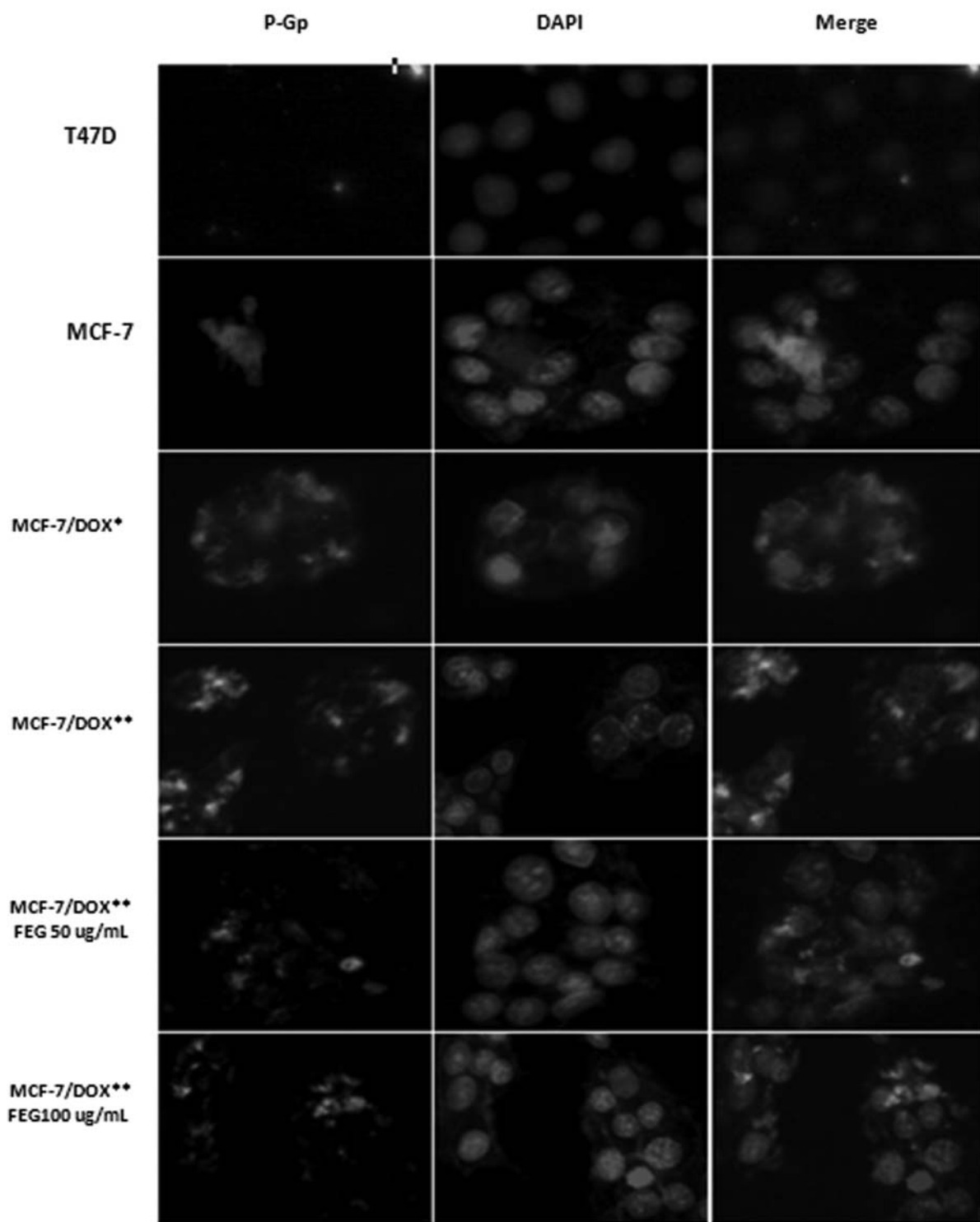


Figure 3. DOX induced P-gp expression on pada MCF-7 cells and FEG prevents the elevation of this protein expression in concentration dependent manner. Cells ($5 \cdot 10^4$ cells/well) were seeded on cover slip at 24-well plate. After treated with or without DOX+FEG for 24 h, then stained with anti- α -Tubulin (green fluorescence) and counterstained with DAPI (blue fluorescence). Cells were observed under fluorescence microscope with 400x magnification.

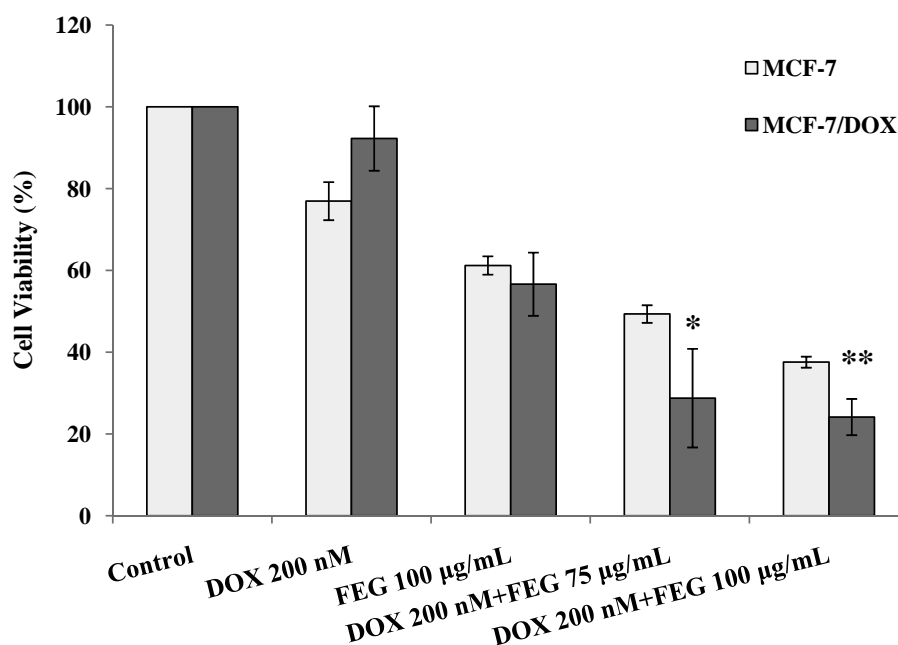


Figure 4. FEG increased the sensitivity of MCF-7/DOX cells to DOX. Cells (10^4 cells/well) were cultured at 96-well plate, then treated with indicated concentration of DOX, FEG and its combination for 24 h incubation. Cells Viability was determined with MTT assay. This result represents from 2 independent experiments with similar results. The data was statistically analyzed by two tailed-T-test analysis. * $p = 0,1$ and ** $p = 0,028$.

failure (Leonessa and Clarke, 2003). This protein has identified as negative prognostic in cancer diseases development.

Sub toxic concentration of DOX for five weeks decreased MCF-7 cells sensitivity to this agent, shown by IC_{50} value become more than 2 fold higher compared with MCF-7 cells. DOX exposure to MCF-7 cells lead to increasing expression of P-gp in time dependent manner. MCF-7 cells after five weeks DOX induction (MCF-7/DOX) shown the significance increasing of this protein level compared with MCF-7 cells. The P-gp level has strong correlation with the low sensitivity of MCF-7/DOX to DOX exposure.

The attempts to overcome drug resistance mediated by *MDR-1* activation and elevation of P-gp level were developed the P-gp inhibitors. The agent that could suppress P-gp expression will increase uptake of chemotherapy agent and may influence its clinical efficacy. Flavonoids

could modulate the expression and activation of P-gp. There are several mechanisms mediate their effect: changing membrane cells permeability, suppressing P-gp expression, inhibition ATPase activity, and occupation substrate binding site at P-gp, then changes its conformation (Arora *et al.*, 2000; Boumendjel *et al.*, 2002; Drori *et al.*, 1995).

Natural compounds such as flavonoids and polyphenol have significant biological properties related to multidrug resistance development on cancer cells. Biochanin A and sylimarin inhibit drug efflux from the cells by P-gp pump, and produce synergistic effect when combined with DOX at MCF-7 and MDA435/LCC6. Both compound decreased IC_{50} value of daunomycin significantly at MCF-7/Adr cells (Zhang and Morris, 2003; Chung *et al.*, 2005). (-)-Epigallocatechin (EGC), (-)-epicatechin gallate (ECG), dan (-)-epigallocatechin gallate (EGCG) increase daunomycin accumulation at P-gp over-expressing cells, KB-C2 (Kitagawa *et al.*,

2004). Flavonoid and polyphenol have a promising properties to be developed as P-gp inhibitor to prevent cell resistance.

FEG treatment enhances DOX cytotoxicity significantly at MCF-7/DOX cells, higher than the effect on MCF-7 cells. The inhibition of cells growth exhibited synergistic effect of combination treatment DOX+FEG in dose dependent manner. FEG treatment decreases P-gp expression level at MCF-7/DOX cells linear to the given concentration. The ethyl acetate fraction of *G. procumbens* (FEG) was suggested have flavon and or flavonol with hydroxyl group modified to be metoksi at 3 and 4', and asetoksi at 7 position (Sugiyanto *et al.*, 2003). The ethyl acetate fraction of *G. procumbens* (FEG) may contain quercetin and kaempferol (Nurulita *et al.*, 2012). FEG may modulate *MDR-1* expression through the same mechanism with quercetin and kaempferol.

Quercetin inhibits the members of *MDR1 family*, such as : P-gp, MRP, and BCRP, competitively (Cooray *et al.*, 2004; Scambia *et al.*, 1994; van Zanden *et al.*, 2005). Kaempferol and quercetin decrease P-gp expression and its activity at MDR KB-V1 cells (Limtrakul *et al.*, 2005). Quercetin and kaempferol prevent cell resistance development through down regulate multidrug resistance gene on human erythroleukemic K562/A cells. It reverses cells sensitivity to adriamycin (Yanqiu *et al.*, 2011). Quercetin suppresses *MDR1* expression and P-gp activity at human pancreatic carcinoma. The net influx of daunorubicin increases when given together with quercetin (Borska *et al.*, 2010). One study on structure activity relationship concludes flavonoid with -OH group at 5 dan 7, with maximal have three total hydroxyl groups, and have strong inhibition on P-gp activity. Double bond at C2 and 3 as shown at flavon generates its planarity, lead to intercalate with hydrophobic amino acids residue of P-gp (Sheu *et al.*, 2010). Quercetin and kaempferol modulate both of *MDR-1* gene and P-gp protein level and also its activity that reverse multidrug resistance on many type of cancer cell lines.

This study found that FEG decrease P-gp expression level at MCF-7/DOX cells. This results were suggested to the decreasing P-gp expression that may affect on the increasing of DOX accumulation at cancer cells. In the next study, monitoring the net uptake of DOX during co-treatment of FEG should be considered. This effect may precede through several possibility, such as suppression on transcriptional activity of *MDR-1* gene, translation, or post translation.

As the conclusion, FEG prevents MCF-7 cells from developing resistance through down-regulating P-gp expression. FEG in combination with DOX potentiates the DOX effect on breast cancer cell growth inhibition. Thus, FEG could be developed as co-chemotherapy agent for reversing multidrug resistance.

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