Journal of Applied Pharmaceutical Science Vol. 8(08), pp 039-043, August, 2018
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2018.8806
ISSN 2231-3354

Dendrophthoe pentandra Leaves Extract Promotes Apoptotic Effects of Doxorubicin in Human Breast Cancer Cell via Modulation of Intracellular Calcium and Survivin

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ARTICLE INFO

Article history:
Received on: 18/05/2018
Accepted on: 09/07/2018
Available online: 31/08/2018

Key words:
apoptosis, intracellular calcium, *Dendrophthoe pentandra* extract, doxorubicin, survivin.

ABSTRACT

Dendrophthoe pentandra is a parasitic plant that has anticancer activity. The leaf of *Dendrophthoe pentandra* traditionally has been used for anticancer agents. The aim of this study was to investigate whether the combination of *Dendrophthoe pentandra* leaves extract (DPE) and doxorubicin reduces intracellular calcium concentration [iCa^{2+}] and survivin levels while increasing the number of apoptotic cells in MCF-7 breast cancer cell lines. MCF-7 cells were cultured and divided into six groups: group 1 is a negative control (without DPE nor doxorubicin); group 2 was treated with doxorubicin alone; groups 3-5 were treated with DPE in doses of either 12.5, 25, or 50 µg/ml immediately after doxorubicin treatment, and group 6 was treated with 50 µg/ml of DPE alone. After 24 hours of treatment, we assessed the concentration of iCa^{2+}, survivin levels and the number of apoptotic cells by flow cytometry. The combination of doxorubicin and DPE significantly reduced the percentage of survivin ($P < 0.05$) and iCa^{2+} concentration ($P < 0.05$); whereas the number of apoptotic cells increased significantly ($P < 0.05$). Therefore, we concluded that the anti-cancer effect of doxorubicin can be enhanced when combined with DPE.

INTRODUCTION

Breast cancer is one of the most common cancers found among women, was diagnosed in an estimated 1.67 million new cases of cancer worldwide in 2012 (Ferlay *et al.*, 2015). Treatment for breast cancer generally involves surgery and may be augmented by radiation or chemotherapy (Kaabel & Baali., 2015).

One of the chemotherapy agents prescribed to treat breast cancer is doxorubicin, which is administered with or without another anti-cancer agent depending on the cancer stage and type (Rivankar, 2014). Despite its anti-cancer activity, doxorubicin has side effects in clinical use, particularly cardiotoxicity and there has been an emergence of resistance (Thorn *et al.*, 2011). These weaknesses have triggered the search for a treatment method to improve the effect of doxorubicin by developing new therapeutic combinations (Staedler *et al.*, 2011).

Dendrophthoe pentandra is a parasitic plant, or mistletoe, that grows on mango trees and is traditionally used as a medicinal plant to treat hypertension, measles, cough, and skin infections, and is prescribed as a diuretic and an anticancer agent (Artanti *et al.*, 2012). The extract of *Dendrophthoe pentandra* Leaves Extract (DPE) contains secondary metabolites including polyphenols, tannins, flavonoids, steroids, triterpenoids, monoterpenoids, and quinines. Flavonoid is one of the secondary metabolites that has an anticancer effect. Furthermore, one of the flavonoid compounds found in DPE is quercitrin (quercetin-3-rhamnose) (Nurfaat & Indriyati., 2016). DPE has been shown significantly inhibit proliferation activity in colon cancer *in vivo* via the p53 independent pathway (Endharti *et al.*, 2016). The activities

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of DPE in inducing apoptosis in human breast (T47D) and leukemia (K562R) cell lines have also been investigated (Widowati *et al.*, 2013; Zamani *et al.*, 2016). In addition, the combination of DPE and doxorubicin has also been shown to enhance the antitumor effect of doxorubicin in liver and breast cancer cells (Wang *et al.*, 2012; Staedler *et al.*, 2011).

In cancer cells, there are imbalances in the apoptosis process as well as uncontrolled cell proliferation (Dubois *et al.*, 2013). Apoptosis can be induced by various conditions, such as the presence of disruption of intracellular calcium homeostasis (Harr & Distelhorst., 2010). Calcium-dependent apoptosis involves two factors: sustained increases in intracellular calcium and a prolonged reduction of calcium in the endoplasmic reticulum (ER) (Dubois *et al.*, 2013). Apoptosis can be inhibited by anti-apoptosis proteins such as survivin, which is overexpressed in a majority of breast cancers (Jha *et al.*, 2012; Jaiswal *et al.*, 2015). The anti-apoptotic activity of survivin is blocked by apoptosis inducer such as p53 protein (Jaiswal *et al.* 2015).

The efficacy of the combination of DPE and doxorubicin as an anti-cancer treatment is unclear. In this study, we investigated the combined effects of doxorubicin and DPE on survivin levels, intracellular calcium concentration, and the number of apoptotic cells in MCF-7 breast cancer cells.

MATERIALS AND METHODS

Plant material and extraction

Dendrophthoe pentandra fresh leaves were collected from Probolinggo, East-Java, Indonesia and the identification of the plants were done at the Department of Biology, University of Brawijaya (specimen No. 0170/Taxonomy Identification/03/2015). The leaves *of Dendrophthoe pentandra* were extracted according to the protocol described by Endharti *et al.* (2016).

Cell cultures

MCF-7 cell line was cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) (Gibco, USA) medium supplemented with 10% Fetal Bovine Serum (FBS) (Atlas Biologicals, Fort Collins, Co, USA), 100 U/ml streptomycin and 100 U/ml penicillin (Gibco, USA). Cells were kept in an incubator in a humidified atmosphere of 5% CO_2 at 37°C.

Measurement of intracellular calcium [iCa2+]

Measurement of [iCa^{2+}] accumulation was labeled by Fluo 3-AM (Bioscience, USA), a fluorescent probe, was applied to determine intracellular calcium, Briefly, MCF-7 cells were plated in 24 well at density 1 × 105 cells per well. After 24 hours of incubation, the culture medium was replaced with medium containing 5 µg/ml doxorubicin (Sigma, USA) with or without DPE and then incubated for 24 hours. Cells were rinsed with PBS followed by dilution in PBS + Ca^{2+} + Mg^{2+} containing 1 mM sodium pyruvate and EGTA. Fluo3-AM was added to obtain a final concentration of 5 µM and then incubated in the darkroom for 30 min at room temperature. The sample was measured by flow cytometer. Intracellular calcium fluorescence was analyzed using Cell Quest ProTM software (BD FACS Calibur) and measured on FL-1 (voltage 600).

Flow cytometry analysis for survivin

Percentage of survivin was determined quantitatively by flow cytometry using the survivin- antibody (D-8) sc-17779 conjugated with FITC (Santa Cruz Biotechnology, Germany) following the manufacturer's protocol. Briefly, MCF-7 cells were plated at a density 1 × 10^5 cells per well and incubated overnight. The cells were treated with doxorubicin 5 µg alone or with DPE (12.5, 25 and 50 µg/ml) for 24 hours followed by trypsinization. Cells were harvested and the pellets were re-suspended in Fixation buffer (Biolegend, San Diego, California, USA) and incubated at 4°C for 30 min. The pellets were incubated with survivin antibody for 20 min. The samples were immediately analyzed by Cell Quest ProTM software (BD FACS Calibur, Becton Dichinson, San Jose, USA).

Apoptosis analysis

Apoptosis cells were determined quantitatively by flow cytometry using the annexin V-conjugated FITC (eBioscience) following the manufacturer's protocol. Briefly, MCF-7 cells were cultured in a 24-well plate at a density of 2 × 10^5/well. The cells were treated with doxorubicin 5 µg alone or with DPE (12.5, 25 and 50 µg/ml) for 24 hours followed by trypsinization. Cells were resuspended in 2.5 µg/ml Annexin-V-FITC and 50 µg/ml propidium iodide (PI) (Biolegend) were added to the cell suspension and incubated for 5 min at room temperature (25°C) in the dark. The apoptosis level was measured by quantifying the population of Annexin V-FITC-positive cells. Flow cytometry data were plotted and analyzed by Cell-Quest software (FACS Calibur TM; Becton-Dickinson).

Statistical analysis

The results were analyzed by the one-way ANOVA test, followed by Post Hoc Tukey test. Data were described as a mean ± standard deviation. Statistical significance of a difference between groups, with $P < 0.05$ being considered significant. Statistical analysis was performed with SPSS software version 11.0 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Results

Doxorubicin combined with DPE decreased Intracellular Calcium.

The [iCa^{2+}] alteration after treatment with a combination of doxorubicin and DPE was observed by flow cytometry (Figure 1). A decline in [iCa^{2+}] was observed as demonstrated by decreasing of fluorescence intensity. The results showed that a combination of doxorubicin and DPE decreased intracellular calcium (Figure 1A &1B). As shown in figure 2B, the doxorubicin treatment combined with DPE at doses 25 µg/ml and 50 µg/ml reduced intracellular calcium concentration significantly at 17% ($P < 0.05$) and 14% ($P < 0.01$), respectively.

Co-treatment doxorubicin and DPE reduced the level of survivin

The results of survivin levels measurement by flow cytometry were shown in Figure 2. Figure 2B exhibit doxorubicin

treatment combined with DPE at doses of 25 µg/ml and 50 µg/ml reduced survivin levels significantly at 27% and 19%, respectively ($P < 0.01$).

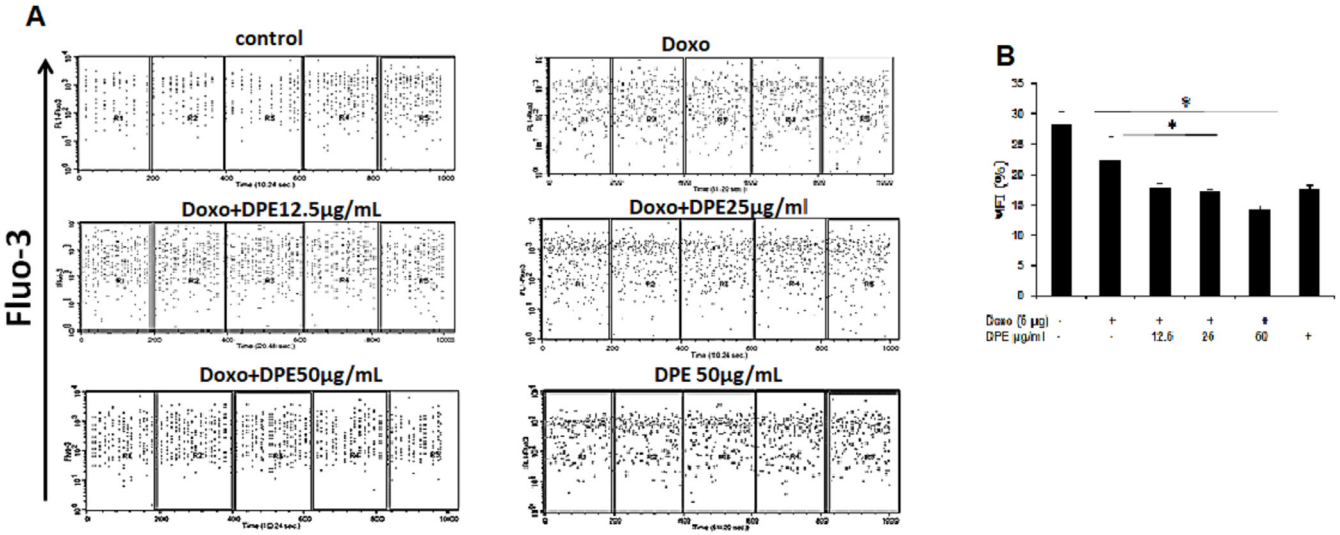

Fig. 1: The combination effect of DPE and doxorubicin decrease [iCa^{2+}] mobilization in MCF-7 cell lines. (A) Calcium intracellular was detected in MCF-7 cells probed with Fluo-3 AM. MCF-7 cell lines loaded with the calcium-sensitive dye Fluo-3 were treated with the combination of doxorubicin 5 µg/ml and DPE (12.5, 25, 50 µg/ml). Fluorescence was recorded every second using flow cytometry. (B) The combination of doxorubicin and DPE decreased the concentration of [iCa^{2+}] in MCF-7 cells. Dot plot showed are representative of four independent experiments. Results shown are mean ± SD, with $n = 4$ replicates in each group. *$P < 0.05$, **$P < 0.001$ versus doxorubicin only group.

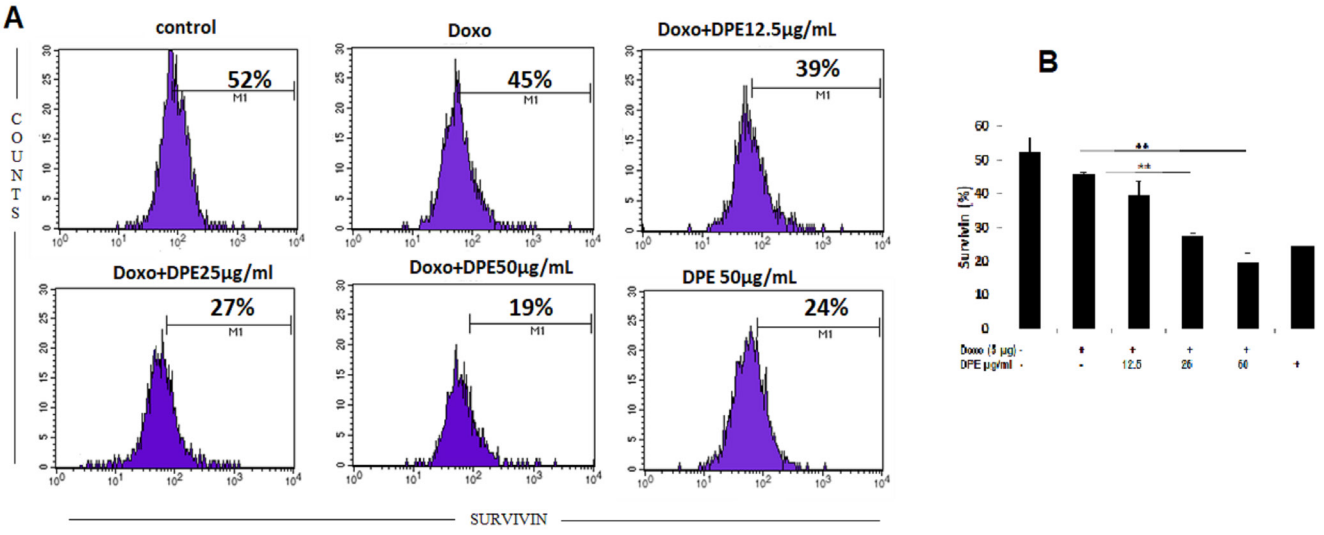

Fig. 2: Survivin levels in MCF-7 cells after doxorubicin treatment 5 µg/ml combined with DPE 12.5, 25, 50 µg/ml. (A) Representative images of survivin levels by flow cytometry. (B) Combination of doxorubicin and DPE reduced survivin levels in MCF-7 cells. Results shown are mean ± SD, with $n = 4$ replicates in each group. *$P < 0.05$, **$P < 0.001$ versus doxorubicin only group.

The combination of doxorubicin and DPE synergistically induced apoptosis

The MCF-7 cell culture was exposed to doxorubicin at a dose of 5 µg/ml alone or combined with DPE at doses of 12.5 µg/ml, 25 µg/ml, 50 µg/ml. Annexin V conjugated fluorochrome-FITC and propidium iodide (PI) (Sigma, Kawasaki, Japan) staining was performed to investigate whether the combination of doxorubicin and DPE induced cell death through apoptosis. The results of flow cytometry showed the percentage of apoptotic cells represented in dot plot (Figure 3A). Doses of 12.5-50 µg/mL

DPE enhanced apoptosis of doxorubicin against MCF-7 cells in a dose-dependent manner. When DPE and doxorubicin were used in combination, the number of apoptosis cells was significantly increased compared with doxorubicin treatment alone (Figure 3B); $P < 0.05$).

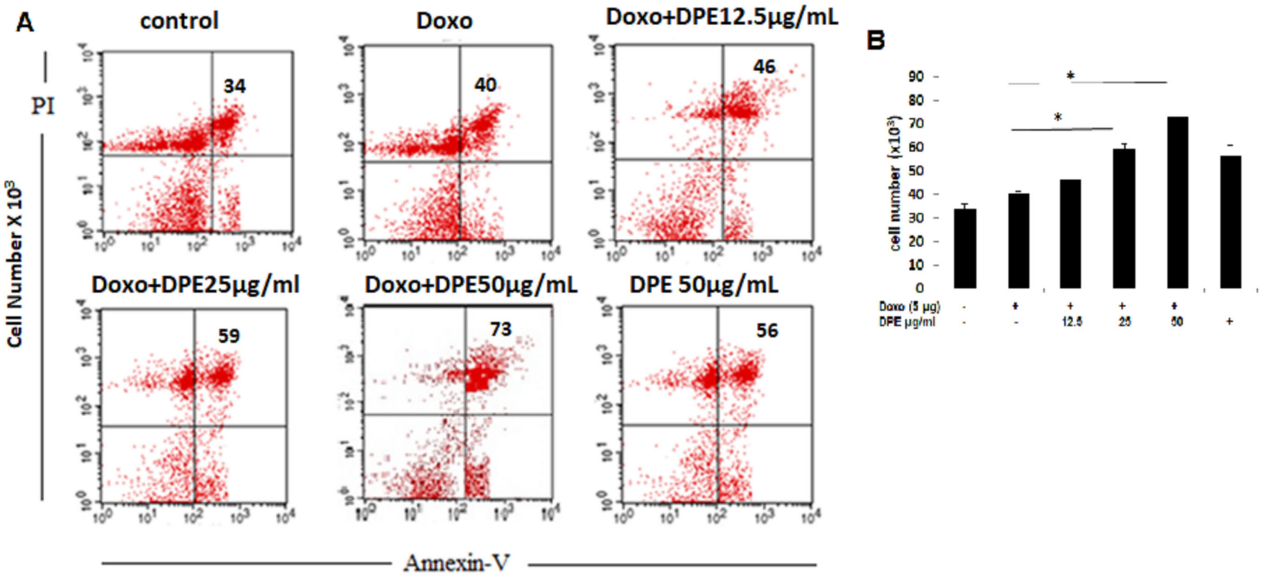

Fig. 3: The apoptotic effects of the combination of doxorubicin 5 µg/ml and DPE (12.5, 25, 50 µg/ml) in MCF-7 cell line. Cells were stained by Annexin V-PI and examined by flow cytometry as described in the Materials and Methods. (A) Representative images of flow cytometric assessment of apoptosis. (B) MCF-7 cell line showed a significant rise in the number of apoptotic cells after treatment for 24 hours. Results shown are mean ± SD, with $n = 4$ replicates in each group. *$P < 0.05$, **$P < 0.001$ versus doxorubicin only group.

Discussion

Various studies have been conducted with the aim of improving the efficacy of existing chemotherapy drugs. One method developed is combining chemotherapy agents either with different chemotherapy agents or with other substances such as herbs. In this study, we investigated the combination effect of doxorubicin and DPE on intracellular calcium concentrations, survivin levels and apoptosis in MCF-7 breast cancer cells.

The results of our study showed that treatment of MCF-7 cells with the combination of doxorubicin and DPE declined the concentration of intracellular calcium. We assume that the mechanisms of this phenomenon because of the treatment of doxorubicin and DPE able to decrease Bcl-2 protein. The decreasing of Bcl-2 protein induces calcium release from the endoplasmic reticulum. The argument corresponds to Tacar *et al.* (2012) reported that doxorubicin stimulates an increase of p53, which blocks the Bcl-2 protein. Moreover, DPE directly decreases the Bcl-2 protein (Duo *et al.*, 2012; Mekahli *et al.*, 2011). Furthermore, DPE has been shown anti-estrogenic effects on breast cancer cell lines (MCF-7) (Yigitaslan *et al.*, 2016), inhibition of estrogen receptor by DPE will reduce Orai3 expression that causes decreasing calcium influx (Faouzi *et al.*, 2010). Here are some of the *mechanisms* suggested to explore in the future research.

In this experiment, the number of apoptotic cells in MCF-7 cell lines was further assessed by flow cytometry. Our results demonstrated that the combination treatment of doxorubicin with or without DPE was able to induce apoptosis. This data prove that the combination of doxorubicin and DPE decreased the survival rate of MCF-7 breast cancer cells. Our results supported by Sharifi *et al.* (2015) and Pilco-ferreto & Calaf. (2016) which showed that doxorubicin induces apoptosis in MCF-7 and MDA-MB-231 Pilco-ferreto & Calaf. (2016) cell lines, DPE was able to increase apoptosis in a human chronic myeloid leukemia cell line (K562) (Zamani *et al.*, 2016).

According to our results, the combination of DPE and doxorubicin directly involve in the decreasing of survivin levels, we supposed that doxorubicin activates p53 lead to suppressing on survivin levels. Whereas DPE decrease surviving level in MCF-7 cells line. Our results are in line with previous report indicating that doxorubicin inhibits topoisomerase 2 enzymes, causing DNA damage (Deng *et al.*, 2013; Thorn *et al.*, 2011) and increase the activation of p53-induced apoptosis pathway (Farfariello *et al.*, 2015) lead to suppress on the survivin level (Tyner *et al.*, 2012; Garg *et al.*, 2016).

In addition, the mechanism of action of. The disruption of calcium homeostasis may induce apoptosis (Harr *et al.*, 2010) while survivin acts as an apoptotic inhibitor (Deng *et al.*, 2013). These findings showed a decrease in intracellular calcium concentration and survivin levels while apoptosis rose. This study suggests that the apoptotic effects of co-treatment of doxorubicin and DPE in MCF-7 cells involve intracellular calcium and survivin.

CONCLUSIONS

Our results demonstrate that DPE effectively promotes the anti-cancer effects of doxorubicin in MCF-7 cells. The apoptotic effects of the combination of doxorubicin and DPE is mediated by reduction of intracellular calcium and survivin levels. Our results indicate that the combination of doxorubicin and DPE may be

beneficial as a potential anti-cancer drug in the future.

ACKNOWLEDGMENTS

We would like to thank the Faculty of Medicine, Brawijaya University, Indonesia for the provided grant for this research. We gratefully acknowledge Heni Endrawati, SSi, Wahyudha Ngatiril Lady, SSi, Ami Maghfiroh, SSi and Bunga Prihardina, SSi for technical assistance

CONFLICTS OF INTEREST

There are no conflicts of interest.

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How to cite this article:
Endharti AT, Wahyuningtyas TE, Hardini, Handono K, Widjajanto E, Permana S. *Dendrophthoe pentandra* Leaves Extract Promotes Apoptotic Effects of Doxorubicin in Human Breast Cancer Cell via Modulation of Intracellular Calcium and Survivin. J App Pharm Sci, 2018; 8(08): 039-043.