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ORIGINAL ARTICLE

Herb Mixture C5E Aggravates Doxorubicin-induced Apoptosis of Human Breast Cancer Cell Lines

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Abstract A number of extracts from Asian traditional medicinal herbs have been successfully used as therapeutic agents against cancers. In this study we assessed the effect of C5E on the proliferation inhibition and apoptosis of breast cancer cell lines. C5E is an ethanol extract from traditional Asian medicinal plants which have anticancer activity. Nonetheless, little is known about the underlying mechanism. Thus, we studied the mechanism of C5E-induced cell death in the human breast cancer cell line MDA-MB-231 and MCF7 cells. The cell survival rate was reduced in a concentration- and time-dependent manner, as assessed by direct cell counting. After incubation for 48 h, typical apoptotic morphological changes were observed by microscope. To determine the synergetic effect with doxorubicin, we co-treated C5E with doxorubicin in breast cancer cells, and flow cytometry revealed that co-treatment obviously enhanced sub-G1 arrest and apoptosis in MDA-MB-231 and MCF7 cells. Furthermore, we showed that pro-apoptotic marker cleaved PARP was synergistically increased with the combined treatment of doxorubicin and C5E in MDA-MB-231, but not in MCF-7. These results suggest that the effect of combined treatment of C5E with doxorubicin on sub-G1 arrest and apoptosis in breast cancer cells could be exerted by the different mechanism and its potential use as a therapeutic agent will be helpful in treatment for breast cancer.

Keywords angelicin · apoptosis · breast cancer · traditional/medicinal herbs

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Introduction

Breast cancer is one of the most common cancers in the world. Although the treatments of breast cancer have been improved, the incidence of breast cancer is still increasing (Kanavos, 2006, Parkin et al., 2009, Kojima and Barron, 2010). About 80% of breast cancer patients have breast tissues removed and adjuvant therapy performed after surgery. Although chemotherapy is successful, tumors can recur within 5 years after therapy. Furthermore, there are many dangerous points including metastasis after surgery. On the other hand, conventional cancer therapy often leads to adverse side effects by damaging normal, healthy cells near the cancer. To overcome this adverse effects of chemotherapy, the extracts from medicinal herbs can be used to give additive or synergistic effects to kill tumors (Cai et al., 2004) and the combined treatment of herbal extracts could lower the required concentration of anticancer drugs, compared to the treatment of anticancer drugs alone.

The extracts from herbs may contain flavonoids, glycosides, polyphenols, and other unidentified secondary metabolites. These extracts exhibit a variety of activities, including antiallergic, antioxidative, and anticancer effects, due to the presence of the various components (Bak et al., 2013). These extracts usually exert their effect by acting on tumor cells directly, or by enhancing immune cell function indirectly. They also show fewer side effects than anticancer drugs. Because of these advantages, including a synergistic action with anticancer drugs and a reduction in the

required concentration of anticancer drug, the antitumor activity of extracts has been studied (Shoemaker et al., 2005, Chu et al., 2009).

Doxorubicin is considered to be the most effective drug for the treatment of breast cancer patients. However this drug also induces chemoresistance like other drugs. The mechanism by which doxorubicin resistance occurs has been studied (Kopp et al., 2012) and the studies have shown that genes involved in a variety of pathways were enhanced. To overcome this resistance, two or more anticancer drugs, cisplatin and taxol, were combined with doxorubicin. In this study we show the anticancer effect of the combined treatment of doxorubicin and C5E on the proliferation inhibition and apoptosis of breast cancer cell lines. This combined treatment contributes a reduction of the required doxorubicin treatment concentration.

Table 1 The composition of C5E

Oriental Name	Country of origin	Grams (dried materials)	%
Ginseng	Korea	166	16.6
Chaga	Russia	166	16.6
Pinellia tuber	China	111	11.1
Sparganium rhizome	China	56	5.6
Alpinia rhizome	China	56	5.6
Cinnamon bark	Vietnam	56	5.6
Astragalus root	Korea	56	5.6
Psoraleae semen	Indida	111	11.1
Evodia fruit	China	111	11.1
Meliae Fructus	China	111	11.1
Total amount		1000	100

Materials and Methods

Cell culture. MDA-MB-231 and MCF-7 human breast cancer cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, USA) supplemented with 10% fetal bovine serum (Gibco BRL, USA). All cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere, unless otherwise specified. Adherent cultures were grown on tissue culture dishes (Falcon, USA). Cells were visualized using an inverted microscope equipped with a DP50 camera system (Olympus, Japan). Cells were harvested and treated with diluted trypsin- ethylenediamine-tetraacetic acid (EDTA) (Gibco BRL, USA) to obtain a single-cell suspension for re-plating or trypan dye exclusion assays.

Oriental medicinal herbs and formulation of C5E. C5E is composed of 10 oriental medicinal herbs (Table 1). The herbal ingredients were obtained from the Oriental Medical Hospital, Dongguk University (Korea) and were kindly authenticated by Dr. Seonghyun Jung (College of Oriental Medicine, Dongguk University). Ethanol extracts from the above listed plants were prepared as follows. The dried and pulverized medicinal herbs were mixed together, and 1 kg batches were soaked in 40% ethanol (3 L). The ethanol extract was concentrated using a rotary evaporator, lyophilized, and then reconstituted in distilled water for the *in vitro* studies described below.

Cell Viability Assay. Cell proliferation was measured by direct cell counting. Briefly, MDA-MB-231 and MCF7 cells were collected and seeded in 12-well plates, each at a density of 1×10^5 cells/well, and were treated with phosphate buffered saline (PBS), as well as various concentrations of herb-mixtures and doxorubicin. After incubation for 24, 48, and 72 h, cells were trypsinized, and viable cells were counted using a hemocytometer after trypan blue staining to remove dead cells.

Immunoblot analysis. MDA-MB-231 and MCF7 cells were lysed with protein lysis buffer [50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% NP-40, and 1 mM phenylmethylsulfonyl fluoride. Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose (Amersham Pharmacia Biotech, USA). Blocking and antibody incubations were performed in 5% low-fat milk in TBS-T [150 mM NaCl, 20 mM Tris (pH 8.0), 0.05% Tween20]; the washing buffer was TBS-T. Blots were developed by ECL (Amersham Pharmacia Biotech) as described previously (Kang et al., 2012).

Propidium iodide staining. After 48 h incubation, cells were xed in 70% ethanol for 1 h, washed with PBS, and treated with 100 mg/mL RNAase A (Sigma, USA) for 1 h at 37°C. Cells were then stained with 25 mg/mL propidium iodide (Sigma), and the samples were analyzed by ow cytometry within 1 h. Flow cytometry was performed using the Flow Cytometry (BD FACS Canto II, USA), and all experiments were performed in triplicate.

Statistical analyses. All experiments were repeated at least three times, and all data were compiled from a minimum of triplicate experiments. The results from treated and untreated control cells were compared using the Student's t-test to assess statistical signicance. A p-value of less than 0.05 was considered statistically signicant.

Results

The chemical content of C5E. C5E is an ethanol extract of complex components including Ginseng, Chaga, Pinellia tuber, Sparganium rhizome, Alpinia rhizome, Cinnamon bark, Astragalus root, Psoraleae semen, Evodia fruit, and Meliae Fructus (Table 1). The components of C5E extract were analyzed using GC-MS (Fig. 1) in an attempt to identify the medicinal components. Compounds were identified by comparison with those in the Wiley 6th edition of MS spectra library. Hits that were >90% probable were viewed as likely hits. Table 2 lists the chemical components of the extracts. Although coumarin is known to have anticancer activity, its amount is only 2.65% of total components. Thus, angelicin in C5E could be the main component to induce apoptosis in addition to coumarin, because angelicin was found to account for 24.54% of total components and is known to have

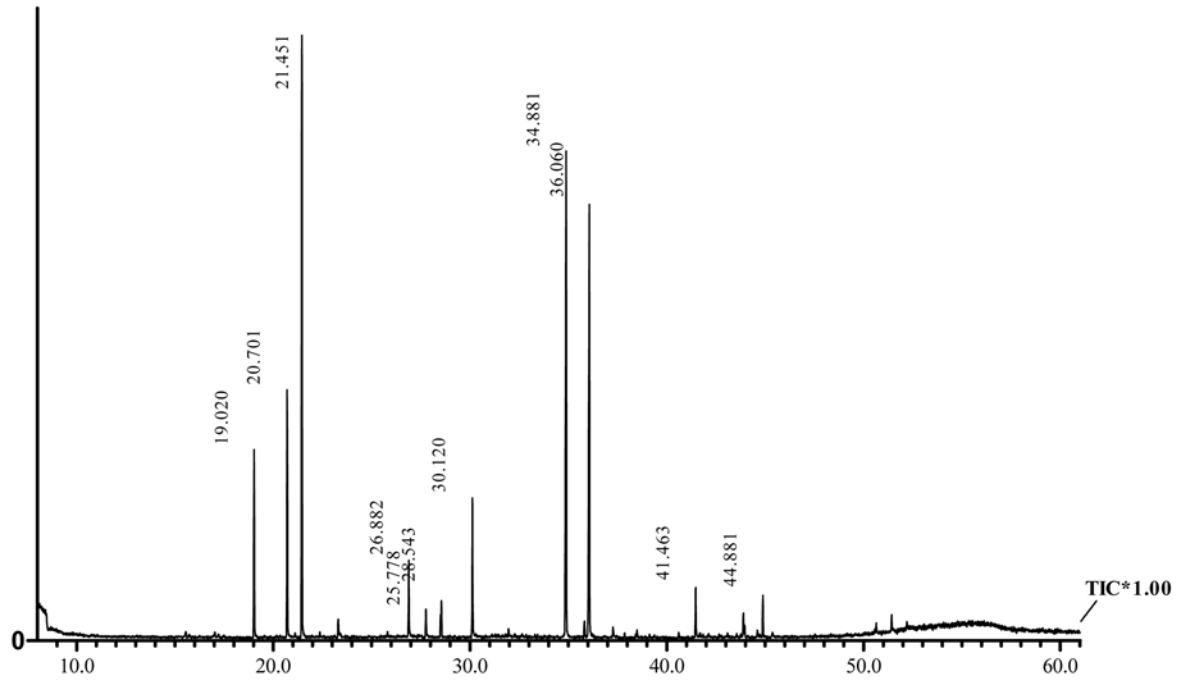

Fig. 1 The composition of C5E was analyzed by GC-MS. Among the volatile compounds in C5E, angelicin is the major component, which has anticancer activity.

Table 2 Volatile compounds identified in C5E

Compounds	Retention time (min)	Relative (%)
3-Phenylpropanal	19.020	7.41
3,4-dimethyl-Benzaldehyde	20.701	9.55
1-Phenyl-3-butanone	21.451	23.53
Coumarin	26.882	2.65
Methyldymron	27.755	1.05
2,4-Di-tert-butylphenol	28.543	0.78
Coniferyl alcohol	30.120	4.93
Angelicin	34.881	24.54
Ficusin	36.060	22.05
unknown	41.463	1.89
Priscoline	44.881	1.61

anticancer activity in previous reports (Rahman et al., 2012); (Shiao Hui-Yi, 2012).

C5E inhibits the proliferation of MDA-MB-231 and MCF-7 cells. To exam whether C5E shows powerful inhibition in breast cancer cell proliferation, the effect of C5E on proliferation of human breast cancer cell lines including estrogen receptor (ER)-positive MDA-MB-231 and ER-negative MCF-7 cells were examined. Both cells were treated with C5E at the indicated concentrations for 48 h. After the treatment of cells with C5E, the relative numbers of viable cells were determined by direct cell counting. C5E inhibited the proliferation of MCF-7 and MDA-MB-231 cells in a dose-dependent manner. The inhibition of proliferation of MCF-7 cell was higher than those of MDA-MB-231 cells (Figs 2A and B). Subsequently, to determine the kinetics of C5E-mediated inhibition of cell proliferation, MDA-MB-231 and MCF-7 cells were treated with C5E at 1 mg/mL. After incubation for the indicated time period, the treated cells were trypsinized, and viable cells were counted with a hemocytometer after trypan blue staining. The inhibition of cell proliferation peaked at 72 h after treatment, and MCF-7 cells were determined to be more susceptible to C5E-induced inhibition compared to MDA-MB-231 cells 48 h after treatment. Furthermore, the morphological changes of cells after treatment were similar to those which occur due to apoptosis (Fig. 2C and D).

Co-treatment of C5E with doxorubicin synergistically inhibits the proliferation of MDA-MB-231 and MCF-7 cells. Doxorubicin is an anticancer drug used in chemotherapy for the treatment of breast cancer. To determine whether C5E is able to enhance doxorubicin-mediated inhibition of cell proliferation, MCF-7 and MDA-MB-231 cells were treated with doxorubicin in the presence of C5E. C5E concentration was fixed at 0.5 mg/mL which had been co-treated with the indicated concentrations of doxorubicin. In MDA-MB-231 cells, the sole doxorubicin treatment of 0.1 μM decreased proliferation by about 60%, and co-treatment with C5E decreased proliferation by about 80%. In MCF-7 cells, the sole doxorubicin treatment of 0.1 μM decreased proliferation by about 50%, whereas co-treatment with C5E decreased proliferation by about 95% (Fig. 3). The co-treatment effect was more dramatic in MCF-7 cells compared to that in MDA-MB-231 cells. These results imply that C5E enhances the effect of the anticancer drug doxorubicin on the inhibition of cell proliferation,

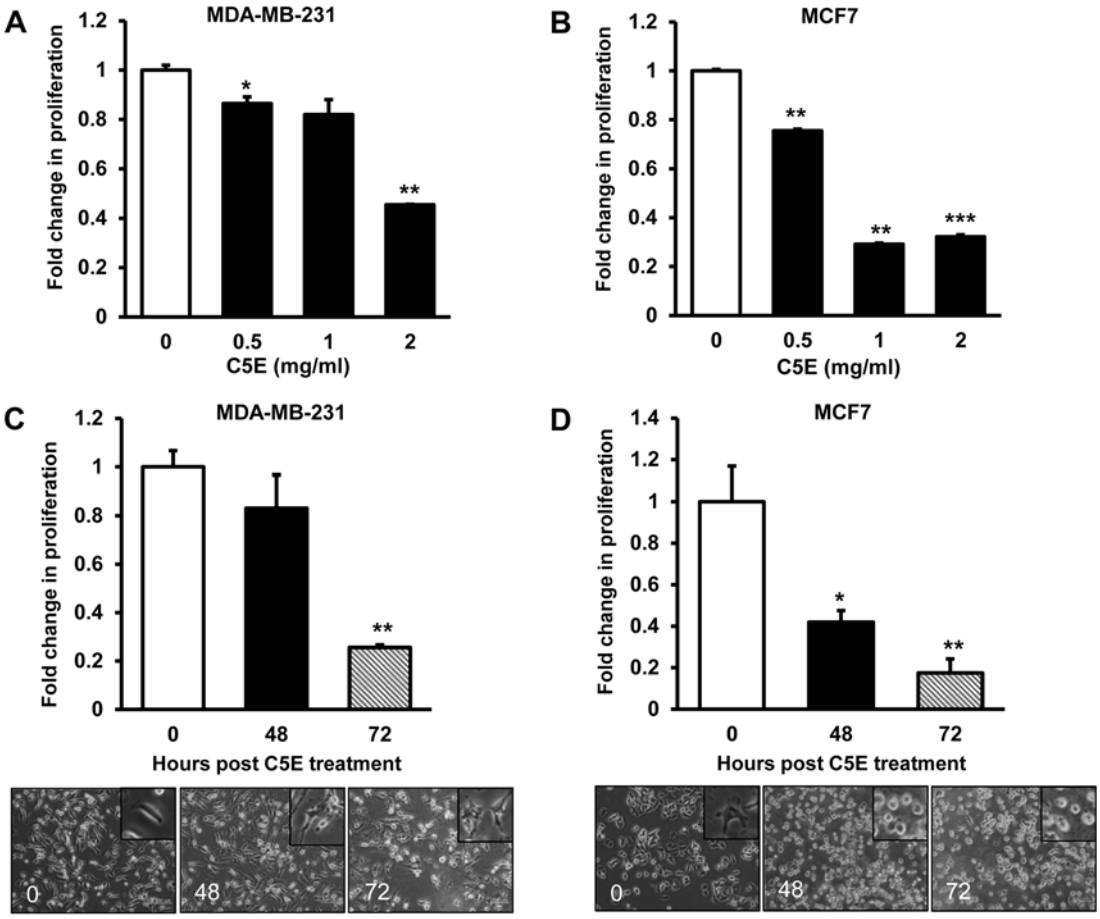

Fig. 2 Effect of C5E on proliferation of breast cancer cell line MCF-7 and MDA-MB-231 cells. (A, B) MCF-7 and MDA-MB-231 cells were treated with the indicated concentration of C5E for 48 h, and proliferation was measured by direct counting. (C, D) MCF-7 and MDA-MB-231 cells were treated with 1 mg/mL of C5E for 48 and 72 h, and proliferation was measured by direct counting. The representative photographs taken in the indicated time are shown in the bottom of C and D. The data were normalized to the control cells and presented as fold change. Data are expressed as the mean ± SD (n = 5). *p <0.05, **p <0.005, ***p <0.001, compared to untreated control.

and that MCF-7 cells are more susceptible than MDA-MB-231 cells to the cell proliferation inhibition by the co-treatment of doxorubicin and C5E.

Co-treatment of C5E with doxorubicin synergistically increases sub-G1 population in MDA-MB-231 and MCF-7 cells. To determine how C5E and doxorubicin exhibit enhanced inhibition of cell proliferation, cell cycle change was examined after co-treatment of doxorubicin and C5E. The two cell lines were co-treated with 0.3 μM doxorubicin and 0.5 mg/mL C5E for 48 h, and were then analyzed by ow cytometry after staining with PI, as described in the Methods section. Intercalation of the propidium iodide to the DNA of cells allows for quantication of the number of cells in a specic cell-cycle phase via uorescence, and the population of each cell cycle phase was also analyzed (G1, S, G2/M, and sub-G1) using the Flow-Jo program. This test was repeated in three separate runs, and the mean value was used. In MDA-MB-231 cells, C5E treatment increased sub-G1 and decreased G2/M populations. Doxorubicin treatment increased sub-G1, S, and G2/M population. Co-treatment of C5E and doxorubicin synergistically increased sub-G1 and S populations (Fig. 4A). In MCF-7 cells, C5E treatment increased sub-G1 and decreased G2/M population. On the other hand, doxorubicin treatment increased both sub-G1 and G2/M populations. The co-treatment of C5E and doxorubicin increased sub-G1 populations (Fig. 4B).

C5E regulates protein expression related to apoptosis. We analyzed sub-G1 population after the co-treatment of C5E and doxorubicin. MDA-MB-231 cells were treated with 0.5 mg/mL of C5E in the absence or presence of 0.3 μM doxorubicin for the indicated time period. The co-treatment of C5E and doxorubicin synergistically enhanced sub-G1 population (Fig. 5A). To explore the potential mechanisms of a sub-G1 population increase by the treatment of C5E and doxorubicin, the expressions of several proteins related to apoptosis were determined in MDA-MB-231 cells. The apoptotic marker, poly ADP ribose polymerase (PARP), and anti-apoptotic marker, inhibitor of apoptosis (c-IAP1), were

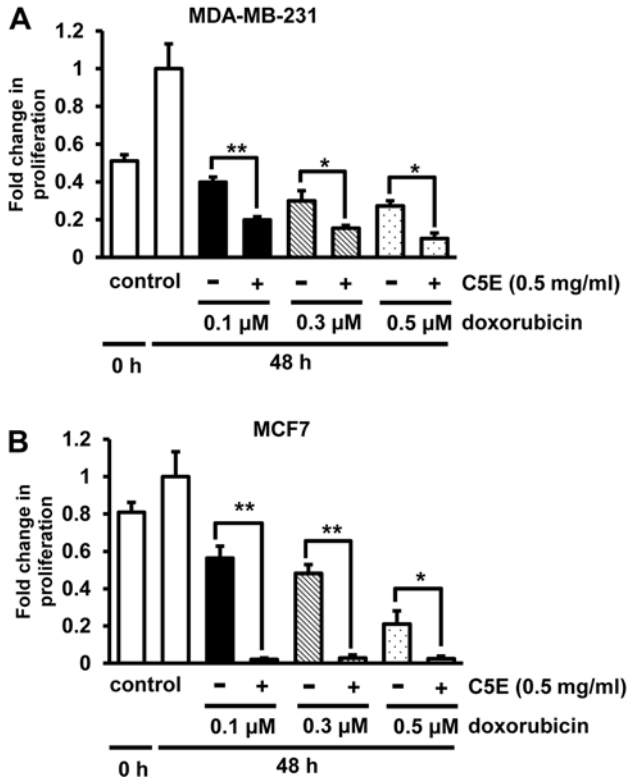

Fig. 3 Effect of combined treatment of C5E with doxorubicin on proliferation of breast cancer cell line MCF-7 and MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with 0.5 mg/mL of C5E in the presence of the indicated concentration of doxorubicin for 48 h, and proliferation was measured by direct counting. (B) MCF-7 cells were treated with 0.5 mg/mL of C5E in the presence of the indicated concentration of doxorubicin for 48 h, and proliferation was measured by direct counting. The data were normalized to the control cells and presented as fold change. Data are expressed as the mean ± SD (n = 5). *p <0.05, **p <0.01, ***p <0.005, compared to untreated control.

examined under the same experimental conditions (Fig. 5A). PARP cleavage was synergistically enhanced 48 h after treatment, whereas the level of c-IAP1 was not significantly changed (Fig. 5B). This finding indicates that C5E and doxorubicin increases apoptosis without increasing anti-apoptotic protein.

Next, we analyzed the effect of C5E and doxorubicin on apoptosis of MCF-7 cells. MCF-7 cells were treated with 0.5 mg/mL of C5E in the absence or presence of 0.3 μM doxorubicin for the indicated time period. The co-treatment of C5E and doxorubicin synergistically enhanced the sub-G1 population, whereas the sub-G1 population was reduced 48 h after the co-treatment (Fig. 5C). PARP cleavage was enhanced 48 h after the treatment, and the level of c-IAP1 was significantly decreased (Fig. 5D). However there was no synergistic increase of apoptotic marker or decrease of anti-apoptotic marker.

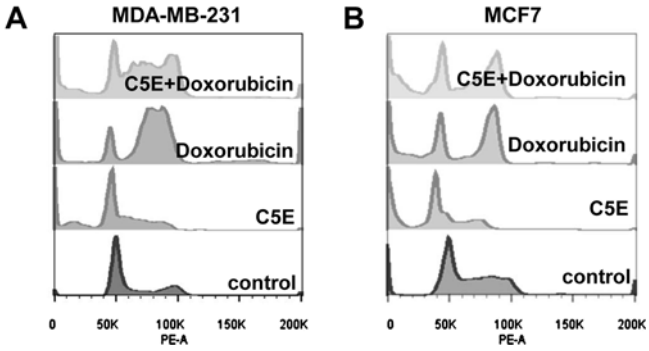

Fig. 4 Effect of combined treatment of C5E with doxorubicin on cell cycle distribution. (A) MDA-MB-231 cells were treated with 0.5 mg/mL of C5E in the presence of 0.3 ìM concentration of doxorubicin for 48 h, followed by cell cycle analysis via flow cytometry. (B) MCF-7 cells were treated with 0.5 mg/mL of C5E in the presence of 0.3 μM of doxorubicin for 48 h, followed by cell cycle analysis via flow cytometry.

Discussion

Traditional medicinal herb extracts, which have been used in Asian countries, have a lower cytotoxic effect on human than synthetic anticancer drugs used for chemotherapy. C5E is an ethanol extract of Ginseng, chaga, Pinellia Tuber, Sparganium rhizome, Alpinia rhizome, Cinnamon bark, Astragalus root, Psoraleae semen, Evodia fruit, and Meliae Fructus. Although there are several reports on the anticancer effects of each material (Yang et al., 2008, Kwon et al., 2010, Ji et al., 2012, Bak et al., 2013), the effect of this mixture C5E was for the first time analyzed in the present study. In addition to the anticancer effect of C5E itself, we also designed our study to determine whether combinational therapy of C5E with the anticancer drug doxorubicin is synergistically effective in killing breast cancer cells, because traditional herbal therapies have been known to have helpful effect on eradication of tumor cells.

Clinical use of doxorubicin, one of the anthracyclines, is limited by its serious adverse effect on cardiomyocytes. To solve this problem, several herbal extracts have been developed (Alkreathy et al., 2012); (Zhao et al., 2013). For example, Tanshinone IIA prevents doxorubicin-induced cardiomyocyte apoptosis through the inhibition of reactive oxygen species generation (Hong et al., 2012). In the present study, we developed a different approach to overcome the adverse effects of doxorubicin. We examined whether C5E could be used with a reduced concentration of doxorubicin, to achieve the same effect as a higher concentration of doxorubicin as a sole treatment. The effect of a reduced concentration of doxorubicin on inhibition of proliferation and apoptosis was enhanced by co-treatment of C5E. These findings support the notion that substantially lower doses of doxorubicin could be used with C5E, without compromising the anti-cancer

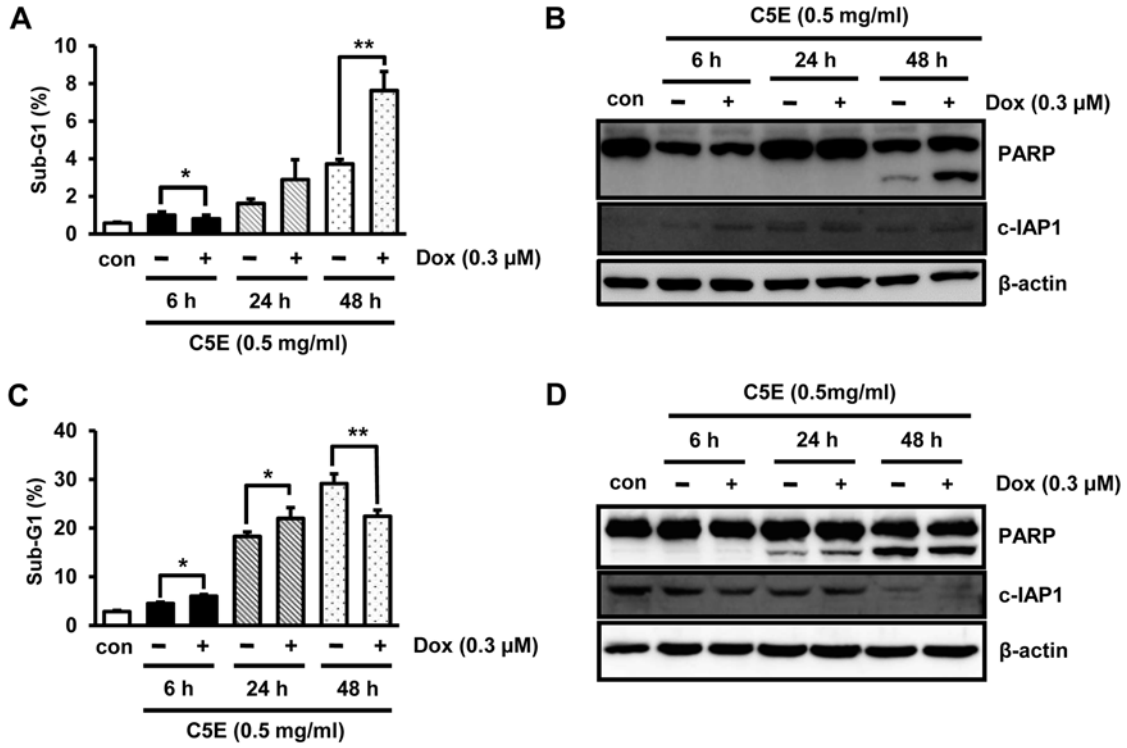

Fig. 5 Effect of combined treatment of C5E with doxorubicin on apoptosis of breast cancer cell lines. (A) MDA-MB-231 cells were treated with 0.5 mg/mL of C5E in the presence of 0.3 μM of doxorubicin for 48 h, followed by cell cycle analysis via flow cytometry. The percent of sub-G1 population was graphed. Quantitation of cell cycle distribution was performed using FACSDIVA software. The data are presented as mean ± standard deviation of three experiments (*p <0.05, **p <0.01). (B) MDA-MB-231 cells were treated with 0.5 mg/mL of C5E in the presence of 0.3 ìM concentration of doxorubicin for 6, 24, and 48 h, and cell lysates were immunoblotted with anti-PARP and anti-c-IAP1 antibodies. (C) MCF-7 cells were treated with 0.5 mg/mL of C5E in the presence of 0.3 μM of doxorubicin for 48 h, followed by cell cycle analysis via flow cytometry. The percent of sub-G1 population was graphed. The data are represented as mean ± standard deviation of three experiments (*p <0.05, **p <0.01). (D) MCF-7 cells were treated with 0.5 mg/mL of C5E in the presence of 0.3 μM concentration of doxorubicin for 6, 24, and 48 h, and the cell lysates were immunoblotted with anti-PARP and anti-c-IAP1 antibodies.

effect, and with lowering detrimental side-effects of doxorubicin. Further study is needed to determine whether C5E has antioxidant effects to prevent doxorubicin-induced side effects.

It is well known that doxorubicin arrests cell cycle at G2/M phase (Koutsilieris et al., 1999). In the present study, doxorubicin arrested MCF-7 cells at G1/S and G2/M phases, whereas MDA-MB-231 cells were arrested at G2/M only. The effect of doxorubicin on G2/M arrest is higher in MDA-MB-231 cells than in MCF-7 cells. This phenomena is partially explained in a previous report (Bar-On et al., 2007). E3 ligase Skp2 is responsible for p27 degradation, and p27 is a cyclin-dependent kinase inhibitor. Thus, it is conceivable that overexpression of Skp2 is associated with aggressive tumor proliferation and poor prognosis in cancer cells. Doxorubicin decreased Skp2 protein levels in MCF-7 cells, but had the opposite effect in MDA-MB-231 cells. In another report, the combined treatment of PI3K inhibitor and doxorubicin enhances G2 with the appearance of a sub-G1 peak, indicating apoptosis/necrosis in MDA-MB-231 cells harboring the p53 mutant, but not in MCF-7 cells harboring wild type p53 (Wang et al., 2008). These data indicate that the effect of doxorubicin and C5E on the cell cycle arrest may be dependent on the status of cells, such as whether they are ER positive or negative, p53 mutant or wild type, among others. Thus, the complete understanding of cell status affecting C5E and doxorubicin-induced cell cycle arrest is necessary for successful tumor therapy.

Volatile sources of C5E contain several complexes such as angelicin, which is known as an apoptotic-inducing chemical, and is structurally related to psoralens and a well-known chemical class of photosensitizers used for its antiproliferative activity in the treatment of different skin diseases. It was also reported that angelicin is an effective apoptosis-inducing natural compound of human neuroblastoma cancer, and analogs of angelicin have been shown to have potent anti-influenza and anti-cancer activities (Rahman et al., 2012); (Shiao Hui-Yi, 2012). Thus, angelicin is likely responsible for anticancer activity and enhanced apoptosis in breast cancer cell lines.

In summary, C5E inhibits breast cancer cell proliferation and increase the sub-G1 population. This effect of C5E on inhibition of breast cancer cell eventually leads to apoptosis. The co-treatment of C5E with doxorubicin enhances doxorubicin-

mediated cell cycle arrest and apoptosis. This finding indicates that C5E can be used with doxorubicin to cure breast cancer. However, it remains to be determined which molecules of C5E show anticancer effects and enhance doxorubicin-induced apoptosis.

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