



# Treatment with Phytoestrogens Reversed Triclosan and Bisphenol A-Induced Anti-Apoptosis in Breast Cancer Cells

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## Abstract

Triclosan (TCS) and bisphenol A (BPA) are endocrine-disrupting chemicals that interfere with the hormone or endocrine system and may cause cancer. Kaempferol (Kaem) and 3,3'-diindolylmethane (DIM) are phytoestrogens that play chemopreventive roles in the inhibition of carcinogenesis and cancer progression. In this study, the influence of TCS, BPA, Kaem, and DIM on proliferation and apoptotic abilities of VM7Luc4E2 breast cancer cells were examined. MTT assay revealed that TCS (0.1–10  $\mu\text{M}$ ), BPA (0.1–10  $\mu\text{M}$ ) and E2 (0.01–0.0001  $\mu\text{M}$ ) induced significant cell proliferation of VM7Luc4E2 cells, which was restored to the control (0.1% DMSO) by co-treatment with Kaem (30  $\mu\text{M}$ ) or DIM (15  $\mu\text{M}$ ). Reactive oxygen species (ROS) production assays showed that TCS and BPA inhibited ROS production of VM7Luc4E2 cells similar to E2, but that co-treatment with Kaem or DIM on VM7Luc4E2 cells induced increased ROS production. Based on these results, the effects of TCS, BPA, Kaem, and DIM on protein expression of apoptosis and ROS production-related markers such as Bax and Bcl-xl, as well as endoplasmic reticulum (ER) stress-related markers such as eIF2 $\alpha$  and CHOP were investigated by Western blot assay. The results revealed that TCS, and BPA induced anti-apoptosis by reducing ROS production and ER stress. However, Kaem and DIM effectively inhibited TCS and BPA-induced anti-apoptotic processes in VM7Luc4E2 cells. Overall, TCS and BPA were revealed to be distinct xenoestrogens that enhanced proliferation and anti-apoptosis, while Kaem and DIM were identified as natural chemopreventive compounds that effectively inhibited breast cancer cell proliferation and increased anti-apoptosis induced by TCS and BPA.

**Key Words:** Kaempferol, 3,3'-diindolylmethane, Triclosan, Bisphenol A, Apoptosis, Reactive oxygen species

## INTRODUCTION

High levels of endogenous estrogen are considered a breast cancer risk factor in women (Missmer *et al.*, 2004; Santen *et al.*, 2007). Estrogen can induce overgrowth of cancer cells by stimulating cell proliferation and disrupting normal cellular processes such as apoptosis and DNA repair via estrogen receptor (ER)-mediated signaling (Lewis-Wambi and Jordan, 2009). In ER-positive breast cancer cells, 17 $\beta$ -estradiol (E2) increased cell growth by promoting cell cycle progression and induced anti-apoptosis by upregulating Bcl-2, an important anti-apoptotic protein (Sakamoto *et al.*, 2010).

Endocrine disrupting chemicals (EDCs) that have similar structures to E2 may exert estrogenic functions and interfere with estrogen signaling, as well as cause cancer in estrogen-responsive organs (Park *et al.*, 2011; Shanle and Xu, 2011;

Kim *et al.*, 2015a). Triclosan (TCS) is a broad-spectrum antibacterial compound commonly used in numerous consumer products (Darbre, 2006) that has been recently identified as a potential EDC (Feng *et al.*, 2016). Previous studies have shown that TCS may play a role in cancer development through its estrogenicity (Dinwiddie *et al.*, 2014), and that it inhibited death of breast cancer cells via regulation of cell cycle and apoptosis-related genes (Kim *et al.*, 2016). Bisphenol A (BPA), which is a typical EDC, is a basic raw material for manufacturing polycarbonate plastics and epoxy resins that is commonly included in plastic, storage containers, and disposable products (Chen *et al.*, 2002; Jeong *et al.*, 2017). Bisphenol A is known to have cancer-related toxicity that leads to cell proliferation effects in estrogen-dependent breast and ovarian cancer cells (Lee *et al.*, 2012, 2014) as well as to have significant effects on cancer development by increasing anti-

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apoptosis (Elswefy *et al.*, 2016; Yin *et al.*, 2017).

Plant-derived natural compounds have been shown to exert significant chemopreventive effects against cancers (Russo *et al.*, 2016). Phytoestrogens are estrogenic chemical compounds derived from plants that have been shown to exert biological effects that prevent tumor development (Coelingh Bennink, 2004; Kim *et al.*, 2014). Phytoestrogens have ER antagonistic actions in the classical ER signaling pathway in breast cancer cells and would be used as selective estrogen receptor modulators (SERMs) (Ahn *et al.*, 2014). Moreover, some experimental studies have shown that phytoestrogens have anti-breast cancer activity (Hilakivi-Clarke *et al.*, 1999; Kang *et al.*, 2013; Hwang and Choi, 2015; Kim *et al.*, 2016; Lee *et al.*, 2016, 2017). As effective anti-cancer agents, phytoestrogens have been studied for their ability to suppress cancer progression by controlling cell proliferation, the cell cycle, and apoptosis (Fang *et al.*, 2016). Phytoestrogens including polyphenols and flavonoids derived from plants such as soybeans and teas have been shown to have antioxidant activities (Santos *et al.*, 2016). Kaempferol (Kaem) is a flavonoid agent found in fruits, teas, and ginkgo leaves that exerts antitumor and anti-oxidation effects in various cancer cells (Liao *et al.*, 2016). Kaem was shown to suppress cell growth by inducing reactive oxygen species (ROS) production, endoplasmic reticulum (ER) stress, and apoptosis (Tatsimo *et al.*, 2012; Lee and Kim, 2016; Liao *et al.*, 2016). Indole-3-carbinol (I3C) is rich in Brassica vegetables such as cauliflower, turnips, kale, and broccoli and may exert protective functions against many types of cancer (Verhoeven *et al.*, 1996). I3C is a precursor of 3,3'-diindolylmethane (DIM) that is converted into DIM via dehydration of acid-catalyzed reactions at low pH (Lee *et al.*, 2016). DIM induces alterations in mitochondrial depolarization and hyperpolarization, which are the early stages of apoptosis (Gong *et al.*, 2006).

Mitochondria-mediated apoptosis as an intrinsic apoptotic pathway is associated with cell death processes and tissue homeostasis (Shi, 2001; Stachon *et al.*, 2015). When cells are stressed and the permeability of mitochondrial membranes is increased, mitochondrial proteins such as second mitochondria-derived activator of caspases (SMACs) are released into the cytosol, where they bind to proteins that inhibit apoptosis, thereby deactivating them and allowing apoptosis to proceed (Fesik and Shi, 2001). Currently, ER stress caused by exposure to reactive oxygen species (ROS) is considered one of the stresses that triggers apoptosis (Kitamura and Hiramatsu, 2010). The ER is an organelle that plays an essential role in the folding of protein molecules and the transport of synthesized proteins to the Golgi apparatus (Soltys *et al.*, 1996). Production of ROS causes oxidative injury, which influences ER homeostasis and induces inappropriate protein folding in the ER (Yang *et al.*, 2016). Under severe oxidative stress, accumulation of unfolded or misfolded proteins, namely the unfolded protein response (UPR), leads to ER stress. This further triggers the pro-apoptotic pathway via activation of transcription factor 6 (ATF6)-CCAAT/enhancer-binding protein-homologous protein (CHOP) (Kitamura and Hiramatsu, 2010). Therefore, CHOP is a major gene associated with ER stress-induced apoptosis (Galehdar *et al.*, 2010).

In the present study, the roles associated with apoptosis of two EDCs, TCS and BPA, as well as two phytoestrogens, Kaem and DIM, in VM7Luc4E2 breast cancer cells, which are estrogen-responsive ER positive cells, were investigated by

examining their effects on the interactions between ROS-induced ER stress and apoptosis. The results of this study will improve understanding of the opposite roles of the EDCs and phytoestrogens in regulation of breast cancer progression.

## MATERIALS AND METHODS

### Reagents and chemicals

17 $\beta$ -estradiol (E2), Irgasan (TCS), bisphenol A (BPA), 3,3'-diindolylmethane (DIM) and kaempferol (Kaem) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). All chemicals were dissolved in 100% dimethyl sulfoxide (DMSO; Junsei Chemical Co., Tokyo, Japan). The culture media used for cell treatment contained a final concentration of DMSO of 0.1%.

### Cell culture and media

VM7Luc4E2 human breast carcinoma cells (VM7Luc4E2 cells), which were recently identified as variant MCF-7 human breast cancer cells (Li *et al.*, 2014), were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories, Inc., Logan, UT, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT, USA), 200 U/ml penicillin G, and 200 mg/ml streptomycin (A&E Scientific, Logan, UT, USA) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> containing air. To prevent the estrogenic effects of some components in DMEM and FBS, phenol red-free DMEM (Sigma Aldrich) supplemented with 5% charcoal-dextran treated FBS was used for detection of the diverse effects of reagents and chemicals on VM7Luc4E2 cells. The cells were detached with 0.05% Trypsin-EDTA (PAA Laboratories, Dartmouth, MA, USA).

### Cell viability assays

VM7Luc4E2 cells were seeded at a density of 3×10<sup>3</sup> cells per well in 96-well plates. After 2-days of incubation, the culture medium was replaced with new medium containing E2 (0.01, 0.001, 0.0001  $\mu$ M), TCS (0.1, 1, 10  $\mu$ M), BPA (0.1, 1, 10  $\mu$ M), and each combination of E2 (0.001  $\mu$ M), TCS (1  $\mu$ M) or BPA (1  $\mu$ M) and Kaem (10, 20, 30, 40  $\mu$ M) or DIM (5, 10, 15, 20  $\mu$ M). The cells were then incubated for an additional 8 days, during which time the medium was changed every three days. The culture medium was then removed, after which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Aldrich) solution was added for 3 h. Following removal of the culture medium, 200  $\mu$ l DMSO (Junsei Chemical Co., Tokyo, Japan) was added per well and the absorbance was measured at 540 nm using an ELISA reader (Epoch, BioTek, VT, USA).

### Western blot assay

After treatment of the VM7Luc4E2 cells with E2 (0.001  $\mu$ M), TCS (1  $\mu$ M), BPA (1  $\mu$ M), Kaem (30  $\mu$ M), DIM (15  $\mu$ M), a combination of E2 (0.001  $\mu$ M), TCS (1  $\mu$ M) or BPA (1  $\mu$ M) and Kaem (30  $\mu$ M), and a combination of E2 (0.001  $\mu$ M), TCS (1  $\mu$ M) or BPA (1  $\mu$ M) and DIM (15  $\mu$ M), the proteins from VM7Luc4E2 cells were harvested with RIPA lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, and 0.1% SDS). Bicinchoninic acid (BCA;

Sigma-Aldrich Corp.) was used for determining protein concentrations. Total cell proteins (50 µg) were separated on 10% SDS-PAGE gel, then transferred to a polyvinylidene fluoride (PVDF) membrane (BioRad Laboratories Inc., Hercules, CA, USA). The membrane was subsequently blocked with 5% skim milk (BioRad Laboratories Inc.) blocking buffer to inhibit the nonspecific interaction with primary antibody for 2 h, then washed four times in 1× TBS buffer (adjusted to pH 7.6 with HCl) containing 0.1% (v/v) Tween 20 (BioRad Laboratories Inc.). Next, the membrane was incubated with mouse monoclonal antibody specific for Bax (1:1000 dilution, Santa Cruz Biotechnology, Dallas, TX, USA), Bcl-xl (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA), CHOP (1:1000 dilution, Cell Signaling Technology), p-eIF2α (1:1000 dilution, Cell Signaling Technology) or GAPDH (1:12000 dilution, Abcam) in 1× TBS with 3% (w/v) BSA (Sigma-Aldrich Corp.) and 0.1% (v/v) Tween 20 overnight at 4°C. The membranes were subsequently probed with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or anti-mouse IgG (1:2000, Thermo Scientific Corp., Rockford, IL, USA) for 2 h at room temperature. Target proteins were detected with a West-Q Chemiluminescent Substrate Plus kit (GenDEPOT, Barker, TX, USA). Quantification of each protein was accomplished by scanning the densities of bands on a transfer membrane using Lumino graph II (ATTO, Amherst, NY, USA).

#### Dichloro-dihydro-fluorescein diacetate (H2DCF-DA) assay

Intracellular ROS generation in VM7Luc4E2 cells was measured by 20,70-dichlorofluorescein diacetate (DCF-DA) assay. VM7Luc4E2 cells were seeded at a density of  $1 \times 10^5$  cells per well in a 6 well plate with 200 µl phenol-free DMEM media containing 5% CD-FBS. After 24 h, the medium was replaced with new medium containing E2 (0.001 µM), TCS (1 µM), BPA (1 µM), Kaem (30 µM), DIM (15 µM), a combination of E2 (0.001 µM), TCS (1 µM) or BPA (1 µM) and Kaem (30 µM), and a combination of E2 (0.001 µM), TCS (1 µM) or BPA (1 µM) and DIM (15 µM), after which cells were incubated for an additional 72 h. Moreover, H<sub>2</sub>O<sub>2</sub> was used as a positive control to detect ROS formation in VM7Luc4E2 cells. Briefly, 3% H<sub>2</sub>O<sub>2</sub> solution was added to the wells with  $1 \times 10^5$  cells of VM7Luc4E2 cells at 1% concentration and incubated for 30 min. Following incubation, the culture medium was removed, and each well was treated with 200 µl H2DCF-DA solution (10 mM in PBS) for 30 min. This plate was then placed in a dark room at room temperature for 30 min, after which the fluorescence intensity of DCF (an oxidized form of H2DCF) was measured and samples were photographed using a fluorescence microscope (IX-73 Inverted Microscopy, Olympus, Japan) to detect ROS production. The ROS production induced by each treatment was quantified using the Cell Sens Dimension software (Olympus).

#### Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling assay

A terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay was processed to detect the fluorescence of apoptotic cells using TdT enzyme with the fluorometric TUNEL assay kit (Promega, Madison, WI, USA). Briefly, VM7Luc4E2 cells were seeded at  $1 \times 10^5$  cells per well in a 6 well plate with DMEM media. After 24 h, the medium was replaced with new medium containing E2 (0.001 µM), TCS (1 µM), BPA (1 µM), Kaem (30 µM), DIM (15

µM), a combination of E2 (0.001 µM), TCS (1 µM) or BPA (1 µM) and Kaem (30 µM), and a combination of E2 (0.001 µM), TCS (1 µM) or BPA (1 µM) and DIM (15 µM). After 48 h, cells were fixed with 4% methanol-free formaldehyde (pH 7.4) for 1 h, permeabilized using lysis buffer (1% Triton X-100 in 1% sodium citrate) for 15 min, and then treated with 50 µL TdT enzyme buffer, which attached to DNA strand breaks. Finally, labeled strand breaks were visualized by the attachment of fluorescein isothiocyanate-5-dUTP. All slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen Life Technologies, Carlsbad, CA, USA), then viewed using a fluorescence microscope (IX-73 Inverted Microscopy, Olympus). Fluorescence by each treatment was quantified with the Cell Sens Dimension software (Olympus).

#### Data analysis

All experiments were conducted at least three times to ensure consistent results. All data were analyzed with the Graphpad Prism software (San Diego, CA, USA). *In vitro* data were expressed as the means ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA) followed by *post hoc* Dunnett's multiple comparison test or by Student's *t*-tests for two-pair comparisons. *p*-values <0.05 were considered statistically significant.

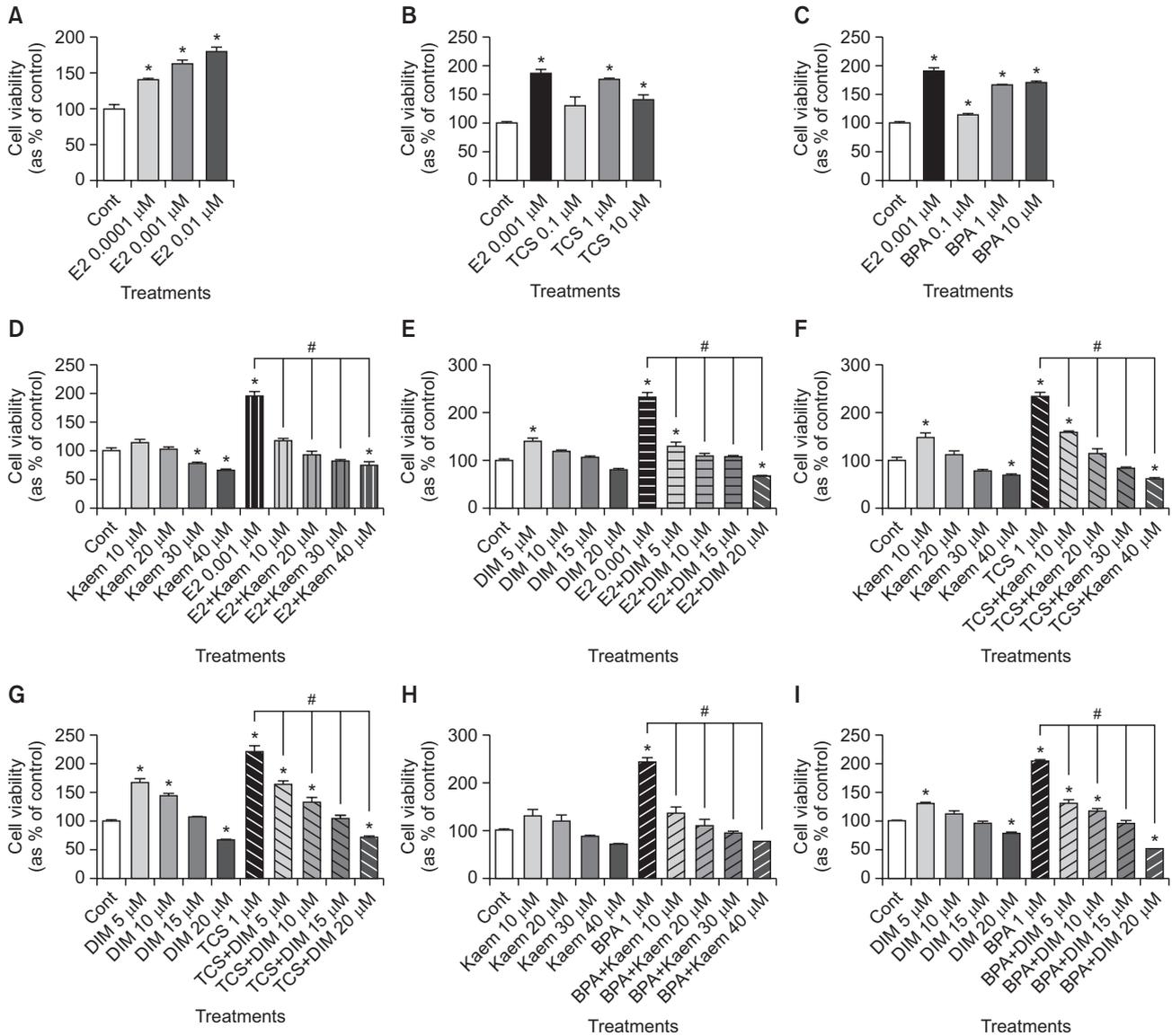
## RESULTS

#### Effect of Kaem and DIM on TCS or BPA induced-VM7Luc4E2 breast cancer cell growth

To evaluate the effects of E2, TCS or BPA on VM7LUC4E2 cell growth, VM7LUC4E2 cells were cultured with E2 (a positive control), TCS or BPA for 8 days. As shown in Fig. 1A-1C, increased VM7LUC4E2 cell viability was observed in response to treatment with E2, TCS or BPA when compared to 0.1% DMSO (a control). The highest increase in cell viability was observed in response to E2 at 0.001 µM in the concentration range tested (Fig. 1A). The cell viability of VM7LUC4E2 cells was the highest at 1 µM TCS, and it was lowered at 10 µM TCS than at 1 µM TCS as shown in Fig. 1B. For BPA, cell viability showed the greatest increase in response to 1 µM BPA in the concentration range of 0.1 µM to 10 µM, and there was no change at two concentrations between 1 µM and 10 µM of BPA (Fig. 1C). These findings indicate that TCS and BPA can induce proliferation of VM7LUC4E2 cells that are ER positive in a fashion similar to that of E2. In addition, the effects of Kaem and DIM on the viability of VM7LUC4E2 cells was identified for the treatment with these compounds alone and in combination with E2, TCS or BPA. When VM7LUC4E2 cells were treated with Kaem or DIM alone, a slight increase in cell viability was observed in response to low doses, while decreased cell viability was observed in response to high doses (Fig. 1D-1I). When cells were co-treated Kaem and DIM and E2, TCS or BPA, E2, TCS or BPA-induced cell proliferation was significantly suppressed in response to all doses (Fig. 1D-1I), indicating that Kaem and DIM exert anti-proliferative activity to abrogate E2, TCS or BPA-induced cell proliferation.

#### Effects of Kaem and DIM on E2, TCS or BPA-induced anti-apoptosis of VM7LUC4E2 breast cancer cells

After VM7LUC4E2 cells were exposed to EDCs and phytoestrogens, apoptotic cells were measured using TUNEL as-

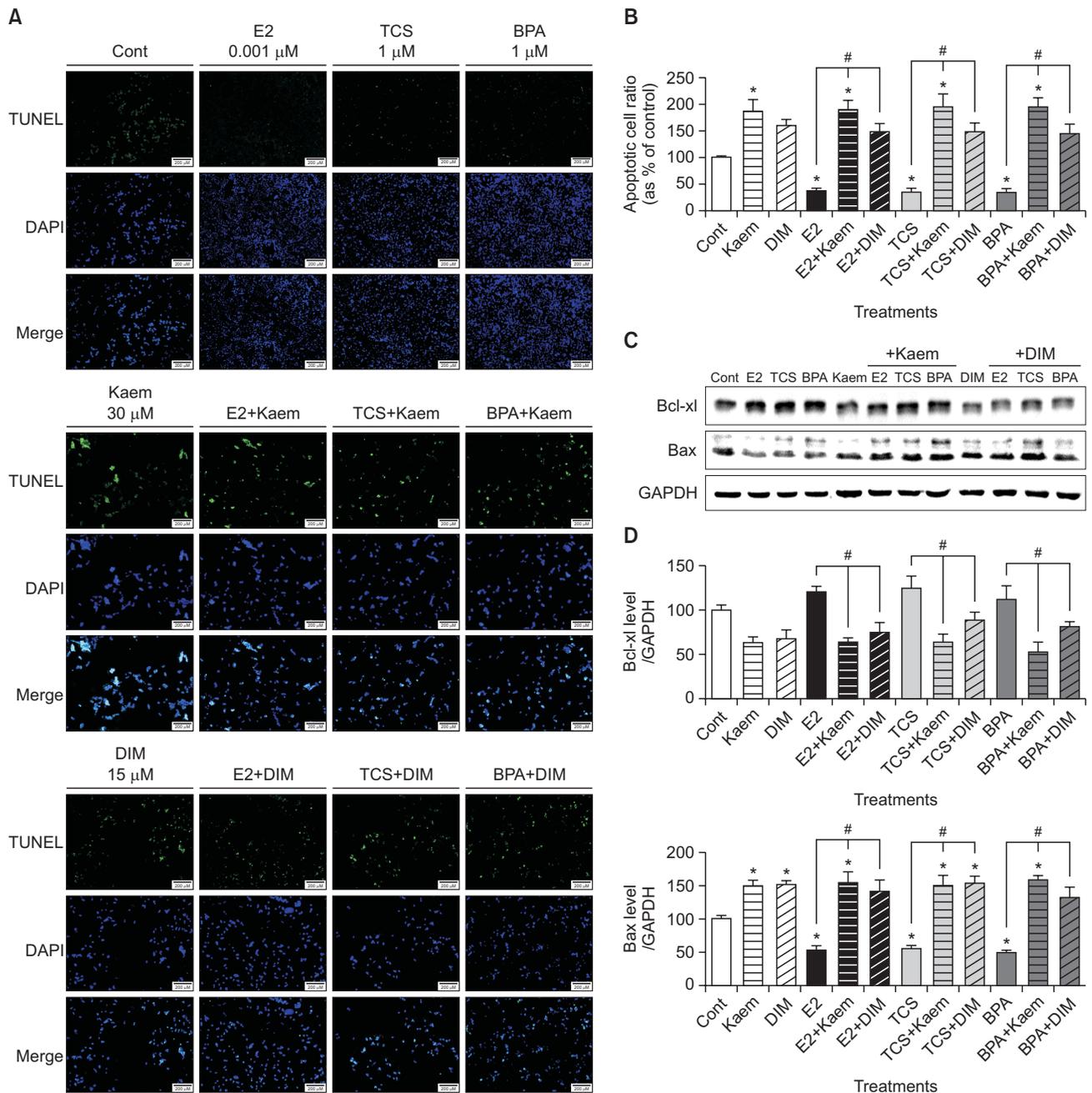


**Fig. 1.** Cell viability of VM7Luc4E2 cells following treatment with E2, TCS, BPA, Kaem, and DIM. VM7Luc4E2 cells were plated at 3,000 cells/well in 200  $\mu$ l of phenol red-free DMEM with 5% CD-FBS medium and then cultured in phenol red-free DMEM with 0.1% DMSO (a control), (A-C) E2 (0.0001-0.01  $\mu$ M), TCS (0.1–10  $\mu$ M) or BPA (0.1–10  $\mu$ M), (D, E) E2 (0.001  $\mu$ M) in the presence or absence of Kaem (10–40  $\mu$ M) or DIM (5–20  $\mu$ M), (F, G) TCS (1  $\mu$ M) in the presence or absence of Kaem (10–40  $\mu$ M) or DIM (5–20  $\mu$ M), and (H, I) BPA (1  $\mu$ M) in the presence or absence of Kaem (10–40  $\mu$ M) or DIM (5–20  $\mu$ M) for 8 days. The cell viability was measured using MTT assay and replicated at least three times to ensure consistent results. Values shown are the means  $\pm$  SD. \*Mean values were significantly different from that of control,  $p < 0.05$  (Dunnett's multiple comparison test). #Mean values were significantly different from that of a single treatment of E2, TCS or BPA,  $p < 0.05$  (Student's *t*-test).

say and fluorescence microscopy. The merged images of E2, TCS or BPA-treated VM7LUC4E2 cells subjected to TUNEL reaction and DAPI staining revealed a decreased number of apoptotic cells compared to the control. TCS and BPA were shown to exert anti-apoptotic effects similar to E2. However, an increased number of apoptotic cells with green fluorescence were detected in cases of co-treatment with Kaem or DIM and E2, TCS or BPA compared to treatment with E2, TCS or BPA alone (Fig. 2).

To investigate the effects of E2, TCS, BPA, Kaem and DIM on protein expression of apoptosis-related markers, a Western blot assay was conducted to measure the proteins extracted

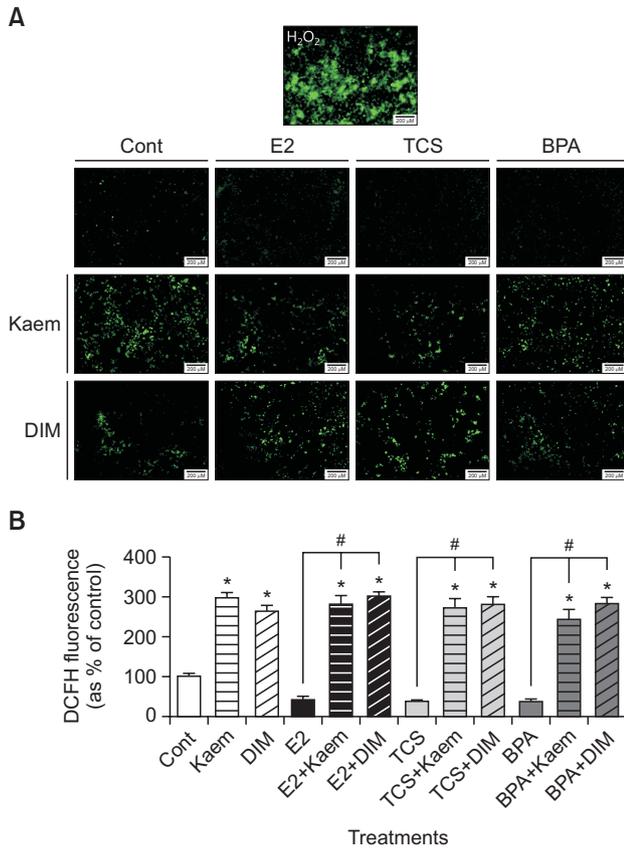
from VM7LUC4E2 breast cancer cells treated with E2 (0.001  $\mu$ M), TCS (1  $\mu$ M), BPA (1  $\mu$ M), Kaem (30  $\mu$ M) or DIM (15  $\mu$ M) for 72 h. The protein expression of Bcl-xl, an anti-apoptotic protein, was decreased by Kaem or DIM compared to the control, while co-treatment of Kaem or DIM and E2, TCS or BPA significantly decreased Bcl-xl expression compared to treatment with E2, TCS or BPA alone (Fig. 2C, 2D). On the contrary, the protein expression of Bax, a pro-apoptotic protein, was decreased by E2, TCS or BPA compared to the control. However, treatment with Kaem and DIM increased the Bax protein expression, and co-treatment with Kaem or DIM and E2, TCS or BPA significantly increased Bax expression com-



**Fig. 2.** Effects of E2, TCS, BPA, Kaem, and DIM on apoptosis of VM7Luc4E2 breast cancer cells. To detect the apoptotic cells, TUNEL assay was examined for VM7Luc4E2 cells treated with (A) 0.1% DMSO (a control), E2 (0.001  $\mu$ M), TCS (1  $\mu$ M), and BPA (1  $\mu$ M), Kaem (30  $\mu$ M) and a mixture of E2 (0.001  $\mu$ M), TCS (1  $\mu$ M) or BPA (1  $\mu$ M) and Kaem (30  $\mu$ M), and DIM (15  $\mu$ M) and a mixture of E2 (0.001  $\mu$ M), TCS (1  $\mu$ M) or BPA (1  $\mu$ M) and DIM (15  $\mu$ M) for 48 h. (B) Fluorescence in response to each treatment was quantified using the Cell Sens Dimension software. (C) VM7Luc4E2 cells were treated with 0.1% DMSO (a control), E2 (0.001  $\mu$ M), TCS (1  $\mu$ M), BPA (1  $\mu$ M), Kaem (30  $\mu$ M), DIM (15  $\mu$ M), a mixture of E2 (0.001  $\mu$ M), TCS (1  $\mu$ M) or BPA (1  $\mu$ M) and Kaem (30  $\mu$ M), and a mixture of E2 (0.001  $\mu$ M), TCS (1  $\mu$ M) or BPA (1  $\mu$ M) and DIM (15  $\mu$ M), respectively, for 72 h. After protein extraction from the cells, each protein band corresponding to Bax and Bcl-xl was detected by Western blot assay. (D) Quantification of each protein was accomplished by scanning the densities of bands on a transfer membrane using Lumino graph II. TUNEL and Western blot assays were replicated at least three times. \*Mean values were significantly different from that of 0.1% DMSO (a control),  $p < 0.05$  (Dunnett's multiple comparison test). #Mean values were significantly different from that of a single treatment of E2, TCS or BPA,  $p < 0.05$  (Student's *t*-test).

pared to treatment with E2, TCS or BPA alone (Fig. 2C, 2D). These results indicate that E2, TCS, and BPA suppressed the apoptosis of VM7LUC4E2 breast cancer cells, but that Kaem

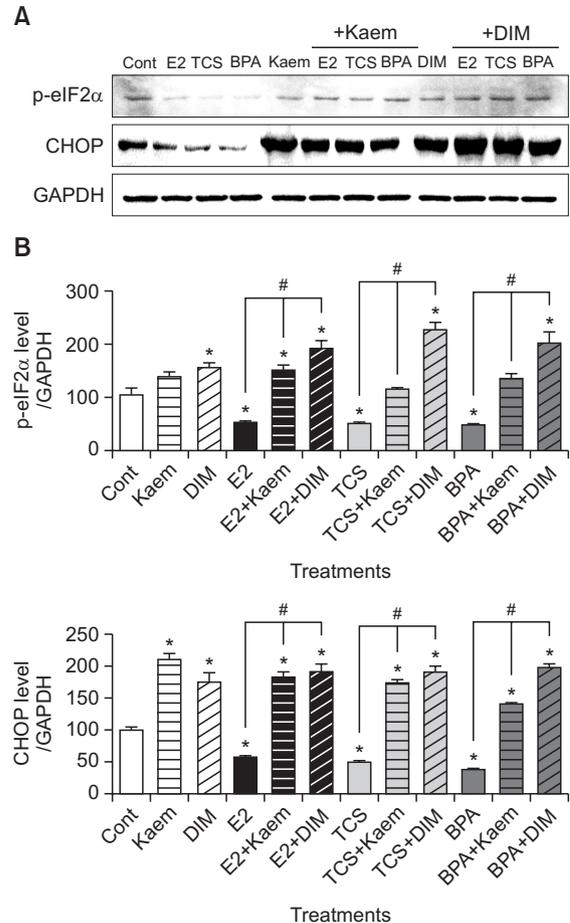
and DIM can induce apoptosis and inhibit the pro-apoptotic effects of E2, TCS or BPA in VM7LUC4E2 cells.



**Fig. 3.** ROS formation in VM7Luc4E2 cells following treatment with E2, TCS, BPA, Kaem, and DIM. (A) VM7Luc4E2 cells were treated with 0.1% DMSO (a control), E2 (0.001  $\mu$ M), TCS (1  $\mu$ M), BPA (1  $\mu$ M), Kaem (30  $\mu$ M), DIM (15  $\mu$ M), a mixture of E2 (0.001  $\mu$ M), TCS (1  $\mu$ M) or BPA (1  $\mu$ M) and Kaem (30  $\mu$ M), and a mixture of E2 (0.001  $\mu$ M), TCS (1  $\mu$ M) or BPA (1  $\mu$ M) and DIM (15  $\mu$ M), respectively, for 72 h. Cells were treated with H<sub>2</sub>O<sub>2</sub> (a positive control for ROS production) for 30 min, after which H2DCF-DA was added to each well for 30 min, and the fluorescence intensity of DCF (an oxidized form of H2DCF) was measured and photographed using a fluorescence microscopy to detect ROS production. H2DCF-DA assay was replicated at least three times. (B) ROS production by each treatment was quantified using the Cell Sens Dimension software. \*Mean values were significantly different from that of 0.1% DMSO (a control),  $p < 0.05$  (Dunnett's multiple comparison test). #Mean values were significantly different from that of a single treatment of E2, TCS or BPA,  $p < 0.05$  (Student's *t*-test). \*Mean values were significantly different from that of 0.1% DMSO (control),  $p < 0.05$  (Dunnett's multiple comparison test). #Mean values were significantly different from that of a single treatment of E2, TCS or BPA, respectively,  $p < 0.05$  (Student's *t*-test).

### Generation of ROS by treatment with E2, TCS, BPA, Kaem, and DIM

To investigate the intracellular ROS generation in VM7LU-C4E2 breast cancer cells a H2DCF-DA assay was conducted to detect ROS based on the appearance of green fluorescence after treatment with E2, TCS, BPA, Kaem, and DIM alone as well as after co-treatment with E2, TCS or BPA and Kaem or DIM for 72 h. We first identified ROS formation from the image of cells treated with H<sub>2</sub>O<sub>2</sub> as a positive control for detection of ROS formation. When VM7LUC4E2 cells were treated with E2, TCS or BPA, a small amount of ROS was detected



**Fig. 4.** Protein expression of ER stress-related markers following treatment with E2, TCS, BPA, Kaem, and DIM. (A) VM7Luc4E2 cells were treated with 0.1% DMSO (a control), E2 (0.001  $\mu$ M), TCS (1  $\mu$ M), BPA (1  $\mu$ M), Kaem (30  $\mu$ M), DIM (15  $\mu$ M), a mixture of E2 (0.001  $\mu$ M), TCS (1  $\mu$ M) or BPA (1  $\mu$ M) and Kaem (30  $\mu$ M), and a mixture of E2 (0.001  $\mu$ M), TCS (1  $\mu$ M) or BPA (1  $\mu$ M) and DIM (15  $\mu$ M) for 72 h. After protein extraction from the cells, the protein band corresponding to CHOP and p-eIF2 $\alpha$ , respectively, was detected by Western blot assay. Western blot assay was replicated at least three times. (B) Quantification of the protein was accomplished by scanning the densities of bands on a transfer membrane using Lumino graph II. \*Mean values were significantly different from that of 0.1% DMSO (a control),  $p < 0.05$  (Dunnett's multiple comparison test). #Mean values were significantly different from that of a single treatment of E2, TCS or BPA,  $p < 0.05$  (Student's *t*-test).

less than that of observed in the control (0.1% DMSO). On the other hand, treatment with Kaem or DIM alone, as well as co-treatment with Kaem or DIM and E2, TCS or BPA significantly increased the ROS formation in VM7LUC4E2 cells compared to the control or treatment with E2, TCS or BPA alone (Fig. 3A, 3B). These results indicate that E2, TCS, and BPA inhibit ROS formation, but Kaem and DIM promote ROS formation in VM7LUC4E2 cells.

### Roles of E2, TCS, BPA, Kaem, and DIM on protein expression of ER stress- and apoptosis-related genes

To examine the effects of E2, TCS, BPA, Kaem, and DIM on ER stress-related apoptotic process, we examined the al-

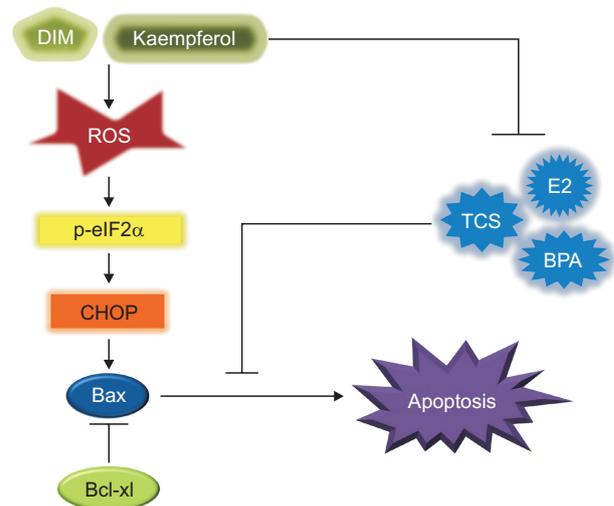
terations in protein expression of p-eIF2 $\alpha$  and CHOP in VM7LUC4E2 cells in response to treatment with these reagents. As shown in Fig. 4, E2, TCS, and BPA suppressed the protein expression of p-eIF2 $\alpha$  and CHOP compared to the control. However, co-treatment of Kaem and DIM significantly increased their expression compared to treatment with E2, TCS, and BPA alone. These results indicated that E2, TCS, and BPA inhibited ER stress-related apoptosis, but Kaem and DIM promoted the apoptotic process by enhancing ER-stress while also inhibiting E2, TCS, and BPA-induced anti-apoptosis.

## DISCUSSION

Apoptosis is a highly regulated programmed cell death for development processes of embryos and homeostasis; however, insufficient apoptosis is closely associated with uncontrolled cell proliferation and cancer (Fuchs and Steller, 2011). Mitochondria-mediated apoptosis is an intrinsic apoptotic pathway, and ER stress is considered to induce this type of apoptosis via UPR and CHOP production (Wang and Kaufman, 2012; Bhat *et al.*, 2017). Stress to the ER generally causes damage to cellular protein-folding, which is responsible for the biosynthesis and modification of many proteins (Gafar *et al.*, 2016). During induction of apoptosis by ER stress via UPR caused by oxidative stress, activated protein kinase R-like endoplasmic reticulum kinase (PERK) phosphorylates eIF2 $\alpha$ , and p-eIF2 $\alpha$  causes selective induction of activating transcription factor 4 (ATF4), leading to the activation of pro-apoptotic CHOP (Kitamura and Hiramatsu, 2010). Eventually, CHOP induces intrinsic apoptosis by regulating BCL2 family protein expression as well as by activating caspase-4 (Bhat *et al.*, 2017). Bax and caspase play crucial roles in mitochondrial permeabilization and apoptosis and they control cytochrome c in mitochondrial inner membrane (Park *et al.*, 2017). And annexin V has been used for detection of apoptosis directly (Kwon *et al.*, 2013).

In the present study, the effects of two EDCs, TCS and BPA, as well as two phytoestrogens, Kaem and DIM, on cell viability of VM7LUC4E2 breast cancer cells were investigated via MTT assay. The results revealed that TCS and BPA significantly induced proliferation of VM7LUC4E2 cells at concentrations of 0.1-10  $\mu$ M similar to E2 as a positive control. The cell proliferation inducing activities of TCS and BPA in ER-positive cancer cells were also identified in our previous studies, in which TCS and BPA were displayed as xenoestrogenic EDCs that can induce breast cancer or ovarian cancer cell proliferation via an ER-dependent pathway in a similar fashion as E2 (Kim *et al.*, 2015b; Lee *et al.*, 2017). These findings support the fact that EDCs have intrinsic estrogenic behavior in estrogen dependent cancers (Dinwiddie *et al.*, 2014). Conversely, Kaem (10-40  $\mu$ M) and DIM (5-20  $\mu$ M) applied in conjunction with E2, TCS, and BPA abrogated the cell proliferation effect of E2, TCS, and BPA, suggesting they have chemopreventive effects against the cancer progression effect of E2 and EDCs.

To elucidate the mechanisms underlying the opposite effects of E2, TCS, and BPA as well as Kaem and DIM on cell viability, we examined their roles in the induction of the apoptotic process in VM7LUC4E2 cells. As shown in Fig. 5, E2, TCS, and BPA inhibited the sequential apoptotic process associated with ROS-induced ER stress in VM7LUC4E2 cells. Conversely, Kaem and DIM promoted ROS formation and apoptosis of VM7LUC4E2 cells in the presence or absence of E2, TCS,



**Fig. 5.** Suppression effect of Kaem and DIM on E2, TCS or BPA-induced anti-ROS formation and anti-ER stress induced apoptosis in VM7Luc4E2 breast cancer cells. As xenoestrogenic EDCs, TCS and BPA inhibited ROS production, ER stress response and apoptosis of VM7Luc4E2 breast cancer cells similar to E2 and down-regulated the expression of ER stress response-related genes such as CHOP and p-eIF2 $\alpha$ . As a result, the protein expression of Bax, a pro-apoptotic gene, was decreased, but the protein expression of Bcl-xl, an anti-apoptotic gene, was increased by E2 and EDCs. Conversely, Kaem and DIM displayed chemopreventive effects against breast cancer progression by effectively producing ROS and ER stress-associated apoptosis in VM7Luc4E2 cells exposed to E2 and EDCs such as TCS and BPA.

and BPA via the induction of ER stress, of which activation was confirmed by increased formation of the phosphorylated form of eIF2 $\alpha$ . As described above, p-eIF2 $\alpha$  induces the activation of pro-apoptotic CHOP by activating ATF4 (Kitamura and Hiramatsu, 2010). The increase in apoptosis by Kaem and DIM was verified by the increased number of apoptotic cells, as well as upregulation of the apoptotic protein BAX via decreased protein expression of Bcl-xl gene. The Bcl-2/Bcl-xl family members controlling the release of pro-apoptotic proteins from the mitochondria intermembrane inhibits programmed cell death by downregulating Bax/Bak, which induce apoptosis (Gross *et al.*, 1999).

Previous studies have also reported apoptosis-inducing anticancer activity via the stimulation of ER stress by Kaem and DIM. Kaem promoted apoptosis via induction of the ER stress-CHOP signaling pathway in HepG2 hepatocellular carcinoma cells (Guo *et al.*, 2016). Highly increased production of intracellular ROS and induction of apoptosis were also identified in MCF-7 breast cancer cells via H2DCF-DA assay (Kim *et al.*, 2008). Moreover, DIM and its derivatives induced apoptosis in Panc-1 and Panc-28 pancreatic cancer cells through ER stress-dependent upregulation of DR5, a CHOP-dependent induction of death receptor (Abdelrahim *et al.*, 2006). Additionally, DIM toxicity toward tumor cells *in vitro* was enhanced when the cells were undergoing nutritional deprivation and ER stress (Sun *et al.*, 2004).

ER stress-associated apoptosis is considered an encouraging chemopreventive approach to treatment of cancer, and potential ER stress-inducing agents have been introduced as an attractive strategy for novel cancer therapy (Bhat *et al.*,

2017). Resveratrol, which is a crucial phytoestrogen derived from grapes and peanuts, was recently found to induce autophagy-mediated apoptosis by triggering ER stress in PC3 and DU145 prostate cancer (Selvaraj *et al.*, 2016). In addition,  $\beta$ -phenethyl isothiocyanate (PEITC) promoted ROS accumulation and UPR-mediated apoptosis in SKOV-3 ovarian cancer cells, while it did not cause any effects in normal ovarian epithelial cells (Hong *et al.*, 2015).

In summary, TCS and BPA, which are considered xenoestrogenic EDCs, were investigated to induce breast cancer progression via the stimulation of cell proliferation, anti-ROS production, anti-ER stress response, and anti-apoptosis by regulating the expression of ROS induced ER stress-associated genes, including p-eIF2 $\alpha$ , CHOP, Bcl-xl and Bax. Conversely, Kaem and DIM significantly inhibited E2, TCS or BPA-induced breast cancer cell proliferation and anti-apoptosis effects by regulating the protein expression of pro- and anti-apoptosis, ROS production, and ER stress response-related genes as shown in Fig. 5. Therefore, Kaem and DIM are effective chemopreventive compounds capable of inducing apoptosis of breast cancer that have the potential for use to prevent tumor aggressiveness and as alternatives to conventional therapeutic agents in environments consistently exposed to diverse EDCs such as TCS and BPA.

## CONFLICT OF INTEREST

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