APOPTOSIS OF MCF-7 CANCER CELL INDUCED BY POMEGRANATE (*Punica granatum* L.) PEEL EXTRACT

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Abstract

Various aspects of pomegranate (*Punica granatum* Linn.) have been documented in traditional medicine. In the present study, pomegranate peel ethanolic extract (PPE) was investigated for many aspects relating to the caspase-3-deficient human breast cancer cell line (MCF-7), especially on the antiproliferative effects, morphological changes, DNA fragmentation, and apoptotic molecule-activated cell death. The results showed that PPE could inhibit the proliferation of MCF-7 cells in a dose- and time-dependent manner. PPE has a strongly positive influence on antiproliferation and apoptotic induction of MCF-7 cells, as seen via morphological changes and oligonucleosomal DNA fragments. Apoptosis was accompanied by bcl-2, caspase-9, caspase-7, and poly (ADP-ribose) polymerase (PARP) levels. PPE induced apoptosis indicating that there was a reduction of bcl-2, procaspase-9, and procaspase-7 activities, but it activated the PARP activity. Moreover, these protein levels were associated with morphological change and DNA fragmentation in the MCF-7 cells. However, caspase-3 deficiency did not affect the PPE-induced levels of bcl-2, caspase-9, caspase-7, and PARP. In conclusion, the PPE had the potential for the induction of antiproliferation and apoptosis in caspase-3-deficient MCF-7 cells.

Keywords: *Punica granatum*, antiproliferation, DNA fragmentation, apoptosis

Introduction

Breast cancer is the commonest cancer in most Asian countries, and it is tending to increase more rapidly than in Western countries. This is caused by changes in lifestyles and diets (Yip, 2009).

Pomegranate peel has been proved to have antioxidant properties (Guo et al., 2003; Manasathien et al., 2011). Pomegranate is composed of phenols and polyphenols, including aliphatic organic acids, hydroxybenzoic acids,
hydroxycinnamic acids, flavan-3-ols, flavonols, flavones, anthocyanins, anthocyanidins, and ellagitannins (Lansky and Newman, 2007). Lansky et al. (2005a; 2005b) found that pomegranate extracts could inhibit proliferation and invasion of human prostate cancers (LNCaP, DU-145, and PC-3 cells), and Malik et al. (2005) have reported that pomegranate fruit extract induced apoptosis and increased pro-apoptotic protein in PC-3 cells. Anthocyanins and ellagitannins, the major phytochemicals of pomegranate peel extract, caused significant inhibition of HT115 colon cancer cell invasion (Coates et al., 2007). Larrosa et al. (2006) have suggested that the anticarcinogenic effect of ellagic acid and ellagitannins induced apoptosis via the mitochondrial pathway with the release of cytochrome c into the cytosol, and activation of the initiator caspase-9 and effector caspase-3, in colon cancer Caco-2 cells.

Apoptosis, a genetically regulated mechanism of cellular suicide that demonstrates a crucial role in homeostasis and development, is a mode of programmed cell death that is coordinated by members of the caspase family of cystein proteases. It is well established that initiator caspases (caspase-2, -8, -9, -10, -11, and -12) are coupled to pro-apoptotic signals. Once activated, these caspases cleave and activate downstream effector caspases (caspase-3, -6, and -7), which in turn execute apoptosis by cleaving cellular proteins (Danial and Korsmeyer, 2004; Degterev et al., 2003).

Caspase-3 is believed to be the primary executioner of apoptosis, since it is activated by many death signals. The major characteristic features of apoptosis such as chromatin condensation, DNA fragmentation, and cleavage of the DNA repair enzyme poly (ADP-ribose) polymerase (PARP), depend on its activation. Previous work has demonstrated that MCF-7 breast carcinoma cells, which lack caspase-3 owing to a functional deletion in the caspase-3 gene, can be killed by apoptotic stimuli, such as tumour necrosis factor (TNF) and staurosporine, without DNA fragmentation and many of the hallmarks of apoptosis. These results demonstrated that caspase-3 was likely to be essential for such hallmarks of apoptotic cell death (Janicke et al., 1998).

This study observed the effects of pomegranate peel ethanolic extract (PPE) on cell proliferation and cell apoptosis of human breast cancer cells (MCF-7). The investigation, in particular, emphasized (i) the alteration of cell proliferation, (ii) the association of morphological change and DNA fragmentation in MCF-7 cells, and (iii) the expression level of important apoptotic molecules. After the study, we expect that such a multi-marker analysis of the apoptosis pathway could be useful for the individualization of therapeutic strategies in the future.

Materials and Methods

Chemicals

Dulbecco’s Modified Eagle’s Medium: Nutrient Mixture F-12 (DMEM/F-12), fetal bovine serum (FBS), penicillin/streptomycin, trypan blue, and resazurin were the Gibco brand, Thermo Fisher Scientific, Waltham, MA, USA. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and Hoechst 33342 were the Molecular Probes brand, Thermo Fisher Scientific, Waltham, MA, USA. Agarose was from Promega Corporation, Madison, WI, USA. Ethidium bromide was from Bio-Rad Laboratories, Inc., Hercules, CA, USA. RNaseA was purchased from AMRESCO LLC, Solon, OH, USA. The DNA ladder and Genomic DNA Extraction Kit were obtained from RBC Bioscience Corp., Taipei, Taiwan. Monoclonal antibodies, the secondary antibody, chemiluminescent substrate, and CL-XPosure film were from Santa Cruz Biotechnology, Dallas, TX, USA. All other chemicals and reagents were of analytical grade.

Pomegranate Collection and Peel Extraction

Pomegranate fruits were collected from a local farm in Klangdong, Saraburi Province, Thailand. The plant was taxonomically identified by the Royal Forest Department of Thailand, specimen voucher no. 080252. The peel was separated from the fruits and then was cleaned, dried, and ground to powder. Then, 50 g of the peel powder was extracted in 500 ml of 70% ethanol for 24 h in a Soxhlet extraction apparatus. The PPE was evaporated, lyophilized, and kept at -20°C for further use.
Cell Culture

Human breast cancer MCF-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in DMEM/F-12 supplemented with 10% FBS and 1% penicillin/streptomycin in a 5% CO₂ atmosphere at 37°C.

Cell Viability Assay

Trypan blue is the staining dye used to discriminate viable and dead cells. Dead cells absorb the dye and appear blue in the cytoplasm of cells when viewed under a microscope, while viable cells are not stained. MCF-7 cells at 10000 cells/well in 100 μl were plated onto a 96-well plate and incubated for 24 h. The medium was then discarded and replaced with PPE at various concentrations and incubation continued for 6-24 h. The treated cells were trypsinized by trypsin-EDTA and 100 μl of the cells were transferred into a 1.5 ml microcentrifuge tube. Trypan blue was added to the tube and mixed thoroughly, and stained for 2 min. The stained cells were transferred onto a hemocytometer. The viable cells were calculated using the following formula and the inhibitory concentration at 50% (IC₅₀ value) was determined using the Probit analysis method (Finney, 1971).

\[
\text{Viable cells (％) =} \left( \frac{\text{Total viable cell per ml}}{\text{Total cells per ml}} \right) \times 100
\]  

Cell Proliferation Assay

Cell proliferation was assayed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT). This is a colorimetric assay based on the conversion of the yellow MTT solution to the purple formazan derivatives by mitochondrial succinate dehydrogenase in viable cells (Hansen et al., 1989). MCF-7 cells at 10000 cells/well in 100 μl were plated onto a 96-well plate and incubated for 24 h. The cultured cells were treated with PPE at various concentrations and continued to incubate for 6-24 h. Dimethyl sulfoxide (DMSO) (0.01% DMSO) was used as the control. The cultured medium was discarded and incubated with 100 μl MTT solution at 5 μg/μl (in phosphate-buffered saline (PBS), pH 7.4) for 4 h. Then, the MTT solution was removed and 150 μl DMSO (100% DMSO) was added. The plate was gently agitated until the formazan precipitate was dissolved. The absorbance was measured at 570 nm with the reference wavelength at 630 nm. A decrease in the absorbance indicated a reduction in cell viability (Babu et al., 2002; Perillo et al., 2000; Zhou et al., 2003; Okonogi et al., 2007). The percentage of antiproliferation activity was calculated using the following formula and the IC₅₀ value was determined using the Probit analysis.

\[
\text{Antiproliferation activity (％) =} \left( 1 - \frac{A_1 - A_0}{A_0} \right) \times 100
\]

where \(A_0\) was the absorbance of the control, \(A_1\) was the absorbance of the treated sample, and \(A_2\) was the absorbance of the treated sample without cells.

Cytoprotective Activity

Resazurin assay is a colorimetric method, based on the conversion of the purple resazurin solution to the red resorufin solution via reduction reactions of metabolically active cells. The MCF-7 cells at 10000 cells/well were plated onto a 96-well plate and incubated for 24 h. The cultured cells were treated with PPE at various concentrations and continued to incubate for 6-24 h. The medium was discarded. Resazurin solution at 100 μl/ml in DMEM/F-12 without FBS was added to each well and incubated for 2 h. The optical absorbance of the color change was measured under a microplate reader at a wavelength of 570 nm with the reference wavelength at 600 nm. A decrease in the absorbance indicated a reduction in cell viability (Page et al., 1993; Goegan et al., 1995; Lancaster and Fields, 1996). The percentage of cell viability was calculated following Equation 2 and the IC₅₀ value was analyzed.

Morphological Observation

Typical nuclear condensation was used as a morphological marker of apoptosis and observed using the Hoechst 33342 dye. MCF-7 cells were treated with various concentrations of PPE and incubated for 24 h. The treated cells were washed twice with ice-cold PBS, pH 7.4. The cells were fixed with 500 μl formaldehyde (10%, v/v) for 5 min, washed...
with PBS, and then stained with 1 μg/μl Hoechst 33342 in PBS for 15 min. The cell morphology was observed and photographed under a fluorescence microscope with an Olympus DP50 digital camera (Olympus Corporation, Tokyo, Japan).

DNA Fragmentation Assay

DNA was isolated from the treated MCF-7 cells using a Genomic DNA extraction kit. RNase A at 10 mg/ml was added to sample lysate and left to stand at room temperature for 30 min. The precipitated DNA was dried under centrifuge at 13,000 rpm for 3 min and eluted in an elution buffer. The purified DNA sample was quantitated spectrophotometrically and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). DNA at 2 μg/well was separated in 1% agarose gel containing ethidium bromide (0.5 µg/ml) in 45 mM Tris, 45 mM boric acid, and 1 mM EDTA at 100 mV for 1.5 h. The gel was visualized under UV fluorescence, and then photographed.

Western Blotting

To detect bcl-2, procaspase-7, procaspase-9, and PARP, intact or apoptotic MCF-7 cells were harvested. The cultured cells were treated with PPE and continued to incubate for 6-24 h. The treated cells were trypsinized and lysed with ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM sodium orthovanadate, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X, and a protease inhibitor cocktail, for 60 min, and then centrifuged at 13000 rpm for 10 min. The lysate protein was quantitated spectrophotometrically by Bradford assay (Bradford, 1976). Next, 30 μg of lysate protein from each sample was subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE gel was transferred onto a poly vinylidene difluoride membrane (PVDF). The blotted PVDF was blocked with 2% (w/v) bovine serum albumin in Tween20-Tris buffered saline (TTBS) containing 0.1% Tween20, 100 mM Tris-HCl, and 150 mM NaCl for 60 min at room temperature. The blocked PVDF was incubated with the first antibodies of anti-mouse monoclonal antibodies, bcl-2 (diluted 1:1000), procaspase-7 (diluted 1:1000), procaspase-9 (diluted 1:500), PARP (diluted 1:500), and actin (diluted 1:500) for 24 h at 4ºC. Actin was used as a loading control in the Western blot analysis. The incubated PVDF was washed in TTBS and incubated with the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse antibody (diluted 1:20 000), for 1 h and then washed in TTBS. Super Signal West Pico chemiluminescent substrate was added for 5 min at room temperature. The developed protein was photographed onto CL-XPosure film (Choudhuri et al., 2002).

Table 1. Viability of MCF-7 cells under different PPE concentrations observed via trypan blue, MTT, and resazurin methods

<table>
<thead>
<tr>
<th>Methods</th>
<th>Conc. (μg/ml)</th>
<th>6 h</th>
<th>12 h</th>
<th>18 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypan blue</td>
<td>300</td>
<td>72.08 ± 5.53</td>
<td>70.36 ± 1.40</td>
<td>73.75 ± 2.23</td>
<td>80.70 ± 1.89</td>
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<tr>
<td></td>
<td>400</td>
<td>57.06 ± 2.41</td>
<td>39.42 ± 7.19</td>
<td>20.68 ± 5.64</td>
<td>20.49 ± 3.21</td>
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<tr>
<td></td>
<td>500</td>
<td>54.06 ± 1.38</td>
<td>9.23 ± 0.15</td>
<td>6.92 ± 0.28</td>
<td>7.39 ± 0.60</td>
</tr>
<tr>
<td></td>
<td>IC50</td>
<td>648.40 ± 37.54a</td>
<td>381.35 ± 13.72b</td>
<td>362.50 ± 13.82b</td>
<td>347.83 ± 11.85b</td>
</tr>
<tr>
<td>MTT</td>
<td>300</td>
<td>93.93 ± 4.89</td>
<td>83.46 ± 1.12</td>
<td>85.49 ± 1.77</td>
<td>80.37 ± 1.32</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>80.63 ± 2.13</td>
<td>58.73 ± 5.75</td>
<td>42.12 ± 4.97</td>
<td>31.49 ± 0.97</td>
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<tr>
<td></td>
<td>500</td>
<td>77.97 ± 1.22</td>
<td>22.11 ± 0.27</td>
<td>17.87 ± 0.50</td>
<td>16.52 ± 0.93</td>
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<tr>
<td></td>
<td>IC50</td>
<td>737.13 ± 35.96a</td>
<td>415.48 ± 24.92b</td>
<td>395.50 ± 15.53b</td>
<td>377.88 ± 13.14b</td>
</tr>
<tr>
<td>Resazurin</td>
<td>300</td>
<td>85.75 ± 1.05</td>
<td>88.64 ± 1.07</td>
<td>85.19 ± 4.70</td>
<td>72.18 ± 1.13</td>
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<tr>
<td></td>
<td>400</td>
<td>82.62 ± 0.32</td>
<td>86.05 ± 2.17</td>
<td>71.41 ± 7.05</td>
<td>52.40 ± 1.81</td>
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<tr>
<td></td>
<td>500</td>
<td>78.95 ± 3.45</td>
<td>65.30 ± 2.75</td>
<td>20.77 ± 2.09</td>
<td>7.85 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>IC50</td>
<td>960.15 ± 43.56a</td>
<td>816.73 ± 31.63b</td>
<td>526.05 ± 17.15c</td>
<td>471.50 ± 13.62d</td>
</tr>
</tbody>
</table>

IC50, median inhibitory concentration. Numbers with different letters within the same row are significantly different (P < 0.05); n=4
Statistical Analysis
Statistical analysis was performed by ANOVA, using the least significant difference test to determine the level of significance at $P < 0.05$. All data were expressed as mean ± standard error (SE).

Results and Discussion

PPE on Antiproliferation of MCF-7 Cells
The cytoprotective activity of PPE on MCF-7 cells at various PPE concentrations could cause a decline in MCF-7 cell growth, and the viability was investigated via the trypan blue exclusion, MTT, and resazurin methods. Based on those methods, PPE could reduce cell proliferation in a dose- and time-dependent manner (Table 1). Similarly, the $IC_{50}$ value was evaluated (Table 1 and Figure 1). According to the trypan blue exclusion method, the $IC_{50}$ values at 6, 12, 18, and 24 h of PPE were 648.40 ± 37.54, 381.35 ± 13.72, 362.50 ± 13.82, and 347.83 ± 11.85 µg/ml, respectively. Consistent with the MTT method, the $IC_{50}$ values at 6, 12, 18, and 24 h of PPE were 737.13 ± 35.96, 415.48 ± 24.92, 395.50 ± 15.53, and 377.88 ± 13.14 µg/ml, respectively. Similarly, under the resazurin method, the $IC_{50}$ values at 6, 12, 18, and 24 h of PPE were 960.15 ± 43.56, 816.73 ± 31.63, 526.05 ± 17.15, and 471.50 ± 13.62 µg/ml, respectively. Based on this experiment, various PPE concentrations induced antiproliferation in MCF-7 cells. The loss of MCF-7 cell viability was also confirmed by inverted microscopy (Figure 2(c)).

The cell antiproliferation was significantly increased as the concentration of PPE increased; therefore, PPE had an antiproliferative effect on MCF-7 cancer cells. PPE could delay cell proliferation in many different human cancer cell lines (Kawai and Lansky, 2004; Mavlyanov et al., 1997; Setheetham and Ishida, 1995). In human prostate cancer cells, DU-145, LNCaP, and PC-3 cells were more sensitive to PPE (Lansky et al., 2005a; Lansky et al., 2005b). The phytochemicals in PPE such as puneric acid, luteolin, ellagic acid, and caffeic acid showed suppression of the invasion of PC-3 prostate cancer cells (Lansky et al., 2005a; Lansky et al., 2005b). Punicalagin, ellagic acid, and total pomegranate tannins showed great antiproliferative activity against HCT116 and HT-29 colon cancer cells (Seeram et al., 2005). PPE showed the most potential against estrogen responsive MCF-7 cells, but had less effect on estrogen negative MDA-MB-231 cells, and no effect on normal breast epithelial cells MCF-10A (Toi et al., 2003; Manasathien et al., 2012). Some chemicals in PPE such as gallic acid, ellagic acid, tannic acid, quercetin, kaempferol, luteolin, and tannins synergistically played to inhibit the proliferation of CaCo-2 and HT-29 colon, MCF-7 and Hs578T breast, and DU145, LNCaP, and PC-3 prostatic cancer cells by inhibiting cell cycle progression, suppressing angiogenic factors, and inducing apoptosis (Saleem et al., 2002; Ackland et al., 2005; Bawadi et al., 2005; Fernandes et al., 2009; Loizzo et al., 2009). This study suggested that PPE could be an agent that induced the death of MCF-7 cells by the apoptotic pathway.

![Figure 1. IC50 of antiproliferation activity of the PPE-treated MCF-7 cells during 6-24 h](image-url)
PPE on Apoptosis Induction of MCF-7 Cells

Based on information of the antiproliferation activity, the optimal concentration for the *in vitro* apoptosis study was 400 µg PPE/ml (Figures 2(c) and 2(d)). On the second day, there were no living cells observed (data is not shown). Using the Hoechst 33342 dye, condensed nuclear DNA was investigated under florescence microscopy after 24 h of 400 µg PPE/ml administration (Figure 2(d)). This study was conducted as 2 additional experiments on the morphological and biochemical feature of the PPE-treated cells to determine whether or not this cell death was apoptosis. Morphological change is a well-known biochemical characteristic of apoptosis (Wyllie, 1988) and dose- and time-dependent condensed nuclei and DNA fragmentation were clearly observed. MCF-7 cells were treated with various PPE concentrations and incubated for 12-24 h. The DNA fragmentation of treated MCF-7 cells after treating with PPE at different concentrations and times is shown in Figure 3. The results indicated that the effector caspases executed the apoptotic signal that was stimulated by the treatment compounds.

PPE induced DNA fragmentation in a dose- and time-dependent fashion. MCF-7 cells lack caspase-3 of apoptosis which is the crucial protease and known as the executioner. Activation of caspase-3 leads to chromatin condensation, PARP cleavage, and DNA fragmentation. Seeram *et al.* (2005); Larrosa *et al.* (2006) and Coates *et al.* (2007) reported that anthocyanins, ellagic acid, punicalagin, and ellagitannins of PPE induced apoptosis in HT-29, HT115, HCT116, and Caco-2 colon cancer cells. Ellagic acid induced cell detachment, decreased cell viability, and induced apoptosis, as measured by DNA strand breaks and alterations of the cell cycle, in SH-SY5Y human neuroblastoma cells (Fjaeraa and Nanberg, 2009). The MCF-7 cell line is known to undergo cell death in response to stimulation of TNF/cycloheximide (TNF-c), staurosporine, and other agents (Tewari *et al.*, 1995; Janicke *et al.*, 1998). TNF/cycloheximide-treated MCF-7 cells resulted in cell death but their ladder nuclear fragmentation was not observed (Mc Gee *et al.*, 2002). Less than 10% of photodynamic therapy (PDT)-treated MCF-7 cells were found to have condensed chromatin.

Figure 2. Morphological alterations of MCF-7 cells after exposure to 400 µg PPE/ml for 24 h. Control MCF-7 cells were observed under inverted microscopy at 400x magnification (A) and staining with Hoechst 33342 (B). PPE-treated MCF-7 cells were observed under inverted microscopy at 400x magnification (C) and staining with Hoechst 33342 (D). The numbers represent 1 ➔: cell detachment; 2 ➔: condensed nuclear DNA and DNA fragmentation.
by 4 or 6 h post-PDT; however, 60% of the PDT-treated MCF-7c3 cells (*casp3*-transfected MCF-7 cells) displayed these apoptotic features (Xue *et al*., 2001). The data supported that caspase-3 was required for ladder DNA fragmentation and an apoptotic body for inducing apoptosis. There were at least 2 phases for DNA fragmentation in apoptotic cells (Walker *et al*., 1993). The first phase involved the generation of large DNA fragments of 50 and 300 kb and the second phase resulted in oligonucleosome-size fragments. PDT-treated MCF-7c3 cells demonstrated a strong DNA band of 50 kb, and the intensity of this band increased with time post-PDT. For PDT-treated MCF-7 cells, the 50 kb DNA band was slightly apparent at 20 h post-PDT, and its intensity was much weaker than that of PDT-treated MCF-7c3 cells at the same time point (Xue *et al*., 2001). The results indicated that the rate and extent of apoptosis were much more reduced in PDT-treated MCF-7 cells than in MCF-7c3 cells due to the deletion of caspase-3.

**PPE on Death Signal**

Two major apoptotic death-signal pathways can be well characterized by (i) the Fas/FasL pathway (Wajant, 2002) and (ii) the mitochondrial pathway (Debatin *et al*., 2002). In this study, the administration of 500 µg/ml of PPE could change the apoptotic proteins of MCF-7 cells in the 12-24 h cultures as compared with untreated cells (Figure 4). The

![Figure 3](image-url)  **Figure 3.** DNA fragmentation on MCF-7 cells after treating with PPE at different concentrations and times. The lanes represent different meanings: DNA marker (lane M); control MCF-7 cells (lane 1); PPE-treated MCF-7 cells at 400-600 µg/ml, 12 h (lanes 2-4); 18 h (lanes 5-7); 24 h (lanes 8-10).

![Figure 4](image-url)  **Figure 4.** Effect of PPE on the expression of PARP, procaspase-9, procaspase-7, and bcl-2 proteins. MCF-7 cells were treated with 500 µg PPE/ml at different time intervals. Proteins of cell lysates (30 µg/well) were subjected to SDS-PAGE and western blot analysis. PARP, procaspase-9, procaspase-7, and bcl-2 proteins were detected by using specific monoclonal antibodies. Control cells (lane C); PPE-treated MCF-7 cells for 12-24 h (lanes 1-4).
decreases of bcl-2, procaspase-9, and procaspase-7 were in a time-dependent manner. Especially, caspase-7-cleaved PARP could be induced by PPE-induced MCF-7 apoptosis after the PPE signal transmission into the cells. As a result, caspase-9 was activated followed by caspase-7 and the cleavage of the PARP (Figure 4).

A weak bax expression could be one of the mechanisms by which MCF-7 cells conferred resistance against chemotherapy induced apoptosis, and by which these agents could induce cell death by a common inhibiting pathway of bcl-2 (Sakakura et al., 1997). Kagawa et al. (2001) showed that bax overexpression produced more DNA fragmentation in casp3 (caspase-3)-transfected MCF-7 cells (24.2%) than it did in untransfected MCF-7 (7.5%). Caspase-3-deficient MCF-7 cells failed to undergo morphological nuclear and DNA fragmentation, whereas casp3-transfected MCF-7 displayed intact nuclear dismantling and DNA fragmentation (Kagawa et al., 2001). Wolf et al. (1999) reported that only caspase-3 and caspase-7 participated to induce DNA fragmentation by inactivating the inhibitor of caspase-activated DNase (ICAD). However, in vitro, caspase-3 inactivated ICAD and promoted DNA fragmentation more effectively than caspase-7. Perhaps in that particular instance, caspase-7 activation did not reach the level to initiate DNA fragmentation. Therefore, it could be assumed that PPE was able to induce apoptosis in MCF-7 cells with untypical apoptotic features. The cells showed slight cytoskeletal rearrangement, nuclei condensation, and DNA fragmentation. The cells were not blebbed. The death of MCF-7 cells would definitely proceed as a result of PPE induction.

It is well known that apoptosis can occur by 2 major pathways; the extrinsic pathway (Fas/FasL or TNF-αR/TNF-α) and intrinsic pathway (mitochondrial pathway). The product of the bcl-2 gene is well known to play a role in promoting cell survival and inhibiting apoptosis in a variety of cancer cells, while the related protein bax accelerates apoptosis and counters the cell survival function of bcl-2 (Korsmeyer, 1992). The bcl-2 gene plays a role in breast cancer development since it is overexpressed in 70% of breast cancer specimens (Silvestrini et al., 1994). Moreover, the bax gene, another apoptosis-promoting element, is downregulated in breast cancer cells leading to cell resistance to apoptosis (Bargou et al., 1995). The bcl-2 gene is also known in an association with the development of breast cancer by inhibiting apoptosis mediated by chemotherapy (Dole et al., 1994), and blocking both the p53-dependent and independent cell death pathway (Kamesaki et al., 1993). Studies of the mechanism of apoptosis in response to other agents are consistent with a role for mitochondria. During the development of a cell-free model of apoptosis, a mitochondrially-enriched cell fraction was found to be an essential component (Newmeyer et al., 1994). Buprenorphine hydrochloride (Bph) induced very rapid apoptosis that related to overexpression of the bcl-2 protein causing inhibition of the Bph-induced apoptosis, in the NG108-15 cell line (Kugawa et al., 2000). Tumor necrosis factor-α (TNF-α) and epigallocatechin-3-gallate (EGCG) induced a much greater increase in the level of reactive oxygen species (ROS). These results indicated that increased ROS generation and decreased mitochondrial membrane potential may be involved in TNF-α and EGCG-induced apoptosis in MCF-7 cells (Hsuuw and Chan, 2007; Siemankowski et al., 1999). TNF-α and EGCG induced apoptosis by modulating the expression of the bcl-2 family members. Bcl-2 functions as a suppressor of apoptosis while bax normally acts as a sensor of cellular damage and stress (Hsuuw and Chan, 2007; Siemankowski et al., 1999). In response to significant damage or stress, bax relocates to the mitochondrial surface and disrupts the normal function of the anti-apoptotic bcl-2 protein. This process leads to loss of membrane potential and efflux of cytochrome c (Green and Reed, 1998). This finding that MCF-7 cells treated with PPE could decrease bcl-2 expression (Figure 4) corresponds with the decline of cell proliferation (Table 1). It could be suggested that bcl-2 responded to PPE-induced apoptosis of MCF-7 cells. Tannins could induce apoptosis of MCF-7 cells via the intrinsic pathway (Soyocak et al., 2011). Consistently, treatment of pomegranate fruit extract on CaCo-2 colon, A549 lung, PC-3 prostate, and MDA-MB-435 breast cancer cells showed an induction of bax (proapoptotic) and down-regulation of bcl-2 (antiapoptotic) (Khan et al., 2007; Kim et al., 2002; Larrosa
et al., 2006; Malik et al., 2005). Therefore, it strongly supports PPE-induced apoptosis in MCF-7 cells through the intrinsic pathway.

Except for the previous study, the apoptosis of MCF-7 by PPE could be possibly via the extrinsic pathway. FasL and TNF-α might activate either c-Jun N-terminal kinase (JNK) leading to the inhibition of bel-2 or caspase-8 cleaving of Bid and truncated Bid (tBid). Cleaved Bid and tBid translocates into mitochondria leading to cytochrome c release (Debatin and Krammer, 2004; Wajant, 2002). Similarly, EGCG and catechin could activate the extrinsic death pathway as demonstrated through an increase of caspase-8 expression levels (Ahmeda et al., 2010; Alshatwi, 2010).

The released cytochrome c forms a complex with apoptosis protease activating factor (apaf-1) and procaspase-9 producing apoptosome. Apoptosome is a key event that triggers the activation of the caspase cascade, including caspases-3, -7 and -6, which execute the programmed cell death (Debatin and Krammer, 2004; Wajant, 2002). Treatment of Caco-2 colon, MDA-MB-231 breast, and PC-3 prostatic cancer cells with ellagitannins, ellagic acid, EGCG, and proanthocyanidins could induce apoptosis via caspase-9 activation (Malik et al., 2005; Larrosa et al., 2006; Kim et al., 2009). EGCG and ellagitannins could lead to apoptosis of MCF-7 via the initiator caspase-9 (Hsuuw and Chan, 2007; Losso et al., 2004). This study indicated that procaspase-9 declined after PPE treatment in a time-dependent manner. These data suggested that PPE induced apoptosis by regulating the initiator caspase-9.

Caspases contributed to the drastic morphological changes associated with apoptosis by proteolyzing a number of key substrates, including the structural proteins such as c-fodrin, gelsolin, PARP, p21-activated kinase, focal adhesion kinase, and DFF45 (Nicholson et al., 1995; Rudel and Bokoch, 1997; Walsh et al., 2008; Wen et al., 1997). It is now well established that the upstream or initiator caspase-9 activates the downstream or effector caspases (caspases-3, -6, and -7). Caspase-3 is believed to be the primary executioner of apoptosis, since it is activated by many death signals and the major characteristics of apoptosis, such as cytoskeletal rearrangement, chromatin condensation, DNA fragmentation, and cleavage of PARP, depend on its activation. Nevertheless, caspase-3 deficiency, on the B6 background in mice, had little discernable impact on cell numbers, and survival (Leonard et al., 2002; Houde et al., 2004; Lakhani et al., 2006). However, mice doubly deficient for casp-3 and casp-7 on the B6 background died immediately after birth because of defective heart development (Lakhani et al., 2006). Caspase-3 and -7 preferentially cleaved DEVD-based peptide substrates with equal efficiency (Mc Gee et al., 2002; Walsh et al., 2008). Furthermore, caspase-3 and -7 both cleaved proteins such as ROCK I, RhoGDI, c-fodrin, PARP, and ICAD equally well (Walsh et al., 2008). However, the majority of substrates such as Bid, gelsolin, XIAP, and caspase-6 were more efficiently cleaved by caspase-3 than caspase-7 (Walsh et al., 2008). Mc Gee et al. (2002) suggested that caspase-7 is involved in the mechanism by which pyrrolo-1,5-benzoxazepine (PBOX-6) induces apoptosis in MCF-7 cells. Caspase-7 activity could be increased from 3 to 5-fold more than the basal levels, signifying an activation of this caspase in MCF-7 cells treated with Phyllanthus extracts (Lee et al., 2011). Therefore, caspase-7 is indeed responsible for the proteolysis of these proteins during the terminal phase of apoptosis in caspase-3-deficient MCF-7 cells. Consistently with the previous study, caspase-9 cleaved procaspase-7 which declined after PPE treatment (Figure 4). Here, this study showed that caspase-9 as an initiator caspase activated procaspase-7 to caspase-7 and evaluated its activation after MCF-7 cells treated with PPE. Therefore, procaspase-9 was activated first, and then it was followed by procaspase-7. It was clearly in a time-dependent manner, 12-24 h after PPE treatment.

The enzyme poly (ADP-ribose) polymerase, or PARP, is an important DNA repair enzyme and was one of the first proteins identified as a substrate for caspases. The ability of PARP to repair DNA damage is prevented following cleavage of PARP by caspase-3 and caspase-7 (Walsh et al., 2008). Treatment of MCF-7 cells with PPE showed cleavage of the PARP, a biochemical hallmark of apoptosis and the processing of caspase-7 into a small active fragment (Figure 4). Similarly, PARP was an excellent substrate for caspase-7 in treated
caspase-3-deficient MCF-7 cells (Walsh et al., 2008). Therefore, it could be suggested that some cleavage of PARP occurred by compensation of caspase-7 in MCF-7 cells.

Conclusions

This study showed that PPE possessed an anticancer property. PPE could induce an antiproliferative effect, apoptotic morphology, and DNA fragmentation in caspase-3-deficient MCF-7 cells. Moreover, PPE could reduce the bcl-2 quantity and activate procaspase-9 to caspase-9. After that, caspase-9 could activate procaspase-7 to caspase-7, which induced it to be capable of cleaving certain substrates such as PARP. Degradation of PARP could reduce the repairing ability of the DNA. Therefore, the MCF-7 apoptosis by PPE administration may be possibly via the intrinsic pathway. Interestingly, PPE, a by-product of the pomegranate juice extraction procedure, might be added value as a natural anticancer source.

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References


