Induction of Apoptosis in SNU-16 Human Gastric Cancer Cells by the Chloroform Fraction of an Extract of Dangyuja (Citrus grandis) Leaves

Jeong Yong Moon¹, Hana Kim¹, Moonjae Cho², Weon Young Chang², Cheong-Tae Kim³, and Somi Kim Cho¹*⁴⁵

¹Faculty of Biotechnology, Cheju National University, Jeju 690-756, Republic of Korea
²Department of Medicine, Cheju National University, Jeju 690-756, Republic of Korea
³Food Safety Research Institute, Nong Shim Co., LTD, Seoul 156-709, Republic of Korea
⁴The Research Institute for Subtropical Agriculture and Biotechnology, Cheju National University, Jeju 690-756, Republic of Korea
⁵Subtropical Horticulture Research Institute, Cheju National University, Jeju 690-756, Republic of Korea

Received January 13, 2009; Accepted March 2, 2009

The anticancer effect of Dangyuja (Citrus grandis Osbeck) leaf extract was investigated using SNU-16 human gastric cancer cells. Maximum cytotoxicity was observed from the chloroform fraction (CF) of the extract. Cell death was dose-dependent (IC₅₀ = ca. 92.15 μg/mL), and was characterized by apoptotic body formation and DNA fragmentation. Flowcytometric analysis showed that treatment of CF resulted in a marked accumulation of cells in the sub-G1 phase. The induction of apoptosis was confirmed by caspase-3 activity assays and immunoblotting using antibodies against Bid, Bel-2, Bax, poly (ADP-ribose) polymerase, caspase-9, caspase-8, caspase-7, and caspase-3. Analyses of the CF by gas chromatography (GC) and GC-mass spectrometry tentatively identified 21 compounds, including linoleic acid, linolenic acid, palmitic acid, α-amyrin, γ-sitosterol, dihydrolanosterol, methyl palmitate, and nobiletin. These results provided the first evidence that the CF of a Dangyuja leaf extract induces apoptosis in SNU-16 cells. Our findings may lead to new strategies for the treatment of human gastric cancer.

Key words: apoptosis, Bel-2 family, caspase-3 activity, Dangyuja, SNU-16 human gastric cancer cell

There is growing interest in the use of plant materials for the treatment of various cancers and the development of safer and more effective therapeutic agents [Ramos et al., 2007]. Several studies have reported that citrus fruits have anticancer effects, including the reduced proliferation of some cancer cells [Arias et al., 2005] and the induction of apoptosis in human gastric and colon cancer cells [Kim et al., 2005]. Much is known about the cancer-preventing potential of the dietary bioactive compounds in citrus fruits: six flavanones including flavanone, 2′-OH flavanone, 4′-OH flavanone, 6-OH flavanone, naringin, and naringenin [Nobili et al., 2009], hesperidin [Tanaka et al., 1997; Yang et al., 1997; Kohno et al., 2001], and limonoids [Poulose et al., 2006]. Citrus grandis Osbeck, of the family Rutaceae, is widely cultivated in warm countries. In Korea, Dangyuja (Citrus grandis Osbeck) is cultivated mainly in Jeju Island, where it has long been used as an herbal medicine. In our previous report, we demonstrated that HF of the extract of immature Dangyuja fruits induces apoptosis in U937 human leukemia cells [Lim et al., 2009]. However, studies on the anticancer effects of citrus leaves are limited.

Apoptosis, an active physiological process resulting in the cellular self-destruction, is characterized by distinct morphologic changes, including cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies [Wyllie et al., 1997;
The Chloroform Fraction of *C. grandis* Leaves Induces Apoptosis

Khan et al., 1999. Initiation of apoptosis occurs through an extrinsic pathway and an intrinsic pathway [Fulda and Debatin, 2004; Jin and El-Deiry, 2005]. Bcl-2 family members and caspases play roles in controlling both pathways [Oltval et al., 1993]. Bcl-2 family proteins control the release of mitochondrial apoptogenic factors, which are associated with death proteases called caspases. Caspases, a class of cysteine proteases, play central roles in the apoptotic process that trigger a cascade of proteolytic cleavage events [Shah et al., 2003]. In the extrinsic (or the death receptor) pathway [Ashkenazi et al., 1998; Chen et al., 2002], procaspase-8 is recruited and cleaved into an active caspase-8, resulting in the caspase-3 activation and subsequently leading to the DNA fragmentation. In the intrinsic (or mitochondrial) pathway [Sun et al., 1999; Green et al., 2000], the activation of caspase-9 is triggered by the formation of apoptosome, further leading to the activation of caspase-3, an important downstream event in apoptosis [Earnshaw et al., 1999], and nuclear damage [Waxman and Schwartz, 2003].

Because cancer is characterized by proliferation disorder and apoptosis obstacle, the inhibition of proliferation and the induction of apoptosis have emerged as attractive approaches for tumor therapy [Thompson, 1995; Kinloch et al., 1999]. Many anti-cancer agents have been found to induce the cell cycle arrest [Kaufmann et al., 1995; Johnson et al., 1997; Bruna et al., 1999]. The therapeutic application of apoptosis is currently being considered as a model for the development of anti-tumor drugs [Hong et al., 2003]. To achieve a more effective use of resources, the ability of Dangyuja leaves to induce apoptosis was examined, and the biochemical mechanisms underlying apoptosis in SNU-16 human gastric cancer cells were investigated. Our results suggest that the chloroform fraction (CF) contained bioactive compounds capable of inducing apoptosis in SNU-16 cells.

**Materials and Methods**

**Reagents.** 'Dangyuja' leaves were obtained from the National Institute of Subtropical Agriculture, Jeju, Korea. RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA, fetal bovine serum (FBS), penicillin, streptomycin, Hoechst 33342 dye were purchased from Invitrogen Life Technologies Inc. (Grand Island, NY). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), RNase A, and caspase activity assay kits were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-Bax, -Bid, -caspase-3, -caspase-9, -cleaved caspase-7, and -PARP antibodies were purchased from Cell Signaling (Beverly, MA), and anti-Bcl-2 antibody was from Santa Cruz (Santa Cruz, CA). Anti-caspase-8 antibody was from R&D systems (Minneapolis, MN), and anti-β-actin antibody was from Sigma. BCA Protein Assay Kit was purchased from Pierce (Rockford, IL), and PVDF membranes were purchased from Millipore (Bedford, MA).

**Preparation of the leaf extracts.** Air-dried Dangyuja leaves were pulverized using a milling machine and extracted with 80% methanol by stirring for 3 days at room temperature (RT). The extract was filtered, concentrated with a vacuum rotary evaporator under reduced pressure, and lyophilized. The dried methanol extracts were then suspended in water (1 L) and further fractionated by additional extraction with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol in a stepwise manner [Moon et al., 2008]. Each solvent was extracted three times at room temperature (RT), evaporated, and then freeze-dried (Fig. 1). The resulting extract powder was dissolved in DMSO and diluted with PBS (pH 7.4) to the desired final concentration.

**Cell culture.** MCF-7, Hep-G2, HCT-15, NCI-H460, SK-N-SH, CCD-25Lu, and SNU-16 cells obtained from the American Type Culture Collection were maintained at 37°C in a humidified atmosphere under 5% CO₂ in RPMI 1640 or DMEM (Invitrogen Gibco BRL, Gaithersburg, MD) containing 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Exponentially growing cells were treated with various concentrations of the solvent fractions, as indicated in Fig. 2.
Cell viability assay. The effect of CF on the viability of various cancer cell lines was determined by the MTT-based assay [Carimichael et al., 1987]. Briefly, the exponential-phase cells were collected and transferred to microtiter plates (10^4 to 10^5 cells/well). The cells were then incubated for 4 days in the presence of various concentrations of CF. After incubation, 0.1 mg of MTT (Sigma) was added to each well. The plates were then incubated at 37°C for 4 h, centrifuged at 2,500 rpm for 20 min at RT, and the medium was carefully removed. DMSO (150 μL) was then added to each well to dissolve the formazan crystals. The plates were read immediately at 570 nm on a microplate reader (Tecan, Salzburg, Austria). The concentration at which growth was inhibited by 50% (the IC_{50} value) was determined in triplicate for each well.

Microscopic observation of cellular morphology and nuclear fragmentation. SNU-16 cells, placed in 6-well plates at 10^5 cells/mL, were treated with an aliquot of CF 5 h after plating. After 24 h, 10 μM of Hoechst 33342, a DNA-specific fluorescent dye, were added to the solution of each well, and the plates were incubated for 10 min at 37°C. The stained cells were then observed under an Olympus fluorescence microscope.

Flowcytometric analysis. For the cell cycle distribution analysis, SNU-16 cells (5 × 10^5 cells/well) were plated in 6-well plates, treated with CF (25-200 μg/mL) for 24 h. After treatment, the cells were collected, fixed in 70% ethanol, and washed in PBS containing 2 mM EDTA. They were then resuspended in 1 mL PBS containing 1 mg/mL RNase and 50 mg/mL propidium iodide, incubated in the dark for 30 min at 37°C, and analyzed by a FACS caliber flow cytometer (Becton Dickinson, San Jose, CA). Data from 10,000 cells were collected for each data file.

Analysis of DNA fragmentation. The SNU-16 cells (1 × 10^5 cells/mL) were treated with 25, 50, 100, and 200 μg/mL of CF for 24 h and then harvested. Genomic DNA was extracted in an extraction buffer (10 mM of Tris, pH 8.0, 0.1 M of EDTA). Sodium dodecyl sulfate was then added to 0.5%, and the mixture was incubated overnight with 0.5 μg/μL proteinase K at 50°C. The mixture was then extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and DNA was precipitated with 5 M NaCl and 2 volumes of absolute ethanol. Equal amount each of the extracted DNA (10 μg) was electrophoresed on a 1.8% agarose gel containing 0.1 μg/mL ethidium bromide and visualized under ultraviolet light.

Immunoblot analysis. After treatment with 25, 50, 100, and 200 μg/mL of CF, the SNU-16 cells were collected and washed twice with cold PBS. The cells were then lysed in a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM NaVO_3, 10 mM NaF, 1 mM DTT, 1 mM PMSE, 25 μg/mL aprotinin, and 25 μg/mL leupeptin) and kept on ice for 30 min. The lysates were then centrifuged at 13,000 × g at 4°C for 30 min, and the supernatants were stored at −70°C until use. The protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Aliquots of the lysates (60-100 μg of protein) were separated by 7.5-15% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, MA) using a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl, pH 8.8, and 20% methanol (v/v)]. After blocking with 5% nonfat dried milk, the membrane was incubated for 2 h with primary antibodies, followed by 30 min with the secondary antibodies in milk containing...
The Chloroform Fraction of *C. grandis* Leaves Induces Apoptosis

Tris-buffered saline and 0.1% Tween 20. Human anti-caspase-3, -cleaved caspase-7, -caspase-9, -PARP, -Bax, -Bid, -caspase-8, and -Bcl-2 antibodies at a 1:1,000 or 1:2,000 dilution and anti-β-actin antibody at 1:10,000 dilution were used as the primary antibodies. Horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG) (Vector Laboratories, Burlingame, CA) was used at a 1:5,000 dilution as the secondary antibody. The membrane was then exposed to the X-ray film. Protein bands were detected using the WEST-ZOL plus Western Blot Detection System (iNtRON, Seongnam, Korea).

**Caspase-3 activity assay.** Caspase-3 activity was assayed by using a commercially available kit (Sigma) according to the manufacturer’s protocol. SNU-16 cells were lysed with chilled lysis buffer after treatment with CF (25, 50, 100, and 200 μg/mL) for 24 h. The protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL). All mixtures were incubated overnight in a humidified environment at 37°C, and the concentration of the p-nitroaniline released from the substrate was measured with a microplate reader (Tecan, Salzburg, Austria) at 405 nm.

**GC and GC-MS analyses.** Gas chromatographic analysis of CF was carried out using an Agilent 6850 system (Santa Clara, CA) equipped with a flame ionization detector (FID) and an HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness). Injector and detector temperatures were maintained at 220 and 280°C, respectively. The column oven temperature was set at 100°C for injection and held for 5 min, programmed at 5°C/min to 200°C and held for 5 min, then at 5°C/min to 300°C, and finally held at 300°C for 10 min. Nitrogen was used as the carrier gas at 1.0 mL/min. Injection volume was 2 μL with a split ratio of 5:1. Quantitative data were obtained from FID area percent data. Gas chromatography (GC)-mass spectrometry (MS) analysis was performed using an Agilent 6890N gas chromatograph coupled to an Agilent 5975N mass spectrometer. An HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness) was used for the gas chromatographic separation of the analyte. GC condition was same as those used in the GC analysis mentioned above. Carrier gas (helium) was set at 1.0 mL/min with an inlet pressure of 10.48 psi. The MS instrument was operated in the electron impact mode with ionization energy of 70 eV. Transfer line was also set at 280°C, quadrupole temperature at 150°C, and source temperature at 230°C. The GC-MS peaks were identified by comparison with the data obtained from the GC chromatogram and the profiles from the Wiley 7th edited library data of the GC-MS system.

**Statistical analysis.** All results were expressed as the mean±standard deviation. One-way analysis of variance using SPSS v 12.0 software package was applied. All assays were performed in triplicate.

### Table 1. Inhibitory effects of the chloroform fraction on human cancer cell growth

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Characteristics</th>
<th>IC₅₀ (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNU-16</td>
<td>Stomach cancer cells</td>
<td>92.15</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast cancer cells</td>
<td>145.45</td>
</tr>
<tr>
<td>HepG2</td>
<td>Hepatoblastoma cells</td>
<td>157.82</td>
</tr>
<tr>
<td>HCT-15</td>
<td>Colon cancer cells</td>
<td>147.08</td>
</tr>
<tr>
<td>NCI-H460</td>
<td>Lung cancer cells</td>
<td>104.62</td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>Neuroblastoma cells</td>
<td>120.29</td>
</tr>
</tbody>
</table>

Cells were treated with CF for 4 days. IC₅₀ is defined as the concentration at which 50% decrease in cell numbers occurred.

---

**Fig. 3. Nuclear Hoechst staining and DNA fragmentation.**

A, cells were treated with DMSO or with the indicated concentration of CF for 24 h and then stained with Hoechst 33342. The stained nuclei were visualized under a fluorescence microscope. B, DNA fragmentation was analyzed by 1.8% agarose gel electrophoresis. The cells were incubated with the indicated concentration of the CF for 24 h.
Results and Discussion

Effect of CF on cell viability. Four organic solvents were used in a stepwise manner to extract the anticancer components from the methanol extract of Dangyuja leaves (Fig. 1). The effects of various concentrations of the resulting fractions n-hexane, CF, ethyl acetate, n-butanol, and aqueous residue on the growth of SNU-16 cells were examined by the MTT-based assay. Among the extracts tested, CF appeared to have the most potent effect. The percent viabilities of SNU-16 cells exposed to CF at 25, 50, 100, and 200 μg/mL were 78.3±7.9, 60.9±3, 22.5±0.4, and 21.5±1.1, respectively, compared with the controls. Moreover, the inhibition of cell growth was dose-dependent (Fig. 2A). We next investigated the effect of CF on the growth of the other cancer cell lines. The IC₅₀ values varied depending on the cell line, and the SNU-16 cells were the most susceptible to the CF-induced toxicity, whereas CCD-25Lu (normal lung fibroblast) cells were not (Fig. 2B, Table 1).

Induction of apoptosis by the CF. To elucidate whether the CF-induced decrease in viability was attributable to apoptosis, we performed nuclear staining with Hoechst 33342 and DNA fragmentation assays. Nuclear staining with Hoechst 33342, a fluorescent DNA-binding dye, revealed fragmented and condensed nuclei in the CF-treated cells in a dose-dependent manner (Fig. 3A). The cells treated with increasing concentrations of CF showed a progressive accumulation of the fragmented DNAs (Fig. 3B). Taken together, these data

Table 2. The percentage of SNU-16 cells in the sub-G1 fraction

<table>
<thead>
<tr>
<th>Concentration (μg/mL)</th>
<th>Time (h)</th>
<th>Sub-G1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25</td>
<td>5.25</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>11.38</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>37.15</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>77.34</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>7.94</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>15.56</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>21.99</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>23.38</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>37.15</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>77.34</td>
</tr>
</tbody>
</table>

The results are presented as the means±SD. *p<0.05 compared with the control.

Table 3. Compounds tentatively identified in the chloroform fraction of Dangyuja leaves

<table>
<thead>
<tr>
<th>No.</th>
<th>RT (min)</th>
<th>Compound</th>
<th>RC (%)</th>
<th>Homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25.423</td>
<td>(-)-Loliolide</td>
<td>2.95±0.22</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td>26.355</td>
<td>2H-1-Benzopyran-2-one, 7-hydroxy- (Coumarin, 7-hydroxy-)</td>
<td>1.73±0.18</td>
<td>68</td>
</tr>
<tr>
<td>3</td>
<td>27.362</td>
<td>Hexadecanoic acid, methyl ester (Methyl palmitate)</td>
<td>2.49±0.93</td>
<td>98</td>
</tr>
<tr>
<td>4</td>
<td>29.365</td>
<td>n-Hexadecanoic acid (Palmitic acid)</td>
<td>4.48±0.26</td>
<td>97</td>
</tr>
<tr>
<td>5</td>
<td>33.380</td>
<td>9,12-Octadecadienoic acid, (Z,Z)- (Linoleic acid)</td>
<td>13.28±2.05</td>
<td>93</td>
</tr>
<tr>
<td>6</td>
<td>34.610</td>
<td>9,12,15-Octadecatrienoic acid, (Z,Z,Z)- (Linolenic acid)</td>
<td>8.92±1.49</td>
<td>98</td>
</tr>
<tr>
<td>7</td>
<td>34.863</td>
<td>Borane, diethylmethyl-</td>
<td>1.39±0.12</td>
<td>83</td>
</tr>
<tr>
<td>8</td>
<td>38.824</td>
<td>9-Octadecenamide, (Z)- (Oleic acid amide)</td>
<td>1.55±0.46</td>
<td>99</td>
</tr>
<tr>
<td>9</td>
<td>41.554</td>
<td>Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (Palmitin, 2-mono-)</td>
<td>1.52±0.06</td>
<td>86</td>
</tr>
<tr>
<td>10</td>
<td>42.079</td>
<td>Dixooyl-phthalate</td>
<td>1.39±0.23</td>
<td>80</td>
</tr>
<tr>
<td>11</td>
<td>44.628</td>
<td>9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z) (Ethyl linoleolate)</td>
<td>2.40±0.26</td>
<td>87</td>
</tr>
<tr>
<td>12</td>
<td>50.610</td>
<td>Vitamin E</td>
<td>1.65±0.17</td>
<td>95</td>
</tr>
<tr>
<td>13</td>
<td>52.097</td>
<td>Glucose-quinol</td>
<td>1.44±0.25</td>
<td>59</td>
</tr>
<tr>
<td>14</td>
<td>52.660</td>
<td>Campesterol</td>
<td>1.40±0.06</td>
<td>53</td>
</tr>
<tr>
<td>15</td>
<td>53.019</td>
<td>(E)-23-ethylcholesta-5,22-dien-3.beta.-ol</td>
<td>1.08±0.14</td>
<td>59</td>
</tr>
<tr>
<td>16</td>
<td>53.902</td>
<td>Gamma-Siosterol</td>
<td>3.64±0.06</td>
<td>99</td>
</tr>
<tr>
<td>17</td>
<td>54.340</td>
<td>Lanost-8-en-3-ol, (3.beta.); Dihydrolanosterol</td>
<td>2.74±0.09</td>
<td>90</td>
</tr>
<tr>
<td>18</td>
<td>54.942</td>
<td>3',4',5,6,7,8-Hexamethoxyflavone; Nobiletin</td>
<td>2.37±0.12</td>
<td>90</td>
</tr>
<tr>
<td>19</td>
<td>55.535</td>
<td>Vimalanol; .alpha.-Amyrin</td>
<td>3.73±0.11</td>
<td>70</td>
</tr>
<tr>
<td>20</td>
<td>55.953</td>
<td>Vitamin E; .alpha.-Tocopherol</td>
<td>1.95±0.29</td>
<td>84</td>
</tr>
<tr>
<td>21</td>
<td>58.229</td>
<td>N-ethyl-1,3-dithiosioindoline</td>
<td>1.26±0.43</td>
<td>43</td>
</tr>
</tbody>
</table>

aRetention time (min)

bCompounds are listed in increasing order of the retention time.

cRelative area percentage (peak area relative to the total peak area %)
The Chloroform Fraction of *C. grandis* Leaves Induces Apoptosis

**Effects of CF treatment on cell cycle progression.** We also quantified the cells in the sub-G1 population after CF treatment as the apoptosis index. Treatment with CF resulted in a concentration- and a time-dependent increase in the sub-G1 cell population (Table 2). The treatment with CF for 24 h increased the percentage of viable cells in the sub-G1 phase from 2.10 (0 μg/mL) to 11.38% (50 μg/mL), and at the same duration of time with 100 μg/mL of CF, the sub-G1 population further increased to 37.15%. The cells treated with 100 μg/mL of CF for a longer time period resulted in an obvious increase of the sub-G1 phase population, which increased from 7.94 (0 h) to 43.40% (24 h) in a time-dependent manner. Thus, these results showed that the inhibition of growth observed in response to CF is associated with the sub-G1 arrest of the cell cycle.

**Western blot analysis of apoptosis-related proteins.** To determine the mechanism of CF-induced apoptosis, the expressions of anti- and pro-apoptotic proteins following the CF treatment was examined by immunoblotting. The expression of Bcl-2 was significantly inhibited in a concentration-dependent manner, whereas that of Bax was relatively constant in the CF-treated SNU-16 cells (Fig. 4A). Our results suggested that CF induced apoptosis by shifting the Bax/Bcl-2 ratio in favor of apoptosis. Western blotting results also showed that after the SNU-16 cells were treated with CF for 24 h, the expressions of procaspase-3 and -9 decreased significantly, indicating the involvement of the mitochondrial pathway in the CF-induced apoptosis. Moreover, the gradual activation of caspase-8 suggested that the CF-induced apoptosis may also involve an extrinsic death pathway. CF also increased the expression of active subunits of caspases-7, as well as the proteolytic cleavage of Bid and PARP in a concentration-dependent manner. The caspase-3 activity was also increased by CF in a dose-dependent manner (Fig. 4B). These results indicated that the apoptotic effects of the CF in SNU-16 cells are associated with the increases in the Bax/Bcl-2 ratio and the caspase activation.

**Compositional analysis of CF.** The CF composition of Dangyuja leaves was analyzed by GC-FID and GC-MS. Twenty-one compounds were tentatively identified: linoleic acid (13.28%), linolenic acid (8.92%), palmitic acid (4.48%), α-amyrin (3.73%), γ-sitosterol (3.64%), (-)-loliolide (2.95%), dihydrolanosterol (2.74%), methyl palmitate (2.49%), 9,12,15-octadecatrienoic acid, ethyl ester (2.40%), 3',4',5,6,7,8-hexamethoxyflavone (2.37%), vitamin E (1.65%), glaucine-quinol (1.44%), and campesterol (1.40%) (Table 3). Linoleic acid, linolenic acid, palmitic acid, and methyl palmitate, shown to possess anticancer activity [Phoon et al., 2001], were present in large amounts in CF [Nano et al., 2003; Kwon et al., 2008]. In addition, the apoptotic activity of CF could be attributed to the presence of nobiletin, which has been shown to possess anti-proliferation, anti-inflammatory,
and anti-tumorigenic activities [Akao et al., 2008; Ho and Lin, 2008; Luo et al., 2008]. Furthermore, β-sitosterol has been identified as a potential candidate for cancer chemotherapy [Moon et al., 2008]. However, not much is known about the apoptotic activity of γ-sitosterol, which is the 24S-epimer of β-sitosterol. In our previous work, we showed that the n-hexane fraction (HF) of an immature Dangyujia fruit extract inhibited the proliferation of U937 cells (histiocytic lymphoma) via the induction of apoptosis [Lim et al., 2009]. SNU-16 cells were much less susceptible to HF of an immature Dangyujia fruit extract than U937 cells. Comparison of the composition of CF and HF revealed several compounds common in both fractions, whereas linoleic acid, linolenic acid, nobiletin, and α-amyrin were identified only in CF of Dangyujia leaves. The possibility that various phytochemicals in each fraction could interact among themselves, resulting in a synergistic or cancelling effect on their anticancer action, is still open. We are currently exploring the interactive effects of these phytochemicals and the potential of these compounds as therapeutic agents for the prevention of cancer.

In conclusion, our study showed that CF inhibited SNU-16 human gastric cancer cell proliferation caused by the apoptosis through demonstration of the induction of caspase-3 activity and the observation of cells containing fragmented nuclei and DNA. Additional studies are needed to determine the molecular mechanisms of the active components and to evaluate the potential in vivo anticancer activity of the extract.

Acknowledgment. This study was supported by the Technology Development Program of the Ministry of Agriculture and Forestry, Republic of Korea, and the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2007-712-J0502).

References

Arias BA and Ramon-Laca L (2005) Pharmacological prop-
erties of citrus and their ancient and medieval uses in the Mediter-
anean region. J Ethnopharmacol 97, 89-95.
Ashkenazi A and Dixit VM (1998) Death receptors: signal-
ing and modulation. Science 281, 1305-1308.
Bruna P, Lorenza B, Marco T, Valeria M., Gerry M, and
Antonio G (1999) Pachitaxel induces apoptosis in Saos-2 cells with CD95L upregulation and Bel-2 phosphoryla-
Carimichael J, Degnaff WG, Gazdar AF, Minna JD, and
signaling pathways. Apoptosis 7, 313-319.
Ho SC and Lin CC (2008) Investigation of heat treating
Jin Z and El-Deiry WS (2005) Overview of cell death sig-
naling pathways. Cancer Biol Ther 4, 139-163.
Johnson N, Ng TTC, and Parkin JM (1997) Camptothecin
causes cell cycle perturbations within T-lymphoblastoid cells followed by dose-dependent induction of apopto-
sis. Leukemia Res 21, 961-972.
Kaufmann WK, Levedakou EN, Grady HL, Pauls RS, and
Khan MR and Mlungwana SM (1999) γ-Sitosterol, a cyto-
toxic sterol from Markhamia zanzibarica and Kigelia
africana. Fitoterapia 70, 96-97.
blanco induces apoptosis in human gastric cancer cells
Kinloch RA, Treherne JM, Fumess LM, and Hajimolah-
Pharmacol Sci 20, 35-42.
Kohno H, Taita M, Sumida T, Azuma Y, Ogawa H, and
Tanaka T (2001) Inhibitory effect of mandarin juice rich in beta-cryptoxanthin and hesperidin on 4-(methylnitro-
samino)-1-(3-pyridyl)-1-butanone-induced pulmon-
Induction of apoptosis by linoleic acid is associated with
the modulation of Bel-2 family and Fas/FasL system and activation of caspases in AGS human gastric adenocarci-


