Master's Thesis

Induction of apoptosis in SNU-16 human gastric cancer cells by the chloroform fraction of extract of *dangyuja (Citrus grandis* Osbeck) and guava (*Psidium guajava* L.) leaves

Department of Biotechnology

GRADUATE SCHOOL
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당유자 잎과 구아바 잎 Chloroform 분획물에 의한 human 위암 세포주인 SNU-16 세포에서의 apoptosis 유도

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ABSTRACT

The anticancer effect of dangyuja (*Citrus grandis* Osbeck) and guava (*Psidium guajava* L.) leaves extract was investigated using SNU-16 human gastric cancer cells. Maximum cytotoxicity was observed using the chloroform fraction (CF) of the extract. Cell death was dose-dependent and was characterized by apoptotic body formation and DNA fragmentation. Flow cytometric analysis showed that treatment of chloroform fraction of dangyujaleaves (CFD) and chloroform fraction of guava leaves (CFG) resulted in a marked accumulation of cells in sub-G1 phase. The induction of apoptosis was confirmed by caspase-3 activity assays and by immunoblotting using antibodies against Bid, Bcl-2, Bax, poly (ADP-ribose) polymerase (PARP), caspase-9, caspase-8, caspase-7, and caspase-3. Together, our results provide the first evidence that the CFD and CFG extracts induces apoptosis in SNU-16 cells. Our findings may lead to new strategies for the treatment of human gastric cancer.

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1. Introduction

Although many anticancer agents have been developed, unfavorable side effects and resistance are serious problems (Panchal, 1998). Thus, 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, 2007). Several studies have reported that citrus fruits have anticancer effects, including the reduced proliferation of some cancer cells (Arias and Ramon-Laca, 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 (Poulose et al., 2006). However, the studies reporting upon the anticancer effects of *citrus* leaves are limited. *Psidium guajava* L. is commonly known as guava, guyava and kuawa. It ranges widely in subtropical climatic region all over the world as subtropical plant belonging to Myrtaceae. It has been claimed to be useful in the treatment of diarrhea, dysentery, acute gastrointestinal inflammation, and antidiabetic effects (Oh et al., 2005). However, the studies reporting upon the anticancer effects of guava leaves are also limited. In order to achieve a more effective use of resources, we investigated the ability of *dangyuja* and guava leaves to induce apoptosis in SNU-16 human gastric cancer cells.

Apoptosis is one of the most important mechanisms for initiating cell death and deregulation of apoptosis contributes to a variety of diseases, especially cancer (Moon et al., 2007). The therapeutic application of apoptosis is currently being considered as a model for the development of anti-tumor drugs (Hong et al., 2003). Apoptosis is an active physiological process resulting in cellular self-destruction that involves specific morphological and biochemical changes in the nucleus and cytoplasm (Khan and Mlungwana, 1999). It is characterized by distinct morphologic changes, including cell

shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies (Buttke and Sandstrom, 1994; Wyllie, 1997). Bcl-2 family proteins determine whether a cell lives or dies by controlling the release of mitochondrial apoptogenic factors, which are associated with death proteases called caspases (Oltval et al., 1993). Caspases, a class of cysteine proteases, are central players in the apoptotic process that trigger a cascade of proteolytic cleavage events (Shag et al., 2003). There are two well-studied signal pathways for activation of caspases. In the extrinsic or death receptor pathway (Ashkenazi and Dixit, 1998; Chen and Wang, 2002) procaspase-8 is recruited and cleaved into active caspase-8 resulting in caspase-3 activation and subsequently leading to DNA fragmentation. In the intrinsic or mitochondrial pathway (Green, 2000; Sun et al., 1999) the activation of caspase-9 is triggered by the formation of apoptosome, further leading to caspase-3 activation and nuclear damage (Waxman and Schwartz, 2003). The activation of caspase-3 is an important downstream event in apoptosis (Earnshaw et al., 1999).

To determine the species' antiproliferative activity, we examined the effects of various leaf solvent fractions on cell viability using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based assay. Hoechst 33342 staining, analyses of DNA fragmentation, and cell cycle analysis confirmed the induction of apoptosis. The expression of the anti-apoptotic protein Bcl-2 and of the pro-apoptotic proteins Bax, Bid, caspase-8, caspase-7, caspase-3 and PARP was assessed by immunoblotting. Our results suggest that the CFD and CFG are capable of inducing apoptosis in SNU-16 human gastric cancer cells via both extrinsic and intrinsic pathways.

2. Materials and Methods

2. 1. Reagents

Dangyuja was obtained from the National Institute of Subtropical Agriculture in Jeju Province, Korea. *Psidium guajava* L. (guava) was purchased in our laboratory that was cultivated in Jeju Province, Korea. Quercetin (Sigma, MO, USA), RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA, fetal bovine serum (FBS), penicillin, streptomycin, Hoechst 33342 dye, and Trizol were purchased from Invitrogen Life Technologies Inc. (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), MTT, PI, RNase A, and caspase activity assay kits were purchased from Sigma Chemical Co. (St. Louis, MO, USA). An Annexin V-FLOUS Staining Kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Anti-Bcl-2, -Bax, -cleaved caspase-3, -caspase-7, -PARP, and -β-actin antibodies were purchased from Cell Signaling (Danvers, MA, USA) and anti-caspase-8 was from R&D system (MN, USA). A Bradford Protein Assay Kit and polyvinylidene fluoride (PVDF) membranes were purchased from Bio-Rad (Hercules, CA, USA).

2. 2. Preparation of the leaf extracts

Air-dried *dangyuja* and guava 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. Each extract powder was dissolved in DMSO and diluted with phosphate-buffered saline (PBS, pH 7.4) to the desired final concentration.

2. 3. Cell culture

SNU-16 cells were maintained at 37°C in a humidified atmosphere under 5% CO_2 in RPMI 1640 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.

2. 4. Cell viability assay

The effect of the CFD and CFG on the viability of various cancer cell lines was determined by an MTT-based assay (Hansan et al., 1989). Briefly, exponential-phase cells were collected and transferred to a microtiter plate. The cells were then incubated for 72 hours in the presence of various concentrations of the CFD and CFG. After incubation, 5 mg/mL of MTT solution (Sigma, MO, USA) was added to each well and the cells were incubated at 37°C for 4 h. The plates were then 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 Sunrise microplate reader (Sunrise, Tecan, Salzburg, Austria). The concentration at which growth was inhibited by 50% (the IC_{50} value) was determined in triplicate for each well.

2. 5. Microscopic observation of cellular morphology

SNU-16 cells, placed in 6-well plates at 10^5 cells/mL, were treated with an aliquot of the CFD and CFG 5 hours after plating. After 24 h, 10 μM of Hoechst 33342 (Lizard et al., 1997) a DNA-specific fluorescent dye, were added to the solution in each well and the plates were incubated for 10 min at 37°C. The stained cells were then observed under an Olympus fluorescence microscope.

2. 6. Analysis of DNA fragmentation

The SNU-16 cells (1×10^5 cells/mL) were treated with 25, 50, 100 and 200 μg/mL of the CFD and CFG 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 for overnight with 0.5 μg/μL proteinase K at 50℃. The mixture was then extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and the DNA was precipitated with 5 M of sodium chloride and 2 volumes of absolute ethanol. Equal amount of the extracted DNA (10 μg) was electrophoresed on a 1.8% agarose gel containing 0.1 μg/mL ethidiumbromide and visualized under ultraviolet light.

2. 7. Flowcytometric analysis

To determine cell cycle distribution analysis, 1×10^5 cells/mL were plated in 6 well plate, treated with the CFD and CFG (25~200 μg/mL) for 24 h. After treatment, the cells were collected, fixed in 70% ethanol, washed in PBS (2mM EDTA), 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 FACS caliber flow cytometry (Becton Dickinson, USA). Data from 10,000 cells were collected for each data file.

2. 8. Immunoblot analysis

After treatment, the cells were collected and washed twice with cold PBS. The cells were then lysed in 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 PMSF, 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; the supernatants were stored at -70°C until use. The protein concentration was determined by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Aliquots of the lysates (60-100 μg of protein) were separated by 7.5-15% SDS-PAGE and transferred onto a polyvinylidine difluoride (PVDF) membrane (Bio-RAD, HC, USA) 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 secondary antibodies in milk containing Tris-buffered saline (TBS) and 0.1% Tween 20. Human anti-caspase-3, -caspase-7, -caspase-8, -PARP, - Bax (Cell signaling, MA, USA) and -Bcl-2 (Santa cruz, MA, USA) antibodies were used at a 1:1,000 or 1:2,000 dilution as the primary antibodies while horseradish peroxidase-conjugated goat anti-human IgG (Vector Laboratories, Burlingame, CA, USA) was used at a 1:5,000 dilution as the secondary antibody. The membrane was then exposed to X-ray film. Protein bands were detected using the WEST-ZOL® plus Western Blot Detection System (iNtRON, Gyeonggi-do, Korea).

2. 9. 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 the CFD and CFG (25, 50, 100 and 200 μg/mL) for 24 h. The protein concentration was measured using BCA protein assay kit. All mixtures were incubated overnight in a humidified environment at 37°C, and the concentration of the ρ-nitroanyline (ρ-NA) released from the substrate was measured with a Sunrise microplate reader at 405 nm.

2.10. GC and GC-MS analysis

Gas chromatographic analysis of chloroform fraction was carried out with Agilent 6850 system equipped with a flame ionization detector (FID) and HP-5MS capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness). Injector and detector temperature were maintained at 220°C and 280°C, respectively. The column oven temperature was set at 100°C for injection (held for 5 min), then programmed at 5°C /min to 200°C (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 carrier gas with flow rate of 1.0 mL/min. Injection volume was 2 μL with split ratio of 5:1. Quantitative data were obtained from FID area percent data. GC-MS analysis was performed using Agilent 6890N gas chromatography coupled to Agilent 5975N mass spectrometer. A HP-5MS capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness) was used for gas chromatographic separation of the analyte. GC condition was same as those used in the GC analysis above. Carrier gas (helium) was set at a flow rate of 1.0 mL/min with an inlet pressure of 10.48 psi. The MS instrument was operated in the electron impact (EI) mode with an 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 data from the GC chromatogram and the profiles from the with Wiley 7th ed.

2.11. Statistical analysis

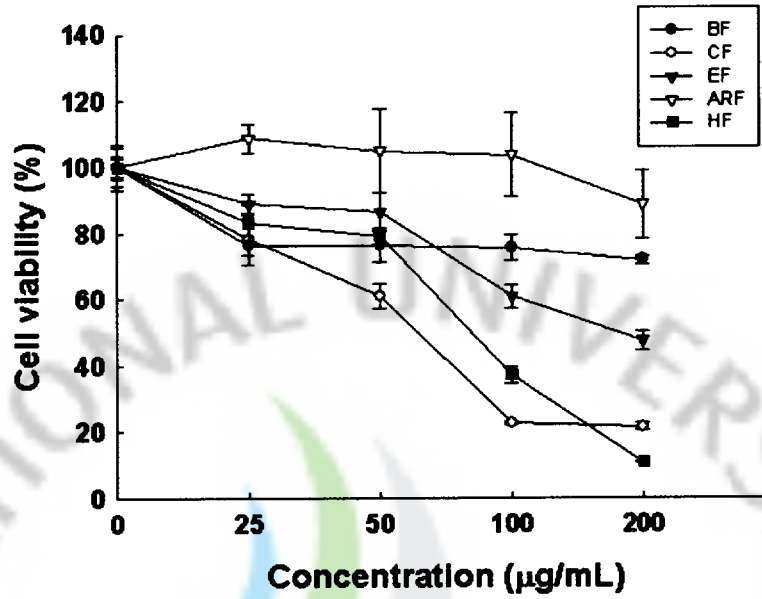
All results were expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) using SPSS v 12.0 software package was applied. A difference at $p < 0.01$ was considered to be statistically significant. All assays were performed in triplicate.

3. Results and Discussion

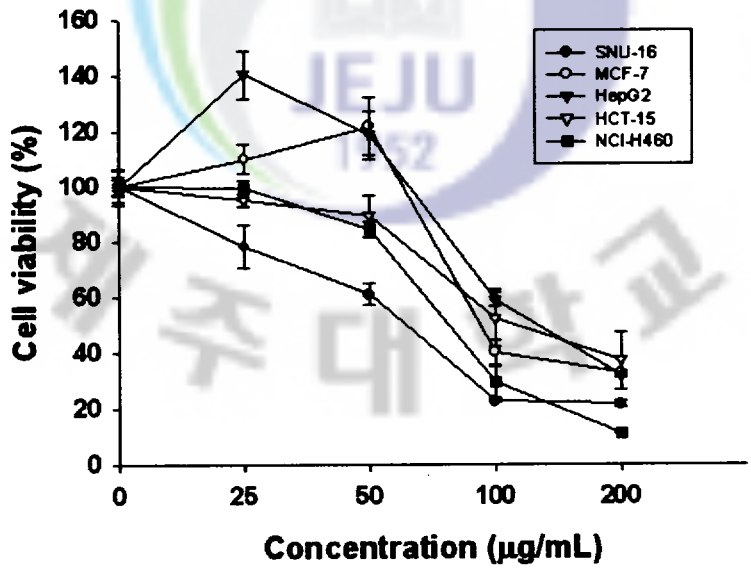
3. 1. Effect of the CF on cell viability

Four organic solvents were used in a stepwise manner to extract the anticancer components from a methanol extract of *dangyuja* and guava leaves. The effect of various concentrations of the resulting fractions (*n*-hexane [HF], chloroform [CF], ethyl acetate [EF], *n*-butanol [BF], and aqueous residue [ARF]) on the growth of SNU-16 cells was subsequently examined by a MTT-based assay. Among the extracts tested, the CF appeared to be most potent (The HF of guava leaves was cytotoxicity to normal lung fibroblast cells). The percent viability of SNU-16 cells exposed to the CFD and CFG at 25, 50, 100, and 200 μg/mL was decreased compared with the controls; moreover, the inhibition of cell growth was dose-dependent (Fig. 1A, 2A). We also investigated the effect of CFs on the growth of other cancer cell lines. Each cell line responded differently to treatment with the CFD (Fig.1B) and CFG (Fig.2B). SNU-16 cells were the most susceptible to CFs-induced cell toxicity whereas CCD-25Lu cells, normal lung fibroblast, was not (Fig. 1C, 2C). Quercetin, the major representative of the flavonol subclass, was included as a positive control as it has been shown to inhibit tumor development in animal colon cancer models (Deschner et al., 1993; Yang et al., 2000; Gee et al., 2002). The percent viability of SNU-16 cells exposed to the quercetin at 25, 50, 100, and 200 μM was 77.59, 54.02, 37.99 and 27.37, respectively (Fig. 3).

A


BF
CF
EF
ARF
HF
Cell viability (%)
140
120
100
80
60
40
20
0
0
25
50
100
200
Concentration (μg/mL)

B


SNU-16
MCF-7
HepG2
HCT-15
NCI-H460
Cell viability (%)
160
140
120
100
80
60
40
20
0
0
25
50
100
200
Concentration (μg/mL)

C

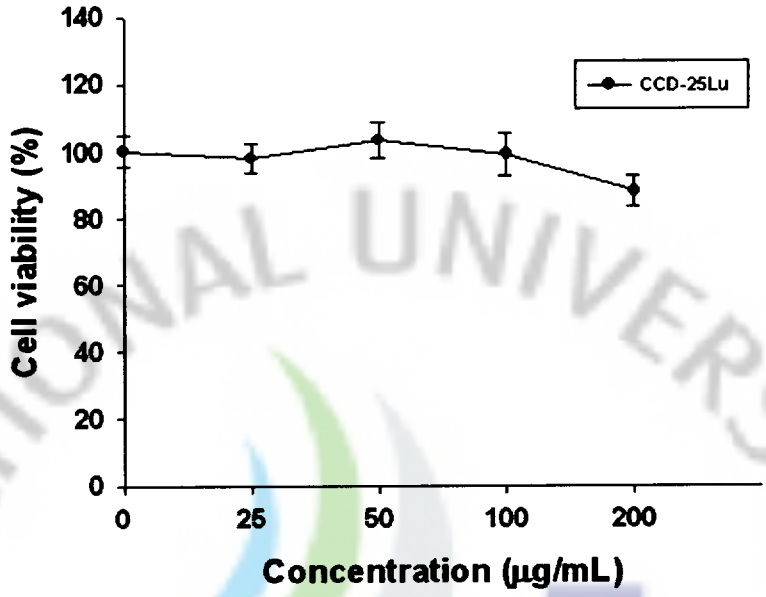
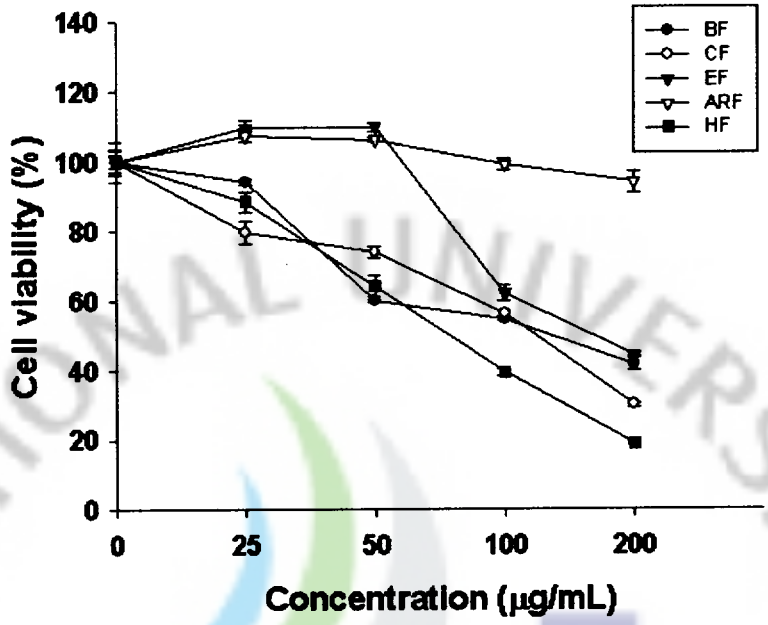
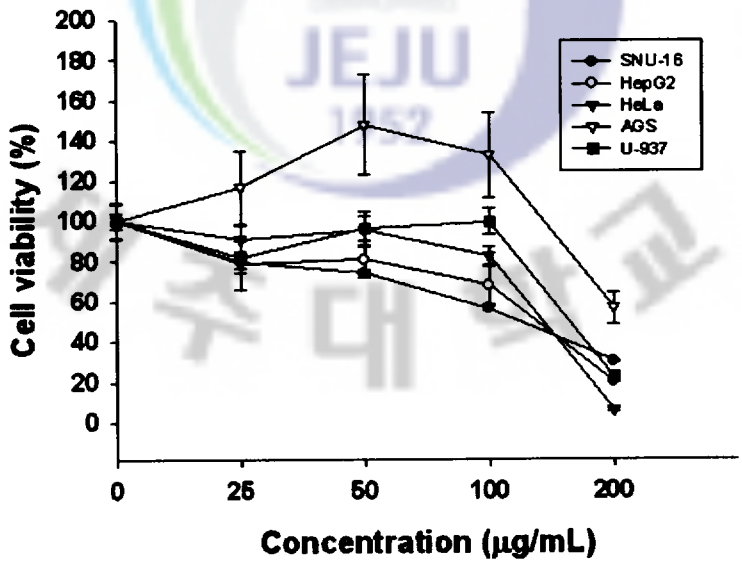


Figure 1. Growth inhibition of CFD in SNU-16 human gastric cancer cells. The various concentration of solvent fractions of *dangyuja* leaves (A). Effect of chloroform fraction on different cells: ●, SNU-16 (human gastric cancer cells); ○, MCF-7 (breast cancer cells); ▼, HepG2 (hepatoblastoma cells); ▽, HCT-15 (colon cancer cells); ■, NCI-H460 (Lung cancer cells) (B). Normal cells treated with chloroform fraction of *dangyuja* leaves (C). The values presented are the means ± SD (n=4).

A


Cell viability (%)
140
120
100
80
60
40
20
0
0
25
50
100
200
Concentration (μg/mL)
BF
CF
EF
ARF
HF

B


Cell viability (%)
200
180
160
140
120
100
80
60
40
20
0
0
25
50
100
200
Concentration (μg/mL)
SNU-16
HepG2
HeLa
AGS
U-937

C

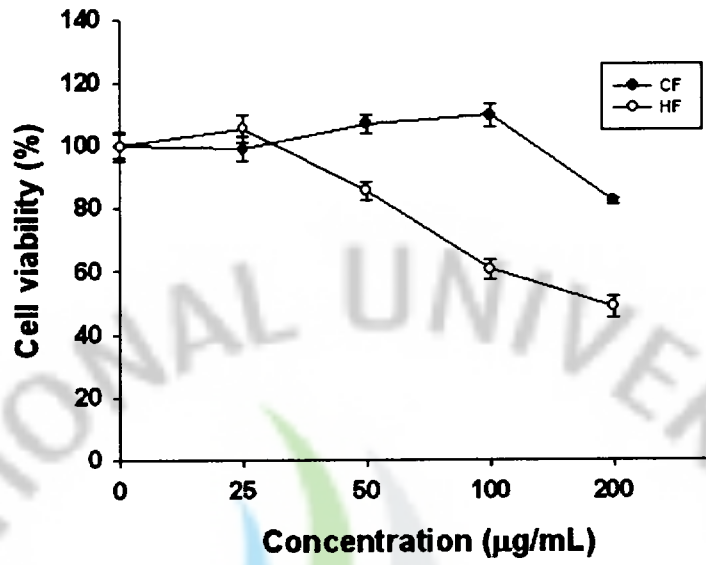


Figure 2. Growth inhibition of CFG in SNU-16 human gastric cancer cells. The various concentration of solvent fractions of guava leaves (A). Effect of chloroform fraction on different cells: ●, SNU-16 (human gastric cancer cells); ○, HepG2 (hepatoblastoma cells); ▼, HeLa (human cervical carcinoma); ▽, AGS (human gastric adenocarcinoma cells); ■, U-937 (human leukemia cells) (B). Normal cells treated with chloroform fraction (CF) and hexane fraction (HF) of guava leaves (C). The values presented are the means ± SD (n=4).

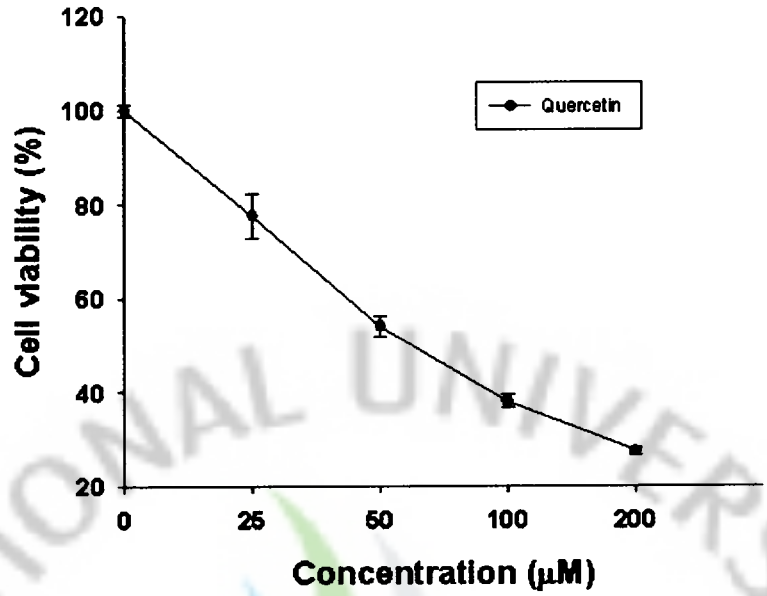
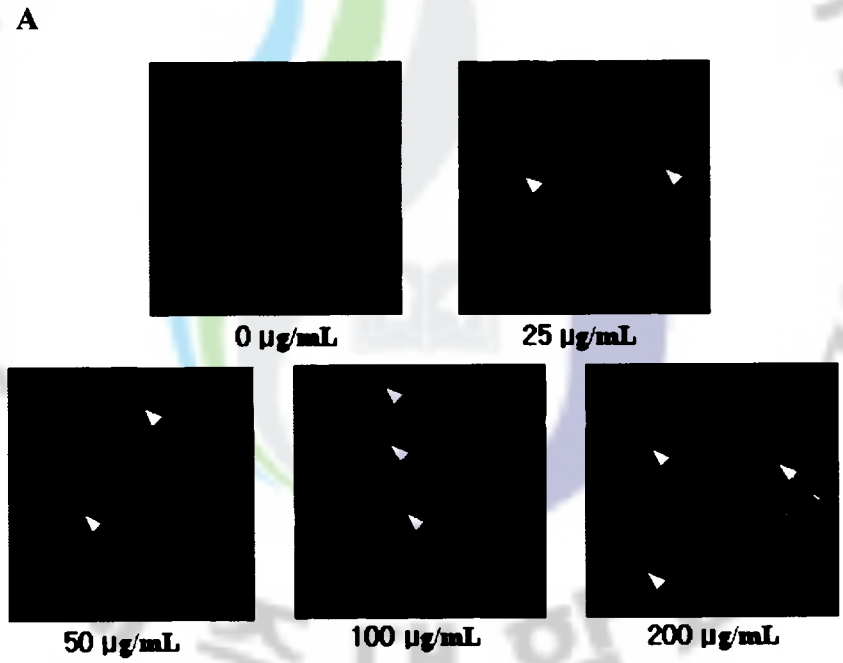


Figure 3. Growth inhibition of quercetin in SNU-16 human gastric cancer cells. SNU-16 cells treated with various concentration of quercetin. The values presented are the means ± SD (n=4).

3. 2. Induction of apoptosis by the CFD and CFG

To elucidate whether the CFD and CFG-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 CFD-, CFG-treated cells in a dose-dependent manner (Fig. 4). The cells treated with increasing concentrations of the CFD and CFG showed a progressive accumulation of fragmented DNA (Fig. 5). Taken together, these data indicate that the CFD and CFG induced apoptosis in SNU-16 cells.



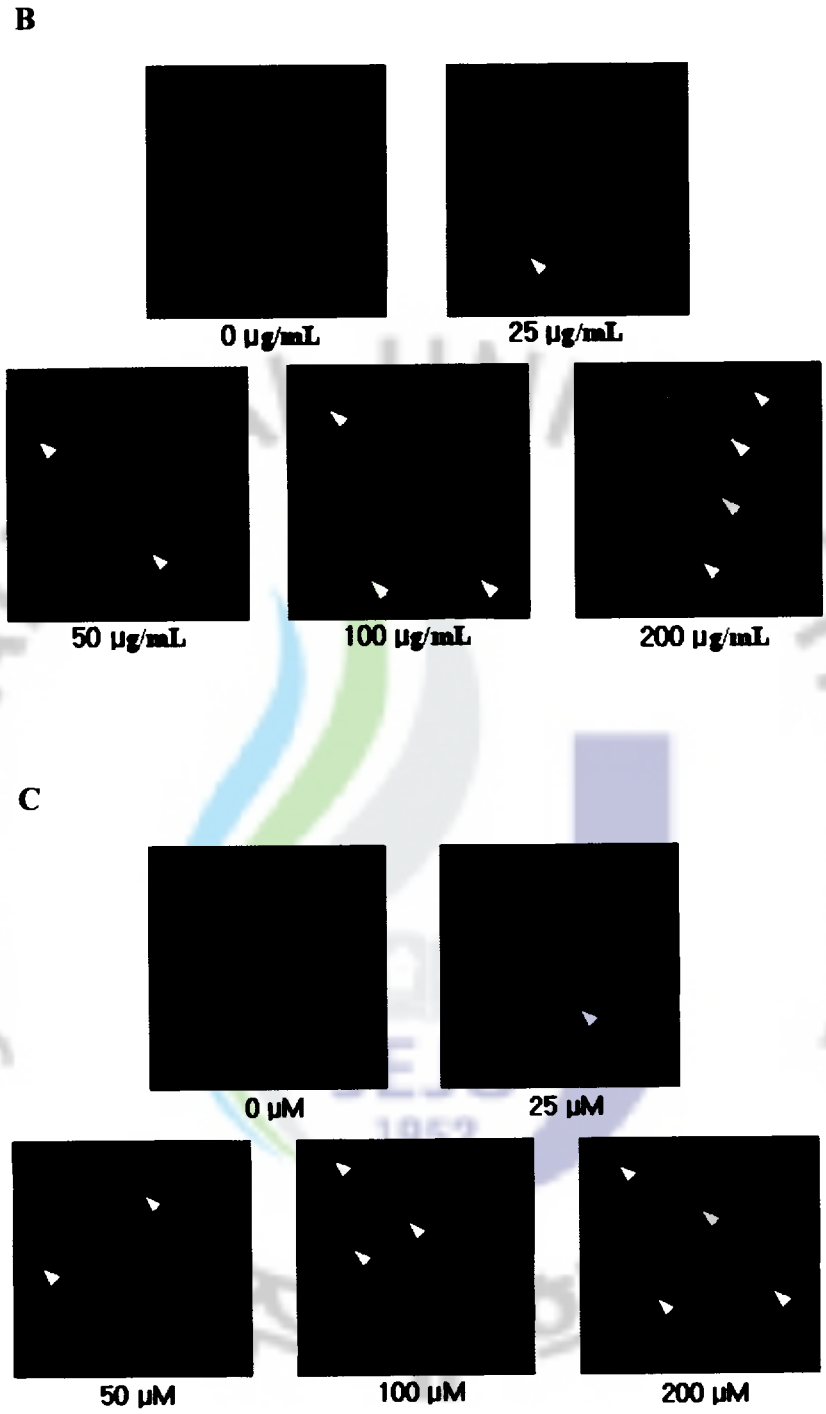


Figure 4. Nuclear Hoechst staining. Cells were treated with various concentration of the CFD (A), CFG (B) and quercetin (C) for 24 h then stained with Hoechst 33342. The stained nuclei were visualized under a fluoresce microscope.

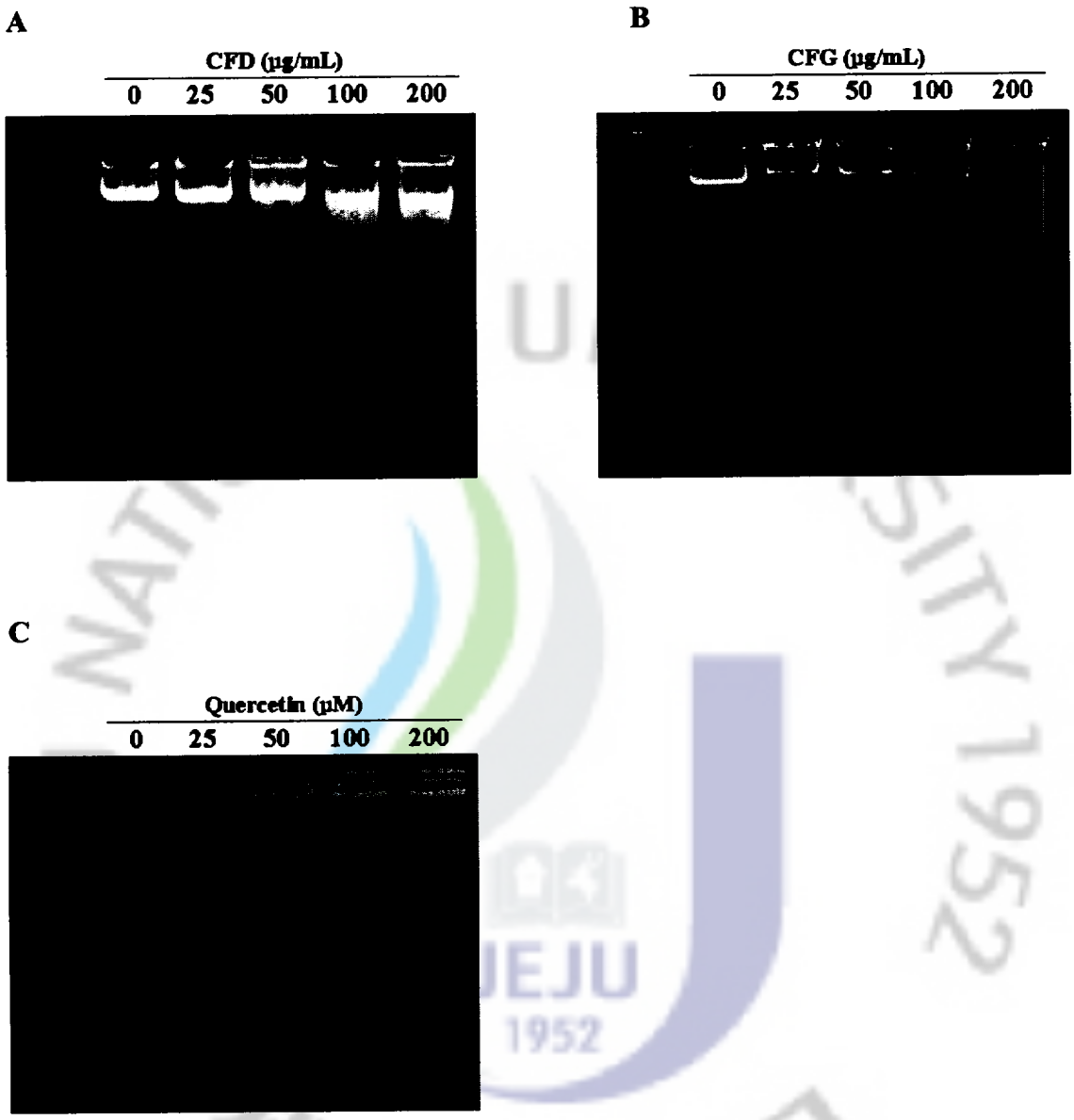


Figure 5. Induction of DNA fragmentation in CFD-, CFG-treated SNU-16 cells. The cells were incubated with the indicated concentration of the CFD (A), CFG (B) and quercetin (C) for 24 h. Genomic DNA samples were prepared and analyzed by 1.8% agarose gel electrophoresis.

3. 3. Effects on cell cycle progression

Cell cycle control is the major regulatory mechanism of cell growth (Gamet-Pavrastre at al., 2000). Many cytotoxic agents and/or DNA damaging agents arrest the cell cycle at the G1, S or G2/M phase and then induced apoptotic cell death. The cell cycle check-point may function to ensure the cells have time for DNA repair (O'Connor et al., 1993). The fractions of the cells in G0/G1, S, and G2/M phase were analyzed using a cell cycle analysis software. The sign of apoptosis was indicated by the accumulation of sub-G1 population in SNU-16 cells after CFs treatment. For example, 200 μg/mL CFD treatment for 24 h resulted in an increase in the percentage of cells in the sub-G1 phase from 2.1 to 77.3%. We quantified the cells in the sub-G1 population after CFD and CFG treatment as the apoptosis index. Treatment with CFD and CFG resulted in a dose-dependent increase in the sub-G1 cell population (Fig. 6). The treatment with CFD and CFG for 24 h increased the percentage of cells in sub-G1 phase, respectively. Thus, these results show that the inhibition of growth observed in response to CFD and CFG is associated with the sub-G1 arrest of the cell cycle.

A

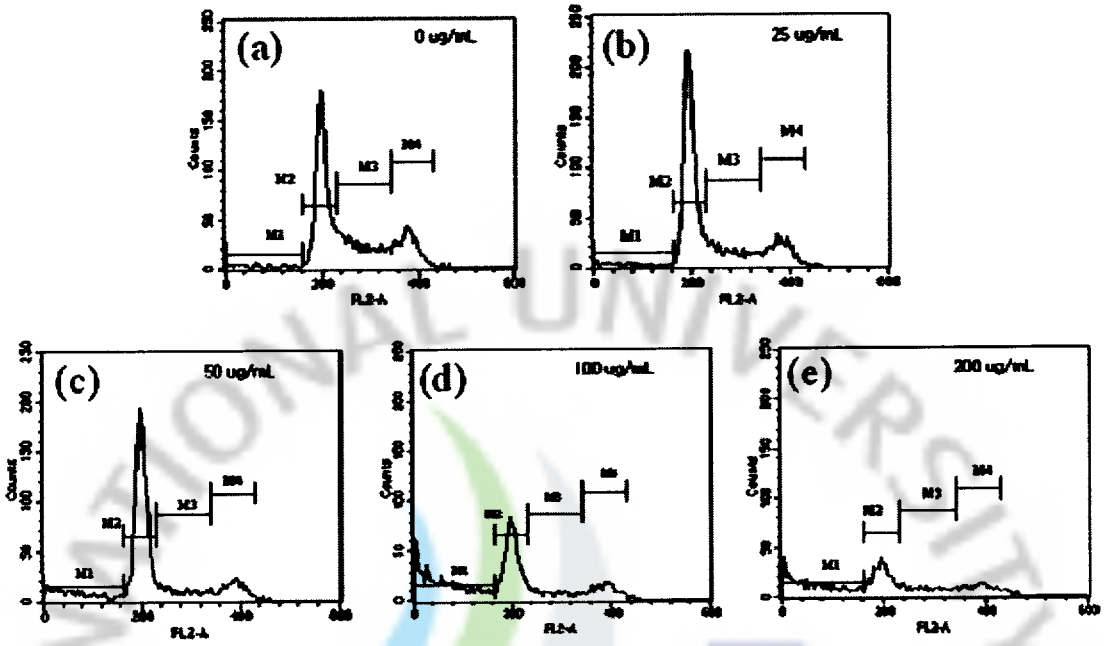


Table 1. Determine of sub-G1 ratio in CFD-treated SNU-16 cells.

Concentration (μg/mL)	0	25	50	100	200
Sub-G1 (%)	2.1±0.1	5.3±1.2	11.4±0.5	37.2±3.2	77.3±7.9

B

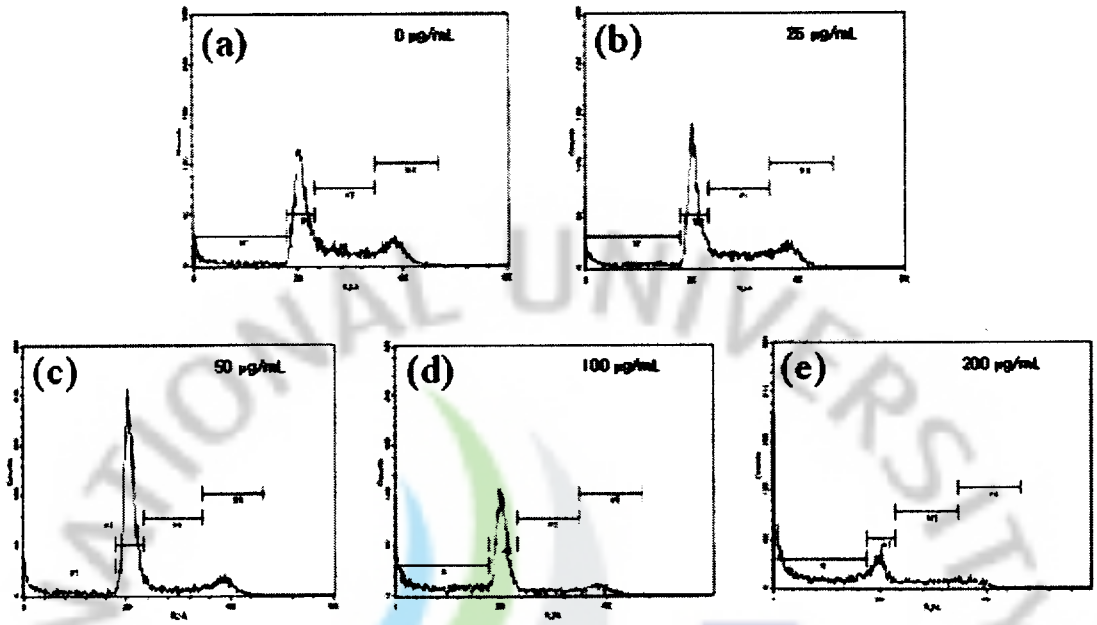


Table 2. Determine of sub-G1 ratio in CFG-treated SNU-16 cells.

Concentration (μg/mL)	0	25	50	100	200
Sub-G1 (%)	7.1±2.9	8.9±2.8	10.1±0.3	31.4±5.8	59.9±3.5

C

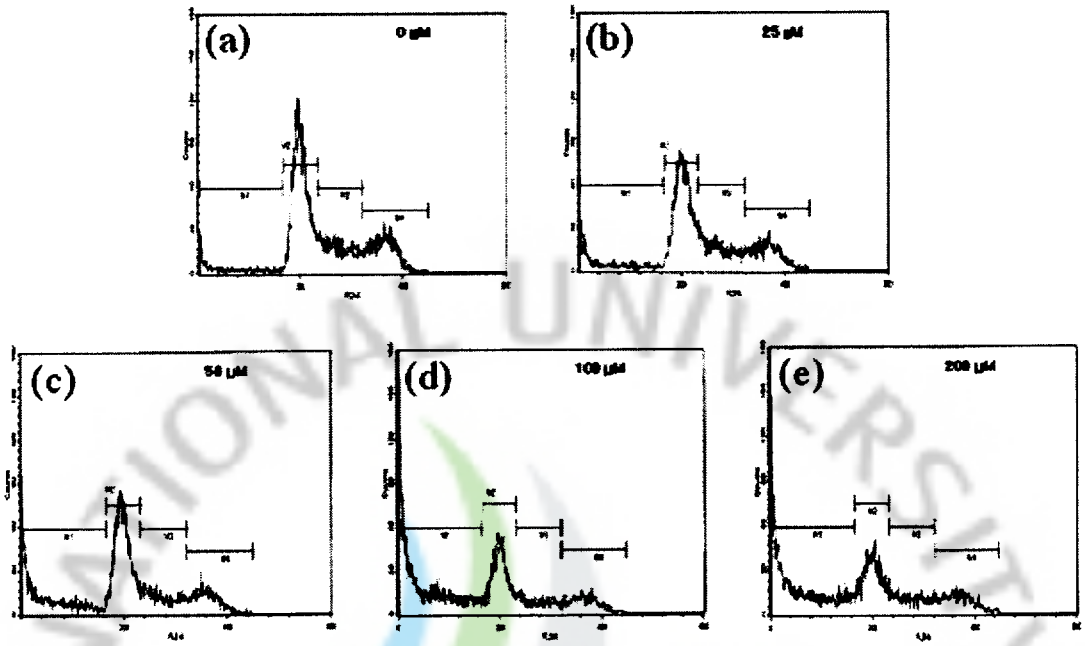


Table 3. Determine of sub-G1 ratio in Quercetin-treated SNU-16 cells.

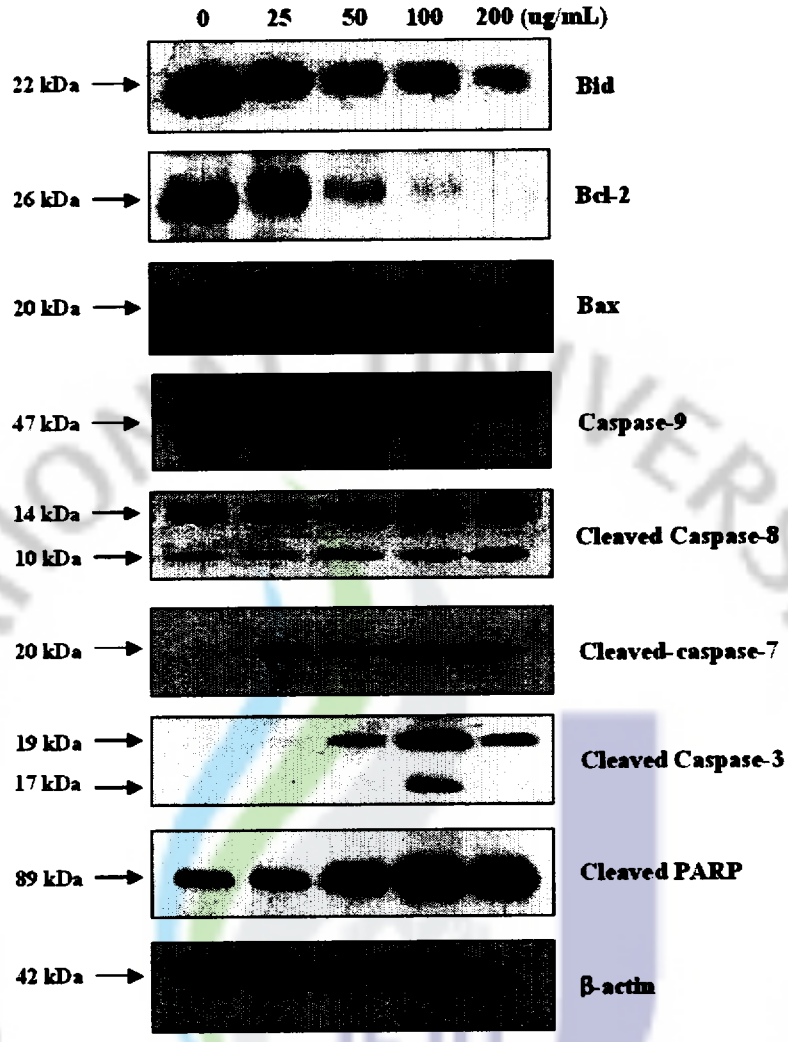
Concentration (μg/mL)	0	25	50	100	200
Sub-G1 (%)	9.9±3.2	13.6±5.1	25.1±4.3	34.9±2.8	35.5±6.0

Figure 6. Cell cycle analysis in treated SNU-16 cells. Histogram patterns of SNU-16 cells cultured with various concentration of CFD (A), CFG (B) and quercetin (C). Concentration: (A, B) Control (a); 25 μg/mL (b); 50 μg/mL (c); 100 μg/mL (d); 200 μg/mL (e). (C) Control (a); 25 μM (b); 50 μM (c); 100 μM (d); 200 μM (e). Percentage of sub-G1 ratio indicated in Table 1, 2, 3.

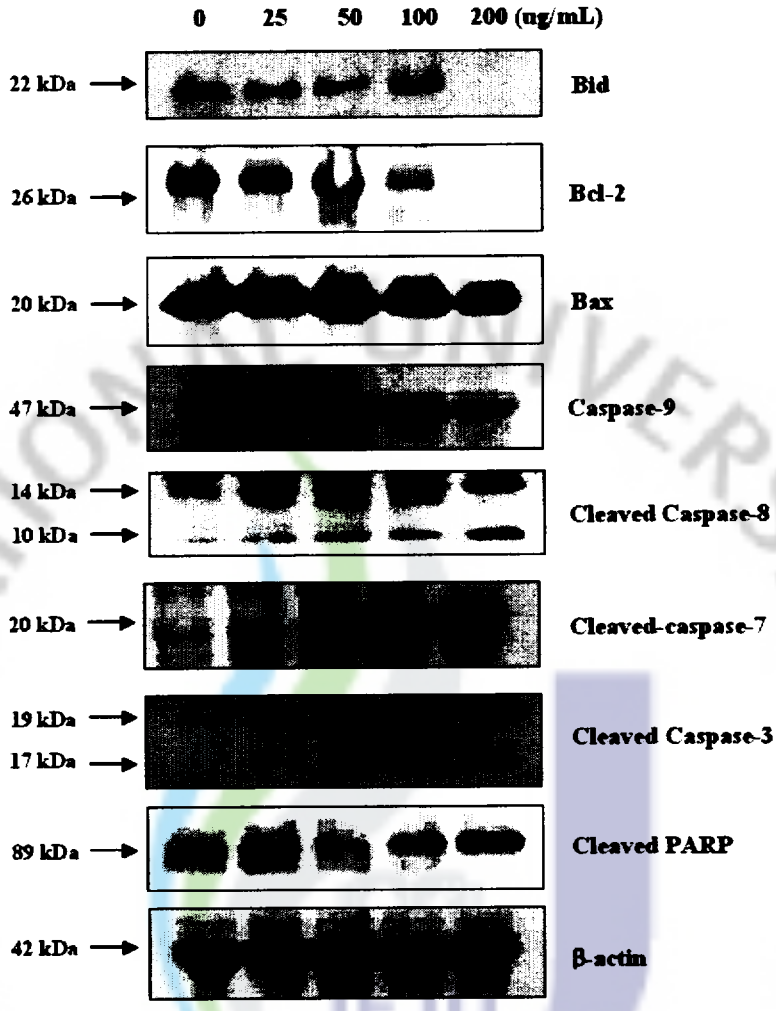
3. 4. Western blot analysis for apoptosis-related proteins

To determine the mechanism of CFD-, CFG-induced apoptosis, the expression of anti- and pro-apoptotic proteins following CFD and CFG treatment was examined by immunoblotting. The expression of anti-, pro-apoptotic members of the Bcl-2 family members regulates the intrinsic pathway. The expression of Bcl-2 was significantly inhibited in a dose-dependent manner whereas the expression of Bax was relatively constant in CFD-, CFG-treated SNU-16 cells (Fig. 7). These results suggest that the CFD and CFG induced apoptosis by shifting the Bax/Bcl-2 ratio in favor of apoptosis. Previous reports have shown that the imbalance between Bax and Bcl-2, causing apoptosis (Cheng et al., 2007). The fractions also increased the expression of the active subunits of caspases-8, -7, and -3, as well as the proteolytic cleavage of Bid and PARP in a dose-dependent manner. The activated capase-8 starts the apoptotic reaction by downstream effector caspases and Bid. These results suggest that the CFD and CFG induced apoptosis through extrinsic pathway. As shown in Fig. 8, caspapse-3 activity was increased by the CFD and CFG in a dose-dependent manner. These results suggest that the apoptotic effects of the CFD and CFG in SNU-16 cells are associated with an increase in the Bax/Bcl-2 ratio and caspase activation.

A



B



C

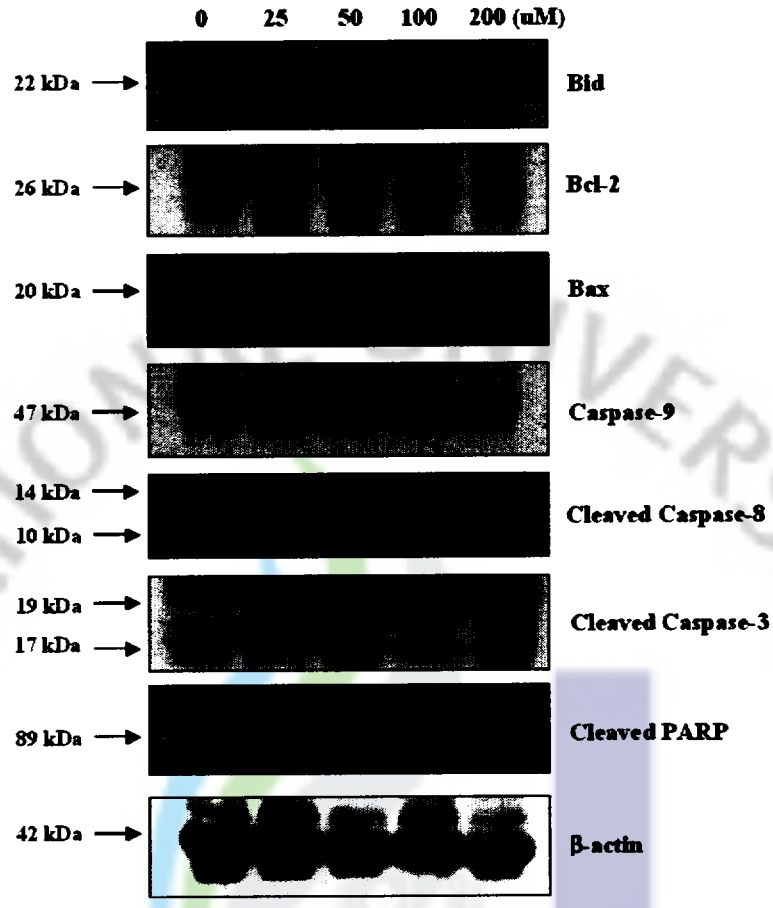


Figure 7. Immunoblot analysis of apoptosis-related protein expression in treated SNU-16 cells. Cells were lysed after 24 h of incubation with the various concentrations of the CFD (A), CFG (B) and quercetin (C). Cellular proteins were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were probed with the indicated primary antibodies then with horseradish peroxidase-conjugated goat anti-rabbit IgG. β-actin was used as an internal control.

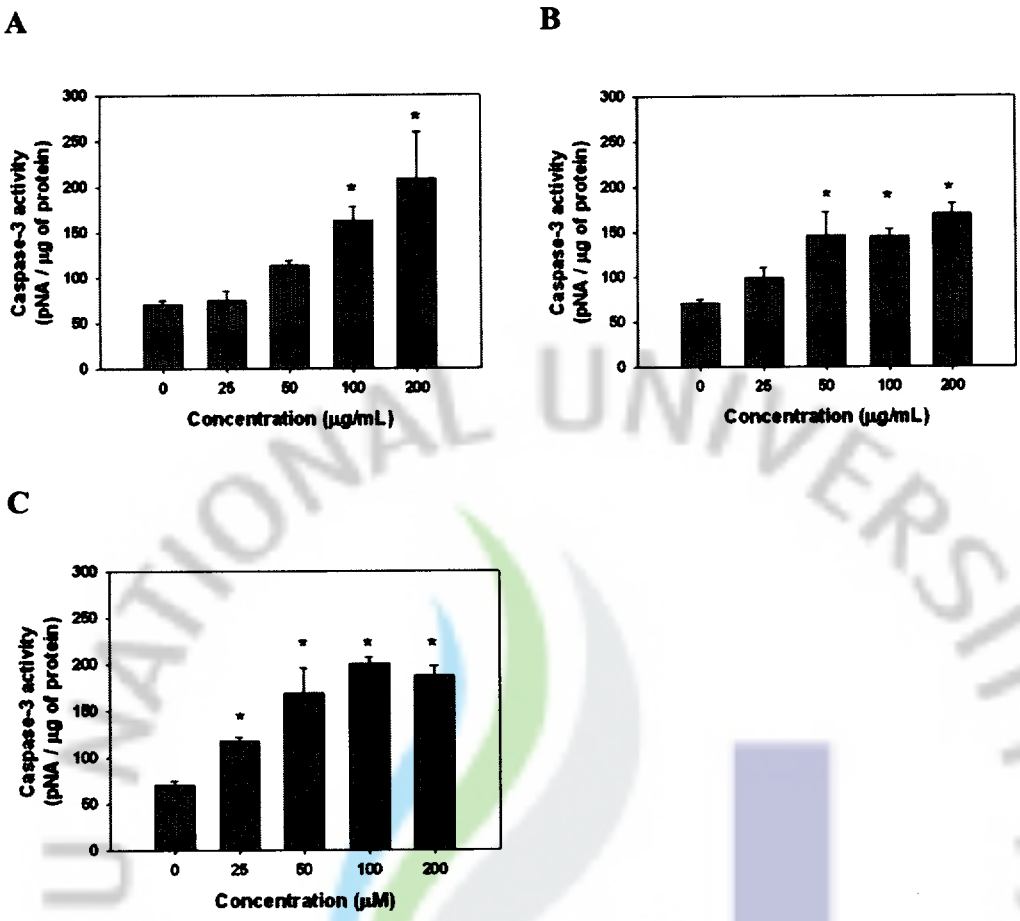


Figure 8. Induction of caspase-3 activity in treated SNU-16 cells. Cell lysates prepared from cells that had been treated with the CFD (A), CFG (B) and quercetin (C) for 24 h were assayed for *in vitro* caspase-3 activity. The rate of cleavage of the caspase substrate DEVD-*p*NA was measured at 405 nm. The results are presented as the mean ± SD. The experiments were done in triplicate. **P* < 0.05 compared with the control.

3. 5. Composition of chloroform fraction

We analysed the composition of the chloroform fraction from Dangyuja leave using GC-FID and GC-MS. We identified 21 major compounds in the fraction, main constituents were 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%) and 3',4',5,6,7,8-hexamethoxyflavone (2.37%); vitamin E (1.65%); glaucine-quinol (1.44%); campesterol (1.40%) (Table 4). Linoleic acid, linolenic acid, palmitic acid and methyl palmitate were a kind of fatty acid and they were reported that there was the anticancer effect (Kwon et al., 2008; Nano et al., 2003; Phoon et al., 2001). α-amyrin, γ-sitosterol, dihydrolanosterol, vitamin E and campesterol were a kind of sterol and they were reported that was possible to cytotoxic effect and inhibit the viability effects (Aragão et al., 2007; Zhang et al., 2007). Nobiletin was a hydroxyl methyl flavonoid and it was also reported that inhibit the viability, anti-inflamatery and anti-tumar effects (Akao et al., 2008; Ho and Lin, 2008; Luo et al., 2008). As to our result, these major components will be able to cause the apoptosis and needed to determine the molecular mechanisms of the major components.

Table 4. Identification of CFD by GC-MS.

Peak no.	RT	Compounds	Area (%)	Homology (%)
1	25.423	(-)-Loliolide	2.95±0.22	94
2	26.355	2H-1-Benzopyran-2-one, 7-hydroxy- (Coumarin, 7-hydroxy-)	1.73±0.18	68
3	27.362	Hexadecanoic acid, methyl ester (Methyl palmitate)	2.49±0.93	98
4	29.365	n-Hexadecanoic acid (Palmitic acid)	4.48±0.26	97
5	33.380	9,12-Octadecadienoic acid, (Z,Z)- (Linoleic acid)	13.28±2.05	93
6	34.610	9,12,15-Octadecatrienoic acid, (Z,Z,Z)- (Linolenic acid)	8.92±1.49	98
7	34.863	Borane, diethylmethyl-	1.39±.12	83
8	38.824	9-Octadecenamide, (Z)- (Oleic acid amide)	1.55±0.46	99
9	41.554	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (Palmitin, 2-mono-)	1.52±.06	86
10	42.079	Diisooctyl-phthalate	1.39±0.23	80
11	44.628	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z) (Ethyl linoleolate)	2.40±0.26	87
12	50.610	Vitamin E	1.65±0.17	95
13	52.097	Glaucine-quinol	1.44±0.25	59
14	52.660	Campesterol	1.40±0.06	53
15	53.019	(E)-23-ethylcholesta-5,22-dien-3.beta.-ol	1.08±0.14	59
16	53.902	gamma.-Sitosterol	3.64±0.06	99
17	54.340	Lanost-8-en-3-ol, (3.beta.)-; Dihydrolanosterol	2.74±0.09	90
18	54.942	3',4',5,6,7,8-Hexamethoxyflavone; Nobiletin	2.37±0.12	90
19	55.535	Viminalol; .alpha.-Amyrin	3.73±0.11	70
20	55.953	Vitamin E; .alpha.-Tocopherol	1.95±0.29	84
21	58.229	N-ethyl-1,3-dithiosloindoline	1.26±0.43	43

In conclusion, our study shows that the CFD and CFG inhibited cancer cell proliferation in a dose dependent manner. Cell death was characterized by chromatin condensation, apoptotic body formation, and DNA fragmentation. The induction of apoptosis was confirmed by caspase-3 activity assay and by immunoblotting using antibodies against Bcl-2, Bax, poly (ADP-ribose) polymerase (PARP), caspase-9, caspase-8, caspase-7, and caspase-3. The molecular mechanism underlying CFD- and CFG-induced apoptosis in SNU-16 cells may involve both intrinsic pathway and extrinsic pathway, as shown by an increase in the Bax/Bcl-2 expression ratio and an increase in the cleaved caspase-8. These results indicate that the CFD and CFG have anticancer activity *in vitro*. Using gas chromatography and gas chromatography-mass spectrometry, we could identify the components of the CFD. The fatty acid, sterol and flavone components were contained in CFD. Additional studies are needed to determine the active components in these extracts and molecular mechanisms of the active compounds and to evaluate the potential *in vivo* anticancer activity of the extract.

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ABSTRACT IN KOREAN

당유자 잎과 구아바 잎의 항암 효능을 인간 위암세포주인 SNU-16 세포를 이용하여 연구하였다. 그 중 각각의 클로로포름 분획에서 가장 큰 세포 성장 억제능을 보였다. 세포 죽음은 농도 의존적으로 일어났고 apoptotic body 형성, DNA 절편과 같은 특징을 보였다. Flow cytometric analysis 로 당유자 잎 클로로포름 분획 (CFD)과 구아바 잎 클로로포름 분획 (CFG) 세포의 Sub-G1 기 축적을 확인하였다. Bid, Bcl-2, Bax, poly (ADP-ribose) polymerase (PARP), caspase-9, caspase-8, caspase-7, and caspase-3 항체를 이용한 면역블로팅과 Caspase-3 활성 측정을 통해 apoptosis 유도를 확인하였다. 동시에 우리는 CFD 와 CFG 분획물이 SNU-16 세포에서 apoptosis 를 유도한다는 첫번째 증거를 증명한다. 우리의 발견은 인간 위 암의 치료를 위한 새로운 전략으로 이어질 수 있을 것이다.

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석사과정을 시작함과 동시에 결혼 생활을 한 저에게 학업과 가정의 양쪽 모두를 충족시키기에는 제 노력과 의지력이 너무도 모자랐나 봅니다. 지나와서 보니 저는 학업에 충실한 것도 아닌 가정에 충실한 것도 아닌 그런 나날들을 보낸 것 같습니다. 하지만 가정에 충실하지 못한 남편이자 이제는 한 아이의 아빠로서 부족한 저를 잘 믿고 따라와주고 이끌어준 민선이에게 감사의 말을 전합니다. 그리고 아무것도 모르는 저를 받아주고 학업에 정진할 수 있도록 지금까지 이끌어주신 김소미 교수님께 깊은 감사를 드립니다. 석사 학위논문을 심사해 주신 김재훈 교수님, 조문제 교수님 그리고 참관해 주신 류기중 교수님께도 진심으로 감사를 드립니다.

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