Master's Thesis

Induction of Apoptosis in HeLa cells by Chloroform Fraction of *Citrus grandis* Osbeck Leaves and its Compositional Analysis

Hana Kim

Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

December, 2009

인체 자궁암 세포주 **HeLa** 세포에서의 당유자 잎 **chloroform** 분획물의 **apoptosis** 유도 및 분획물의 성분 분석

지도교수 김 소 미

김 하 나

이 논문을 이학 석사학위 논문으로 제출함

2009년 12월

김하나의 이학 석사학위 논문을 인준함

심사위원장 _____ 印

위 원 _____ 印

위 원 _____ 印

제주대학교 대학원

2009년 12월

Induction of Apoptosis in HeLa cells by Chloroform Fraction of *Citrus grandis* Osbeck Leaves and its Compositional Analysis

Hana Kim

(Supervised by Professor Somi K. Cho)

A thesis submitted in partial fulfillment of the requirement
for the degree of Master of Biotechnology

December, 2009

This thesis has been examined and approved.

_____

Chairperson of the supervising committee

Professor Dong-Sun Lee, Ph.D., College of Applied Life Science, Jeju National University

_____

Professor Eun-Sook Yoo, Ph.D., College of Medicine, Jeju National University

_____

Professor Somi K. Cho, Ph.D., College of Applied Life Science, Jeju National University

Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Dangyuja is a citrus fruit of Jeju Island in Korea, which is known to have a high content of flavonoids with health-related properties. Although recent experimental data have revealed the anticancer potency of some *Citrus* species, the underlying molecular mechanisms of this apoptotic activity have not yet been studied in detail. In the present study, chloroform fraction (CF) of *Dangyuja* leaves was investigated on the inhibition of growth on human cervical carcinoma cells. It was found that the exposure of HeLa cells to CF resulted in growth inhibition and the induction of apoptosis in a dose-dependent manner. The CF treatment induced the down regulation of anti-apoptotic Bcl-2 expression which was associated with the proteolytic activation of caspases and the concomitant degradation of poly (ADP ribose) polymerase (PARP) protein. Arrested cell growth and the induction of apoptosis were confirmed by flow cytometry and DNA fragmentation analysis. Moreover, a new and rapid high performance liquid chromatography technique with an UV photodiode-array detector was used to analyze the major components of CF which were identified as sinensetin, nobiletin, tangeretin and 4',5,6,7-tetramethoxyflavone by using electrospray ionisation mass spectrometry (ESI-MS). This is the first report, showing the possible mechanism of antiproliferative effect of *Dangyuja* leaves for the prevention of cervical cancer in cell culture models. Our results suggested that CF of *Dangyuja* leaves is excellent

sources of functional polymethoxyflavones that may help prevent female cancer and a potentially useful agent for the treatment of certain malignancies.

요약문

제주도에서 재배되는 당유자 (*Citrus grandis* Osbeck)는 많은 양의 플라보노이드 함량을 포함한다. 최근 연구에서 몇몇 감귤류에서 항암 효과를 가진 것으로 밝혀졌지만, apoptosis를 통한 분자수준의 메커니즘은 아직 자세하게 보고되지 않았다.

이번 연구에서는, 당유자 잎의 클로로포름 분획물 (CF)이 human cervical carcinoma cell인 HeLa 세포의 성장억제에 대하여 조사하였다. 그 결과, CF를 처리한 HeLa 세포에서 농도의존적으로 세포 성장 억제와 apoptosis 유도를 확인하였다. CF가 처리된 HeLa 세포에서 anti-apoptotic 단백질인 Bcl-2의 down regulation과 caspase의 기질인 PARP 단백질의 degradation을 확인하였다. Flow cytometry를 통하여 CF가 처리된 HeLa 세포의 arrest와 DNA fragmentation을 확인하였다. LC-MS/MS를 이용하여 CF의 성분을 분석한 결과, polymethoxyflavone 계통인 sinensetin, 4′,5,6,7-tetramethoxyflavone, nobiletin과 tangeretin을 동정하였다. 이러한 연구 결과는 처음 보고 되는 것으로, 당유자 잎의 클로로포름 분획물 (CF)이 자궁경부암세포모델에서의 세포 성장 억제 및 항암활성의 메커니즘에 관여하는 것으로 사료된다.

본 연구는 여성 암을 예방하고 종양 치료에 도움을 줄 수 있을 것이라고 사료되는 polymethoxyflavone화합물을 제공하는 훌륭한 자원으로서 당유자 잎 클로로포름 분획물의 우수성을 잘 시사해준다.

1. Introduction

Cancer is a class of diseases in which a group of cells display uncontrolled growth, invasion, and sometimes metastasis. These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, and do not invade or metastasize. Cancer may affect people at all ages, even fetuses, but the risk for most varieties increases with age (Cancer Research UK, 2007). Cancer causes about 13 % of all human deaths (WHO, 2006).

According to the American Cancer Society, 7.6 million people died from cancer in the world during 2007 (American Cancer Society, 2007). Cervical carcinoma still remains most common female malignancy worldwide, despite gradual fall in its frequency in Western countries (Greenlee *et al*., 2001; Smith *et al*., 2000). Present treatments include surgery, radiation, drugs and other therapies. However the majority of cases, especially for advanced stage cancer, are still treated with conventional chemotherapy (Smith *et al*., 2000). Many researchers have been trying to find more efficient drugs to treat it.

In the last decade, advances in cancer research have enhanced our understanding of cancer biology and genetics. Among the most important of these is that the genes that control apoptosis have a major effect on malignancy through the disruption of the apoptotic process that leads to tumor initiation, progression, and metastasis. Therefore, one mechanism of

tumor suppression by natural products may be to induce apoptosis thereby providing a genetic basis for cancer therapy by natural products.

Anticancer effects may be exhibited through selective cytotoxicity, antiproliferative actions and apoptosis. Natural products or phytochemicals can be defined as substances found in edible fruits and vegetables that, may exhibit a potential for modulating human metabolism in a manner favorable for the prevention of chronic and degenerative diseases. Nowadays, many studies are carried out on different phytochemicals that may protect against degenerative pathologies, such as cancer and atherosclerosis (Hertog *et al*., 1992; Keys, 1995).

Apoptosis, also called programmed cell death, is a type of cell death regulated in an orderly way by a series of signal cascades under certain situation. It plays an important role in the regulation of cell growth, tissue development and homeostasis (Green and Reed, 1998; Hengartner, 2000; Kaufmann and Hengartner; 2001). It is also an important way by which organisms can maintain a constant amount of cells in order to live successfully. The induction and execution of apoptosis require the cooperation of a series of molecules, receptors, enzymes and gene regulating proteins. Among them, the caspase-cascade signaling system, regulated by various molecules such as the inhibitor including of apoptosis protein, Bcl-2 family proteins, and calpain, is vital in the process of apoptosis (Launay *et al*., 2005).

Apoptotic cells can be recognized by stereotypical morphological changes: the cell shrinks, shows deformation and looses contact to its neighboring cells. Its chromatin condenses and marginates at the nuclear membrane, the plasma membrane is blebbing or budding, and finally the cell is fragmented into compact membrane-enclosed structures, called 'apoptotic bodies' which contain cytosol, the condensed chromatin and organelles (Van Cruchten and Van Den Brock, 2000). Those morphological changes are a consequence of characteristic molecular and biochemical events occurring in an apoptotic cell, most notably the activation of proteolytic enzymes which eventually mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles (Saraste and Pulkki, 2000).

Citrus plants are of great interest because their fruits and leaves accumulate large amount of flavonoid glycosides, whose aglycones are early intermediates in the flavonoid biosynthetic pathway. Most *Citrus* species accumulate abundant quantities of flavonoids and limonoids (Castillo *et al*., 1992) and focus on changes in the flavonoids and limonoid content during fruit growth, and on the level of antioxidants a in the fruits of the species(Sun *et al*., 2005; Mokbel and Hashinaga, 2006; Lim *et al*., 2006). Moreover, Citrus pectin gets attention of researcher for its wonderful activity against various cancer cell lines. Pectin from *Citrus*

has inhibition of metastasis in a rat prostate cancer model and anticancer activity induced apoptosis on several cancer cells (Pienta *et al*., 1995; Olano-Martin *et al*., 2003). In recent literature on *Citrus* flavonoids, a broad spectrum of biological activity including anticarcinogenic and antitumor activities has been discussed (Attaway, 1994; Sugiyama *et al*., 1993; Benavente-Garcı´a *et al*., 1997). *Citrus* flavonoids exert their anticancer effects through a number of these diverse mechanisms (Manthey *et al*., 2001). Flavonoids can be potentially involved in carcinogenesis: 1. DNA damage (induction), 2. Tumor development (promotion), 3. Invasion (proliferation). *In vitro,* flavonoids inhibit several human neoplastic cellular line proliferations: lymphoid and myeloid leukemia (Larocca *et al*., 1990), gastric carcinoma (Yoshida *et al*., 1990), ovarian carcinoma (Scambia *et al*., 1990), prostate carcinoma (Peterson and Barnes, 1993) and squamous-cellular carcinoma (Kandaswami *et al*., 1991). It has been observed that the polymethoxylated flavonoids (nobiletin and tangeretin) inhibit tumoral cell development in a dose-dependent manner. These data indicate, therefore, that the polymethoxylated flavonoids can be considered anticancer substances (Kandaswami *et al*., 1991). Polymethoxyflavones (PMFs) also show chemopreventive potential in antimutagenic and antitumor properties (Li *et al*., 2007; Li *et al*., 2009a, 2009b; Walle, 2007). Various *Citrus* species present different composition of PMFs (Green *et al*., 2006; Hirata *et al*., 2009; Li, Pan, *et al*., 2007; Mizuno *et al*., 1991). As PMFs are widely

distributed in *Citrus* genus with wide dynamic range, the rapid and sensitive characterization of these constituents in such samples is necessary.

Dangyuja, the local name of *Citrus grandis* Osbeck, is cultivated mainly in Jeju Island in South Korea and utilized for a long time as a traditional herb medicine in Jeju. The flesh is usually separated from the skin of the segments and is eaten with or without sugar whereas the leaves are used as food flavoring. Previously, there were some studies that anticancer, antioxidant and antidiabetic activity (Lim, *et al*., 2009; Lim, *et al*., 2006; Kim, *et al*., 2009), metabolomic profiling (Cho *et al*., 2009), analysis of limonoid content (Boo *et al*., 2007) in fruits. However, about the leaves, only a few studies reported biological effect, anti-inflammatory effect, antioxidant, anticancer activity and its compositional analysis using GC and GC-MS (Yang *et al*., 2008; Kim *et al*., 2008; Moon *et al*., 2009). But, approximately 80 % of all known natural compounds are nonvolatile or thermally unstable and therefore incompatible with GC. When coupled with photodiode-array UV–Vis absorbance detection, HPLC began to serve as a powerful tool for the rapid characterization of natural product extracts. HPLC–ESI-MS has been successfully applied to the determination of compounds present in material from a variety of natural product sources. HPLC–ESI-MS offered a significant advance over earlier techniques not only because of the increased stability and efficiency of ionization in the ESI source, but also because it was capable of directly

correlated with the corresponding UV–Vis absorbance and MS information.

The aim of this study was to investigate whether *Dangyuja* leaves inhibit cell proliferation and to determine the biochemical mechanisms via apoptosis in human cervical carcinoma cell line, HeLa cells. Therefore we conducted the effect of various fractions of *Dangyuja* leaves on cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Through the Hoechst 33342 staining, DNA fragmentation assay and determination of cell cycle analysis, we confirmed that $CHCl_3$ fraction induced apoptosis in HeLa cells. The expression of apoptosis-related protein was verified by western blotting. Moreover to determine which compounds were constituted in $CHCl_3$ fraction and what compounds contributed to its anticancer activity, a compositional analysis of $CHCl_3$ fraction was conducted by a liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS).

2. Materials and methods

2. 1. Materials

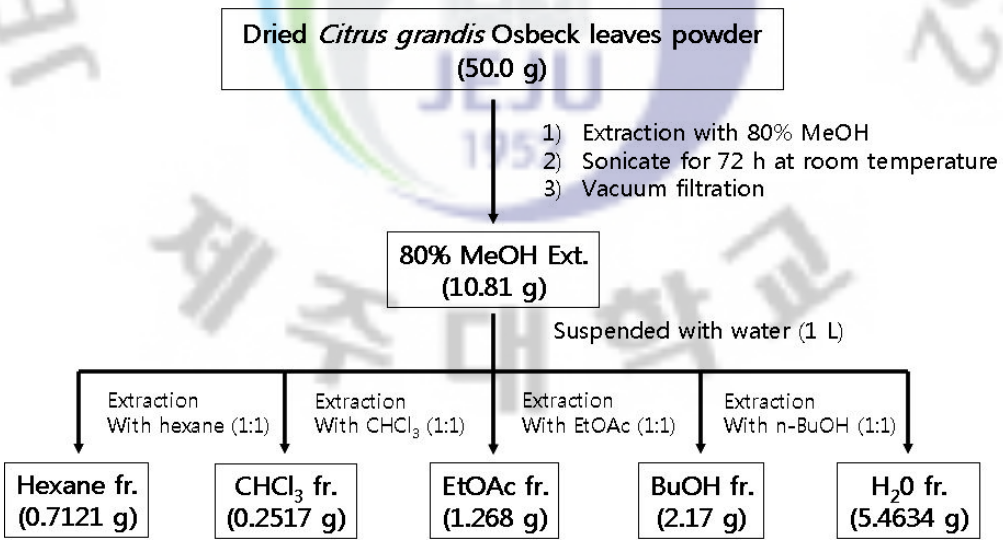
Dangyuja (*Citrus grandis* Osbeck) leaves were collected from the National Institute of Subtropical Agriculture, Jeju Province, Korea. Botanical samples were taxonomically identified previously (Kim, 1988), and a voucher specimen (number SKC.070531) was deposited in the laboratory of Dr. S.K. Cho at the College of Applied Life Sciences, Jeju National University.

Solvents, HPLC grade acetonitrile, water and trifluoroacetic acid (TFA) was purchased Merck (Darmstadt, Germany) and Fisher Scientific Ltd. (MA, USA). 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. (NY, USA). Dimethyl sulfoxide (DMSO), MTT was purchased from Amresco (OH, USA). Quercetin, propidium iodide (PI) and caspase-3 activity assay kit were purchased from Sigma (MO, USA). Antibodies for western blotting were purchased from Cell signaling (MA, USA), R&D systems (MN, USA) and Santa cruz, (MA, USA). Nobiletin as a standard was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were used in analytical grade.

2. 2. Preparation of extracts

Air-dried *Dangyuja* (*Citrus grandis* Osbeck) leaves were pulverized and extracted with 80 % methanol by sonicating for 3 days at room temperature (RT). After filtered, the extract was concentrated with a vacuum rotary evaporator under reduced pressure, and lyophilized.

The methanol extract was suspended in water and further fractionated with four different solvents in a stepwise manner, as previously described (Lim *et al*., 2006). The resulting fractions were: n-hexane fraction, chloroform fraction, ethyl acetate fraction, n-butanol fraction and water fraction. The extract powder was dissolved in dimethyl sulfoxide (DMSO) and diluted with phosphate buffered saline (PBS, pH 7.4) to give the final concentrations (Scheme 1).



Scheme 1. Flow diagram of fractionization of *Dangyuja* leaves extract.

2. 3. Cell culture

The HeLa cells, human cervical cancer cells, were purchased from the Korea Cell Line Bank (Seoul, Korea). HeLa cells were maintained in a nutrient medium, DMEM, supplemented with 10 % (v/v) heat-inactivated FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin in a humidified incubator at 37 °C in a 5 % CO_2 atmosphere.

2. 4. Cell viability assay

The effect of the fractions of *Dangyuja* leaves on the HeLa cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (Mosmann, 1983). In brief, cells were plated in 96-well plates at an initial density of 2×10^3 cells per well. After 16 h, cells were incubated for 72 h in the presence of various concentrations (from 50 to 200 μg/mL) of the *Dangyuja* leaves fractions. Then 0.1 mg of MTT was added to each well and the cell incubated at 37 °C for 4 h. The medium was removed, and then 200 μL of DMSO was added to each well to dissolve the formazan crystals. Absorbance was read at 570 nm on a microplate reader (Tecan, Salzburg, Austria).

2. 5. DNA fragmentation assay

HeLa cells (5×10^4 cells per well) were treated with different concentration of chloroform fraction of *Dangyuja* leaves (CF) for 24 h. After harvested, cells were washed with PBS and then centrifuged. Lysis buffer [10 mM of Tris-HCl (pH 8.0), 0.1 M of EDTA and 0.5 % of sodium dodecyl sulfate] and protease K were added to the pellet, then pellet was incubated for overnight at 50 °C. DNA was extracted adding phenol: chloroform: isoamylalcohol (25:24:1) and precipitated with 5 M sodium chloride and absolute ethanol. Same amounts of extracted DNA were separated by 1.8 % agarose gel. To visualize the agarose gel was containing ethidium bromide.

2. 6. Analysis of morphological changes

HeLa cells (1×10^4 cells per well) were treated with CF in a dose-dependent. After 24 h, 10 μM of Hoechst 33342, a DNA-specific fluorescent dye, was added to medium, and the cells were incubated for 10 min at 37 °C. Then the stained cells were observed using Olympus fluorescence microscope.

2. 7. Western blot analysis

HeLa cells (5×10^5 cells) treated with various concentration of CF were harvested and washed with PBS twice. The cells were lysed in a lysis buffer containing 40 mM of Tris-HCl (pH 8.0), 120 mM of NaCl and 0.1 % NP-40. We used the following protease inhibitors: 25 μg/mL of aprotinin, 25 μg/mL of leupeptin and 0.2 mM of PMSF. The cells were kept on ice for 30 min. After centrifugation for 30 min at 4 ℃, the supernatant was collected. Protein concentrations were determined using the BCA assay (Pierce, IL, USA). For Western blotting analysis, equal amounts of protein (μg) were separated by 8-12 % SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) microporous membrane (Millipore, MA, USA) with a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20 % MeOH (v/v)]. The block was blocked in blocking buffer (5 % nonfat dried milk) for 2 h at room temperature, and then incubated with primary antibodies for 2 h followed by incubation with the secondary antibody for 30 min in TBS-Tween buffer. Human anti –caspase 9, -caspase 7, –caspase 3, -poly ADP-riboxyl polymerase (PARP), -cleaved caspase 9, -Bid (Cell signaling), -caspase 8 (R&D systems), -Bcl-2, -Bax (Santa cruz), antibodies were used at 1:1000 dilution and HRP - goat anti-rabbit IgG (H+L) conjugate and HRP – goat anti-mouse IgG (Invitrogen) was used at 1:5000 dilution as secondary antibody. The PVDF membrane was then exposed to X-ray film (AGFA, Mortsel,

Belgium), and the protein bands were detected using a WEST-ZOL® plus Western Blot Detection System (iNtRON, Gyeonggi-do, Korea).

2. 8. Flow cytometry analysis

HeLa cells (5×10^4 cells per well) treated with CF were harvested and washed with PBS twice. The cells were fixed in ice-cold 70 % ethanol and kept at 4 ℃. Then cells were centrifuged, washed twice with 2 mM EDTA-PBS. The supernatant discarded, cells were resuspended in 0.5 ml of DNA staining solution (40 μg/mL of propidium iodide, 250 μg/mL of RNase in PBS-2 mM EDTA) for 30min at 37 ℃. DNA contents were analyzed by FACScan flow cytometer (BD Bioscience, CA, USA). The population of cells in each cell-cycle phase was determined using CellQuest software (BD Bioscience). The sub-G1 population indicated apoptosis-associated chromatin degradation (Tounekti *et al*., 1995)

2. 9. Caspase-3 colorimetric assay

Caspase-3 activity was assayed by using a commercially available kit (Sigma) according to the manufacturer's protocol. The caspase-3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in the release of the p-nitroaniline (pNA) moiety. p-Nitroaniline has a high absorbance at 405 nm. The concentration of the pNA released from the substrate is calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined pNA solution. HeLa cells were lysed with cold lysis buffer after treatment with CF (0, 100, 200 μg/mL) for 24 h. The protein concentration was measured by BCA protein assay kit (Pierce). 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) at 405 nm. The values were calculated using a p-nitroaniline calibration curve. The caspase-3 activity was calculated following formula:

Activity = (μmol pNA × d)/(t×v)

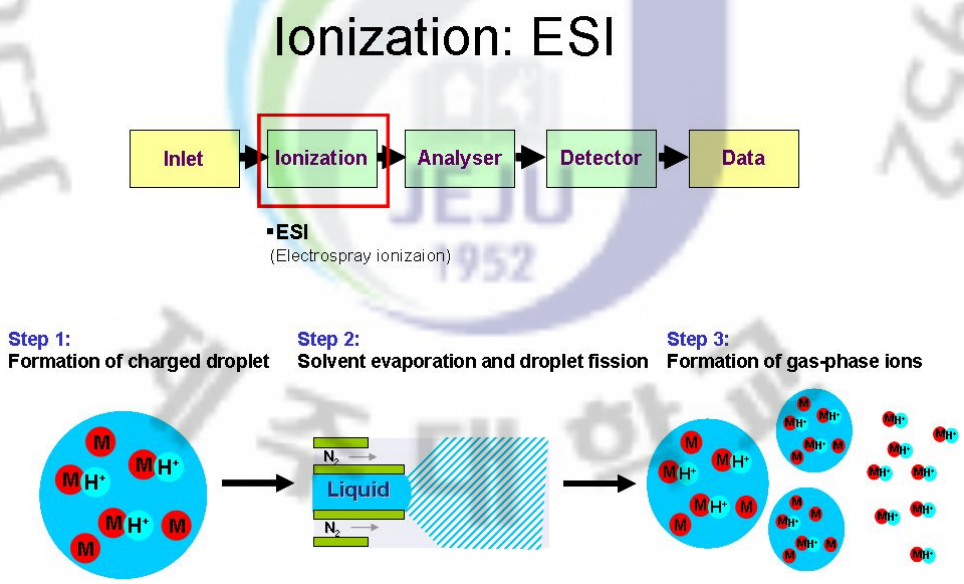
v = volume of sample in mL

d = dilution factor

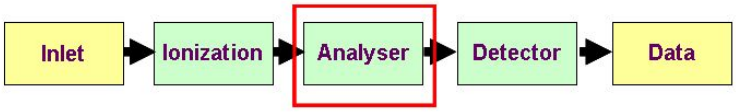
t = reaction time in minutes

2. 10. LC-ESI-MS/MS analysis

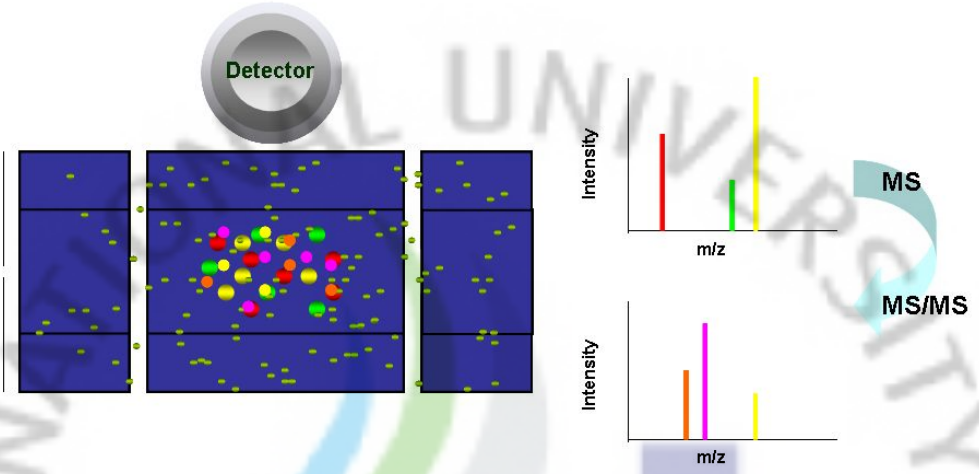
The LC-MS/MS used for this study was LXQ system (Thermo Scientific Inc., MA, USA) equipped with electrospray ionization ion source (ESI). ESI is provided by a hollow needle through which effluent flows, and an electric field at the tip of the needle produces a cone-shaped meniscus from which a spray of highly charged droplets emerges, with subsequent evaporation of the droplets resulting in the formation of ions which can be measured through the use of a variety of detection systems such as quadrupole, ion trap, and time-of-flight. High purity nitrogen was used as the nebulizer and auxiliary gas; helium was used as the collision gas.



Analyser: Ion trap



▪ Ion Trap



2. 10. 1. HPLC

HPLC system : Thermo Scientific Surveyor™ MS pump with Surveyor autosampler

Column : 100×2.1 mm ID. packed with 1.9 µm silica particles

Mobile phase : A:- Water with TFA 0.05 %

B:- Acetonitrile with TFA 0.05 %

Run time : 45 min.

UV-detector : PDA, D_2 and W lamps, 10 mm flow cell, 20Hz.

Scans : 200 -500 nm with Channel A (220nm), Channel B (280 nm) and Channel C (350nm)

Column Temp : 40 °C

Injection : 2 µL from 25 µL sample loop.

2. 10. 2. Gradient

Time (mins.)	% A (Water)	%B (ACN)	Flow rate (µL/min.)
0	90	10	200
25	40	60	200
27	40	60	200
35	2	98	200
37	2	98	200
43	90	10	200
45	90	10	200

2. 10. 3. Mass Spectrometer conditions

Mass spectrometer: LTQ linear ion trap mass spectrometer

Ionization mode: Positive and Negative electrospray ionization (ESI)

Capillary temperature: 350 °C

Spray voltage: 4.93 kV

Sheath gas: 30 Units.

Sweep gas: 10 units.

2. 10. 4. Software

Instrument operation and data collection and manipulation was achieved through the use of commercially available software such as Xcalibur (Finnigan Corp., CA, USA).

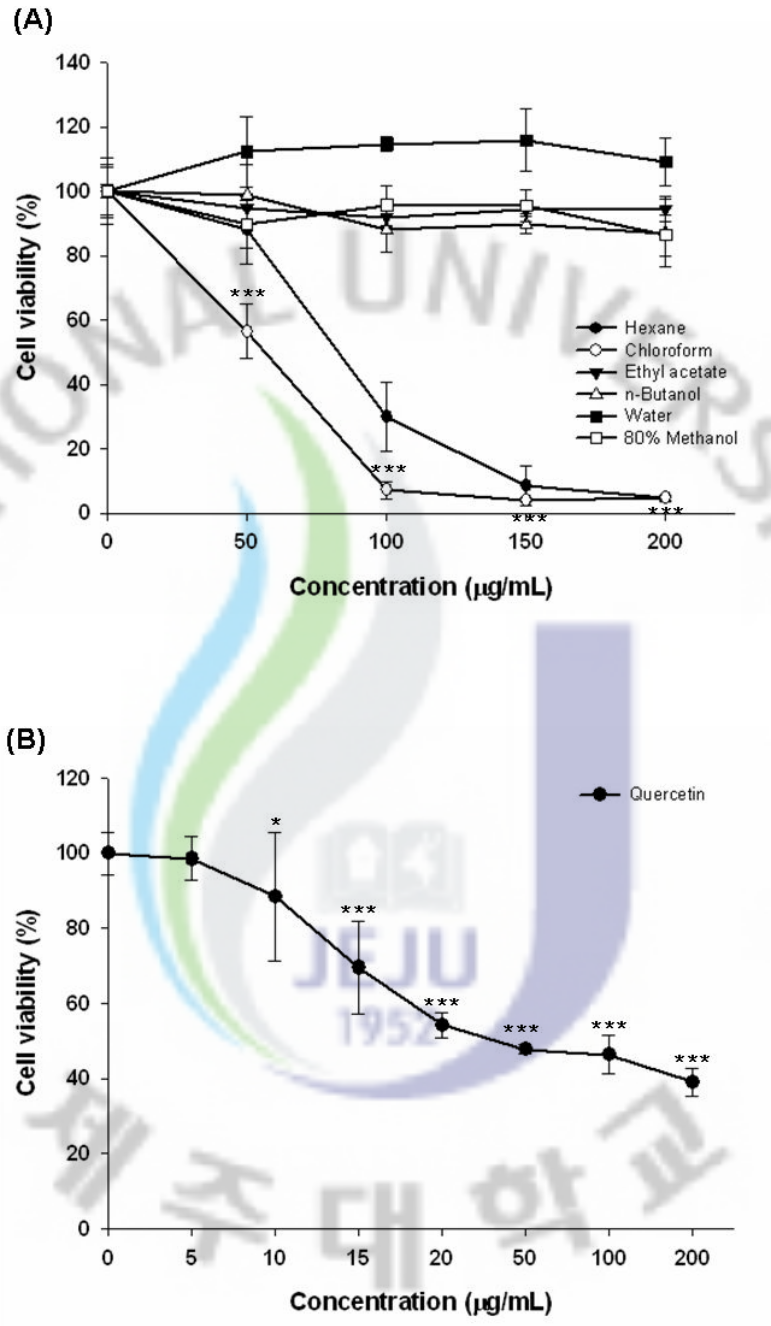
2. 11. Statistical analysis

All results were expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) with a Tukey's test using SPSS v. 12.0 software package was applied. A difference at *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ was considered to be statistically significant. All assays were performed in triplicate.

3. Results and discussion

3. 1. Effect of fractions from *Dangyuja* leaves on cell viability in human cell line HeLa cells

To investigate the effect of *Dangyuja* leaves fraction on HeLa cell viability, MTT assay was conducted. HeLa cells were incubated with different concentration of various fractions from *Dangyuja* leaves and quercetin, well-known that protected cancer, for 72 h (Fig. 1 (A) and (B)). As shown in Fig. 1 (A), the cell viability reduced dose-dependently by hexane and chloroform fraction. The cell viabilities of HeLa cells treated with 100 μg/mL of hexane, chloroform, ethyl acetate, n-butanol, water, 80 % methanol fractions and quercetin were 29.84 %, 7.15 %, 91.92 %, 87.98 %, 114.59 %, 95.68 % and 46.40 %, respectively when cell viability in the absence of treatment was taken as 100 % (Fig. 1). The IC_{50} value of chloroform fraction was 68.08 μg/mL and that of quercetin was 117.61 μg/mL. The chloroform fraction had the most effect to inhibit cell proliferation. Fig. 1 (C) showed that CF didn't show any toxicity on CCD25Lu cells, lung fibroblast. Table 1 showed the IC_{50} value of *Dangyuja* leaves fractions on HeLa cells. We conducted next experiments examined apoptosis with chloroform fraction from *Dangyuja* leaves (CF).



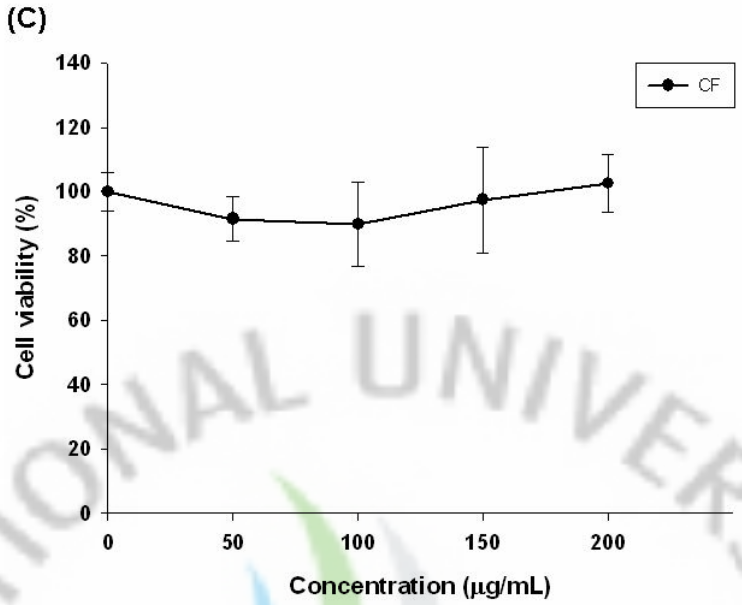


Figure 1. Effect of *Dangyuja* leaves fraction on the cell viability. Cells were treated for 72 h in the presence of various fractions (A) and quercetin (B), as a positive control, in the medium. Chloroform fraction of *Dangyuja* leaves had an effect decreased cell viability during incubation. Cell viability was determined by MTT assay. Cell viability without treatment was taken as 100 %. Data expressed as mean ± S.D. (n=5). (C) CCD25Lu cells, lung fibroblast, were treated with CF dose-dependently. (*: $p<0.05$, ***: $p<0.001$ compared with non-treatment (0 μg/mL))

Table 1. IC_{50} value of *Dangyuja* leaves fractions on HeLa cells.

Fractions of *Dangyuja* leaves	Concentration at IC_{50} value
Hexane	92.95 μg/mL
Chloroform	68.08 μg/mL
Ethyl acetate	< 100 μg/mL
n-Butanol	< 100 μg/mL
Water	N/A*
80 % Methanol	< 100 μg/mL

*: N/A : not available

3. 2. Effect of CF on DNA laddering

While apoptosis, DNA was cleaved into oligonucleosomal fragments (Saraste and Pulkki, 2000). To confirm that CF induced cell apoptosis, we conducted DNA fragmentation assay. Exposure of HeLa cells to CF from 50 to 200 μg/mL for 24 h led to evident DNA fragmentation as indicated by the formation of DNA ladder in the agarose gel containing ethidium bromide. From cells treated with 50 μg/mL of CF, ladder pattern was shown, whereas control indicated no evident DNA ladder (Fig. 2). This result suggested that CF induced apoptosis in HeLa cells.

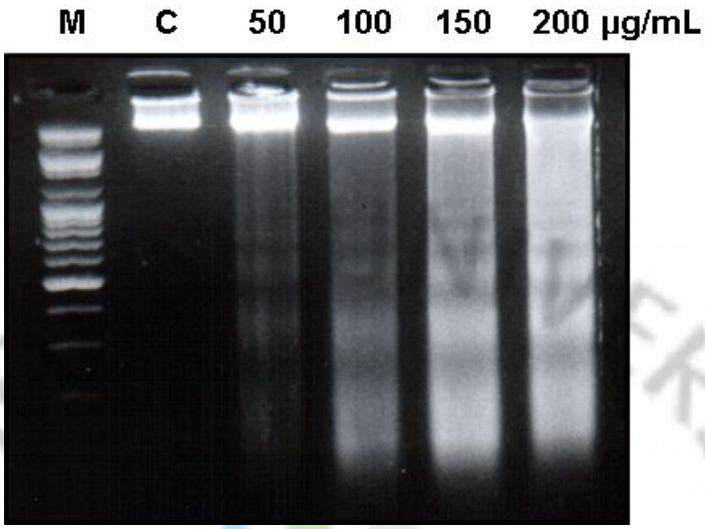


Figure 2. Effect of CF on DNA fragmentation in HeLa cells. DNA fragmentation of HeLa cells treated with CF in a dose-dependent manner for 24 h. Ladder patterns reflecting the presence of DNA fragment were viewed on agarose gel stained ethidium-bromide.

3. 3. Effect of CF on the cell morphology

Morphological changes such as cell shrinkage, condensed and fragmented chromatin, associated with apoptotic cell death. In order to characterize the apoptosis induced by CF, we treated with various concentration of CF for 24 h. The morphology and chromatin of the cells were examined by fluorescence microscopy after Hoechst 33342 staining. In a dose-dependent manner, results indicated that morphological changes of treated cells were found. Fig. 3 resulted that treated cells had cell shrinkage, condensed and fragmented chromatins and apoptotic bodies (arrows) by contrast with non-treated cells (control).

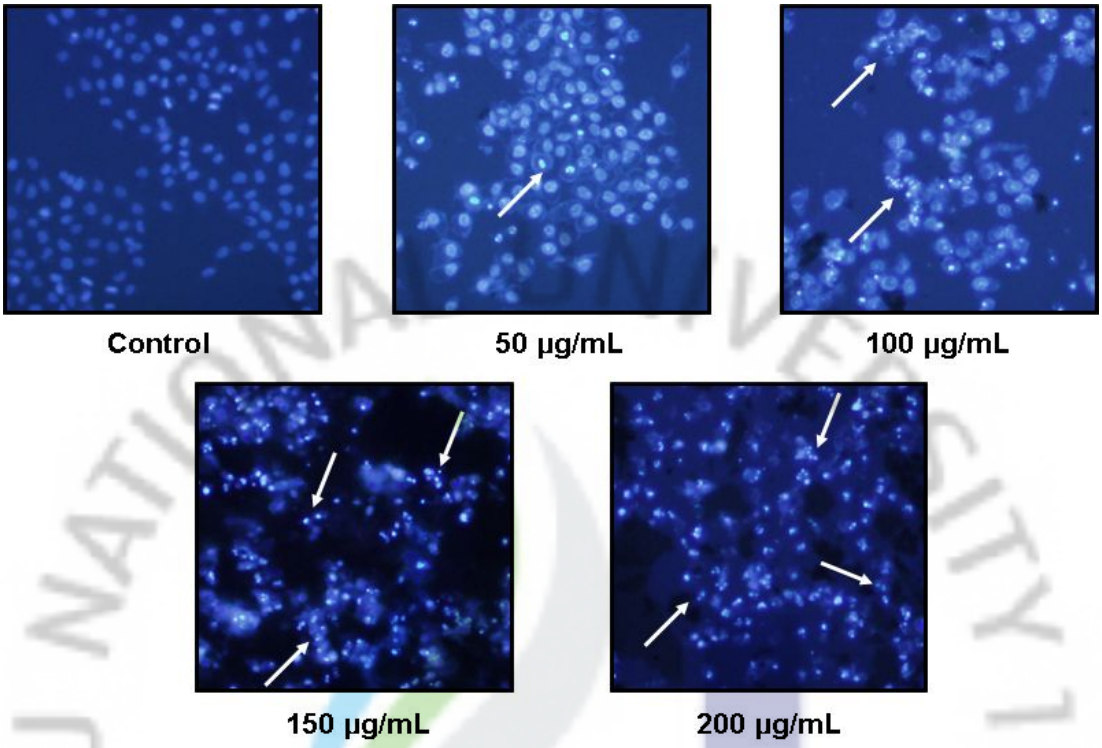


Figure 3. Effect of CF on cell morphological change in HeLa cells. Morphological changes of HeLa cells after CF treatment for 24 h followed Hoechst 33342 staining. Arrows indicate that cells shrinkage condensed and fragmented chromatins such as apoptotic bodies.

3. 4. Effect of CF on expression of apoptosis-related proteins

Activation of a caspase cascade occurs in apoptotic events. As an initiation of apoptosis, procaspase-8 is cleaved to create an active form of caspase-8. Once activated, caspase-8 cleaves procaspase-3, 7 to create an active form of caspase-3, 7 for execution of apoptosis. Procaspase-9 formed massive complex known as apoptosome with cytochrome c and oligomerized Apaf-1 together. Thereby, apoptosome activated initiator procaspase protein, procaspase-9. And then, the activated caspase-9 molecules activated down stream executioner procaspase-3, 7. The active caspase-3 cleaved 116 kD of PARP protein into 89 kD fragment, that is characteristic marker of the execution of apoptosis. Caspase-8 cleaved Bid into tBid. tBid translocated to mitochondria, where it inhibits anti-apoptotic Bcl2 proteins, and triggered the aggregation of pro-apoptotic BH123 proteins, thereby amplifying the death signal. To certify whether CF induces the activation these caspases, HeLa cells treated with various concentrations of CF for 24 h. Western blotting results showed that precursors of caspase-9, caspase-8, caspase-3, caspase-7 were decreased to active and proteolytic cleavage of PARP resulted in the characteristic shift of the protein from 116 to 89 kDa. These results suggested that caspase-9, 8, 3, 7 and PARP were involved in CF-induced cell death. BH3-only protein, Bid, decreased cleavaging to tBid by caspase-8, and inactivated anti-apoptotic Bcl-2 protein. β-actin was used internal control (Fig. 4).

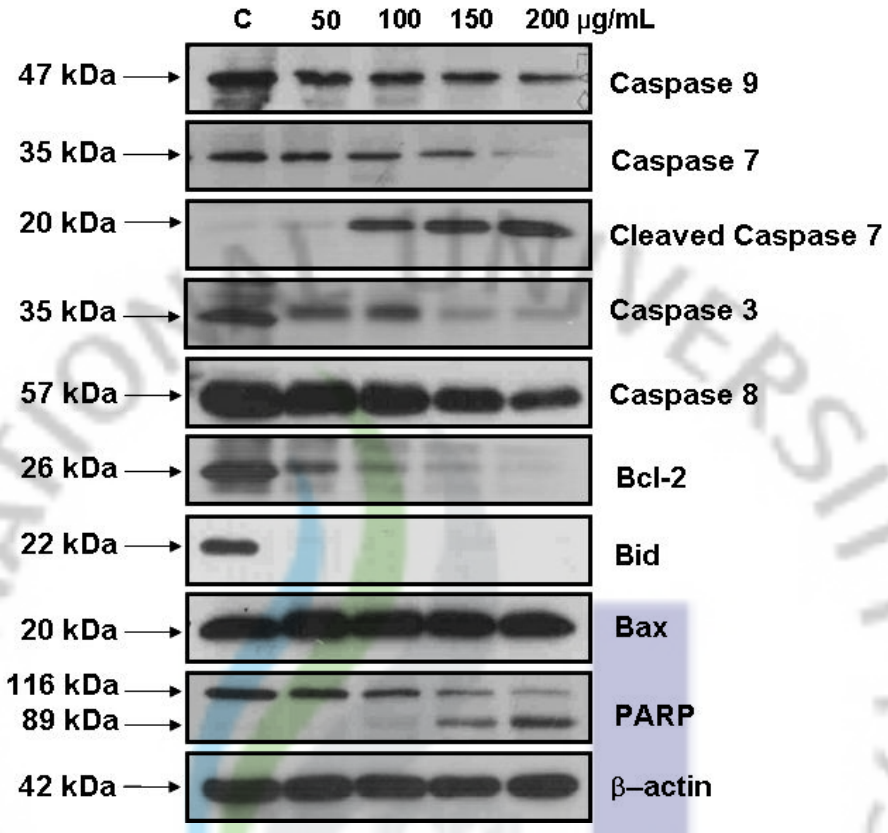


Figure 4. Effect of CF on apoptosis-related protein expression in HeLa cells. Western blotting analysis of protein extract obtained from HeLa cells treated with 0, 50, 100, 150, 200 μg/mL of CF, respectively. Total protein lysates were prepared after treatment for 24 h, and analyzed with antibodies to caspase-9, caspase-8, caspase-3 and caspase-7, Bcl-2, Bid and PARP. The β-actin was used as internal control.

3. 5. Dose-dependent effect of CF on cell cycle distribution

To investigate that CF induces apoptosis in HeLa cells, we evaluated DNA content and cell-cycle phase distribution by flow cytometry with PI staining. The percentage of cells in G0/G1, S and G2/M phases were calculated using CellQuest software. When cells undergo apoptosis, sub-G1 phase was increased. Sub-G1 population indicated apoptotic-associated chromatin degradation (Tounekti *et al*., 1995). HeLa cells were treated with CF in a dose-dependent course (0, 50, 100, 150, 200 μg/mL for 24 h). As compared to control, the sub-G1 phase significantly increased after expose of HeLa cells to CF (Fig. 5). These results suggested that chloroform fraction could induce HeLa cell cycle to be arrested in G2/M phase and trigger apoptosis in HeLa cells with dose-dependent manner. Tables indicated each of sub-G1 phase ratios (Table 1).

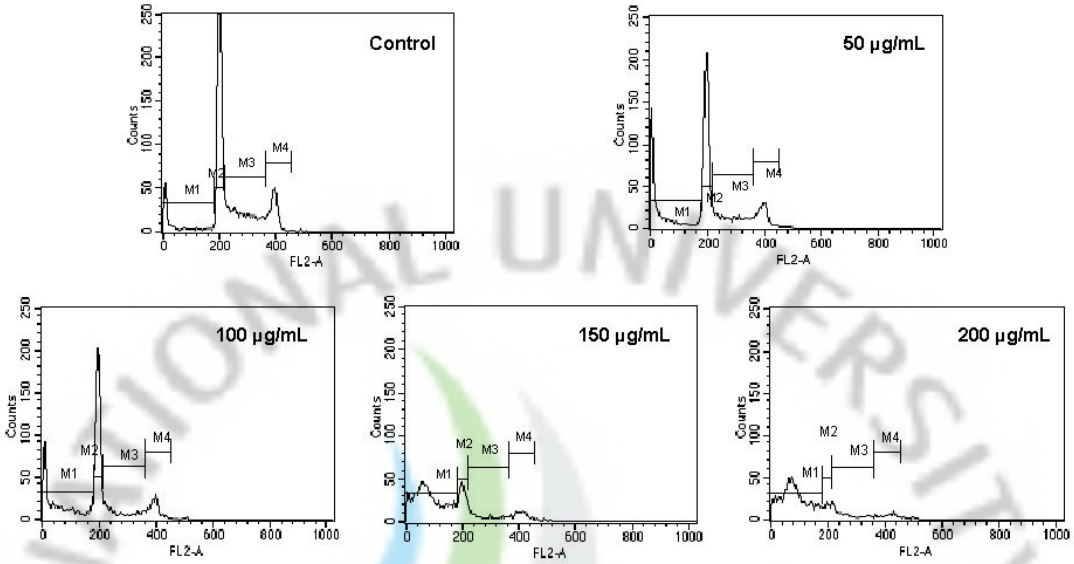


Figure 5. Dose-dependent effect of CF on cell cycle distribution using flow cytometry. HeLa cells were treated with different concentration of CF for 24 h analyzed. Sub-G1 phase increased in a dose-dependent manner.

Table 2. Sub-G1 phase ratio of HeLa cells treated CF in a dose-dependent manner.

Concentration	Sub-G1 (%)
0 μg/mL	9.32 ± 1.76
50 μg/mL	30.36 ± 1.44**
100 μg/mL	38.34 ± 3.46***
150 μg/mL	50.72 ± 11.43***
200 μg/mL	71.74 ± 1.41***

**: $p<0.01$, ***: $p<0.001$ compared with non-treatment (0 μg/mL)

3. 6. Effect of CF on caspase-3 activity

As shown in Fig. 7, caspase-3 activity was also increased by the CF in a dose-dependent manner. These results suggest that the apoptotic effects of the CF in HeLa cells are associated with an increase in the Bax/Bcl-2 ratio and caspase activation.

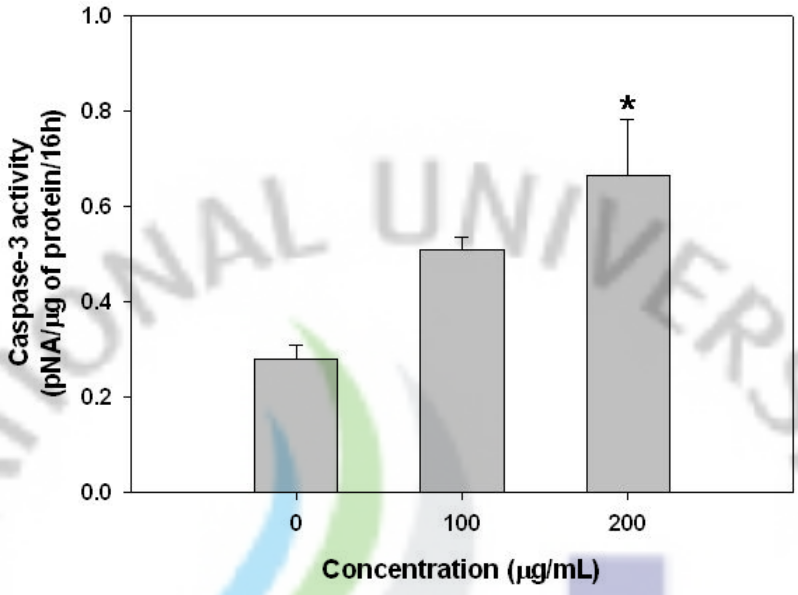


Figure 6. Effect of CF on caspase-3 activity in HeLa cells. Cell lysate prepared from cell treated with CF for 24 h were assayed for *in vitro* caspase-3 activity. The rate of cleavage of the caspase substrate DEVD-*p*NA was measured at 405 nm. The results showed as the mean ± SD. *: $p<0.05$ compared with non-treatment (0 μg/mL; control).

3. 7. Compositional analysis of CF by LC-MS/MS

To know what are the responsible active ingredients in CF of *Dangyuja* leaves, HPLC-PAD-MS was used. Among the four different solvent fractions, chloroform fraction (CF) exhibited anticancer activity. Therefore, CF was used for further investigations towards identification by HPLC-PAD-MS. The HPLC-PAD analysis of CF of *Dangyuja* leaves revealed the presence of phenolic acids including polymethoxy flavone compounds. The MS/MS parameters like ion mode, capillary voltage, cone voltage, collision energy and dwell times were optimized to maximize the overall sensitivity, as estimated from the peak areas of the analytes. All compounds were identified by matching retention times and mass spectra with those of the reported and comparing with the web-based database.

Table 3 showed chromatographic and spectral (MS and UV) information of compounds which were identified based on their UV spectra, molecular and fragmented ions. The presence of these compounds was observed distinctly in CF. CF represented the most diverse source due to presence of polymethoxylated flavones (PMFs), nobiletin and tangeretin, along with some phenolic acids in its leaves extract.

PMFs have the basic aglycone structure with maximum seven substituents such as methoxyl group (OCH_3) and/or hydroxyl group (OH) on its A, B and C rings. In addition, PMFs have characteristic dissociation pattern, they can lose one or two methyl radicals

($CH_3^{\bullet}$) to produce radicals $[M+H-15.0235]^+$ or$[M+H-2\times15.0235]^+$ as predominant fragments. The regular elemental composition and characteristic dissociation pattern, determined by exact mass measurements, should be used to form the diagnostic mass spectrometric fingerprint (MSFP) of the species for structural identification. A typical chromatographic profile of CF is shown in Fig. 8 where major identified compounds were reconstructed with their corresponding molecular mass ion and matched with the total scans of ESI positive mode (150-1000 m/z). The ions 164.1, 197.2, 371.4, 354.2, 373.24, 343.2, 403.2, 373.26 and 389.5 were the representative parent ions $[M+H]^+$ of p-amino-cinnamic acid, methyl veratrate, 7-acetoxy-3,3'4' trimethoxy flavone, chlorogenic acid, sinensetin, 4',5,6,7-tetramethoxyflavone, nobiletin, tangeretin and geneposide, respectively; using single ion monitoring approach (HPLC/ESI/SIM/MS).

Fig. 9 showed a representative chromatogram of the PMFs of CF from *Dangyuja* leaves, monitored at 280 nm and four PMF, sinensetin, 4',5,6,7-tetramethoxyflavone, nobiletin and tangeretin were detected. These compounds have been identified according to their retention time, UV pattern and the spectral characteristics of their peaks compared to those of published data as well as by spiking the sample with standards. Nobiletin and tangeretin were detected to be the major PMF component in the CF contributing about 11.3 % and 2 % to the total amount, respectively,

The MS spectra showed the protonated molecule $[M+H]^+$ and sodium-adduct molecule $[M+Na]^+$ of the PMFs analyzed (Fig. 10). The accurate mass and isotopic pattern of precursors and their fragmented ions were observed closely to the theoretical value, which means that they can reflect the exact elemental composition. Moreover, the diagnostic dissociation pattern of PMFs was acquired too. $[M+H-CH_3^{\bullet}]^+$ and $[M+H-2\times CH_3^{\bullet}]^+$ were the most predominant fragment ions in MS/MS spectra for both PMF references. The MS data of sinensetin, 4',5,6,7-tetramethoxyflavone, nobiletin and tengeretin proveed that the characteristic MSFP for PMFs, consisting of elemental composition and dissociation pattern, were obtainable by exact mass measurements using HPLC-ESI/MS.

Early reported methods for analysis of PMFs were based on high-performance liquid chromatography (HPLC) separation coupled with ultraviolet (UV) detection Rouseff and Ting, 1979; Mouly *et al*., 1998]. The methods are limited to the detection of a number of known compounds with purified standard. Recently, the methods based on LC–MS/MS have overcome the limitation and allow the identification of PMFs in crude and partially purified samples even without any need for purified standard (Li *et al*., 2006; Wang *et al*., 2007; Zhou *et al*., 2007). The accurate mass data is a key information for structural elucidation using mass spectrometry, as which can confirm the molecular formula of a compound (Gross, 1994). Therefore, HPLC-PAD-ESI-MS/MS provides an attractive alternative. Since the

combination of HPLC-PAD-MS/MS offers high chromatographic resolution with exact mass measurement for both UV and MS/MS, then provides significant advantages concerning flexibility, selectivity, sensitivity, accuracy and speed for rapid screening for target compounds in plant samples. We have performed a direct and rapid method for qualitative analysis of flavonoids in a *Citrus* leaves extract, with a very simple sample treatment. Confirmation of results was achieved by applying LC/MS technique

Although many studies on PMFs from different *Citrus* species (e.g. *Citrus aurantium*, *Citrus sinensis*) have been reported and most PMFs have been confirmed by UV, IR, MS, 1H NMR and 13C NMR (Hirata *et al*., 2009; Kurowska and Manthey, 2004; Li *et al*., 2007; Li *et al*., 2006; Li *et al*., 2007; Wang *et al*., 2007), there is no systematic study on PMFs from *Dangyuja*. In order to screen resources possessing high functional PMF content, the present study performs the isolation of PMFs in *Dangyuja* leaves based on high-speed chromatography and preparative high performance liquid chromatography, identification of structures by electrospray ionisation mass spectrometry (ESI-MS).

To our knowledge this is the first study that reports PMF presence in a leaves of *C. grandis* Osbeck. Due to the rapid sample preparation and analysis, this method can be applied in quality assurance routine analysis for flavonoids detection in *Citrus* species.

Table 3. List of phytochemicals identified in CF of *Dangyuja* leaves in LC-MS/MS.

Chemical analysis of $CHCl_3$ fraction of *Dangyuja* (*C. grandis* Osbeck) leaves from HPLC-PAD-MS/MS data

Peak	Rt (min)	UV λmax	ESI $[M+H]^+$	MS/MS_2 (rel. Int)	Compound	Molecular formula	Relative area (%)	Ref.
1	6.16	-	164.1	-	p-amino-cinnamic acid	$C_9H_9NO_2$	3.20	SDBS[a] and Lit[c].
2	7.73	-	197.2	-	Methyl veratrate	$C_{10}H_{12}O_4$	1.32	SDBS and Lit.
3	10.41	-	371.4	-	7-acetoxy-3,3',4'-trimethoxyflavone	$C_{20}H_{20}O_{11}$	1.41	SDBS and Lit.
4-8	11.0-15.25	-	-	-	Unknown	-	-	-
9	15.45	-	354.2	-	Chlorogenic acid	$C_{16}H_{18}O_9$	3.86	Mass bank[b] and Lit.
10	15.78	-	-	-	Unknown	-	3.68	-
11	16.6	264, 333	373.24	-	Sinensetin	$C_{20}H_{20}O_7$	1.73	Mass bank and Lit.
12	17.10	270, 333	343.20	-	4',5,6,7-Tetramethoxyflavone (Scutellarein tetramethylether)	$C_{19}H_{18}O_6$	1.25	Mass bank and Lit.
13	18.22	249, 270, 334	403.20	388, 373	Nobiletin	$C_{21}H_{22}O_8$	11.40	Mass bank and Lit.
14	20.10	271, 324	373.26	-	Tangeretin	$C_{20}H_{20}O_7$	1.99	Mass bank and Lit.
15	21.21	-	389.5	-	Geneposide (iridoid glycoside)	$C_{17}H_{24}O_{10}$	1.18	Mass bank and Lit.

[a] SDBS: Spectral Database for Organic compounds; [b] Mass bank: High resolution mass spectral database; [c] Lit: Literatures

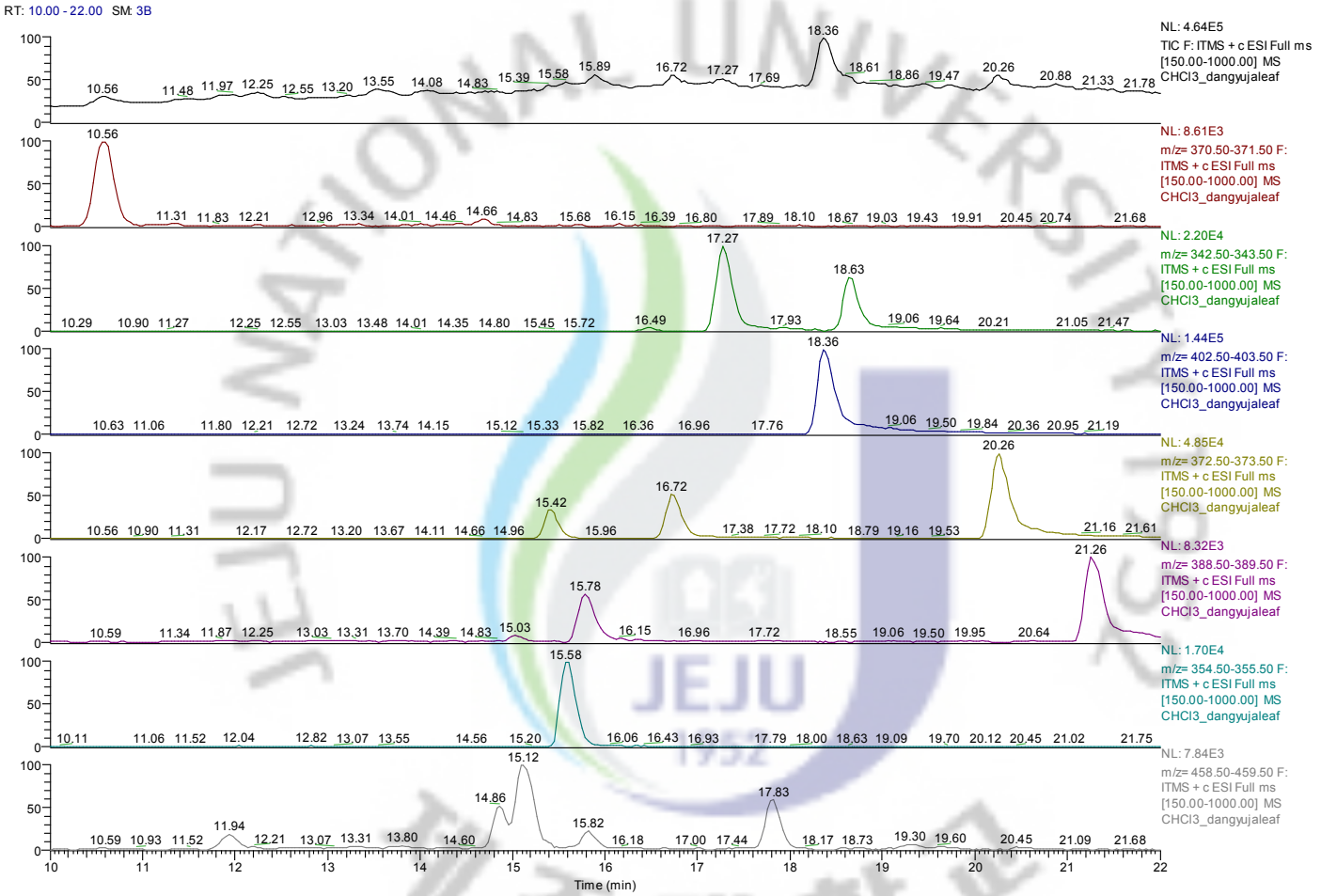


Figure 7. TIC spectrum of positive ESI of CF with reconstructed mass ion of identified phytochemicals.

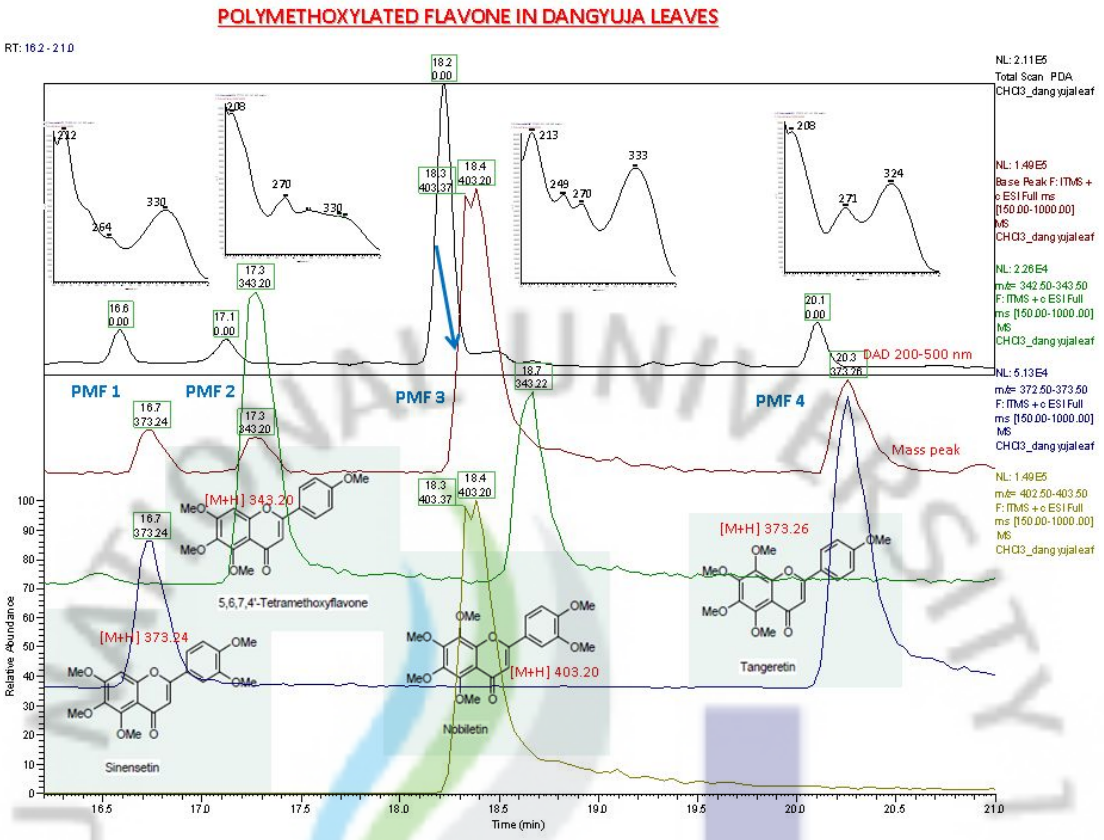
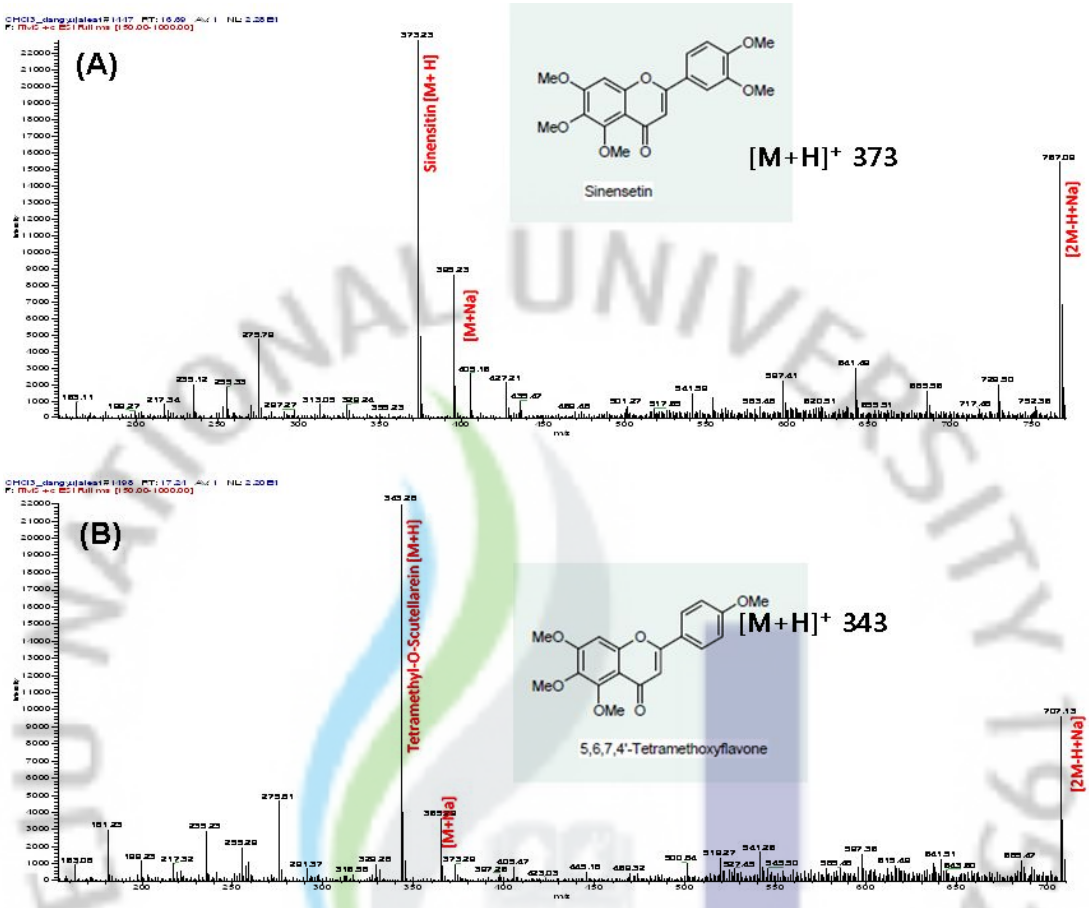


Figure 8. Expanded and 3D view of PDA and re-constructed total ion chromatograms of four major polymethoxyflvones from CF of *Dangyuja* leaves.



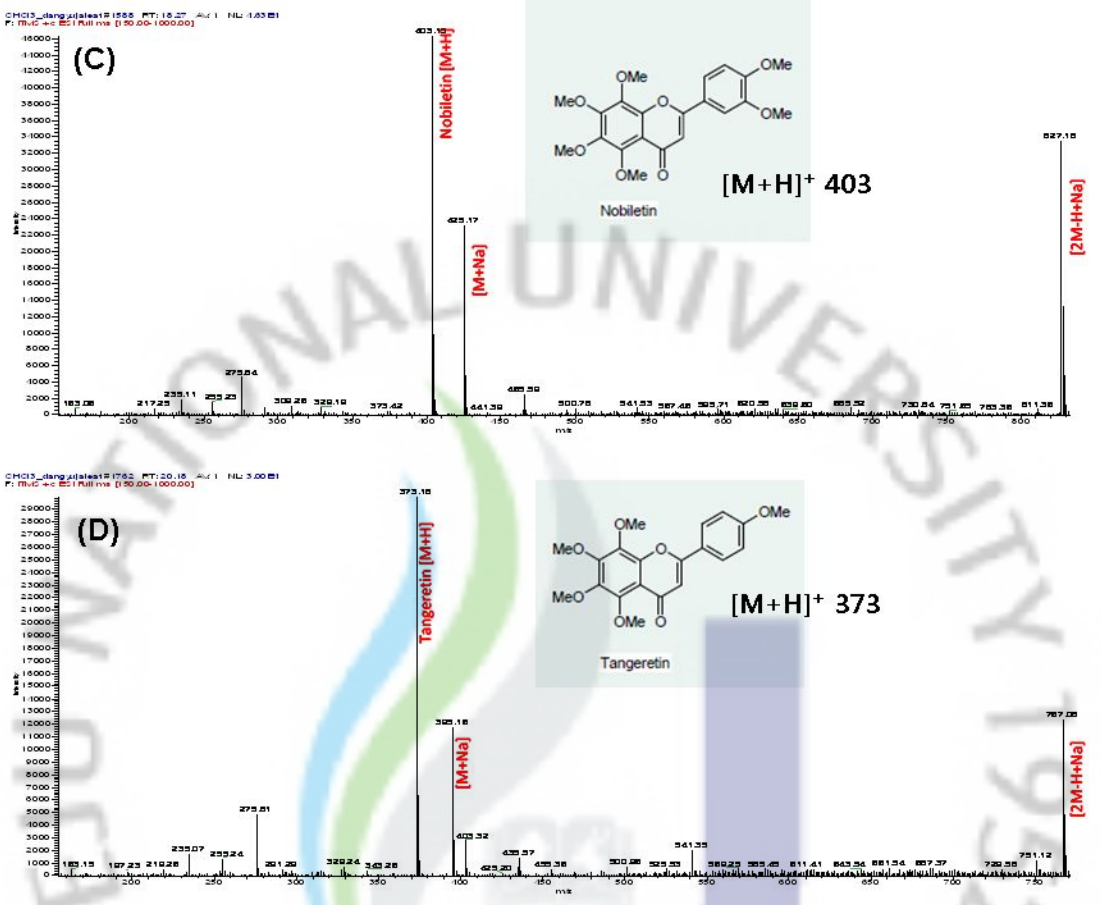


Figure 9. Product ion mass spectra of $[M+H]^+$ of sinensetin ($[M+H]^+$; 373) (A), 4',5,6,7-tetramethoxyflavone ($[M+H]^+$; 343) (B), nobiletin ($[M+H]^+$; 403) (C) and tangeretin ($[M+H]^+$; 373) (D) of CF.

.

4. Conclusion

In conclusion, present study showed that CF of *Dangyuja* leaves had antiproliferation activity in a dose-dependent manner. It was confirmed that CF had effects of DNA fragmentation, chromatin condensation and apoptotic body formation in HeLa cells. Through the western blotting, it was verified that the level of caspase-9, -7, -3, -8 and PARP decreased. The ratio of pro-apoptotic protein, Bax/pro-survival protein, Bcl-2 was increased. The caspase-3 activity was increased. In cell cycle, subG-1 phase increased in a dose-manner.

As a result of this, it was demonstrated that CF of *Dangyuja* leaves induced apoptosis in HeLa cells through the intrinsic and extrinsic pathway. In addition, phytochemical analysis was conducted by LC-MS/MS spectrometry and 4 polymethoxyflavones, sinensetin, 4',5,6,7-tetramethoxyflavone, nobiletin and tangeretin, were identified including few phenolic acids. The apoptotic activity of CF of *Dangyuja* leaves could be attributed to the presence of various phytochemicals that might to interact among themselves, resulting in a synergistic or canceling effect on anticancer action. These results suggested that Dangyuja leaves is excellent sources of functional polymethoxyflavones that may help prevent female cancer and a potentially useful agent for the treatment of certain malignancies. However, additional studies are needed to determine the molecular mechanisms of the active components and to evaluate the potential in vivo anticancer activity of extract.

5. Reference

American Cancer Society (December 2007). “Report sees 7.6 million global 2007 cancer deaths.”

Anagnostopoulou, M.A., Kefalas, P., Kokkalou, E., Assimopoulou, A.N. and Papageorgiou, V.P., 2005. Analysis of antioxidant compounds in sweet orange peel by HPLC-diode array detection-eletrospray ionization mass spectrometry. Biomedical Chromatography 19, 138–148.

Attaway, J.A, 1994. Citrus juice flavonoids with anticarcinogenic and antitumor properties. In Food Phytochemicals for Cancer Prevention I; Maple Press: York, PA, pp 240-248.

Baldi, A., Rosen, R.T., Fukuda, E.K. and Ho, C.T., 1995. Identification of nonvolatile components in lemon peel by high-performance liquid chromatography with confirmation by mass spectrometry and diode-array detection. Journal of Chromatography A 718, 89-97.

Benavente-Garcia, O., Castillo, J. and del Rio Conesa, J.A., 1993. Changes in neodiosmin

levels during the development of Citrus aurantium leaves and fruits postulation of a neodiosmin biosynthetic pathway. Journal of Agricultural and Food Chemistry 41, 1916-1919.

Benavente-Garcı´a, O., Castillo, J., Marin, F.R.; Ortun˜o, A., Del Rı´o, J.A., 1997. Use and properties of Citrus flavonoids. Journal of Agricultural and Food Chemistry 45, 4506-4515.

Boatright, K.M. and Salvesen, G.S., 2003. Mechanisms of caspase activation. Current Opinio in Cell Biology 15, 725-731.

Boo, K.H., Kim, H., Riu, K.Z., Kim, Y.W., Cho, M. and Cho, S.K., 2007. Analysis of the limonoid contents of *Dangyuja* (*Citrus* spp.) by liquid chromatography-mass spectrometry (LC-MS). Journal of the Korean Society for Applied Biological Chemistry 50, 238-243.

Cancer Research UK (January 2007). “UK cancer incidence statistics by age.”

Castillo, J., Benavente, O. and Del Rio, J.A., 1992. Naringin and neohesperidin levels during development of leaves, flower buds, and fruits of *Citrus aurantium*. Plant Physiology 99, 66–73.

Castillo, J., Benavente, O. and del Rio, J.A., 1993. Hesperetin 7-O-glucoside and pruning in Citrus species (*C. aurantium* and *C. paradisi*). A study of their quantitative distribution in immature fruits and as immediate precursors of neohesperidin and naringin in *Citrus aurntium*. Journal of Agricultural and Food Chemistry 41, 1920-1924.

Cho, S.K., Yang, S.-O., Kim, S.-H., Kim, H., Ko, J.S., Riu, K.Z., Lee, H.-Y. and Choi, H.-K., 2009. Classification and prediction of free-radical scavenging activities of *Dangyuja* (*Citrus grandis* Osbeck) fruit extracts using ^{1}H NMR spectroscopy and multivariate statistical analysis. Journal of Pharmaceutical and Biomedical Analysis 49, 567-571.

Cory, S. and Adams, J.M., 2002. The Bcl 2 family: regulators of the cellular lifeor-death switch. Nature Reviews Cancer 2, 647-656.

Dhuique-Mayer, C., Tbatou, M., Carail, M., Caris-Veyrat, C., Dornier, M. and Amiot, M.J., 2007. Thermal degradation of antioxidant micronutrients in *Citrus* juice : kinetics and newly formed compounds. Journal of Agricultural and Food Chemistry 55, 4209-4216.

El-Shafae, A.M., 2002. Bioactive polymethoxyflavones and flavanone glycosides from the

peels of Citrus deliciosa. Zhonghuá yáoxué zázhì 54, 199-206

Gattuso, G., Barreca, D., Caristi, C., Gargiulli, C. and Leuzzi, U., 2007. Distribution of flavonoids and furocoumarins in juices from cultivars of *Citrus bergamia* Risso. Journal of Agricultural and Food Chemistry 55, 9921–9927.

Gil-Izquierdo, A., Riquelme, M.T., Porras, L. and Ferreres, F., 2004. Effect of the Rootstock and interstock grafted in lemon tree (*Citrus limon* (L.) Burm.) on the flavonoid content of lemon juice. Journal of Agricultural and Food Chemistry 52, 324-331.

Green, C.O., Wheatley, A.O., Osagie, A.U., Morrison, E.Y. St. A. and Asemota, H.N., 2006. Determination of polymethoxyflavones in peels of selected Jamaican and Mexican citrus (*Citrus spp.*) cultivars by high-performance liquid chromatography Biomedical Chromatography 21, 8–54.

Green, D.R. and Reed, J.C., 1998. Mitochondria and apoptosis. Science 281, 1308-1312.

Greenlee, R.T., Hill-Harmon, M.B., Murray, T. and Thun, M., 2001. Cancer statistics. A

Cancer Journal for Clinicians 51, 15-36

Gross, M.L., 1994. Accurate masses for structure confirmation. Journal of the American Society for Mass Spectrometry 5, 57.

Guthrie, N. and Carroll, K.K., 1998. Inhibition of mammary cancer by citrus flavonoids. Advances in Experimental Medicine and Biology 439, 227-236

He, X., Lian, L., Lin, L. and Bernart, M.W., 1997. High-performance liquid chromatography-electrospray mass spectrometry in phytochemical analysis of sour orange (*Citrus aurantium* L.). Journal of Chromatography A 791, 127–134.

Hengartner, M.O., 2000. The biochemistry of apoptosis. Nature 407, 770-776

Hertog, M.G.L., Hollman, P.C.H. and Katan, M.B., 1992. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. Journal of Agricultural and Food Chemistry 40, 2379-2383.

Hirano, T., Abe, K., Gotoh, M. and Oka, K., 1995. Citrus flavone tangeretin inhibits leukaemic HL-60 cell growth partially through induction of apoptosis with less cytotoxicity on normal lymphocytes. British Journal of Cancer 72, 1380-1388.

Hirata, T., Fujii, M., Akita, K., Yanaka, N., Ogawa, K. and Kuroyanagi, M. *et al.*, 2009. Identification and physiological evaluation of the components from Citrus fruits as potential drugs for anti-corpulence and anticancer Bioorganic and Medicinal Chemistry 17, 25–28.

Huppertz, B., Frank, H.G. and Kaufmann, P., 1999. The apoptosis cascade-morphological and immunohistochemical methods for its visualization. Anatomy and Embryology 200, 1–18.

Hyun, P.W., Hee, C.Y., Won, J.C., Oh, P.J., Kim, K., Hyuck, I.Y., Lee, M.H., Ki, K.W. and Park, K., 2003. Arseni trioxide inhibits the growth of A498 renal cell carcinoma cells via cell cycle arrest or apoptosis. Biochemical and Biophysical Research Communications 300, 230-235.

Justesen, U., Knuthsen, P. and Leth, T., 1998. Quantitative analysis of flavones, and

flavanones in fruits, vegetables and beverages by high-performance liquid chromatography with photo-diode array and mass spectrometric detection. Journal of Chromatography A 799, 101-110.

Kandaswami, C., Perkins, E., Soloniuk, E.S., Drzewiecki, G. and Middleton, E., 1991. Antiproliferative effects of citrus flavonoids on a human squamous cell carcinoma in vitro. Cancer Letters 56, 147–152.

Kaufmann, S.H. and Hengartner, M.O., 2001. Programmed cell death: alive and well in the new millennium. Trends in Cell Biology 11, 526-534.

Keys, A., 1995. Mediterranean diet and public health: personal reflections. The American journal of Clinical Nutrition 61, 1321S-1323S.

Kim, H.Y., 1988. Distribution, taxonomy, horticultural characters of the local *Citrus* spp. in Cheju and the genetic markers among them. Ph.D thesis, Chonnam National University 1988.

Kim, G.-N., Shin, J.-G. and Jang, H.-D., 2009. Antioxidant and antidiabetic activity of *Dangyuja* (*Citrus grandis* Osbeck) extract treated with *Aspergillus saitoi*. Food Chemistry 117, 35-41

Kim, Y., Cho, M. and Cho, S.K., 2008. In vitro antioxidant and cytoprotective activities of the extract of *Dangyuja* (*Citrus grandis* Osbeck) leaves. Food Science and Biotechnology 17, 1086-1091.

Kobayashi, T., Sawa, H., Morikawa, J., Zhang, W. and Shiku, H., 2000. Bax induction activates apoptotic cascade via mitochondria cytochrome c release and Bax of the expression enhances apoptosis induced by chemotherapeutic agents in DLD-1 colon cancer cells. Japanese Journal of Cancer Research 91, 1264-1268.

Kurowska, E.M. and Manthey, J.A., 2004. Hypolipidemic effects and absorption of citrus polymethoxylated flavones in hamsters with diet-induced hypercholesterolemia. Journal of Agricultural and Food Chemistry 52, 2879-2886.

Larocca, L.M., Piantelli, M., Leone, G., Sica, S., Teofili, L. and Benedetti-Panici *et al.*, 1990. Type II oestrogen binding sites in acute lymphoid and myeloid leukaemias: Growth inhibitory effect of oestrogen and flavonoids British Journal of Haematology 75, 489-495.

Launay, S., Hermine, O., Fontenay, M., Kroemer, G., Solary, E. and Garrido, C., 2005. Vital functions for lethal caspases. Oncogene 24, 5137-5148.

Li, S., Lambros, T., Wang, Z., Goodnow, R. and Ho, C.T., 2007. Efficient and scalable method in isolation of polymethoxyflavones from orange peel extract by supercritical fluid chromatography Journal of Chromatography B 846, 291–297.

Li, S., Lo, C.Y., Ho and C.T., 2006. Hydroxylated polymethoxyflavones and methylated flavonoids in sweet orange (*Citrus sinensis*) peel. Journal of Agricultural Food Chemistry 54, 4176-4185.

Li, S., Pan, M.H., Lai, C.S., Lo, C.Y., Dushenkov, S. and Ho, C.T., 2007. Isolation and syntheses of polymethoxyflavones and hydroxylated polymethoxyflavones as inhibitors of HL-60 cell lines Bioorganic and Medicinal Chemistry 15, 3381–3389.

Li, S., Pan, M.H., Lo, C.Y., Tan, D., Wang Y. and Shahidi, F. *et al.*, 2009b. Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review Food Chemistry 104, 466–479.

Li, S., Pan, M.H., Lo, C.Y., Tan, D., Wang, Y. and Shahidi, F. *et al.*, 2009a. Chemistry and health effects of polymethoxyflavones and hydroxylated polymethoxyflavones Journal of Functional Foods 1, 2–12.

Li, X., Xiao, H., Liang, X., Shi, D. and Liu, J., 2004. LC-MS/MS determination of naringin, hesperidin and neohesperidin in rat serum after orally administrating the decoction of *Bulpleurum falcatum* L. and *Fractus aurantii*. Journal of Pharmaceutical and Biomedical Analysis 34, 159–166.

Lim, H.K., Moon, J.Y., Kim, H., Cho, M. and Cho, S.K., 2009. Induction of apoptosis in U937 human leukaemia cells by the hexane fraction of an extract of immature *Citrus grandis* Osbeck fruits. Food Chemistry 114, 1245-1250.

Lim, H.K., Yoo, E.S., Moon J.Y., Jeon Y.J. and Cho, S.K., 2006. Antioxidant activity of extracts from *Dangyuja* (*Citrus grandis* Osbeck) fruit produced in Jeju Island. Food Science and Biotechnology 15, 312-316.

Manthey, J.A., Guthrie N. and Grohmann, K., 2001. Biological properties of citrus flavonoids pertaining to cancer and inflammation. Current Medicinal Chemistry **8,** 135–153.

Marchetti, P., Susin, S.A., Decaudin, D., Gamen, S., Castedo, M., Hirsch, T., Zamzami, N., Naval, J., Senik, A. and Kroemer, G., 1996. Apoptosis associated derangement of mitochondrial function in cells lacking mitochondrial DNA. Cancer Research 56, 2033-2038.

Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., LaFace, D.M. and Green, D.R., 1995. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. Journal of Experimental Medicine 182, 1545-1556.

Matsumoto, H., Ikoma, Y., Sugiura, M., Yano, M. and Hasegawa, Y., 2004. Identification and quantification of the conjugated metobolites derived from orally administered hesperidin

in rat plasma. Journal of Agricultural and Food Chemistry 52, 6653–6659.

McDonnell, T.J., Beham, A., Sarkiss, M., Andersen, M.M. and Lo, P., 1996. Importance of the bcl-2 family in cell death regulation. Experientia 52, 1008-1017.

Meterissian, S.H., Kontogiannea, M., Al-Sowaidi, M., Linjawi, A., Halwani, F., Jamison, B. and Edwardes, M., 2001. Bcl-is a useful prognostic marker in Duke's B colon cancer, Annals of Surgical Oncology 8, 533–537.

Miyagi, Y., Om, A.S., Chee, K.M. and Bennik, M.R., 2000. Inhibition of azoxymethane-induced colon cancer by orange juice. Nutrition and Cancer 36, 224-229.

Mizuno, M., Iinuma, M., Ohara, M., Tanaka, T. and Iwamasa, M., 1991. Chemotaxonomy of the genus citrus based on polymethoxyflavones Chemical and Pharmaceutical Bulletin 39, 945–949.

Mokbel, M.S. and Hashinaga, F., 2006. Evaluation of the antioxidant activity of extracts from buntan (*Citrus grandis* Osbeck) fruit tissues. Food Chemistry 94, 529–534.

Moon, J.Y., Kim, H., Cho, M., Chang W.Y., Kim, C.-T. and Cho, S.K., 2009. Induction of apoptosis in SNU-16 human gastric cancer cells by the chloroform fraction of extracts of *Dangyuja* (*Citrus grandis*) leaves. Journal of the Korean Society for Applied Biological Chemistry 52, 168-175.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survivals: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55-63.

Mously, P., Gaydou, E.M. and Auffray, A., 1998. Simultaneous separation of flavanone glycosides and polymethoxylated flavones in citrus juices using liquid chromatography. Journal of Chromatography A 800, 171-179.

Nicholson, D.W. and Thornberry, N.A., 1997. Caspases: killer proteases. Trends in Biochemical Sciences 22, 299-306.

Olano-Martin, E., Rimbach, G.H., Gibson, G.R. and Rastall R.A., 2003. Pectin and pectin-oligosaccharides induce apoptosis in in vitro human colonic adenocarcinoma cells. Anticancer Research 23, 341-346.

Peterson, G. and Barnes, S., 1993. Genistein and biochain A inhibit the growth of human prostate cancer cells but not epidermal growth factor receptor tyrosine autophosphorylation. Prostate 22, 335–345.

Pienta, K.J., Naik, H., Akhtar, A., Yamazaki, K., Replogle, T.S., Jeffrey, L., Terry, L., Donat, L.T., Victor, H. and Avraham, R., 1995. Inhibition of spontaneous metastasis in a rat prostate cancer model by oral administration of modified citrus pectin. Journal of the National Cancer Institute 87, 348-353.

Poulose, S.M., Harris, E.D. and Patil, B.S., 2005. Citrus limonoids induce apoptosis in human neuroblastoma cells and have radical scavenging activity. Journal of Nutrition 135, 870-877

Reed, J.C., 2000. Mechanisms of apoptosis. American Journal of Pathology 157, 1415–1426.

Sa´nchez-Rabaneda, F., Ja´uregui, O., Casals, I., Andre´s-Lacueva, C., Izquierdo-Pulido, M. and Lamuela Revento´ s, R. M., 2003. Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa (Theobroma cacao). Journal of Mass Spectrometry 38, 35–42.

Rouseff, R.L. and Ting, S.V., 1979. Quantitation of polymethoxylated flavones in orange juice by high-performance liquid chromatography. Journal of Chromatography B 176, 75-87.

Sa´nchez-Rabaneda, F., Ja´uregui, O., Lamuela-Ravento´ s, R. M., Bastida, J., Viladomat, F. and Codina, C., 2003. Identification of phenolic compounds in artichoke waste by high-performance liquid chromatography-tandem mass spectrometry. Journal of Chromatography A 1008, 57–72.

Saraste, A. and Pulkki, K., 2000. Morphologic and biochemical hallmarks of apoptosis. Cardiovascular Research 45, 528-537.

Scambia, G., Ranelletti, F.O., Benedetti-Panici, P., Piantelli, M., Bonanno G. and De Vincenzo, R. *et al.*, 1990. Inhibitory effect of quercetin on OVCA 433 cells and presence of type II oestrogen binding sites in primary ovariun tumors and cultured cells, British Journal of Cancer 62, 942–946.

Smith, H.O., Tiffany, M.F., Qualls, C.R. and Key, C.R., 2000. The rising incidence of adenocarcinoma relative to squamous cell carcinoma of the uterine cervix in the United States-a 24-year population-based study. Gynecologic Oncology 78, 97-105

Sugiyama, S., Umehara, K., Kuroyanagi, M., Ueno, A., Taki, T., 1993. Studies on the differentiation inducers of myeloid leukemic cells from Citrus species. Chemical and Pharmaceutical Bulletin 41, 714-719.

Sun, C.D., Chen, K.S., Chen, Y. and Chen, Q.J., 2005. Contents and antioxidant capacity of limonin and nomilin in different tissues of citrus fruit of four cultivars during fruit growth and maturation. Food Chemistry 93, 599–605.

Thompson, C.B., 1995. Apoptosis in the pathogenesis and treatment of disease. Science 267,

1456-1462.

Thornberry, N.A., 1998. Caspases: key mediators of apoptosis. Chemistry and Biology 5, R97-R103.

Tounekti, O., Belehradek Jr., J. and Mir, L.M., 1995. Relationships between DNA fragmentation, chromatin condensation, and changes in flow cytometry profiles detected during apoptosis. Experimental Cell Research 217, 506-516.

Van Cruchten, S. and Van Den Broeck, W., 2000. Morphological and biochemical aspects of apoptosis, oncosis and necrosis. Anatomia, Histologia, Embryologia 31, 214-223.

Vanamala, J., Leonardi, T., Patil, B.S., Taddeo, S.S., Murphy, M.E. and Pike, L.M., 2006. Suppression of colon carcinogenesis by bioactive compounds in grapefruit. Carcinogenesis 27, 1257-1265

Vander Heiden, M.G. and Thompson, C.B., 1999. Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis. Nature Cell Biology 1, 209-216.

Walle, T., 2007. Methoxylated flavones, a superior cancer chemopreventive flavonoid subclass Seminars in Cancer Biology 17, 354–362.

Wang, G., Chen, H.F., Huang M.H., Wang N.L., Zhang, J.C., Zhang, Y., *et al.*, 2006. Methyl protodioscin induces G2/M cell cycle arrest and apoptosis in HepG2 liver cancer cells, Cancer Letters 241, 102-109

Wang, D., Wang, J., Huang, X., Tu, Y. and Ni, K., 2007. Identification of polymethoxylated flavones from green tangerine peel (*Pericarpium Citri Reticulatae Viride*) by chromatographic and spectroscopic techniques. Journal of Pharmaceutical and Biomedical analysis 44, 63-69.

WHO (February 2006). "Cancer" World Health Organization.

Yang, E.-J., Kang, G.-J., Lee, H.-J., Park, S.-S., Yoon, W.-J., Kang, H.-K., Kim, S.-M. and Yoo, E.-S., 2008. Anti-inflammatory effect of *Citrus grandis* Osbeck (*Dangyuja*) leaves in LPS-stimulated raw 2764.7 cells. Cytokine 43, 330.

Yoo, K.M., Lee, K.W., Park, J.B., Lee H.J. and Hwang, I.K., 2004. Variation in major antioxidants and total antioxidant activity of yuza (Citrus junos Sieb ex Tanaka) during maturation and between cultivars. Journal of Agricultural and Food Chemistry 52, 5907-5913.

Yoshida, M., Sakai, T., Hosokawa, N., Marui, N., Matsumoto, K. and Nishino, H. *et al.*, 1990. The effect of quercetin on cell-cycle progression and growth of human gastric cancer cells, FEBS Letters 260**,** 10–13.

Zhou, D.Y., Chen, D.L., Xu, Q., Xue, X.Y., Zhang, F.F. and Liang, X.M., 2007. Characterization of polymethoxylated flavones in *Fructus aurantii* by liquid chromatography with atmospheric pressure chemical ionization combined with tandem mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis 43, 1692-1699.

Zhong, W.B., Wang, C.Y., Ho, K.J., Lu, F.J., Chang, T.C. and Lee, W.S., 2003. Magnolol induces apoptosis in human leukemia cells via cytochrome c release and caspase activation. Anti-Cancer Drugs 14, 211-217.

ACKNOWGEMENT

5년 전 꿈나무로부터 시작하여,

지금은 실험실의 고참으로써

부족한 저를 여기까지 이끌어주시고 지켜 봐주신 모든 분들께

지면으로나마 감사의 말을 전합니다.

감사합니다.