Master's Thesis

Antioxidant and Anticancer Activity of Extracts from *Dangyuja* (*Citrus grandis* Osbeck) Leaves Produced in Jeju Island

Department of Biotechnology

Graduate School

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Antioxidant And Anticancer Activity of Dangyuja (*Citrus grandis* Osbeck) Leaves Produced in Jeju Island.

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ABSTRACT IN KOREAN

지구상 대부분의 생명체는 공기 중의 산소를 호흡하여 산화시켜 얻어지는 에너지를 이용하여 생명을 유지하는데, 이런 산소가 필요한 대사과정에서 불가피하게 세포를 파괴시키는 독성물질들이 부산물로 만들어지는데 이것을 활성산소라고 한다. 활성산소는 생체 조직을 공격하여 세포를 산화, 손상시키는 주범이며 유해산소라고도 한다. 활성산소는 세포나 세포소기관에 손상을 초래하기도 하며 생체 내 여러 단백질의 아미노산을 산화시켜 단백질의 기능 저하를 초래한다. 핵산에도 손상을 주는데 핵산 염기의 변형, 핵산 염기의 유리, 결합의 절단, 당의 산화 분해 등을 초래하여 돌연변이나 암의 원인이 되기도 한다. 이로 인해 우리 몸이 노화되고 손상되는 것을 막아주는 물질을 항산화제 (antioxidants) 라고 한다.

항산화제는 인체 내에 자연적으로 존재하는 것과 외부에서 투여해 주는 것으로 나눌 수 있다. 인체 내에 자연적으로 존재하는 항산화제로는 SOD, glutathione, peroxidase 등의 효소와 요산, 빌리루빈 등이 있으며 외부에서 투여해 주는 것으로 비타민 E, C, 베타카로틴이 있으며 미네랄 중에는 셀레니움이 대표적이다. 이들 항산화제들은 활성 산소의 독작용을 제거하여 생체를 보호하고 있으며 항산화 물질이 활성산소를 적절히 제거하지 못할 경우 축적되는 활성산소에 의해 여러 가지 질병이나 노화가 초래된다고 알려져 있다.

과일과 채소의 섭취는 여러 가지 암의 발생률을 낮춘다는 역학조사가 많이 보고되고 있다. 과일이 질병을 억제 하는 이유 중의 하나는 여러 가지 영양 성분 중의 하나인 비타민 C와 E가 있기 때문인 것으로 알려져 왔으나 최근의 연구들은 항산화 역할을 하는 것은 비타민뿐만 아니라 과일에 많이 들어 있는 폴리페놀화합물, 플라보노이드류 등이라는 것이 보고되고 있다.

당유자는 제주에서 왕귤 또는 뎅유지라고 불리우며, 인체에 기능성을 가지는 성분으로 limonene, obacunone, nomiline, naringin 등을 함유하고 있는데, 특히 limonoid 성분이 다량 함유되어 있어 암세포 증식 억제 및 예방에 효과가 있고, 임상실험 결과 간, 폐, 장, 피부암 억제에 효과가 있는 것으로 알려져 있다. 당유자는 쓴맛과 향기가 강하여 당 절임한 당유자청 등을 이용한 음료에 이용되어 왔고, 주로 과육만 이용하는 다른 감귤류와는 달리 과육과 과피를 모두 이용하는 과일이므로 과피 부분에 많이 함유되어 있는 생리활성 성분을 귤이나 오렌지 같은 과일보다 용이하게 섭취할 수 있는 장점을 가지고 있다. 당유자 껍질은 두텁고 맛은 달며, 독이 없고 위속에 악기를 없애며 술독을 풀어주고 입맛을 좋게 하는 것으로 알려져 있으며, 최근 당유자 추출물을 이용한 실험에서 항바이러스 효과 및 항산화 효과가 있는 것으로 보고되었다.

특히 당유자 미숙과에는 성숙되었을 때에 비하여 비타민 C 함량은 적지만, citric acid가 2배 이상 높아 상큼한 맛을 낼 수 있고, 항알러지 작용 및 항산화 효과가 있는 것으로 알려진 flavonoid 성분인 naringin이 5배, hesperidin이 10배 더 많은 것으로 보고되었다. 민간에서는 과즙을 다려서 복용하면 위장장애, 천식 동맥경화 등에도 효과가 있는 것으로 알려져 있다. 그러나 이러한 당유자의 과육, 과피에 대한 연구는 되어 있으나 잎에 대한 연구는 아직 없으며, 이러한 활성 및 그 기전에 대한보고 또한 없는 실정이다.

따라서 본 연구는 당유자 잎의 여러 가지 용매 추출물의 항산화 활성을 살펴보고 암세포의 세포사멸 효과에 대한 가능성을 제시하고자 한다. 당유자 잎 유기용매 분획물의 DPPH 라디칼 소거활성은 항산화제로 널리 알려져 있는 BHT 및 녹차 추출물과 비교하였을 때, 500 μg/mL의 농도에서는 *n*-butanol과 ethyl acetate 분획(EF)에서 각각 71.92%와 71.86%로 높은 활성을 나타내었지만 50 μg/mL 농도에서는 EF에서 가장 높은 활성을 나타냈다. *t*-BHP 처리로 유발된 HepG2 세포의 ROS 수준, 지질 과산화, DNA 손상에 대한 억제 효과는 EF의 낮은 농도에서도 유의하게 나타났다. 또한 MTT assay를 통한 세포 보호 효과에서도 500 μg/mL의 농도에서 약 95%의 우수한 세포 보호효과를 가지는 것을 확인하였다. 또한 HepG2 세포를 EF 처리 후, *t*-BHP로 apoptosis를 유도한 결과, *t*-BHP만을 처리한 세포에서는 분획물을 처리하지 않은 대조구에 비해 apoptotic body가 많이 관찰되었으며, p53, cleaved caspase 3, cleaved caspase 7, 및 cleaved PARP의 발현이 증가하는 것을 확인하였다. 이에 비해 EF를 처리한 세포에서는 농도 의존적으로 apoptotic body의 생성을 억제하였으며, apoptosis에 관여하는 여러 단백질들의 발현 또한 억제하였다. 또한 당유자 잎의 ethyl acetate 분획물의 항암 활성을 알아보기 위하여, HepG2 세포에 EF를 72시간 동안 처리하였다. 그 결과 농도의존적으로 세포 성장을 억제하였으며, 정상 세포주에서는 아무런 영향을 미치지 않았다. 또한 DNA 손상 및 apoptotic body 형성에 있어서도 농도 의존적으로 apoptosis를 일으키는 것으로 나타났다. 마지막으로 apoptosis에 관계된 여러 가지 단백질들의 발현을 western blotting으로 확인하였다. 그 결과 pro-apoptotic 단백질인 Bax와 Bad의 발현은 증가시켰고, 이와는 반대로 anti-apoptotic 단백질인 Bid의 발현은 감소시켰다. 또한 caspase 3 및 PARP의 발현 역시 감소됨을 확인하였다.

이러한 결과를 종합해 볼 때, 향후 당유자 잎의 생리활성 성분을 이용한 다양한 제품 개발을 통해 당유자의 소비 증진과 소비자의 건강 증진에 기여할 수 있을 것이라 사료된다.

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INTRODUCTION

Free radicals are normal oxidant by-products of aerobic metabolism, and under normal metabolic conditions about 2–5% of O_2 consumed by mitochondria is converted to reactive oxygen species (ROS) (Cadenas and Davies, 2000). ROS include three major radical species: superoxide anion radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), highly reactive hydroxyl radical ($^{\cdot}OH$), and peroxyl radicals ($ROO^{\cdot -}$) (Halliwell et al., 1995).

These reactive ROS cause destructive and irreversible damage to the components of a cell, such as lipids, proteins and DNA (Lopaczyski and Zeisel, 2001). Although normal cells possess antioxidant defense systems against ROS, the continuous accumulation of damage to the cells induces diseases such as cancer and aging (Matés and Sánchez-Jiménez, 2000). The continuous antioxidant dose also plays a preventive role against these diseases by removing the ROS in biological systems (Sgambato et al., 2001).

Anticancer agents, on the other hand, are mainly related to their curative role in a damaged system. Under normal conditions, the cells in which the DNA or other components are irreversibly damaged by various causes undergo apoptotic cell death, which is a self-destructive metabolism according to the genetically encoded cell death-signal (Korsmeyer, 1995). However, cancer cells, which are already irreversibly developed, obtain the capability to evade apoptosis by various ways. The aim of anticancer agents is to trigger the apoptosis signaling system in these cancer cells while disturbing their proliferation (Bold et al., 1997).

Antioxidants have been used to inhibit apoptosis because apoptosis was initially thought to be mediated by oxidative stress (Lopaczyski and Zeisel 2001). Many antioxidant materials have anticancer or anticarcinogenic properties (Johnson et al., 1994). For example, resveratrol in grapes and other food products has been shown to protect cells from oxidative damage and cell death (Jang et al., 1997; Chanvitayapongs et al., 1997) and to prevent carcinogenesis in a murine model (Clement et al., 1998). Curcumin,

a yellow coloring ingredient present in turmeric (*Curcuma longa Linn*, Zingiberaceae), has a diarylheptanoid moiety and has anticarcinogenic or antimutagenic effects in diverse animal models and in cultured cells (Rao et al., 1995).

Plants have many phytochemicals with various bioactivities, including antioxidant, anti-inflammatory and anticancer activities. For example, some studies have reported that extracts from natural products, such as fruits, vegetables and medicinal herbs, have positive effects against cancer, compared with chemotherapy or recent hormonal treatments (Jang et al., 1997). Therefore, many plants have been examined to identify new and effective antioxidant and anticancer compounds, as well as to elucidate the mechanisms of cancer prevention and apoptosis (Pietta et al., 1998; Kim et al., 1998 and Swamy and Tan, 2000). In particular, oriental medicinal plants are considered to be one of the most promising sources due to their variety of species and applications. In addition, their therapeutic effect has been demonstrated by their clinical uses in Asia for many decades.

A great number of different traditional herbs have been tested for their antioxidant activity, however, there are still many plants, which were not examined on this matter or the knowledge about their antioxidative properties are very insufficient. *Dangyuja* (*Citrus grandis* Osbeck) is distributed solely in Jeju island in south Korea and the fruit has been utilized for a long time as a traditional herb medicine in Jeju island. Most *Citrus* species accumulate abundant quantities of flavonoids during the development (Benavente-Garca et al., 1993; Castillo et al., 1992, 1993). Much is known about the cancer-preventing potential of dietary citrus flavonoids (Guthrie and Carroll, 1998; Miyagi et al., 2000; Poulose et al., 2005; Vanamala et al., 2006) and the antioxidant activity of citrus fruit extracts (Lim et al., 2006; Jung et al., 2007; Yoo et al., 2004). However, few studies report the biological effects of the citrus leaf on the oxidative stresses induced by either *tert*-butylhydroperoxide (*t*-BHP) or hydrogen peroxide (H_2O_2) in HepG2 cells.

The present study was therefore designed to investigate whether *dangyuja* leaf inhibits

t-BHP-induced oxidative stress and to characterize the mechanism of its anticancer effects in human hepatoma cell line (HepG2).

Part Ⅰ

Antioxidant Activity of Ethyl Acetate Fraction of *Dangyuja* (*Citrus grandis* Osbeck) Leaves Produced in Jeju Island

Part Ⅰ

Antioxidant Activity of Ethyl Acetate fraction of *Dangyuja* (*Citrus grandis* Osbeck) Leaves Produced in Jeju Island

1. ABSTRACT

The oxidative stress has been demonstrated to play an important role in the apoptotic cell death in a variety of cell types. The aim of this work is to investigate the hepatoprotective effects of a dangyuja (*Citrus grandis* Osbeck) leaf on the oxidative damages induced by *tert*-butylhydroperoxide (*t*-BHP) in human hepatoma cell line, HepG2 cells. The antioxidant activities of methanol extract and five different solvent fractions (i.e., hexane- (HF), chloroform- (CF), ethyl acetate- (EF), n-butanol- (BF), and aqueous-fraction (AF)) were evaluated. The EF exhibited the greatest 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity among all the fractions and its activity increased with increasing concentration of the EF. Highest flavonoid content (429.8 mg rutin equivalent/g dried extract) was noticed in EF of dangyuja leaf. Generation of reactive oxygen species (ROS) was reduced whereas lipid peroxidation inhibitory capacity was increased in the HepG2 cells pretreated with EF prior to oxidative damages. Furthermore, the EF exhibited anti-apoptotic actions on the oxidative damaged HepG2 cells; it inhibited chromosomal DNA degradation in a dose dependent manner.

2. MATERIALS AND METHODS

2. 1. Materials

Dangyuja (*C. grandis* Osbeck) leaves were obtained from the National Institute of Subtropical Agriculture, Jeju Province, Korea. All chemicals and reagents were purchased from Sigma Chemicals (St. Louis, MO, USA) and Invitrogen Gibco (Grand Island, NY, USA).

2. 2. Preparation of extracts

Air-dried *dangyuja* leaves were pulverized using a milling machine and extracted with 80% methanol by stirring for 3 days at room temperature (RT). The extract was filtered, concentrated with a vacuum rotary evaporator under reduced pressure, and lyophilized. The methanol extract (ME) was suspended in water and further fractionated with four different solvents in a stepwise manner, as previously described (Lim et al., 2006). Each solvent was extracted three times at RT, evaporated and then freeze -dried (Fig. 1). The resulting fractions were: hexane fraction (HF), chloroform fraction (CF), ethyl acetate fraction (EF), *n*-butanol fraction (BF), and water fraction (WF). The extract powder (s) were dissolved in dimethyl sulfoxide (DMSO) and diluted with phosphate buffered saline (PBS, pH 7.4) to give the final concentrations.

2. 3. Free-radical scavenging activity

DPPH radical scavenging activity was assessed according to the method of Blois (1958) with minor modification. Initially, 10 μL of the test extracts in DMSO, yielding a series of extracts with different concentrations (50, 125, 250, 500 μg/mL) in each reaction, were mixed with 190 μL of 150 μM DPPH in ethanol. The resulting solutions were vigorously mixed and then left to stand in the dark at RT for 30 min. The absorbance of the remaining DPPH was measured at 517 nm using a microplate ELISA reader (MRX II, DYNEX Technologies, Chantilly, VA, USA). Butylated hydroxytoluene (BHT) and 80%

MeOH extract of green tea, with different concentrations (50, 125, 250, 500 μg/mL), were used as positive controls. The negative control lacked the sample. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following equation:

Radical scavenging activity (%) = ([A_0-A_1]/A_0)x100%,

where A_0 is the A517 of DPPH without the sample (control), and A_1 is the A517 of DPPH with the sample.

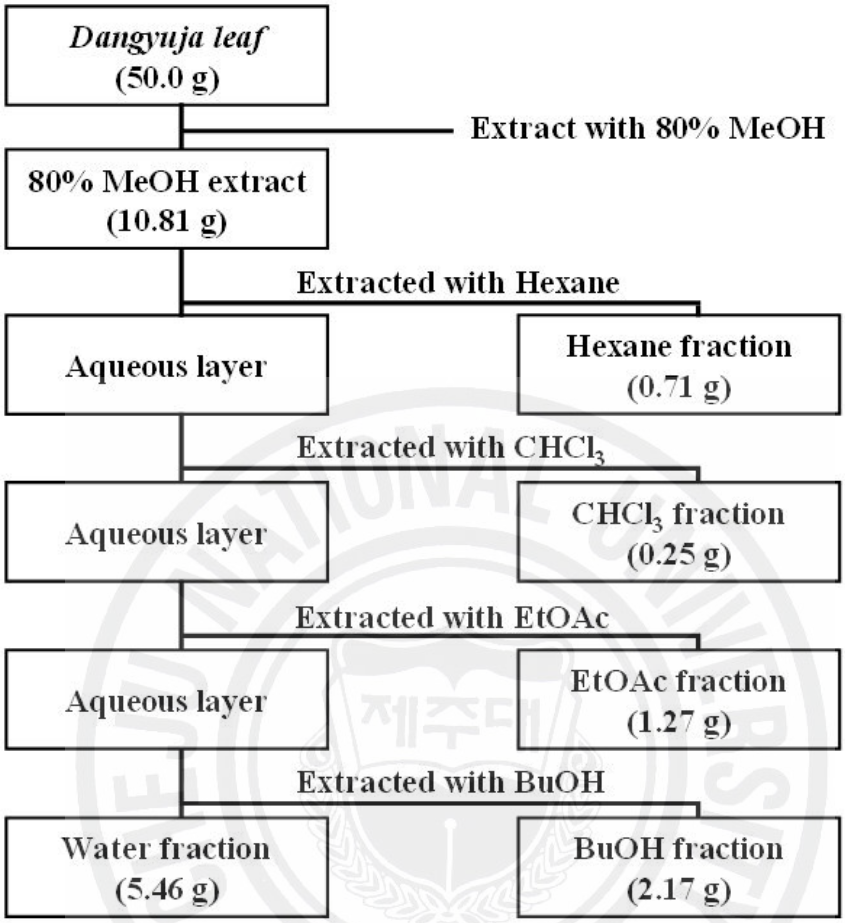

Fig. 1-1. Flow diagram of fractionation of *dangyuja* leaf extract.

2. 4. Cell culture

Human hepatoma, HepG2 (KCLB No.58065) cells were purchased from the Korea Cell Line Bank (Seoul, Korea). The cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in a humidified atmosphere of 95% air, 5% CO_2 at 37 ^{o}C. The culture medium was changed twice a week, and the cells were subcultured at a ratio of 1:4 once a week.

2. 5. Determination of ROS generation

Cellular ROS were quantified using the dichlorofluorescein (DCFH) assay (Wang and Joseph, 1999), in which the esterified form of DCFH-DA diffuses through the cell membrane and is enzymatically deacetylated by intracellular esterases. The resulting compound, DCFH, is reactive with ROS to give an oxidized fluorescent compound, DCF (Datta et al., 2002). HepG2 cultures were pre-treated with EF for 30 min, and then the cultures were washed twice and incubated with 200 μM *t*-BHP for 3 h. DCFH-diacetate was added to the culture plates at a final concentration of 25 μM, and DCF fluorescence was detected over a period of 30 min at 37 ^{o}C at an excitation wavelength of 485 nm and emission wavelength of 530 nm, using a Genios multiwell, fluorescence plate reader (Genios, Tecan, Salzburg, Austria).

2. 6. Inhibition of Lipid Peroxidation

Lipid peroxidation was determined through malondialdehyde (MDA) determinations, according to the method reported by Ohkawa et al. (1979). Lipid peroxides react with thiobarbituric acid (TBA) and develop a pink color due to the formation of TBA reactive substances (TBARS). In this method, cells were exposed to the EF at various concentrations for 1 h, followed by incubation with 200 μM *t*-BHP for 3 h. Oxidation of phospholipids and evaluation of TBARS formation was achieved in a single 96-well

microplate, and the amount of MDA formed as a breakdown product was measured at 532 nm using a microplate ELISA reader (MRX II, DYNEX Technologies, Chantilly, VA, USA).

2. 7. Determination of DNA damage (Comet assay)

The alkaline Comet assay was conducted according to Singh et al. (1995) with slight modification. The cells in a 24-well plate were treated with the extract, as described above. The cell suspension was mixed with 100 μL 0.5% low -melting point agarose (LMPA) and added to slides pre-coated with 1.0% normal melting agarose (NMA). After solidification of the agarose, the slides were covered with another 100 μL of 0.5% LMPA and then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% sodium laurylasarcosine; 1% Triton X-100 and 10% DMSO) at 4 ^{o}C for 1 h. The slides were then placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 min. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied at 4 ^{o}C for 20 min. The slides were washed three times with a neutralizing buffer (0.4 M Tris-HCl, pH 7.5) at 4 ^{o}C for 5 min, and then treated with ethanol for another 5 min before staining with 50 μL ethidium bromide (20 μg/mL). Measurements were made by image analysis (Kinetic Imaging, Komet 5.0, UK) and fluorescence microscopemicroscopy (LEICA DMLB, Germany), determiningto determine the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of two replicate slides).

2. 8. Cytotoxicity assay

To evaluate the cytotoxicity of EF, a MTT colorimetric assay was performed to determine the cell viability (Hasan et al., 1989). HepG2 cells were cultured at a density of 1×10^5 cells/mL on 96-well microplates for 16 h, washed twice using PBS, and pretreated

with EF. After 30 min incubation, *t*-BHP solution was added to the wells, and the cells were re-incubated for 3 h. MTT reagent (5 mg/mL) was added to each well, and the plate was incubated at 37 ℃ for an additional 4 h. The media were then removed, and the intracellular formazan product was dissolved in DMSO. Absorbance at 570 nm of the mixture was detected using a micro plate ELISA reader (MRX II, DYNEX Technologies, Chantilly,VA, USA). The results were determined by three independent experiments.

2. 9. Staining with Hoechst 33342

Apoptotic cells are characterized by nuclear condensation of chromatin and/or nuclear fragmentation (Lizard et al., 1997). HepG2 cells were cultured at a density of 1×10^4 cells/mL on 6-well plates for 16 h, washed twice using PBS, and pretreated with various concentrations of *dangyuja* leaf extracts. After 30 min incubation, 200 μM *t*-BHP solutions were added to the wells, and the cells were re-incubated for 3 h. Then, Hoechst 33342, a DNA specific fluorescent dye was added into the culture medium at a final concentration of 10 μM and plate was incubated for another 20 min at 37 ℃. The morphological aspect of cell nuclei was observed under an Olympus fluorescence microscope.

2. 10. Western Blotting

We cultured cells in 100-mm dishes and treated them with extract as described above. Cells were lyses for 40 min at 4 ℃ with a lysis buffer containing 40 mM of Tris-HCl, pH 8.0, 120 mM of NaCl, 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. We removed the insoluble material by centrifugation at 13,000 rpm for 30 min, and we determined the protein content with the BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amount of lysates (60-100 μg of protein) were separated by a 7.5-15% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, HC, USA) with a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH

8.8), 20% MeOH (v/v)]. After blocking the nonspecific site with 5% nonfat dried milk, the membrane was then incubated with primary antibody against PARP (1:2000), caspase 3 (1:1000), Cleaved caspase-7 (1:1000) Bcl-2 (1:1000) and β-actin (1:100000) antibodies (Santa Cruz Biotech and Cell signaling, MA, USA) and further incubated with a secondary peroxidase-conjugated goat IgG (1:5000, Vector, CA, USA). The PVDF membrane was exposed to X-ray film, and the protein bands were detected using a WEST-ZOL® plus Western Blot Detection System (iNtRON., Gyeonggi-do, Korea).

2.11. Statistical analysis

All results were expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) using the SPSS v 12.0 software package was applied. A difference at $p < 0.05$ was considered to be statistically significant. All assays were performed in triplicate.

3. RESULTS

3. 1. DPPH free radical scavenging activity of extract

The free radical scavenging activities of the various solvent-extracted-fractions of *dangyuja* leaves were determined with the DPPH assay, and the results are shown in Fig. 1-2. The percentage DPPH radical scavenging activities of all the extracts were dose - dependent, although the HF showed minimum activity at almost all-tested concentrations. At a concentration of 500 μg/mL, the 80% MeOH extract of *dangyuja* leaves and their derived fractions decreased in the following order: BF (71.92%) > EF (71.86%) > WF (57.56%) > ME (38.68%) > HF (16.79%) > CF (14.04%). Both the EF and BF showed good inhibitory effects, which were comparable to that of 80% MeOH extract of green tea. However, at a concentration of 50 μg/mL, EF showed better performance against the DPPH radical than BF. These results indicate that the EF of *dangyuja* is a good potential source of antioxidants.

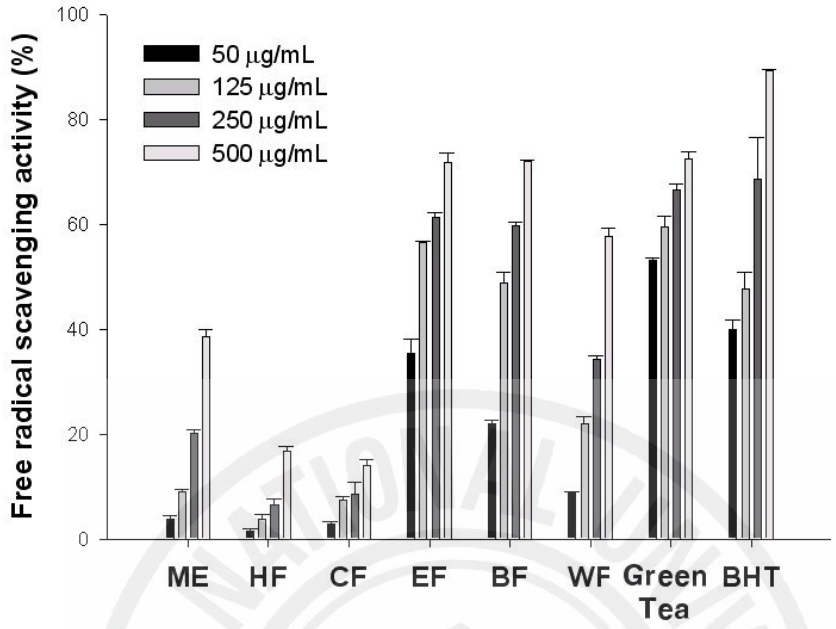

Fig. 1-2. DPPH radical scavenging activity of extracts of *dangyuja* leaves. All extracts were added at 50–500 μg/mL. ME: 80% methanol extract, HF: hexane; EF: ethyl acetate; CF: chloroform; BF: butanol; WF: water -extracted fractions; BHT: butylated hydroxytoluene. Values are means ±SD (n=3).

3. 2. Effect of EF on *t*-BHP-induced intracellular ROS generation

Direct evaluation of intracellular ROS levels is a very good indication of the oxidative damage to living cells (Wang and Joseph, 1999). A prooxidant, such as *t*-BHP, can directly oxidize DCFH to fluorescent DCF, and it can also decompose to peroxyl radicals and generate lipid peroxides and ROS, thus increasing fluorescence (Alía et al., 2006). As shown in Fig. 1-3, increased ROS generation in cultured HepG2 cells submitted to an oxidative stress by *t*-BHP was completely inhibited by pre-treatment with 50 μg/mL of EF for 3 h. These results suggest that the natural antioxidant EF strongly inhibits the generation of ROS induced by *t*-BHP in cultured HepG2 cells.

3. 3. Effects of EF on *t*-BHP-induced lipid peroxidation

The levels of lipid peroxidation evaluated through the malondialdehyde (MDA) concentration in the cultured cells. Thiobarbituric acid reactive substances are produced as by-products of lipid peroxidation–induced by *t*-BHP. The incubation of HepG2 cells for 3 h with 200 μM of *t*-BHP-induced significant increase of MDA level in the cell lysates, about 150.49 ± 2.7%. The EF revealed that the dose-dependent protective effect against lipid peroxidation induced by *t*-BHP (Fig. 1-4). Also, the EF showed the highest inhibition of lipid peroxidation, about 107.5 ± 0.9% at a lower concentration (125 μg). These results indicate that EF has powerful effect against lipid peroxidation induced by *t*-BHP.

3. 4. Effects of EF on *t*-BHP-induced DNA damage

The incubation of HepG2 cells for 3 h with 200 μM of *t*-BHP induced significant DNA damage without cell death, conditions that can be used to assess effects of extracts against DNA damage by the comet assay. As shown in Fig. 7, HepG2 cells were treated with EF, formation of DNA damage induced by the 200 μM *t*-BHP was completely prevented at 500 μg/mL concentrations of EF (Fig. 1-5). The level of DNA strand breaks in cells exposed to *t*-BHP was significantly higher than that in control cells, while the level of DNA strand breaks in cells exposed to *t*-BHP in the presence of EF did not differ significantly from that in the control cells.

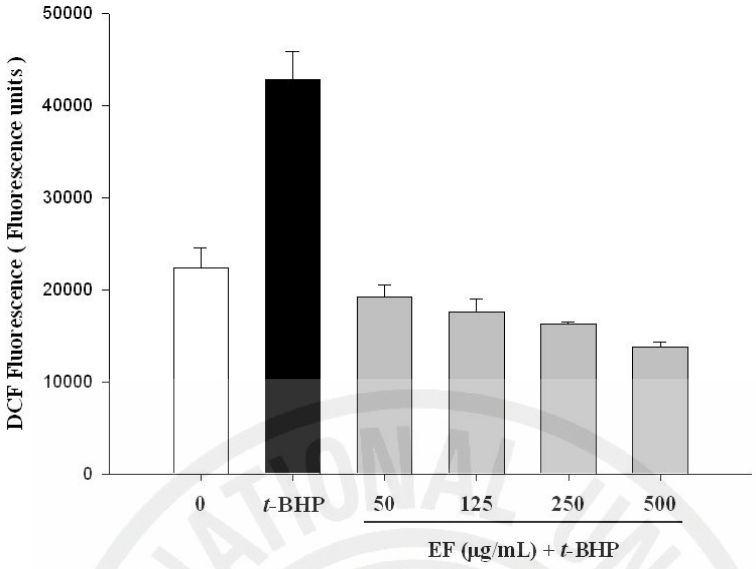

Fig. 1-3. Protective effects of EF treatment on *t*-BHP-induced intracellular reactive oxygen species (ROS) generation in HepG2 cells. HepG2 cells were incubated with 50~500 μg/mL EF for 1 h before the addition of *t*-BHP (200 μM, 3 h) and then the level of intracellular ROS was determined using the peroxide-sensitive fluorescent probe DCFH-DA. Values are presented as means SD (n=4).

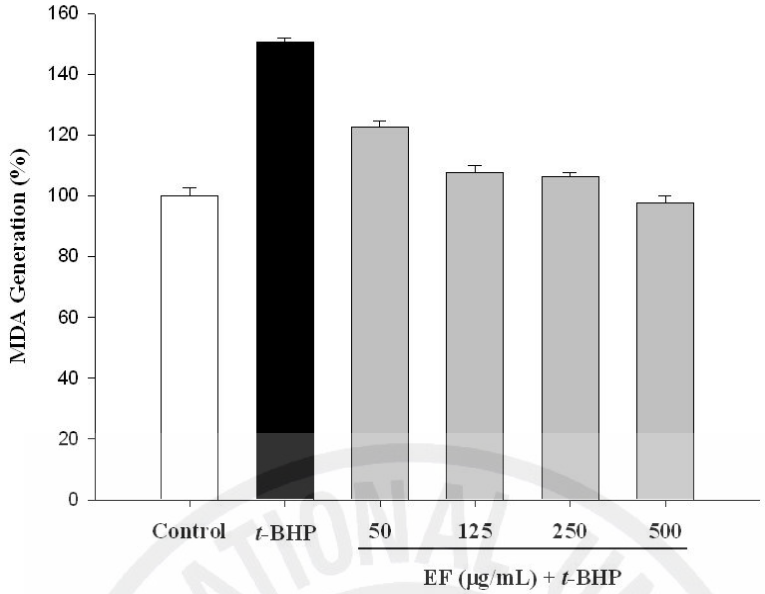

Fig. 1-4. Protective effects of EF treatment on *t*-BHP-induced lipid peroxidation in HepG2 cells. Cultures were treated for 1 h with the noted concentrations of EF and then incubated with 200 μM *t*-BHP for 3 h. Values are presented as means SD (n=4).

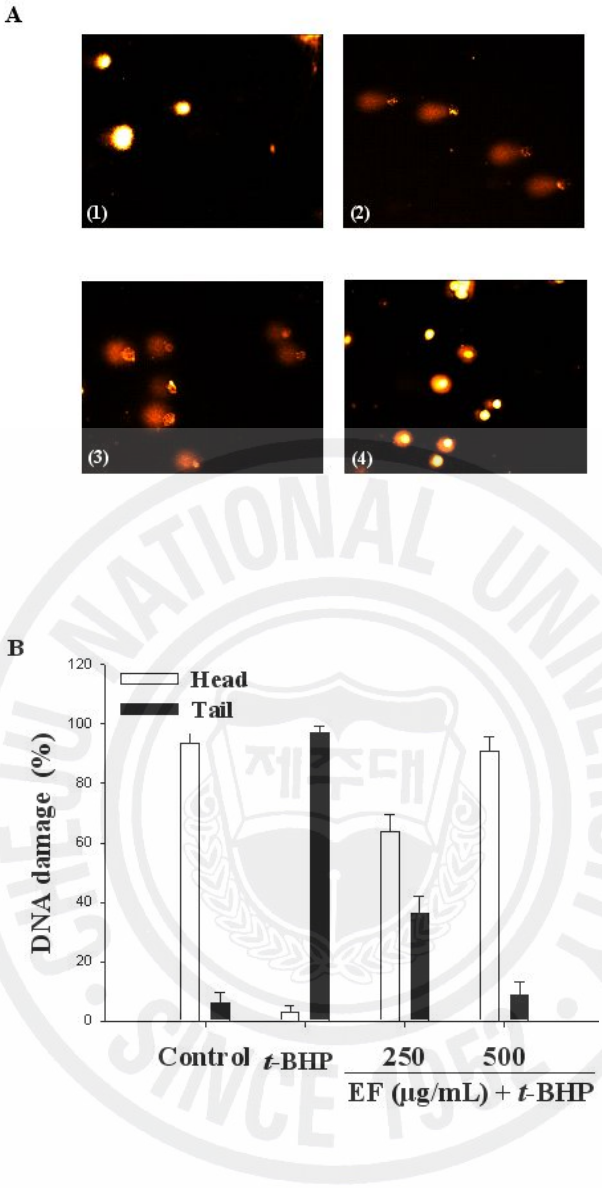

Fig. 1-5. Comet assay of *t*-BHP-induced DNA damage in HepG2 cells. Cells were pretreated with indicated amount of EF for 1 h prior to the incubation of *t*-BHP (200 uM) for 3 h. (A) HepG2 control (1); *t*-BHP only (2); incubation with EF (250 μg/mL) prior to the addition of *t*-BHP (3); incubation with EF (500 μg/mL) prior to the addition of *t*-BHP (4). (B) Graphical representation of Comet Assay. Data represent the % of DNA head and tail analyzed by Comet assay of n=3 independent experiments.

3. 5. EF protected HepG2 cells against *t*-BHP-induced cytotoxicity

MTT was used as an indicator of cytotoxicity induced by *t*-BHP in HepG2 cells in culture. A 3 h treatment with 50, 100, 200 and 400 μM *t*-BHP induced cell death (Fig. 1-6). A dose-dependant increase of cell death was observed with *t*-BHP. Treatment with 200 and 400 μM *t*-BHP shown to induce oxidative damage to HepG2 cells causing about 50% of cell death. No significant cell death was observed in incubations of HepG2 cells with EF (Fig. 1-7). As shown in Fig. 1-8, EF protected against cell death in a dose-dependent manner.

3. 6. Effect of EF on *t*-BHP-induced Nuclear Damage

To clarify the inhibitory effect of EF against cytotoxicity of *t*-BHP, we investigated the effect on the nuclear morphological changes observed in the *t*-BHP treated cells. Nuclear staining with Hoechst 33342 demonstrated that control HepG2 cells had regular and round-shaped nuclei. In contrast, the shrinkage and condensation of nuclei characteristic of apoptotic cells were evident in HepG2 cells treated with 200 μM for 3 h at 37 ℃ (Fig. 1-9). However, *t*-BHP-induced nuclear damage was prevented by EF.

3. 7. Effect of EF on apoptosis-related protein levels

To understand the molecular mechanism by which the effect of EF against *t*-BHP-induced cytotoxicity, we examined various apoptosis-related proteins. *t*-BHP-induced apoptotic cell death was also examined by modulating the expression of p53, Caspase, PARP (Piret et al., 2004; Lee et al., 2005). HepG2 cells were cultured in media containing with or without of EF (50, 125, 250 and 500 μg/mL) for 1 h, and then treatment of 200 μM *t*-BHP for 3 h. At each concentration, total protein was isolated and p53, cleaved caspase 3, cleaved caspase 7, and cleaved PARP (poly ADP-ribosyl polymerase) protein expression levels were measured by Western blotting (Fig. 1-10). HepG2 after treating the HepG2 cells with 200 μM *t*-BHP for 3 h, results showed an induced expression of p53, cleaved caspase 3, cleaved caspase 7, and cleaved PARP protein. The pro-apoptotic protein, p53 was decreased in a dose-dependent manner in response to extract. And cleaved caspase 3 and cleaved caspase 7 levels were also decreased. To investigate the enzymatic activation of caspase 3, we measured the cleaved PARP, which is a caspase-3 substrate. When cells were treated with EF, a dose-dependent decrease in the formation of the 89 kDa fragment of PARP. These results show that EF was significantly depressed the *t*-BHP-induced cell death-related protein expression in dose-dependent manner.

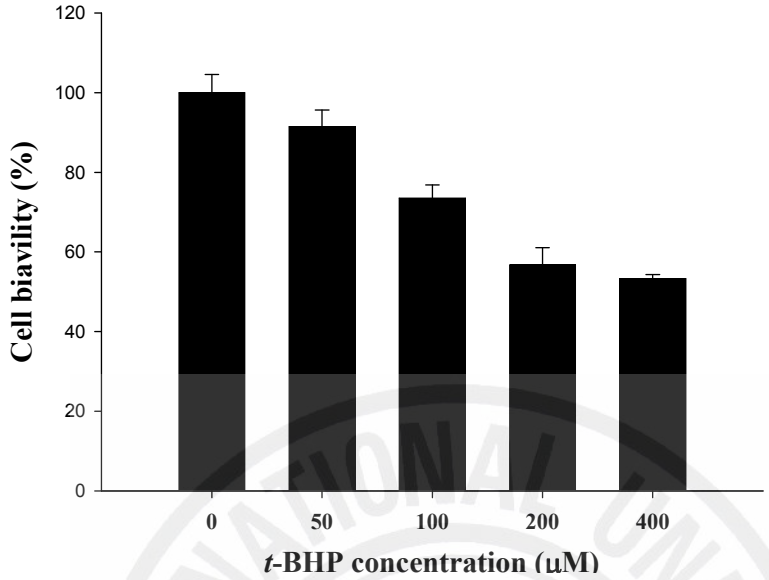

Fig. 1-6. Effects of *t*-BHP-induced cytotoxicity in HepG2 cells. Cultures were treated for 3 h with the noted concentrations of *t*-BHP. Values are presented as means SD (n=3)

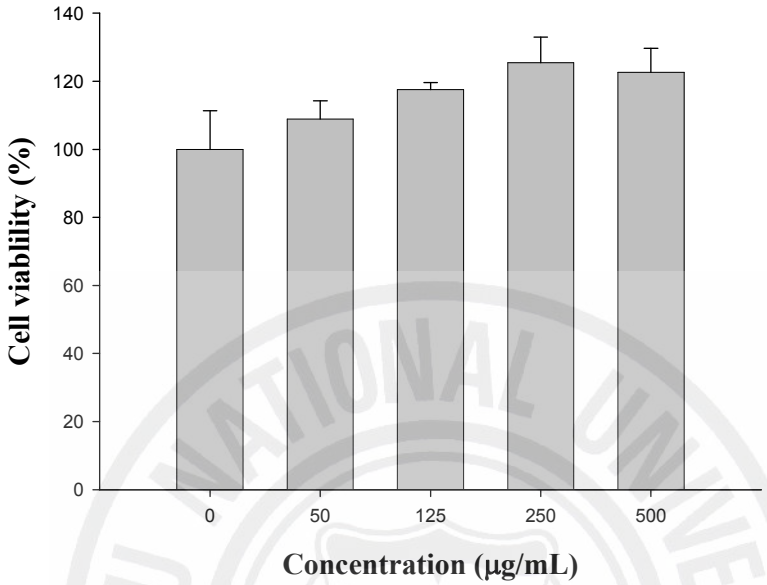

Fig. 1-7. Effects of EF in HepG2 cells. Cultures were treated for 4 h with the noted concentrations of EF. Values are presented as means SD (n=3)

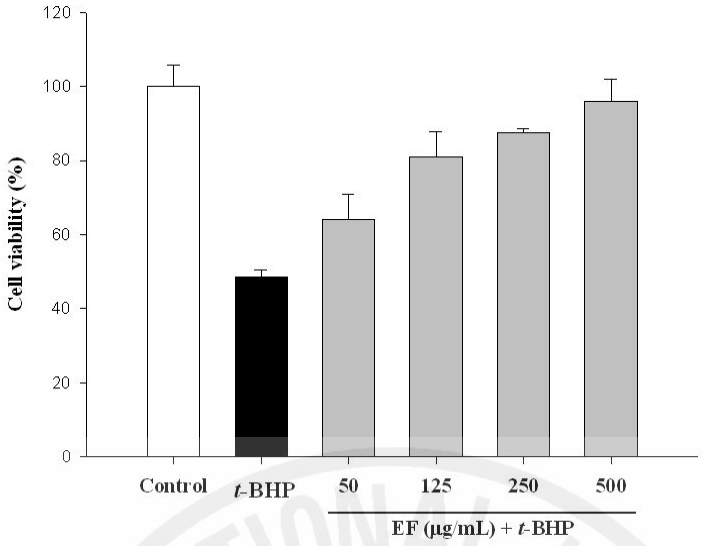

Fig. 1-8. Protective effects of EF on *t*-BHP-induced cytotoxicity in HepG2 cells. Cultures were treated for 1 h with the noted concentrations of EF and then incubated with 200 μM *t*-BHP for 3 h. Values are presented as means SD (n=3)

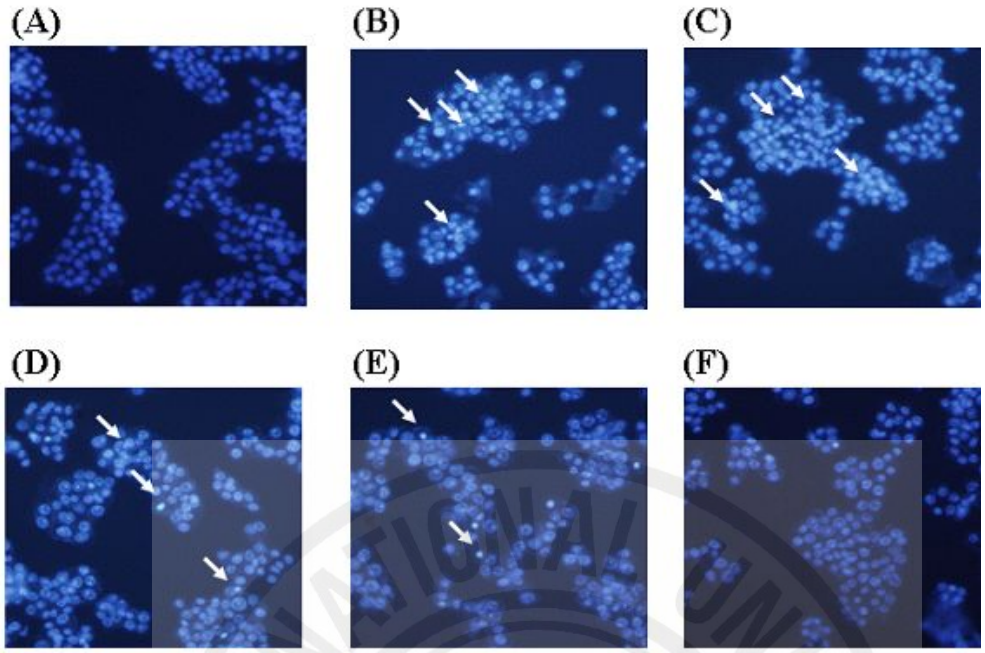

Fig. 1-9. Protection effects of EF treatment on *t*-BHP-induced chromosomal DNA condensation in HepG2 cells. Cultures were treated for 1 h with the noted concentrations of EF and then incubated with 200 μM *t*-BHP for 3 h, stained with Hoechst 33342, and observed by fluorescence microscopy. Cells were treated in the absence of (A) or in the presence of (B) 200 μM *t*-BHP, (C) 200 μM *t*-BHP + 50 μg/mL, (D) 200 μM *t*-BHP + 120 μg/mL, (E) 200 μM *t*-BHP + 250 μg/mL, and (F) 200 μM *t*-BHP + 500 μg/mL of EF.

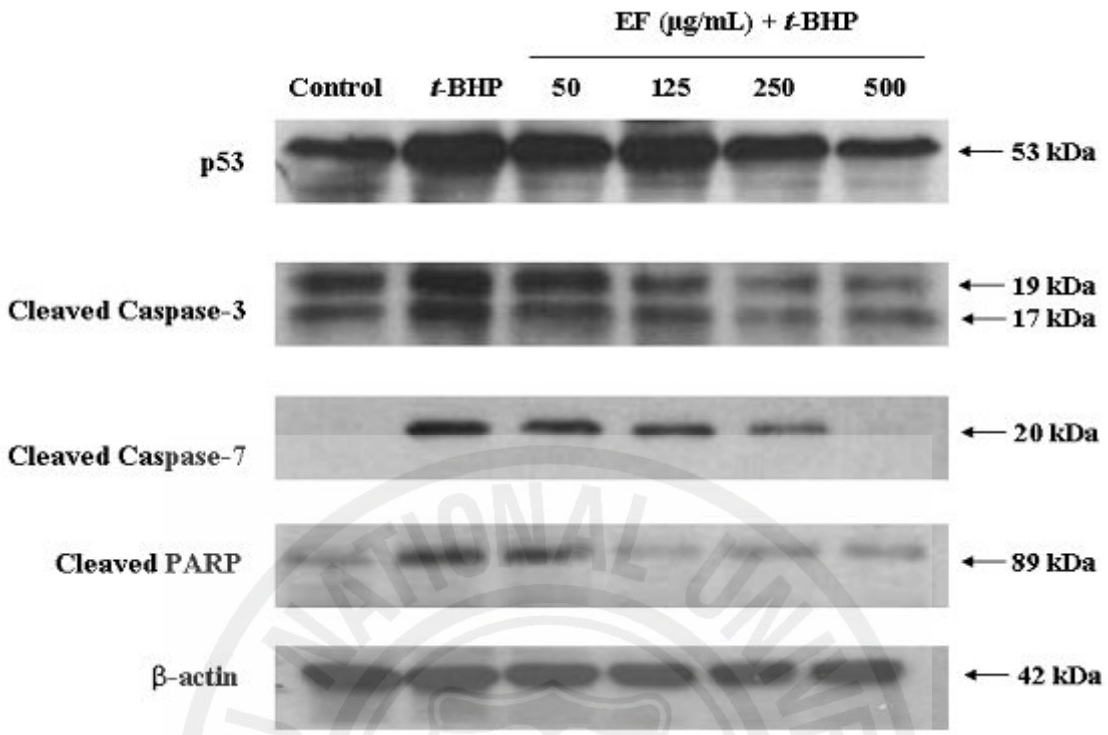

Fig. 1-10. Effects of EF treatment on apoptosis-related proteins in HepG2 cells by Western blotting analysis. β-actin was used as an internal control to normalize the amounts of proteins loaded in each lane.

4. Disccusion

Citrus have served as a good source of antioxidant agents. A large number of citrus possessing anticancer properties have been documented (Guthrie and Carroll, 1998; Miyagi et al., 2000; Poulose et al., 2005; Vanamala et al., 2006). *Dangyuja*, one of *Citrus* species, was traditionally used in the treatment of coughs.

There is significant current interest in the cytoprotective effects of natural antioxidants against oxidative stress and the different defense mechanisms involved. This study is the first to report on the cytoprotective effect of ethyl acetate-extracted fraction from the *dangyuja* (*Citrus grandis* Osbeck.) leaf using a human hepatoma cell line (HepG2 cells) model. This study demonstrates that ethyl acetate-extracted fraction from *dangyuja* leaf has the ability to protect the cell against an oxidative damage by modulating cell proliferation, intracellular ROS level, MDA production, DNA damage, nuclear damage and protein expression related to cell death.

The use of DPPH free radicals is a general scheme to evaluate antioxidant activities. The DPPH free radical scavenging capacity of the various solvent-extracted-fractions of *dangyuja* leaves at different concentrations (50, 125, 250, 500 μg/mL) are present in Fig. 1-2. The various solvent fractions of *dangyuja* leaf at a concentration of 0.5 mg/mL showed the DPPH radical scavenging activity in the decreasing order of EF BF (71.92%) > EF (71.86%) > WF (57.56%) > ME (38.68%) > HF (16.79%) > CF (14.04%). Both the EF and BF showed good inhibitory effects, which were comparable to that of 80% MeOH extract of green tea. However, at a concentration of 50 μg/mL, EF showed better performance against the DPPH radical than BF. These results suggest that the antioxidant activity of *dangyuja* leaf is mainly attributable to EF.

Through the well known antioxidant and free radical scavenging activity of dietary flavonoids, these substances can inhibit oxidative damage to DNA and, therefore, prevent or reduce cellular oxidative (Duthie et al., 1997; Lipkin et al., 1999).

Direct evaluation of the intracellular ROS levels a very good indication of the oxidative damage to living cells (Wang et al., 1999). Based upon the fact that nonfluorescent 2′,7′-dichlorofluorescin (DCFH) crosses cell membranes and is oxidized by intracellular ROS to highly fluorescent DCF (LeBel et al., 1992), the intracellular DCF fluorescence can be used as an index to quantify the overall oxidative stress in cells (Wang et al., 1999; Alía et al., 2005). A prooxidant such as *t*-BHP can directly oxidize DCFH to fluorescent DCF, and it can also decompose to peroxyl radicals and generate lipid peroxides and ROS, thus

increasing fluorescence. In this study, increased ROS generation in cultured HepG2 submitted to an oxidative stress by *t*-BHP was completely inhibited by a pretreatment for 3 h with doses of EF in the upper physiological range and an almost complete inhibition was obtained with other doses of pretreatment (Fig. 1-3). This result clearly shows that the natural antioxidant EF strongly inhibits the generation of ROS induced by *t*-BHP in cultured HepG2.

t-BHP is genotoxic, capable of inducing oxidative damage, including lipid peroxidation, formation of butoxyl radicals, promote intracellular ROS production (Nieminen et al., 1997; Amoroso et al., 1999; Martin et al., 2001; Annunziato et al., 2003). In our experimental conditions, a treatment of HepG2 cells with 200 μM *t*-BHP induced a remarkable decrease in the concentration of MDA which was dose-dependent prevented by a pretreatment with four doses of EF for 3 h (Fig. 1-4). This protection against lipid peroxidation in a cell culture by EF, reported here for the first time to our knowledge, is in concert with previous studies that showed a similar protection by tea catechins (Chen et al., 2002; Murakami et al., 2002a, 2002b) in the same cell line. Thus, our results show that the EF-treated cells were significantly protected against the oxidative damage.

Also, present study presents evidences that EF has a strong protective effect against *t*-BHP -induced oxidative DNA damage in HepG2 cells. Consistent with this protective effect, EF displayed a significant dose-dependent protection against *t*-BHP -induced DNA breaks, as verified by the comet assay (Fig. 1-5).

The choice of *t*-BHP over other common prooxidants such as hydrogen peroxide is based on previous research (Alía et al., 2005), where it was shown that a condition of cellular stress was evoked when HepG2 in culture were treated with *t*-BHP. The role of EF in the protection of the HepG2 cells from *t*-BHP -induced oxidative stress was evaluated by MTT assay. The HepG2 cell line is a dependable model, well characterized, and widely used for biochemical and nutritional studies where many antioxidants and conditions can be assayed (Alía et al., 2005). When human hepatoma HepG2 cells were pretreated with EF for 1h prior to being submitted to an oxidative, the cell toxicity was significantly inhibited in a dose-dependent manner (Fig. 1-8). When the cell exposed various concentration of *t*-BHP for 3hr without pretreatment of extracts, cell proliferation was inhibited in a dose-dependent manner by EF. However, cell viability was restored back to about 90% by pretreatment of 0.5 mg/mL of EF, indicating that EF-treated cells were protected against the oxidative damage.

Apoptosis is a highly regulated cell death program that is induced in cells as a

conditions or exposure to pro-apoptotic external stimuli (Chen et al., 2002). Some characteristics of apoptotic cells include exposure of *t*-BHP on the outer plasma membrane (Hasan et al., 1989), changes in mitochondrial activity, activation of caspase, chromatin condensation, DNA laddering, and cell shrinkage (Alía et al., 2006). Also, the direct interaction between ROS and chromatin is certainly dangerous, as it causes DNA strand breakages and a structural relaxation of chromatin. These changes may allow a more intimate binding of nuclear probes to DNA and this explains the correlation observed, at single cell level, between nuclear ROS and Hoechst 33342 fluorescence intensity.

In the present study, we further found that treatment with EF attenuated the biochemical alternations associated with *t*-BHP-induced apoptotic cell death. Present study presents evidences that EF has a dose-dependent protective effect against *t*-BHP -induced chromatin DNA condensation in HepG2 cells (Fig. 1-9). Also, EF prevented the *t*-BHP -induced expressions of the pro-apoptotic proteins p53, cleaved caspase-3, caspase-7 and PARP (Fig. 1-10). It is well known that ROS-induced oxidative DNA damage can active p53 and cause cell cycle arrest and subsequent apoptotic cell death. Thus, the inhibitory effect of EF on ROS-induced p53 expression could have a marked preventative effect on *t*-BHP -induced cell death. The activation of caspase 3, caspase 7, and PARP, a family of the cysteine proteases, is believed to induce apoptosis in hepatocytes (Sanchez et al., 1996). The suppressive effect of EF on the expression of activated caspase-3, 7, and PARP further suggests that the inhibitory effect of EF on the cell death is related to its antioxidant property.

The results of our study show that both EF of Dangyuja leaf have strong antioxidant activity as measured by DPPH radical scavenging acitivity, ROS generation, and inhibition of lipid peroxidation. Moreover, EF significantly reduced HepG2 cell proliferation, and induced apoptosis evidenced by chromosomal DNA degradation and apoptotic body appearance. Also, apoptotic cell death-related proteins (p53, cleaved caspase 3 & 7, cleaved PARP) expression was induced by EF from *dangyuja* leaf treatment. These results suggest that EF from *dangyuja* leaf have both antioxidant properties and protective effect about the *t*-BHP in HepG2 cells.

Part Ⅱ

Anticancer Activity of Ethyl Acetate Fraction of *Dangyuja* (*Citrus grandis* Osbeck) Leaves Produced in Jeju Island

Part Ⅱ

Anticancer Activity of Ethyl Acetate Fraction of *Dangyuja* (*Citrus grandis* Osbeck) Leaves Produced in Jeju Island

1. ABSTRACT

Ethyl acetate-extracted fraction from *dangyuja* (*Citrus grandis* Osbeck.) leaf extract (EF) contains significant amounts of flavonoid and exhibits powerful antioxidative activity against oxidative damage by *tert*-butyl hydroperoxid (*t*-BHP) in human hepatoma cell line, HepG2. Therefore, the purpose of this study is anticancer activity and the mechanism of cell death exhibited by the EF against HepG2. Treatment of HepG2 cells with various concentrations of EF resulted in growth inhibition in HepG2 cells. However, the EF did not exert any significant cytotoxic effect on normal mammalian cell line CCD-986sk, confirming the cancer-selective cytotoxicity. Also, EF-treated cancer cells were induction of apoptosis in a dose-dependent manner as determined by chromatin condensation and DNA damage in HepG2 cells. Moreover, the EF induced the Bax and Bad protein levels in a time-dependent manner, whereas the Bid, caspase-3 and PARP protein levels were slightly decreased. Our findings showed that EF exhibited potential anticancer activity against hepatocellular carcinoma in vitro through DNA damage effect, chromatin condensation and apoptosis induction of cancer cells.

2. MATERIALS AND METHODS

2. 1. Materials

Dangyuja (*C. grandis* Osbeck) leaves were obtained from the National Institute of Subtropical Agriculture, Jeju Province, Korea. All chemicals and reagents were purchased from Sigma Chemicals (St. Louis, MO, USA) and Invitrogen Gibco (Grand Island, NY, USA).

2. 2. Preparation of extracts

Air-dried *dangyuja* leaves were pulverized using a milling machine and extracted with 80% methanol by stirring for 3 days at room temperature (RT). The extract was filtered, concentrated with a vacuum rotary evaporator under reduced pressure, and lyophilized. The resulting methanol extract (ME) was suspended in water. The methanolic extracts were further portioned in separating using n-hexane (HF), chloroform (CF), ethyl acetate (EF), n-butanol (BF), and water (WF) as described (Lim et al., 2006). The extract powders was dissolved in dimethyl sulfoxide (DMSO) and diluted with phosphate buffered saline (PBS, pH 7.4) to give the final concentrations (Fig. 1-1).

2. 3. Cell culture

Human hepatoma, HepG2 (KCLB No.58065) cells were purchased from the Korea Cell Line Bank (Seoul, Korea). The cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in a humidified atmosphere of 95% air, 5% CO_2 at 37 ^{o}C. The culture medium was changed twice a week, and the cells were subcultured at a ratio of 1:4 once a week.

2. 4. Cytotoxicity assay

To evaluate the cytotoxicity of EF, a MTT colorimetric assay was performed to determine the cell viability (Hasan et al., 1989). HepG2 cells were cultured at a density of 1×10^4 cells/mL on 96-well microplates for 16 h, washed twice using PBS, and incubation of cells with the EF for 24 h. MTT reagent (5 mg/mL) was added to each well, and the plate was incubated at 37 ℃ for an additional 4 h. The media were then removed, and the intracellular formazan product was dissolved in DMSO. Absorbance at 570 nm of the mixture was detected using a micro plate ELISA reader (MRX II, DYNEX Technologies, Chantilly,VA, USA). The results were determined by three independent experiments.

2. 5. Determination of DNA damage (Comet assay)

The alkaline comet assay was conducted according to Singh et al. (1995) with slight modification. HepG2 cells were cultured at a density of 1×10^5 cells/mL on 24-well plates for 16 h, washed twice using PBS, and incubation of cells with the EF for 24 h. The cell suspension was mixed with 100 μL of 0.5% low melting point agarose (LMPA), and added to the slides precoated with 1.0% normal melting agarose (NMA). After solidification of the agarose, slides were covered with another 100 μL of 0.5% LMPA and then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% sodium laurylasarcosine; 1% Triton X-100 and 10% DMSO) for 1 h at 4 ℃. The slides were next placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 min for DNA unwinding. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 min at 4 ℃. The slides were washed three times with a neutralizing buffer (0.4 M Tris-HCl, pH 7.5) for 5 min at 4 ℃, and then treated with ethanol for another 5 min before staining with 50 μL of ethidium bromide (20 μg/mL). Measurements were made by image analysis (Kinetic Imaging, Komet 5.0, UK) and fluorescence microscope (LEICA DMLB, Germany), determining the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of two replicate slides).

2. 6. Staining with Hoechst 33342

Apoptotic cells are characterized by nuclear condensation of chromatin and/or nuclear fragmentation (Lizard et al., 1997). HepG2 cells were cultured at a density of 1×10^5 cells/mL on 6-well plates for 16 h, washed twice using PBS, and incubation of cells with the EF for 24 h. Then, Hoechst 33342, a DNA specific fluorescent dye was added into the culture medium at a final concentration of 10 μM and plate was incubated for another 20 min at 37 ℃. The morphological aspect of cell nuclei was observed under an Olympus fluorescence microscope.

2. 7. Western Blotting

HepG2 cells were cultured at a density of 1×10^5 cells/mL on 100 ϕ dish for 16 h, washed twice using PBS, and incubation of cells with the EF for 24 h. Cells were lysed for 40 min at 4 ℃ with a lysis buffer containing 40 mM of Tris-HCl, pH 8.0, 120 mM of NaCl, 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. We removed the insoluble material by centrifugation at 13,000 rpm for 30 min, and we determined the protein content with the BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amount of lysates (60-100 μg of protein) were separated by a 7.5-15% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, HC, USA) with a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)]. After blocking the nonspecific site with 5% nonfat dried milk, the membrane was then incubated with primary antibody against Bax (1:1000), Bad (1:1000), Caspase 3 (1:1000), PARP (1:2000), and β-actin (1:100000) antibodies (Santa Cruz Biotech and Cell signaling, MA, USA) and further incubated with a secondary peroxidase-conjugated goat IgG (1:5000, Vector, CA, USA). The PVDF membrane was exposed to X-ray film, and the protein bands were detected using a WEST-ZOL® plus Western Blot Detection System (iNtRON., Gyeonggi-do, Korea).

2. 8. 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 $p < 0.05$ was considered to be statistically significant. All assays were performed in triplicate.

3. RESULTS

3. 1. Cytotoxic activity of EF on HepG2 cells

The effects of the EF from *dangyuja* leaf on the growth of cancer cells were assessed using a MTT assay. HepG2 cells were cultured for 24 h in a medium containing of the EF at concentrations of 50, 125, 250, and 500 μg/mL. As the result in Fig. 2-2, the viabilities of cells exposed to the EF at concentrations of 50 and 125 μg/mL were 92.7±1.0% and 83.2±4.6%, respectively. However, cell viabilities of 250 and 500 μg/mL were significantly reduced to 68.7±1.9% and 48.9±1.7% compared with control group ($P < 0.001$). The effects of the EF of *dangyuja* leaf in growth of U937 (human histiocytic lymphoma cells), SNU-16 (human stomach cancer cells), and HeLa (human cervical carcinoma cells), cells were cultured for 24 h in a medium containing of the EF at concentrations of 50, 125, 250, and 500 μg/mL. The result illustrate that, EF showed a dose-dependent inhibitory effect on the growth of U937, SNU-16, HeLa and HepG2 cells (Fig. 2-2). And, this result displayed similar tendency when compared with HepG2. However, No significant cell death was observed in incubations of CCD25Lu (human normal lung fibroblast) with EF (Fig. 2-1).

3. 2. Induction of DNA damage by EF on HepG2 cells

To confirm the induction of DNA damage by treatment of the EF in HepG2 cells, cells cultured with various concentrations of the EF, and DNA was then isolated and analyzed by agarose gel electrophoresis. Following agarose gel electrophoresis of HepG2 cells treated with various concentrations of the EF for 24 h, a typical ladder pattern of internucleosomal fragmentation was observed in cells treated with 125 μg/mL the EF (Fig. 2-3). These data demonstrate that the EF induces DNA damage of HepG2 cells.

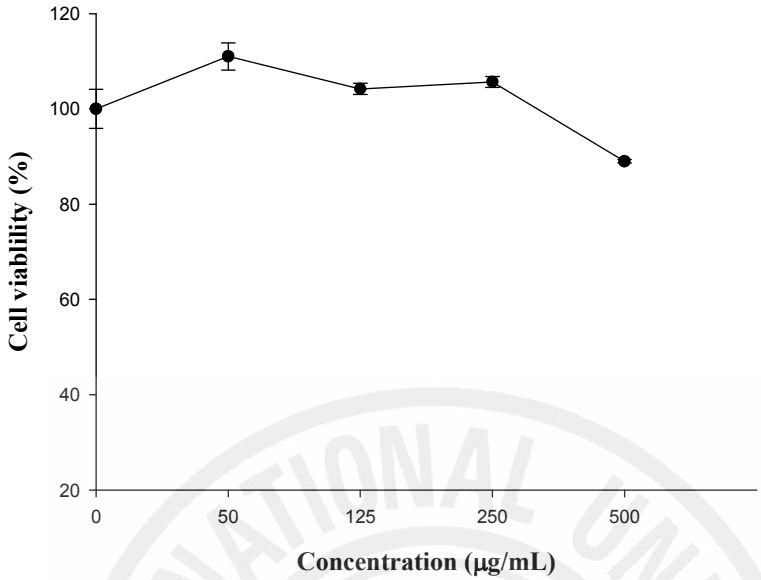

Fig. 2-1. Effects of EF on growth of human normal lung fibroblast, CCD25Lu. CCD 25Lu cells, plated at density of 1 × 10^4 cells/mL, were incubated with various concentration of EF (50-500 μg/mL). Viable cells were evaluated by MTT assay. Data show the only viable cells and are expressed as means S.D. ± (n = 4).

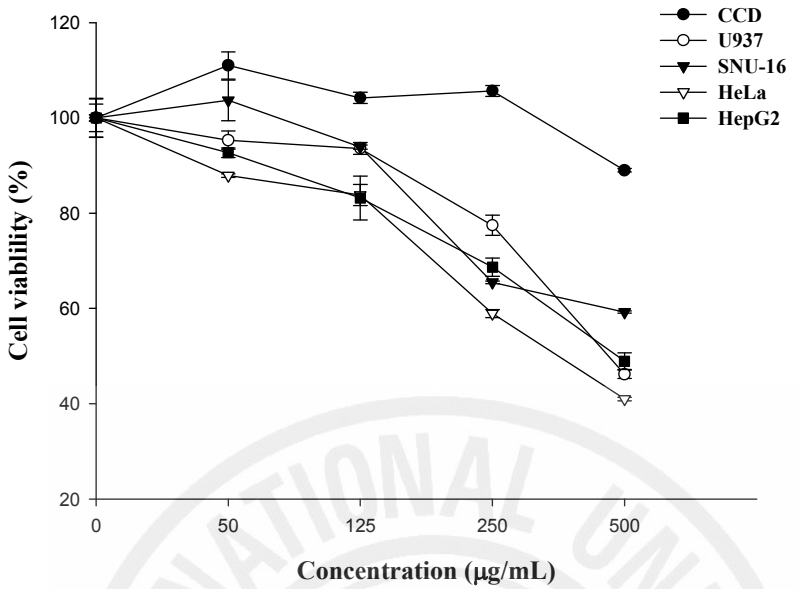

Fig. 2-2. Effects of EF on growth of U937, SNU-16, HeLa, and HepG2. The various cell lines, plated at density of 1 × 10^4 cells/mL, were incubated with various concentration of EF (50-500 μg/mL) for 24h. Viable cells were evaluated by MTT assay. Data show the only viable cells and are expressed as means S.D. ± (n = 4).

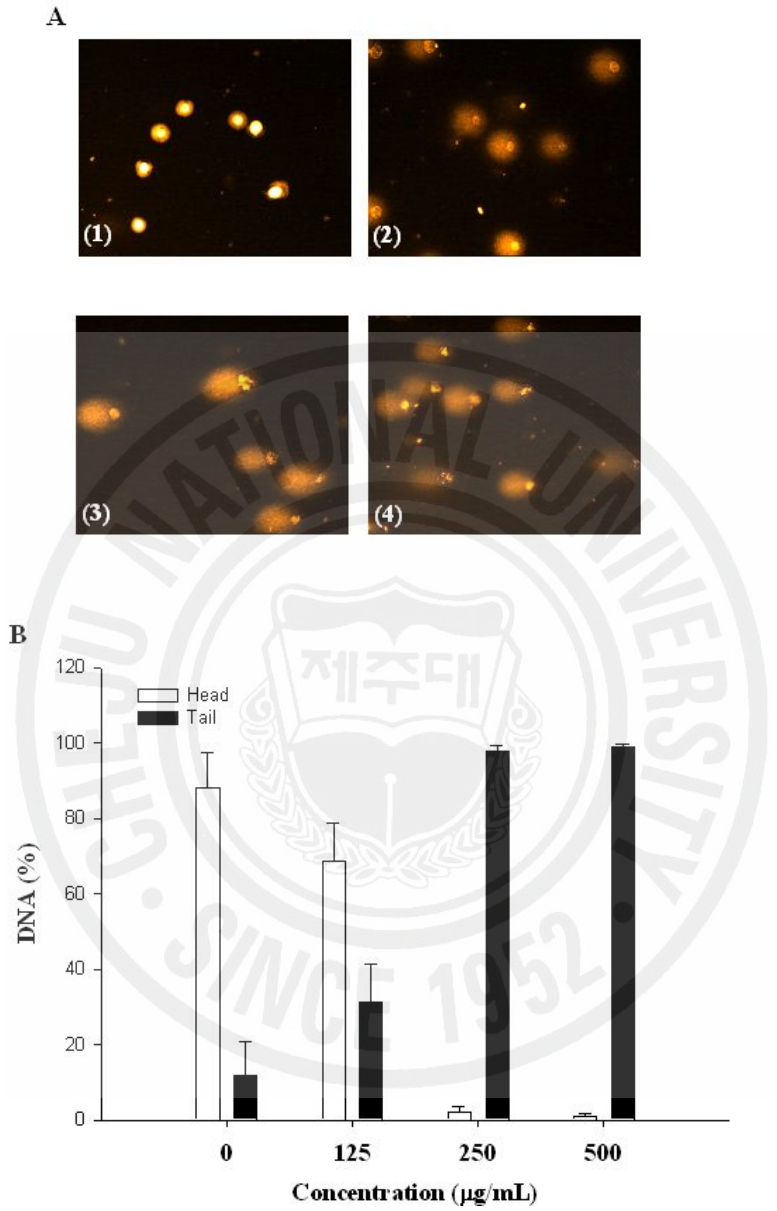

Fig. 2-3. Comet assay of EF induced DNA damage in HepG2 cells. Cells were treated with indicated amount of EF for 24 h. (A) HepG2 control (1); incubation with EF (125 μg/mL) (2); incubation with EF (250 μg/mL) (3); incubation with EF (500 μg/mL) (4). (B) Graphical representation of Comet Assay. Data represent the % of DNA head and tail analyzed by Comet assay of n=3 independent experiments.

3. 3. Induction of apoptosis by EF on HepG2 cells

The nuclear morphological changes of HepG2 cells was analyzed using fluorescence microscopy analyses of cell nuclei stained with the DNA-specific dye Hoechst 33342 after exposure to various concentration of EF for 24 h. Nuclear staining with Hoechst 33342 demonstrated that control HepG2 cells had regular and round-shaped nuclei. In contrast, the shrinkage and condensation of nuclei characteristic of apoptotic cells were evident in HepG2 cells treated with various concentration of EF for 24 h at 37 ℃ (Fig. 2-4).

3. 4. Effect of EF on apoptosis-related protein levels

To understand the molecular mechanism by which the apoptosis by the treated of the EF in HepG2 cells, the various apoptosis-related proteins were analyzed by western blotting. HepG2 cells were cultured in media containing with or without of EF (50, 125, 250 and 500 μg/mL) for 24 h. At each concentration, total protein was isolated and Bax, Bad, Bid, Caspase 3 and PARP (poly ADP-ribosyl polymerase) protein expression levels were measured by Western blotting (Fig. 2-5). The results showed an induced expression of pro-apoptotic protein, such as Bax and Bad. The anti-apoptotic protein, Bid was decreased in a dose-dependent manner in response to extract. Also, caspase 3 and PARP levels were also decreased. These results show that EF was significantly induced apoptosis in dose-dependent manner.

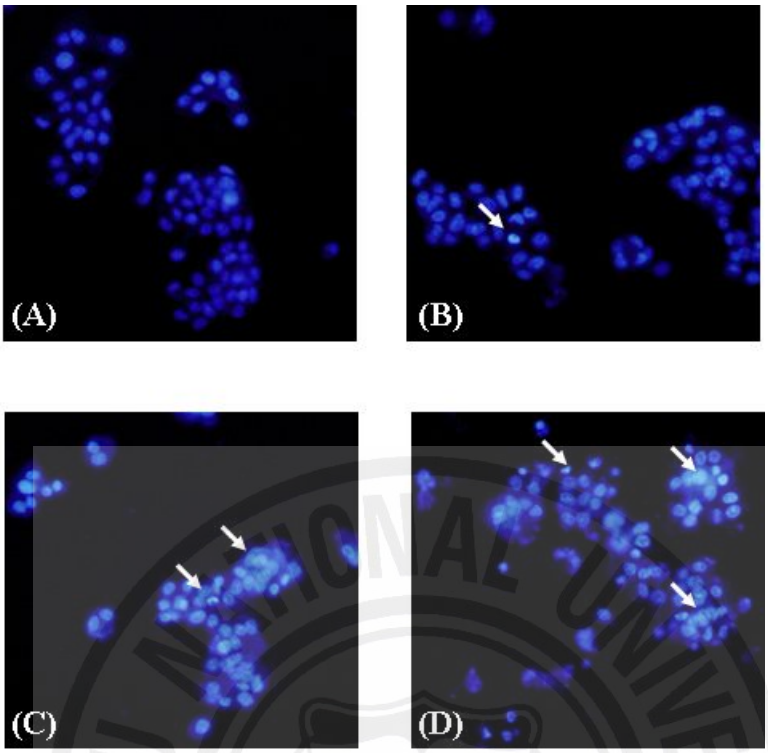

Fig. 2-4. EF induce apoptosis in HepG2 cells. HepG2 cells, plated at density of 1×10^4 cells/mL, were grown on chamber-slides and treated for 24 h with various concentration of EF; (A) 0 μg/mL, (B) 125 μg/mL, (C) 250 μg/mL, and (D) 500 μg/mL. After washing twice with PBS, cells were stained with Hoechst 33342 and visualized by fluorescence microscopy. White arrows show nuclei with typical apoptotic features.

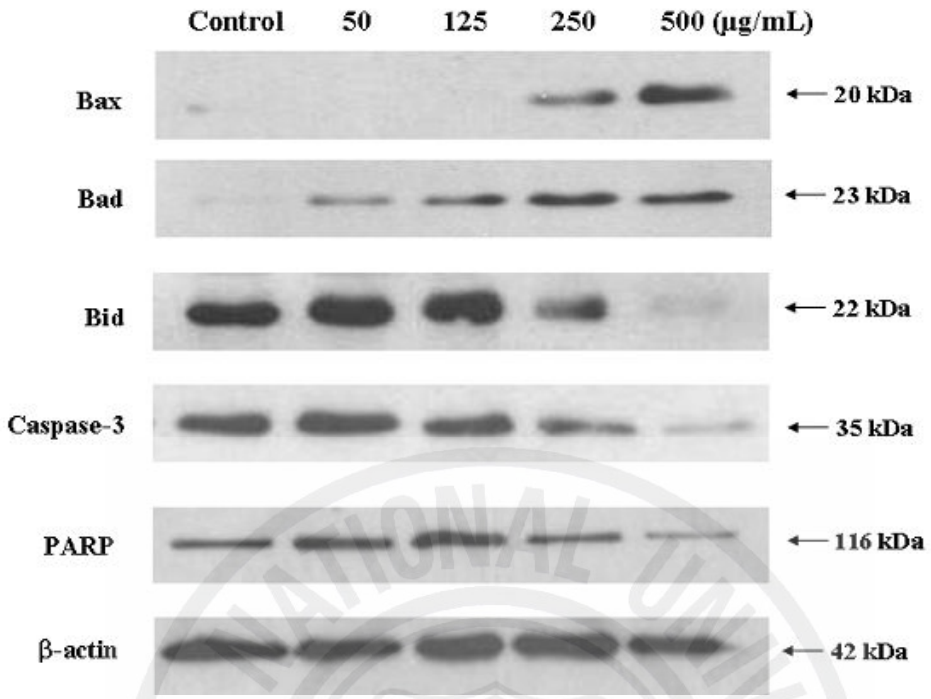

Fig. 2-5. Effects of EF treatment on apoptosis-related proteins in HepG2 cells by Western blotting analysis. β-actin was used as an internal control to normalize the amounts of proteins loaded in each lane.

4. Disccusion

Citrus have served as a good source of antioxidant agents. A large number of citrus possessing anticancer properties have been documented (Guthrie and Carroll, 1998; Miyagi et al., 2000; Poulose et al., 2005; Vanamala et al., 2006). *Dangyuja*, one of *Citrus* species, was traditionally used in the treatment of coughs.

There is significant current interest in the cytoprotective effects of natural antioxidants against oxidative stress and the different defense mechanisms involved. This study is the first to report on the anticancer effect of ethyl acetate-extracted fraction from the *dangyuja* (*Citrus grandis* Osbeck.) leaf using a human hepatoma cell line (HepG2 cells) model. This study demonstrates that ethyl acetate-extracted fraction from *dangyuja* leaf has the ability to anticancer activity by DNA damage, shrinkage and condensation of nuclei characteristic of apoptotic cells, and protein expression related to cell death.

Apoptosis is a genetically controlled cell-death process, which is characterized by chromatin condensation, DNA fragmentation to nucleosome-sized pieces, membrane blebbing, cell shrinkage, and compartmentalization of the dead cells into membraneenclosed vesicles or apoptotic bodies (Darzynkiewicz et al., 1997). Apoptosis is tightly regulated by a number of gene products that promote or block cell death at different stages; the most extensively studied and perhaps the most important is Bcl-2 family (Reed, 1997). In mammals, Bcl-2 has at least 20 relatives, including 4 other anti-apoptotic proteins and 2 groups of protein that promote cell death. Bax family consists of Bax, Bak, and Bok; and BH3-only family comprises Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, and Puma (Zamzami and Kroemer, 2001).

Bax and adenine nucleotide translocator cooperate within the permeability transition pore complex to increase the mitochondrial membrane permeability, and thereby discharge a number of apoptogenic molecules into the cytosol (Marzo et al., 1998). The most notorious apoptogenic factor released from permeabilized mitochondria is the respiratory component cytochrome c, which recruits apoptosis protease activating factor (Apaf-1) and procaspase-9 to form apoptosome; caspase-9 is thus activated, and orchestrates caspase-3 and other effector molecules for the cell death (Rodriguez and Lazebnik, 1999).

In this respect, many studies were performed for screening of apoptosis inducing compounds from plants (Shimizu et al., 2006; Deepak and Salimath, 2006; Corbiere et al., 2004). In this present study, it was found that the ethyl acetate extract of dangyuja leaf

showed significant cytotoxic activity and induction of apoptosis on HepG2 cells. The cells exposed to EF exhibit the morphological and biochemical changes that characterize apoptosis as shown by loss of cell viability (Fig. 2-1, 2-2, 2-3), chromatin condensation (Fig. 2-4), (Kaufmann and Hengartner, 2001; Reed, 2001), which can be evaluated by MTT assay, Comet assay, and Hoechst 33342 staining analysis, respectively.

In Western blot analysis, EF was shown to activate a serial proteins involved in apoptosis. Caspases, a family of aspartate-specific cysteine proteases, play an essential role in the execution of apoptosis (Kolenko et al., 2000; Shi, 2002). Caspases can be divided into two groups. Initiator caspases, such as caspases-1, -2, -8, -9 and -10, act in early stages of the proteolytic cascade. Caspase-3, one of the another group of executioner caspases containing caspases-3, -6 and -7, cleaves specific intracellular substrates, such as polyADP-ribose polymerase (PARP), lamins and inhibitor of caspase activated DNase (ICAD) in downstream of cascade, resulting in programmed cell death (Esposti, 2002; Korsmeyer et al., 2000; Luo et al., 1998). In our study, it was found that EF caused the reduced the activation of Bid, caspases-3 and PARP in HepG2 cells.

In conclusion, this study clearly demonstrates that the ethyl acetate extract of dangyuja leaf strongly inhibits cell proliferation and induces apoptosis in HepG2 cells. EF induced apoptosis through the mitochondrial pathway, involving cytochrome C release from the mitochondria, the activation of Bax and Bad, and degradation of Bid, caspase 3 and PARP. Because apoptosis was regarded as a new target in discovery of anticancer drugs, these results confirm the potential of *dangyuja* leaf as an agent of chemotherapeutic and cytostatic activity in HepG2. However, to elaborate this nascent possibility, further investigation of its activity including in vivo and purification of bioactive compounds is now in progress.

SUMMARY

ROS include three major radical species: superoxide anion radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), highly reactive hydroxyl radical ($^{\cdot}OH$), and peroxyl radicals ($ROO^{\cdot -}$) (Halliwell et al., 1995). These reactive ROS cause destructive and irreversible damage to the components of a cell, such as lipids, proteins and DNA (Lopaczyski and Zeisel, 2001).

Natural antioxidants have a wide range of biochemical activities, including inhibition of ROS generation, direct or indirect scavenging of free radicals, and alteration of intracellular redox potential (Finkel and Holbrook, 2000). Antioxidants have been used to inhibit apoptosis because apoptosis was initially thought to be mediated by oxidative stress (Hockenhery et al., 1993). Many antioxidant materials have anticancer or anticarcinogenic properties (Johnson et al., 1994; Dragsted et al., 1993). *Dangyuja* (*Citrus grandis* Osbeck) is distributed solely in Jeju Island in south Korea and the fruit has been utilized for a long time as a traditional herb medicine in Jeju Island. Most *Citrus* species accumulate abundant quantities of flavonoids during the development (Benavente-Garcia et al., 1993; Castillo et al., 1992, 1993).

The present study was therefore designed to investigate whether *dangyuja* leaf inhibits *t*-BHP-induced oxidative stress and to characterize the mechanism of its anticancer effects in human hepatoma cell line (HepG2).

The EF was confirmed to exhibit antioxidant and anticancer activities. Among the various solvent extracted fractions of *dangyuja* leaves, the ethyl acetate-extracted fraction (EF) showed the greatest 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity. Also, the generation of reactive oxygen species (ROS) was reduced and the level of lipid peroxidation inhibitory capacity was increased in cells pretreated with EF before *t*-BHP treatment. Their MTT assay showed that the EF reduced *t*-BHP-induced cytotoxicity dose-dependently in human hepatoma HepG2 cells. Furthermore, the EF exhibited anti-apoptotic actions on the oxidative damaged HepG2 cells; it inhibited chromosomal DNA degradation and chromatin condensation and reduced the expression of apoptosis-related proteins such as p53, cleaved caspase-3, cleaved caspase-7, and

cleaved PARP in a dose dependent manner. These results suggest that EF from *dangyuja* leaf have both antioxidant properties and protective effect about the *t*-BHP in HepG2 cells.

Furthermore, to confirm the induction of apoptosis by treatment of the EF in HepG2 cells, cells cultured with various concentrations of the EF for 24 h. Treatment of HepG2 cells with various concentrations of EF resulted in growth inhibition in HepG2 cells. However, the EF did not exert any significant cytotoxic effect on normal mammalian cell line CCD25Lu, confirming the cancer-selective cytotoxicity. Also, EF-treated cancer cells were induction of apoptosis in a dose-dependent manner as determined by chromatin condensation and DNA damage in HepG2 cells. Moreover, the EF induced the Bax and Bad protein levels in a time-dependent manner, whereas the Bid, caspase-3 and PARP protein levels were slightly decreased. Our findings showed that EF exhibited potential anticancer activity against hepatocellular carcinoma in vitro through DNA damage effect, chromatin condensation and apoptosis induction of cancer cells.

Based on these data, it can be concluded that the EF of *dangyuja* leaf may be useful as an antioxidant and anticancer agent.

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먼저, 연구와 강의로 바쁘신 가운데도 논문이 완성되기까지 바른 길로 인도해 주시고 연구방향에 대하여 넓은 안목으로 키워주시며 지도해주신 김소미 교수님께 진심으로 깊은 감사를 드립니다. 석사 학위논문을 심사해주신 김재훈, 전유진 교수님, 그리고 석사 과정 동안 저의 학문적 지식을 넓혀주신 학과 교수님들께 진심으로 감사를 드립니다.

처음 실험실 생활을 시작하며, 생물학에 대한 기초가 부족했던 저에게 학부 때부터 생물학이라는 학문에 재미를 느끼도록 해주시고, 연구하는 사람의 자세를 일깨워 주신 오문유 교수님께 감사드립니다. 부족한 제가 처음 실험실에 들어가기로 결심했을 때부터 이 길을 걸어오며, 잠시 방황의 길을 걷을 때나, 힘에 겨울 때, 늘 최선을 다하라며 격려를 아끼지 않으셨던 생물종다양성 연구소 정용환 박사님께 감사드립니다.

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