현호색 메탄올 추출물이 Bcl-2와 Bcl-XL 발현 억제를 통해 유방암 세포의 자멸사에 미치는 영향

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ABSTRACT

*Corydalis Tuber* Induces Apoptosis in MCF-7 Cells, Via Inhibition of Bcl-2 and Bcl-XL Expression

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목 적: 본 연구는 현호색의 메탄올 추출품 (*Corydalis Tuber* Extract; CTE)이 인간 유방암 세포인 MCF-7 세포의 자멸사에 미치는 영향을 알아보고자 하였다.

방 법: 현호색 메탄올 추출품으로 인간유방암에서 유래된 MCF-7세포를 처리하였다. CTE의 세포자멸사 유도 효과는 MTT, FACS, TUNEL, DNA laddering and immunoblot assay를 통하여 측정하였다.

결 과: 본 연구에서 현호색 메탄올 추출품은 MCF-7 세포의 활성을 감소시켰다. CTE로 처리한 MCF-7 세포는 응용에 따라 세포 자멸사 갑작스러울 증가하였다. CTE에 노출하였을 때, Bcl-2와 Bcl-XL의 발현을 억제하고, 미토콘드리아로부터 세포질로 cytochrome-c를 방출하며, caspase와 PARP의 절단을 유발하여 세포자멸사가 증가했다. 현호색 메탄올 추출품에 존재하는 성분중에서 coptisine이 세포 활성을 효과적으로 감소시킨 것으로 사료된다.

결 론: 실험결과 현호색 메탄올 추출품이 유방암의 임상 치료에 있어 가능성을 있는 치료 방법이 될 수 있을 것으로 사료된다.

**Key Words:** *Corydalis Tuber*, apoptosis, caspase-3, Bcl-2, Bcl-XL, Cytochrome-c.
I. Introduction

Breast cancer is one of the most common cancers in women\(^1\), accounting for approximately 32% of all new cancer cases in a year\(^2\). Although various cancers initially respond to chemotherapeutic drugs, resistances against the drugs often develop\(^3\). Because lots of present breast cancer chemotherapeutic agents in the present market have undesirable side effects\(^4\), the scientists' efforts for discovering new drugs for the cancer are continuing.

*Corydalis Tuber* (*C. Tuber*) has been frequently used for treating female afflictions and extravasated blood as well as cardiovascular diseases such as hypertension and cardiac arrhythmia in the traditional oriental medicine\(^5\). Several studies have shown that *C. Tuber* has anti-cholinesterase, anti-inflammatory activities, anti-hypertensive effects, and analgesic effects\(^6\). Other studies also have shown that *C. Tuber* has neuroprotective effects in heat-stroke rats\(^8\) and regulatory effects on glutamate level in rat brain through activation of glutamate dehydrogenase\(^9\). In spite of the extensive studies on the pharmacological effects of *C. Tuber* on cardiovascular and inflammatory diseases, the anti-tumor effects of *C. Tuber* in female disorders have rarely been studied. There is a previous study which demonstrated that *C. Tuber* has apoptosis-inducing effects in uterine leiomyoma cells\(^10\).

The present study was designed to determine the anti-tumor effect of *C. Tuber* methanolic extract (CTE) and to clear up its mechanism of action in MCF-7 cells.

The effect of CTE on inducing apoptosis was monitored by MTT. Fluorescence-activated cell sorting (FACS). Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) and DNA laddering assay. and anti/pro-apoptotic protein expressions in the cells were assessed by immunoblot analysis. As a result, we found that CTE significantly induced apoptosis in MCF-7 cells by inhibiting Bcl-2 and Bcl-xL, the representative anti-apoptotic protein expression. In addition, we evaluated the effects of coptisine, dehydrocorydaline, corydaline, tetrahydropalmatine and protopine, the representative alkaloids in CTE on inducing the apoptosis in MCF-7 breast cancer cells.

II. Materials and Methods

1. Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from BioWhittaker (Walkersville, MD, USA) and Life Technologies (Gaithersburg, MD, USA), respectively.

[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT) and other reagents in the molecular studies
were supplied from Sigma Chemical (St. Louis, MO, USA).

2. Preparation of the methanolic extract of C. Tubor (CTE)

CTE was prepared by extracting 200 g of C. Tuborin 1 L of methanol for 72 h. The methanolic extracts were filtered through a 0.2 μm filter (Nalgene, New York, NY, USA). lyophilized and stored at -20°C until use. The amount of CTE was estimated by the dried weight of lyophilized CTE. The yield of lyophilized CTE was 7.34%.

3. Cell culture

MCF-7 cells, a human breast adenocarcinoma cell line (Korean Cell Line Bank, Seoul, Korea), were cultured in Dulbecco's modified Eagle's medium (Cambrex Bio Science, MD, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. MCF-7 cells were plated at a density of 2 x 10^5/mL and pre-incubated for 24 h at 37°C. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2. For all experiments, cells were grown to 80-90% confluence, and subjected to no more than 20 cell passages. MCF-7 cells were incubated in the medium without 10% FBS for 24 h and then exposed to CTE for the indicated time period.

4. MTT cell viability test

MCF-7 cells were plated at a density of 5 x 10^4 cells/well in a 96 well plate to determine cytotoxic concentrations of CTE. Cells were exposed to CTE in the concentrations of 0.03 through 0.30 mg/mL at 37°C under 5% CO2. After incubation of the cells in the presence of CTE for 24 or 48 h, viable cells were stained with MTT (0.5 mg/mL) for 4 h. The media were then removed and formazan crystals produced in the wells were dissolved by the addition of 200 μl of dimethylsulfoxide (DMSO).

Absorbance was measured at 570 nm using a Titertek Multiskan Automatic ELISA microplate reader (Model MCC/340, Huntsville, AL, USA). The cell viability was defined as the % of untreated control cells [i.e. viability (% control) = 100 x (absorbance of MCE-treated sample)/(absorbance of control)].

5. Preparation of total cell lysates

Cells were lysed in the buffer containing 20 mM Tris HCl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM b-glycerophosphate, 2 mM sodium pyrophosphate, 1 mg/mL leupeptin, and 1 mM phenylmethylsulfonylfluoride (PMSF). Total cell lysates were prepared by vortexing every 5 min for 1 h on ice and then centrifuging at 10,000 x g for 10 min to remove cell debris.

6. Immunoblot analysis

Expressions of apoptosis-related proteins
were immunochemically monitored in the lysate fraction of MCF-7 cells using each antibody. Bad, Bax, Bcl-2, Bcl-xl and actin antibodies were purchased from Zymed (Zymed Laboratory, San Francisco, CA, USA) and procaspase-3 and -9 antibodies were obtained from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The band of each protein was visualized using ECL western blotting detection reagents (Amersham, USA) according to the manufacturer’s instruction.

7. Poly-ADP Ribose Polymerase (PARP) cleavage

Expression of PARP was determined from nuclear extracts. Nuclear extracts were prepared according to previously published methods.11) MCF-7 cells were suspended in the hypotonic buffer containing 10 mM HEPES (pH 9.0), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM DTT and 0.5 mM PMSF. After the incubation on ice for 10 min, the lysates were centrifuged at 7,200×g for 5 min at 4°C. Pellets containing crude nuclei were resuspended in the extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10 mM DTT and 1 mM PMSF, and then incubated for 30 min on ice. The samples were centrifuged at 15,000×g for 10 min to obtain supernatants containing nuclear fractions. Proteins in nuclear fractions were resolved on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with an anti-PARP antibody (Zymed Laboratory, San Francisco, CA, USA). The immune complexes were visualized as described in immunoblot analysis.

8. Cytochrome-c analysis from cytosolic fractions

MCF-7 cells were washed with PBS and resuspended with lysis buffer containing 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 1 mg/mL leupeptin, and 1 mM PMSF. The cells were incubated for 20 min on ice and homogenized with 15 strokes of Dounce homogenizer. The homogenates were centrifuged at 1,000×g for 10 min and then the supernatants were recentrifuged at 15,000×g for 20 min to obtain cytosolic fractions (supernatants). The proteins from cytosolic fractions were resolved on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with an anti-cytochrome-c antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immune complexes were visualized as described in immunoblot analysis.

9. TUNEL assay (Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay)

MCF-7 cells were plated at a density of 1×10⁵ cells/well in 4 well chamber
slide and incubated with CTE for 24 h. To detect apoptotic cells, In Situ cell death detection kit–POD (Roche, Mannheim, Germany) was used according to the manufacturer’s instruction. Briefly, the cells were washed with PBS, and then fixed in 4% paraformaldehyde for 20 min. The fixed cells were blocked with 0.3% H2O2 and treated with 0.1% triton X-100/0.1% sodium citrate to make permeable state. The permeable cells were labeled with TdT-enzyme solution for 1 h and treated with Coverter- peroxidase(POD) for 30 min at 37°C. Apoptotic cells developed color after the addition of DAB substrate and were analyzed under light microscope.

10. Caspase-3 activity assay
Caspase-3 is a key mediator of apoptosis and responsible for the proteolytic cleavage of many key proteins such as PARP. Activation of caspase-3 requires proteolytic processing of procaspase-3 into activated p17 and p12 fragments. To measure caspase-3 cleavage, MCF-7 cells were exposed to CTE with various concentrations for 24 h. and then collected by washing with PBS and scraping with ice-cold cell lysis buffer supplied in the cleaved caspase-3 detection kit (Cell Signaling, MA, USA). The cells scraped off the plate were sonicated on ice, and microcentrifuged for 10 minutes at 4°C. The supernatant was collected and applied to cleaved caspase-3 sandwich ELISA kit (Cell signaling, MA, USA) according to the manufacturer’s instructions.

11. Flow cytometric analysis
Detection of apoptotic and/or necrotic cells was assessed with the green fluorescent dye FITC annexin V entering apoptotic cells and the red fluorescent dye PI not entering (Molecular Probes, Eugene, OR, USA). Briefly, cells were harvested, washed in cold phosphate buffered saline (PBS), and adjusted to the density of ~1×10^5 cells/mL in PBS. Cells were incubated on ice with PI (0.1 μg/mL) and FITC annexin V for 20 min. The stained cells were analyzed by a flow cytometer (PAS system, Partec, Münster, Germany).

12. DNA laddering assay
The genomic DNA from MCF-7 cells was purified with the DNA purification kit (Nucleogene, Seoul, Korea). The cultured cells with CTE were collected and lysed with lysis buffer supplied in the kit for 10 min at 56°C. DNA was extracted with ethanol by filtering and washing as recommended in the manufacturer’s instructions. DNA samples (20 μg) were electrophoretically separated in 1.8% agarose gel containing 0.1 mg/mL ethidium bromide.

13. Statistical evaluation
Data are expressed as mean ± S.D. for all experiments. The t-test was used to
assess statistical significance between means. \( p < .05 \) was considered significant.

III. Results

1. CTE-induced apoptosis in MCF-7 cells

To assess the concentration-dependent effect of CTE on the cell viability, MCF-7 cells were serum starved for 12 h and then incubated with 0.03, 0.1 and 0.3 mg/mL of CTE for 24 h. There was decrease of cell viabilities induced by CTE at 0.03 - 0.3 mg/mL on the concentration dependent manner (Fig 1). As we prolonged the incubation time of CTE to 48 h, CTE further increased the extent of cell death at time dependent manner. We next compared the apoptosis with necrosis in cell death by CTE. MCF-7 cells were treated with CTE for 24 h and then stained with FITC annexin V and PI. The population was separated into three groups: live cells showing only a low level of fluorescence; apoptotic cells showing green fluorescence; necrotic cells showing both red and green fluorescence. Treatment of CTE (0.3 mg/mL) increased to 73.28% of green fluorescence representing apoptotic cells (Fig 2A). As calculated the fold increase of apoptotic death cell, we observed the CTE treatment potently increased the apoptosis of MCF-7 cells on the concentration dependent manner (Fig 2B). In addition, TUNEL assay, the enzyme terminal deoxynucleotidyl transferase labels 3'-OH DNA ends, generated during apoptosis, confirmed the CTE treatment induced apoptosis in the cells. CTE increase the dark violet spots representing condensed nuclei in the assay (Fig 3). DNA purified from cells treated with different concentrations of CTE for 24 h was subjected to agarose gel electrophoresis to assess DNA fragmentation. DNA fragmentation was observed and then was dose-dependent (Fig 4).

![Fig. 1. The effects of CTE on the changes of cell viability MCF-7 cells were exposed to CTE (0.03-0.30 mg/mL), for 24-48 h. Cell viability was assessed by MTT assay. Data represent the mean ± S.D. with eight separate experiments. (significant compared with untreated control at the same treated time. CTE: *Corydalis Tuber Extract*)](image-url)
Fig. 2. Flow cytometric profile of green versus red fluorescence of MCF-7 cells stained with FITC Annexin V and PI. MCF-7 cells were treated with CTE for 24 h. The population was separated into three groups: live cells showing only a low level of fluorescence, apoptotic cells showing green fluorescence and necrotic cells showing both red and green fluorescence (A), and the fold increase of apoptotic death cell was calculated (B). Data represent the mean ± SD of six separate experiments. (**Significant at p < 0.001 compared with vehicle treated. CTE: *Corydalis Tuber Extract*)

Fig. 3. TUNEL assay of CTE-treated MCF-7 cells. MCF-7 cells treated with 0.03-0.30 mg/mL of CTE for 24 h. The TUNEL assay confirms that the CTE treatment induces apoptotic cell death. The dark violet spots are condensed nuclei. (CTE: *Corydalis Tuber Extract*)
2. CTE-induced activation of caspase

We determined the molecular basis of CTE induced apoptosis, as characterized by the PARP cleavage by caspase cascade. Treatment of cells with CTE at 0.1 and 0.3 mg/mL resulted in the significant increase of the 85 kDa cleavage form of PARP (Fig. 5A and B). We investigated CTE-induced activation of caspase-3 and -9, the upregulators of PARP cleavage, by immunoblot analysis. CTE treatment of the cells caused a reduction of procaspase-3 (~61%) and procaspase-9 (~37%) on the dose dependent manner (Fig. 5A and B).

3. CTE-induced modulation of pro/anti-apoptotic protein in mitochondria

Another characterization of apoptosis is the alteration of pro/anti-apoptotic protein in mitochondria and the subsequent release of cytochrome-c from
mitochondria. First, we performed western blot analysis for Bax and Bcl-2/Bcl-xl, the pro- and anti-apoptotic proteins, respectively. There was no significant change in the pro-apoptotic protein Bax after CTE treatment. However, Bcl-2 and Bcl-xl expressions as anti-apoptotic proteins were potently reduced when cells treated with 0.03 - 0.3 mg/mL of CTE for 24 h (Fig 6A and B). To evaluate the effect of CTE on the release of cytochrome-c from mitochondria, we prepared cytosolic fractions of the cells with or without 0.3 mg/mL of CTE for 24 h. Treatment of CTE significantly increased the protein level of cytochrome-c in cytosol suggesting release from mitochondria (Fig. 7A and B).

![Figure 6. The effects of CTE on the levels of pro/anti-apoptotic proteins (in total lysate)](image)

MCF-7 cells were exposed to CTE (0.03-0.30 mg/mL) for 24 h. Bcl-2, Bcl-xl, Bax and Bad proteins were immunoblotted using the respective antibodies in the total lysate. Bad is a loading control (a). The density of proteins was calculated using densitometer (b). Results were confirmed by repeated experiments. The 1 is control, 2 is 0.03 mg/mL, 3 is 0.10 mg/mL, and 4 is 0.30 mg/mL of CTE. (CTE: Corydalis Tuber Extract)

![Figure 7. The effects of CTE on the levels of cytochrome-c protein associated with apoptosis (in cytosolic fraction)](image)

MCF-7 cells were exposed to CTE 0.30 mg/mL for 24 h. Cytochrome-c was immunoblotted using the cytochrome-c antibody in the cytosolic fraction. The density of cytochrome-c was calculated using densitometer. Results were confirmed by repeated experiments. (**Significant at p<.001 compared with vehicle treated. CTE: Corydalis Tuber Extract)

4. Active compounds in CTE to induce apoptosis

C. Tuber comprises a variety of alkaloids as major constituents, which include coptisine, d-corydaline (corydal A), protopine (corydal C), dl-tetrahydropalmatine (corydal B), d-glaucine, dehydrocorydaline and corydal G. To assess the major constituents of C. Tuber on the cell viability, MCF-7 cells were incubated with 25, 50 and 100M of the components. Decrease of cell viabilities induced by coptisine was the most significant (IE 50 ~ 60% at 25 - 100M) (Fig 8). In
the case of corydaline, treatment of 25 and 50 M have low apoptotic effects, although 100 M of corydaline treatment induced 70% decrease of cell viability. As a result, coptisine was the candidate of active compound in C. Tuberin inducing apoptosis in MCF-7 cells.

Fig. 8. The effects of Compounds on the changes of cell viability. MCF-7 cells were exposed to compounds (0-100μM), for 24-48 h. Cell viability was assessed by MTT assay. Data represent the mean ± SD with eight separate experiments. (significant compared with untreated control at the same treated time. **p < .001)

IV. Discussion

Breast cancer is one of the most common cancers in women\textsuperscript{9}, accounting for approximately 32% of all new cancer cases in a year\textsuperscript{2}. Common symptoms associated with breast cancer are mass effect, pain, bleeding, breast deformation, skin edema, and ulcer. The cause of breast cancer is unknown but many hypothesizes are thought to be hypersecretion of estrogen\textsuperscript{12}. Although various cancers initially respond to chemotherapeutic drugs, resistances to the drugs often develop\textsuperscript{3,12}. Because lots of present breast cancer chemotherapeutic agents in the present market have undesirable side effects\textsuperscript{4,13}, the scientists’ efforts for discovering new drug for the cancer are continuing. In oriental medicine, cancer is caused by dysfunction of blood and qi (vital energy) circulation or lack of blood and qi. And breast is especially realated to liver and stomach pathways in the body along which qi flows. So in herbal therapy on breast cancer, the key methods are to circulate the blood and qi fluently or to replenish blood and qi\textsuperscript{14}.

Recent Studies in oriental medicine, a single herb medicine such as Ulmi Pumilae Cortex, Sparganii Rhisoma, Euonymus alatus(Thumb.) Sieb and mixed herb medicine such as Ikiyangyoung -Tang, Guichuixiaoting-Tang, Whalahyoryoung -Dan have anti proliferative effect on MCF-7 cells\textsuperscript{15-20}.

Apoptosis is a programmed cell death that occurs in various physiological situations when cells and tissues normally develop\textsuperscript{14}. The apoptosis involves a series of biochemical events leading to a characteristic cell morphology and death. It also leads to a variety of morphological changes, including blebbing and changing in the cellular membrane, resulting in cell shrinkage, nuclear fragmentation and chromosomal DNA fragmentation\textsuperscript{14}. In recent cancer researches, one of the most crucial progresses is the cognition that apoptosis importantly
involved in regulation of tumor development and formation. When tumor cells propagate for the first time, they must endure a variety of extremely harsh microenvironments, such as hypoxic conditions or nutrient deprivation, as well as the host anti-tumor responses. Under these stress conditions, mutation of genes encoding apoptosis-inducing molecules can be produced in cancer cells. Furthermore, it is well known that killing of tumor cells by most anticancer strategies currently used has been shown to depend on induction of apoptosis in target cells\textsuperscript{21,22}.

In oriental medicine, \textit{C. Tuber} has effects on promoting blood circulation and making qi go through blood freely so that it can remove bad aggregation of blood and release pain\textsuperscript{23-27}. So \textit{C. Tuber} has been frequently used for treating female afflictions and extravasated blood as well as cardiovascular diseases such as hypertension and cardiac arrhythmia in the traditional oriental medicine\textsuperscript{5}. \textit{C. Tuber} was shown that it has anti-cholinesterase, anti-inflammatory activities, anti-hypertensive effects, and analgesic effects\textsuperscript{5,7}.

In the present study, we investigated chemotherapeutic effects of CTE in human breast cancer MCF-7 cells. CTE inhibited the growth of MCF-7 cells and causes apoptotic DNA fragmentation and an increase of the sub-G1 population, the representative apoptotic phenomena. These results mean CTE induces apoptosis in breast cancer cells, which is one of the most important anticancer strategies.

PARP cleavage is a common process in apoptotic cell death, and the caspase-3 activation plays a pivotal role in several apoptotic progresses\textsuperscript{28}. Caspase-3 activation is reported to mediate activation of caspase-9 that stimulates perturb mitochondria\textsuperscript{29}.

In the present study, we demonstrated that CTE causes apoptotic cell death that the path of cell death involves degradation of procaspase-3 and -9, and PARP cleavage. In addition, the bcl-2 family of proteins plays a crucial role in apoptosis, either as activators (Bax) or as inhibitors (Bcl-2 and Bcl-X\textsubscript{L})\textsuperscript{30}.

In the current study, we observed that the level of Bcl-2 and Bcl-X\textsubscript{L} decreased in the cells treated with the CTE, and that the inhibition of Bcl-X\textsubscript{L} caused release of cytochrome-c from the mitochondria. However, researcher could not see a notable change in the level of mitochondrial Bax. These results suggest that CTE decrease the release of mitochondrial cytochrome-c as a result of decreases in Bcl-2 and Bcl-X\textsubscript{L}(Fig. 9).

\textit{C. Tuber} comprises a variety of alkaloids as major constituents, which include coptisine, d-corydaline (corydalis A), protopine (corydalis C), d-tetrahydrocorydaline (corydalis B), d-glaucine, dehydrocorydaline and corydalis G. In these compounds, coptisine was the most significant in inducing the decrease of cell viabilities. In the previous reports, coptisine was
also demonstrated that it blocked cell cycle progression in vascular smooth muscle cells\textsuperscript{31}, and had cytotoxic effects on hepatoma and leukemia cell growth\textsuperscript{32}. Thus, coptisine was regarded as the most active component for the anti-tumor effects of CTE on inducing apoptosis in MCF-7 cells.

In summary, CTE induces apoptosis in MCF-7 cells demonstrated by MTT, FACS, TUNEL and DNA laddering assay, and anti-apoptotic protein expressions such as Bcl-2 and Bcl-XL were decreased in the CTE-pretreated cells. The observation that \textit{C. Tuber} has apoptosis-inducing effects in breast cancer cells provides a possible therapeutic approach to the chemotherapy for various tumors including breast cancer.

\section{V. Conclusion}

CTE induces apoptosis in MCF-7 cells demonstrated by MTT, FACS, TUNEL and DNA laddering assay, and anti-apoptotic protein expressions such as Bcl-2 and Bcl-XL were decreased in the CTE-pretreated cells. The observation that \textit{C. Tuber} has apoptosis-inducing effects in breast cancer cells provides a possible therapeutic approach to the chemotherapy for various tumors including breast cancer.

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