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# 비쑥의 휘발성물질에 의한 KB 세포의 세포사멸 유도

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〈ABSTRACT〉

## Induction of Apoptosis in Human Oral Epidermoid Carcinoma Cell by The Essential Oil of *Artemisia Scoparia* WALDST. et KITAMURA

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*Artemisia scoparia (A. scoparia)*, perennial herb is indigenous to Korea and has been traditionally used in liver damage. We investigated the effect of the essential oil obtained from *A. scoparia* on apoptosis of KB cells. Cytotoxicity and cellular DNA content were analyzed by MTT assay and flow cytometry, agarose gel electrophoresis, and Hoechst 33258 staining. The caspase-3 and poly (ADP-ribose) polymerase (PARP) proteins were estimated by Western blotting method. We found that the essential oil induced the apoptosis of the KB cells by concentrations of 0.4 to 0.2 mg/ml which was verified by DNA fragmentation, apoptotic bodies, and the sub-G0/G1 ratio. The essential oil also transient caspase-9 and caspase-3 activity and cleavage of PARP in KB cells for 24 h. The essential oil-induced apoptotic cell death was accompanied by up-regulation of Bax and down-regulation of Bcl-2. In conclusion, we demonstrated that the essential oil of *A. scoparia* induces apoptosis in KB cells.

*Key words* : *Artemisia scoparia,* Essential oil, Apoptosis, Cell cycle, Caspase-3, PARP

## I. INTRODUCTION

Apoptosis, or programmed cell death, is involved in a wide range of biological and pathological process such as embryogenesis, immune responses, and progression of cancer[1,2]. Apoptosis, distinct from necrosis, is an active process of cell destruction with specific defining biochemical and morphological features characterized by activation of endogenous proteases (caspase) and endonuclease, nuclear/chromatin condensation, internucleosomal cleavage of DNA (DNA ladder), cell shrinkage, dilated endoplasmic reticulum, membrane blebbing, and formation of apoptotic body[3-5]. Caspases can be divided into ini-

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tiator caspases (caspase-2, -8, -9, and -10) and effector caspases (caspase-3, - 6, and -7)[6]. Moreover, the effector caspases cleave intracellular substrates, such as poly-ADP- ribose polymerase (PARP), and the cleavage of the protein results ultimately in cellular morphological and biochemical alterations characteristic of apoptosis[7,8].

Essential oils have been found to be antibacterial, antifungal, anti-inflammatory properties and therapeutic in cancer treatment[9-12]. *Artemisia scoparia* (oriental wormwood) plants are distributed around sandy areas along the seacoast fuming perennial plant communities[13]. *A. scoparia*, perennial herb is indigenous to Korea and has been traditionally used in liver damage[14]. The crude extract of *A. scoparia* has been extensively studied and is known to exhibit multiple pharmacological activities including antitumor, antiallergic anti-inflammatory, and hepatoprotective effects[15-18].

The aim of this work was to identify the apoptosis induction of natural essential oil of *A. scoparia* in KB cells. We demonstrated that the essential oil of *A. scoparia* induce apoptosis in KB cells via morphology, DNA fragmentation, cell cycle, and mitochondria stress and caspase-9 and -3 activity, and cleavage of PARP.

## II. MATERIALS AND METHODS

### 1. Plant material

The aerial parts of *A. scoparia* were collected in September 1998 from the area of around Jebumose of Hwaseong-si in Korea. The identity was confirmed by Dr. Bong-Seop Kil, College of Natural Science, Wonkwang University. A voucher specimen (DJ-98-A23) was deposited at the herbarium of the College of Natural Science, Wonkwang University.

### 2. Isolation of the essential oil

The crushed materials of *A. scoparia* (1 kg) were subjected to steam distillation for 3 h, using a modified Clevenger-type apparatus in order to obtain essential oil. Overall yield was about 0.75%. The essential oil was stored on deep freezer (-70 ℃) to minimize the loss of volatile compounds. In a previous study, we isolated the essential oil from *A. scoparia*, and identified by GC/MS representing 92.65% (area per cent) of the total oil, mainly comphor (10.99%), 1.8-cineole (21.50%), terpinen-4-ol (3.04%), $\alpha$-terpineol (17.60%), borneol (4.72%), and $\beta$-caryophyllene (6.75%)[19].

### 3. Cell culture

KB cells, human oral epidermoid carcinoma cell line (ATCC CCL-17; American Type Culture Collection, Rockville, MD, USA) were grown in Dulbecco's Modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U/ml penicillin and 10 $\mu$g/ml streptomycin. KB cells were maintained as monolayers in plastic culture plate at 37 ℃ in a humidified atmosphere containing 5% $CO_2$

### 4. Cell viability

Cell viability was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO, USA) staining as described[20]. Briefly, KB cells were plated at a density of $10^5$ cells/well into 24-well plates and treated with different concentrations of the essential oil for 24 h. After incubation, medium was replaced by the one containing 50 $\mu$l MTT (5 mg/ml) with phosphate buffered saline (PBS) washing in between, cells were incubated for a further 4 h. The purple crystals, produced from the reduction of MTT by metabolically active cells, were solubilized by DMSO. After a few minutes at room temperature, to ensure that all the crystals were dissolved, the plates were read on a microplate reader (Molecular Devices Sunnyvale Ca. USA) at a wavelength of 540 nm.

### 5. Cell cycle analysis

Flow cytometric analysis was performed as previously described[21]. KB cells treated with different concentrations of the essential oil for 24 h were collected and fixed with 70% ethanol at 4℃ for 1 h. After washing with PBS, the cells were treated with RNase A (1 mg/ml) and stained with propidium iodide (Sigma, 50 $\mu$g/ml) for 30 min at 4℃ in the dark. The stained cells were quantitatively analyzed by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NH, USA).

### 6. Nuclear morphology analysis

Morphological changes of apoptotic cells were determined by fluorescence microscopy as previously described[22]. KB cells treated with different concentrations of the essential oil for 24 h were collected and fixed in 100% ethanol, and stained with Hoechst-33258 (Sigma, 0.5 $\mu$g/ml) for 15 min at 37℃, then visualized under a fluorescence microscope (Olympus BX50, Japan) with UV excitation at 300-500 nm. The cells with nuclei containing condensed chromatin or with fragmented nuclei were defined as apoptotic cells.

### 7. DNA extraction and DNA gel electrophoresis

The characteristic ladder pattern of DNA break was analyzed by agarose gel electrophoresis. KB cells treated with different concentrations of the essential oil for 24 h were collected, washed with PBS twice, and DNA from KB cells was isolated by a Wizard Genomic DNA purification kit (Promega Co., Wisconsin Madison, WI, USA). Isolated genomic DNA was subjected to 2.0% agarose gel electrophoresis at 100 V for 1 h. DNA was visualized by staining with ethidium bromide under UV light.

### 8. Preparation of cytosolic and mitochondrial extract

The harvested pellets were suspended in 5 vol of buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol (DTT), 1 $\mu$g/ml aprotinin, 100 $\mu$g/ml phenylmethylsulfonylfluoride (PMSF), and 250 mM sucrose). After incubate on ice for 10 minutes, homogenize cells in an ice-cold dounce tissue grinder (45 strokes) until 70-80% of the nuclei did not have the shiny ring and centrifuge at 700×g for 10 minutes at 4℃. The supernatant was collected and further centrifuged at 10,000×g for 30 minutes at 4℃ to isolate cytosolin fraction. Cytosolic fraction was stored at -80℃ until ready for Western blotting.

### 9. Western blotting

Western blotting was carried out using standard techniques. Briefly, proteins in the cell lysates were separated on 8-12% SDS-polyacrylamide minigels and transferred to immobilon polyvinylidenedifluoride membranes (Millipore Co., Bedford, MA, USA). The membrane was blocked with 1% BSA in PBS-0.3% Tween20 (PBST) for 1 h at room temperature, and incubated with anti-PARP (Oncogene, Darmstadt, Germany) and anti-caspase-9, -3, Bcl-2, Bax, cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and actin (Sigma) monoclonal antibodies. After washing with PBST three times, the blot was incubated with secondary antibodies and bound antibodies were detected by ECL kit (Amersham Biosciences, Little Chalfont Buckinghamshire, England), exposing blots to Hyperfilm (Amersham pharmacia biotech, Little Chalfont Buckinghamshire, England).

### 10. Statistical analysis

All the data are expressed as a mean ± standard error (SE). One-way ANOVA using SPSS ver. 10.0 software was used for multiple comparisons. A $P < 0.05$ was considered significant.

## III. RESULTS

### 1. Essential oil of A. scoparia induces cytotoxicity in a dose-dependent manner

KB cells were treated with the essential oil at various concentrations for 24 h, and the cell viability was determined as described above by MTT assay. As shown in Fig. 1, essential oil inhibited the growth of KB cells in a dose-dependent manner. Cell viability was obviously inhibited at the concentration of 0.2 mg/ml in essential oil($P < 0.05$).

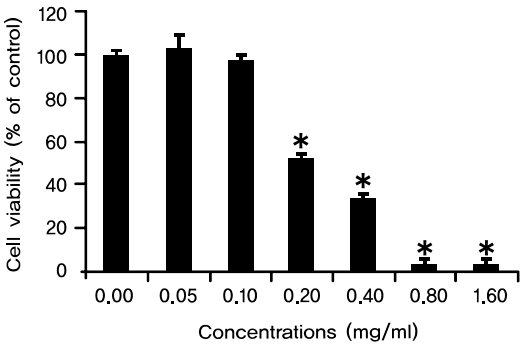


Fig. 1. Effect of essential oil extracted from *Artemisia scoparia* on cell proliferation in KB cells. KB cells were plated into 24 well plates and treated with different concentrations of the essential oil for 24 h. Cell proliferation was determined by the MTT assay and expressed as percentage of the absorbance value obtained without essential oil. The results are expressed as the mean ± S.E. from three different experiments with triplicate cultures. * $p < 0.05$ *compared with control.*

### 2. Effect of the essential oil on cell cycle phase distribution in KB cells

The redistribution of cell cycle phases was analyzed after the treatment with various concentrations of the essential oil for 24 h. The proportion of cells in G0/G1 and S-phase was increased and in G2/M-phase was decreased in essential oil-treated cells when compared with control (0 mg/ml). The cells with sub-G1 DNA content, a hallmark of apoptosis, were seen in essential oil-treated group following 24 h exposure at concentrations of 0.2 and 0.4 mg/ml in KB cells (Fig. 2)

### 3. Effect of the essential oil on determination of morphological changes in KB cells

Nucleic acid staining with Hoechst 33258 revealed typical apoptotic nuclei, which exhibited highly fluorescent condensed chromatin in cells treated with the essential oil. The typical apoptotic characters such as condensation and/or the fragmentation of nuclei and cellular shrinkage were detected in the cells treated with 0.2 and 0.4 mg/ml of the essential oil for 24 h (Fig. 3).

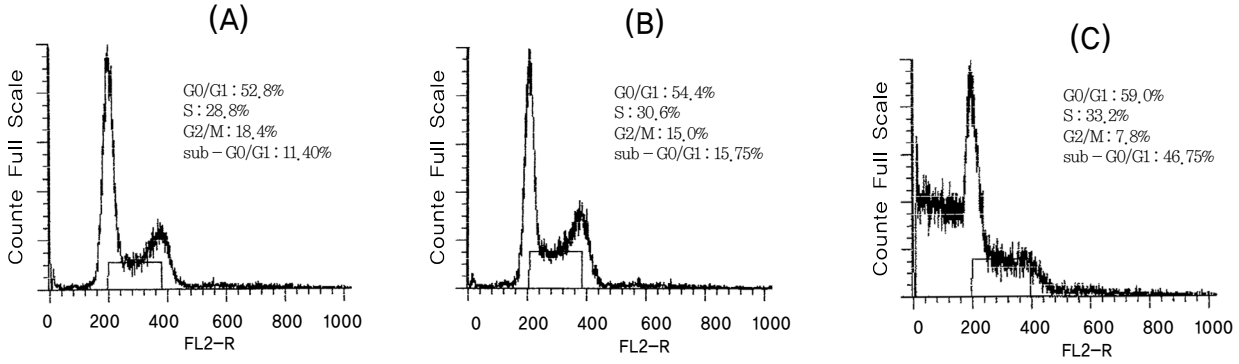


Fig. 2. *Artemisia scoparia* essential oil-induced apoptosis in KB cells. KB cells were treated with the essential oil 0.2 mg/ml (B) and 0.4 mg/ml (C) for 24 h. Non-treated cells were used as a negative control (A). Following the treatment, cells were collected for three kinds of experiments for apoptosis induction. Flow cytometric analysis. Percentages of cells in each phase are shown. The percentages of apoptotic cells were determined by propidium iodide staining followed by flow cytometric analysis.

### 4. Effect of the essential oil on DNA fragmentation in KB cells

To determine whether the essential oil, induces apoptosis in KB cells, we investigated the DNA fragmentation, which is a biochemical hallmark for apoptosis. The results demonstrated that the essential oil induced endonucleolytic DNA cleavage in a dose-dependent manner (Fig. 4). The efficient induction of apoptosis was observed at concentrations of 0.2 and 0.4 mg/ ml essentail oil-treated KB cells for 24 h.

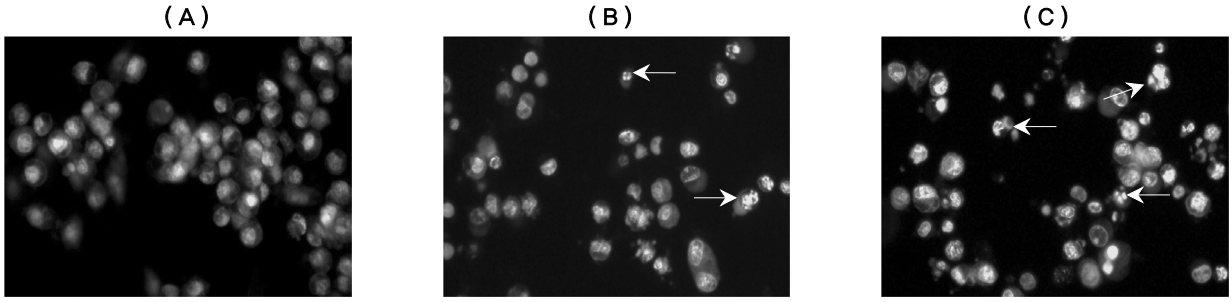


Fig. 3. *Artemisia scoparia* essential oil-induced apoptosis in KB cells. KB cells were treated with the essential oil 0.2 mg/ml (B) and 0.4 mg/ml (C) for 24 h. Non-treated cells were used as a negative control (A). Following the treatment, cells were collected for three kinds of experiments for apoptosis induction. The morphologic change analysis under fluorescence microscope after staining with Hoechst 33258. The apoptotic cells are indicated with arrows. Normal nuclear morphology is observed in untreated cells; in contrast, small, fragmented, and condensed nuclei with typical apoptotic morphology are observed in treated cells.

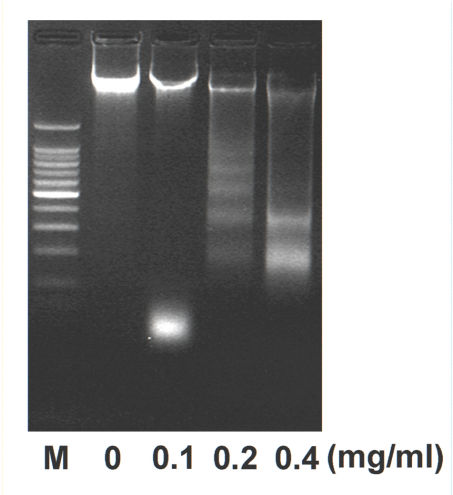


Fig. 4. Artemisia scoparia essential oil-induced apoptosis in KB cells. KB cells were treated with the essential oil for 24 h. Non-treated cells were used as a negative control. Following the treatment, cells were collected for three kinds of experiments for apoptosis induction. DNA fragmentation analysis. Intracellular DNAs were isolated and analyzed by agarose gel (2.0%) electrophoresis.

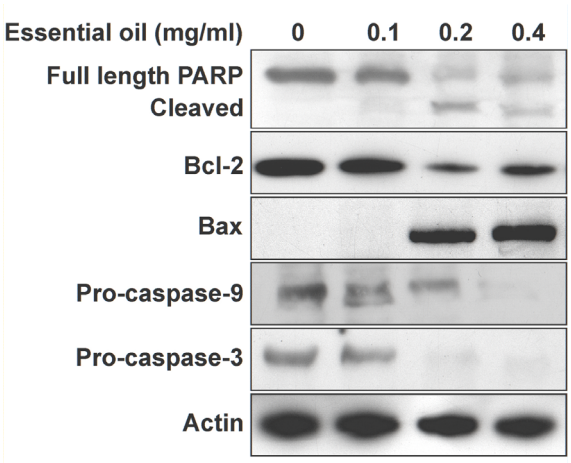


Fig. 5. Artemisia scoparia essential oil-induced apoptosis involves mitochondrial dysfunction pathway and activation of caspase-3, -8, -9, and PARP cleavage. KB cells were treated with different concentrations of the essential oil for 24 h. Cytosolic fractions were isolated as described in Materials and methods. Cytochrome *c* was detected by Western blotting analysis with cytochrome *c* antibody. Whole-cell lysate was used for Western blotting analysis with Bcl-2 and Bax and caspase-3, -8, -9, and PARP antibody, respectively. Cytochrome *c* release, Bcl-2 reduction, and Bax truncation and activation of caspase-3, -8, -9, and PARP cleavage were increased as a dose-dependent manner.

### 5. Mitochondrial stress and activation of caspase-9 and -3 and cleavage of PARP in the essential oil-mediated apoptosis of KB

In particular, the release of cytochrome c into the cytosol plays an important role in the execution of apoptosis in a number of different cell types, which is tightly regulated by the equilibrium between the anti-apoptotic Bcl-2 and pro-apoptotic Bad and Bax[23-25]. Essential oil treatment increased the intensity of the bands corresponding to Bax protein in mitochondrial fractions but significantly reduced the level of Bcl-2 protein(Fig. 5). In addition, the amount of cytosolic cytochrome c was apparently higher in the cells treated with 0.2 and 0.4 mg/ml of the essential oil for 24 h.

The caspase activities were measured by Western blot analyses because the activation of caspases such as caspase-3, and -9, is one of the most common processes occurring in the apoptotic signaling events[26]. As shown in Fig. 5, the dose-dependent activation of caspase-9 and -3 was observed after treating KB cells with the essential oil. In particular, approximately 30% and 90% degradation of procaspase-9 and -3 was observed when the cells were exposed to 0.2 and 0.4 mg/ml of the essential oil for 24 h, respectively. These results strongly suggest the involvement of caspase-dependent pathway in the essential oil-mediated apoptosis of KB cells. To confirm further the apoptosis induced by the essential oil, we investigated the cleavage of PARP in KB-treated cells. The KB cells treated with the essential oil caused a proteolytic cleavage of PARP, with accumulation of the characteristic 85-kDa fragments and a concomitant disappearance of the full-length 116-kDa protein(Fig. 5).

## IV. DISCUSSION

The use of essential oils obtained plants has been examined in various fields, such as developing effective anticancer, chemopreventive approaches and substitute medicines[27, 28]. Moreover, the essential oils can induce sensitive growth inhibition and apoptosis in cancer cells[27]. The essential oil of *A. scoparia* is a highly purified volatile extract that contains a variety of aromatic components[19].

In this study, we examined whether or not the essential oil of *A. scoparia* has potential in a cancer chemoprevention using KB cells. The essential oil-induced cell death was accompanied by nuclear condensation and DNA fragmentation characteristic of apoptosis. And also, we observed that the KB cells treated with the essential oil cause morphologic changes such as apoptotic bodies and chromatin condensation as well as DNA fragmentation into oligonucleosomal-sized fragments. Apoptosis-associated nuclear condensation is usually accompanied by oligonucleosomal DNA fragmentation into oligomers of 180 base pairs[29]. In addition, cytometric analysis showed that the essential oil-treated KB cells with those agents were accumulated in the G0/G1-phase and S-phase for 24 h, compared to control. Sub-G1 peaks, which represent the cell population containing apoptotic nuclear fragments dramatically increased after treatment with essential oil in a dose-dependent manner for 24 h.

Apoptosis is a tightly regulated progress under the control of several signaling pathways, such as caspase and mitochondrial pathways[4, 23, 24, 26]. Cytochrome c release from mitochondrion to cytosol, which causes caspase-9-dependent activation of caspase-3 and cleavage of the DNA reparatory protein PARP[4,8,26]. The essential oil treatment caused activation of caspase-9 and -3 in a dose-dependent manner that is consistent with the results of PARP inactivation and DNA fragmentation. A dose-dependent release in mitochondrial cytochrome c and a concomitant increase in cytosolic cytochrome c were also observed in the essential oil-treated KB cells. In this study, we found that the essential oil induced the release of cytochrome c and the activation of caspase-9 and -3. Furthermore, the essential oil also caused specific activation by cleavage of the caspase-3 substrate, PARP provides further evidence of apoptosis. PARP, a nuclear protein implicated in DNA repair, is one of the earliest proteins targeted for a specific cleavage to the

signature 85-kDa fragment during apoptosis. PARP cleavage can serve as a sensitive parameter for identification of early apoptosis[8].

Overexpression of Bcl-2 can protect against chemotherapy-induced release of mitochondrial cytochrome c, caspase activation, and DNA fragmentation[23, 24]. In the present study, the increase of Bcl-2 expression in the esssential oil-induced apoptosis was associated with an decrease in levels of Bax protein. Our study demonstrated that the essential oil may alter the ratio of Bcl-2 and Bax and, therefore, lead to the apoptosis of KB cells.

In conclusion, these results also indicate the possibility of exploitation of the essential oil of *A. scoparia* as induction of apoptosis in KB cells. Induction of cancer cell apoptosis or death, without affecting healthy cells or producing side effects, is a major goal for development of new therapeutic agents. Even though more precise efficiency and safety data are required to accurately evaluate the amount of the essential oil that could be used for the preventive or therapeutic purpose of some kind of cancers, our results suggest that the essential oil of *A. scoparia* can be a candidate for anticancer agent.

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