

Induction of Apoptosis in Human Oral Epidermoid Carcinoma Cells by Essential Oil of *Chrysanthemum boreale* Makino

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Abstract The effect of the essential oil obtained from *Chrysanthemum boreale* Makino on the apoptosis of KB cells was investigated. Cytotoxicity and cellular DNA content were analyzed by MTT assay, 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. The various cytotoxic effects of the essential oil which are hallmarks of apoptosis, including DNA fragmentation, apoptotic body formation, and sub-G1 DNA content, all progressed in a dose-dependent manner. Treatment with an apoptosis-inducing concentration of the essential oil caused rapid and transient induction of caspase 3 activity. Further, the efficacious induction of PARP cleavage and caspase-3 activation was observed at an essential oil concentration of 0.1 and 0.2 mg/mL for 12 hr.

Keywords: *Chrysanthemum boreale* Makino, essential oil, apoptosis, caspase-3, PARP

Introduction

Apoptosis, or programmed cell death, is involved in a wide range of biological and pathological processes, such as embryogenesis, immune responses, and progression of cancer (1-3). 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 bodies (4-6). Strasser *et al.* (7) reported that accomplishment of apoptosis requires the activation of caspases, which are ubiquitously and constitutively expressed as inactive zymogens in the cytosol. Caspases can be divided into initiator caspases (caspase-2, -8, -9, and -10) and effector caspases (caspase-3, -6, and -7) (8, 9). Moreover, Decker *et al.* (10, 11) reported that effector caspases cleave intracellular substrates, such as poly-ADP-ribose polymerase (PARP), and that the cleavage of the protein results ultimately in the cellular, morphological and biochemical alterations characteristic of apoptosis.

Chrysanthemum boreale Makino is a perennial plant, which belongs to the Compositae family and is an aromatic shrub with yellow flowers. Kim *et al.* (12-15) have shown that the aerial parts of *C. boreale* and other related species such as *Chrysanthemum indicum* L. and *Chrysanthemum lavandulaefolium* Makino have been used in oriental traditional medicine for the treatment of several infectious diseases, such as bacteria colitis, stomatitis,

fever and sores, and have been used to treat cancer, vertigo and hypertensive symptoms, as well as being a common folk liquor in Korea. Jang *et al.* (12, 13, 16) reported that the major compounds of the essential oil obtained from *C. boreale* are monoterpene camphor, α -thujone, *cis*-chrysanthanol, 1,8-cineole, α -pinene and sesquiterpene β -caryophyllene. The guaianolide sesquiterpene lactones, flavonoids, and polyacetylenes isolated from *C. boreale* were recently shown to induce strong cytotoxic activity and apoptosis against several cancer cells (16). Lee *et al.* (17) showed that borenolide isolated from the flowers of *C. boreale* was associated with the apoptosis modulator. The essential oil of several plants was previously reported to induce the apoptosis of cultured hepatocarcinoma cells, human breast cancer cells, human melanoma cells, and human ovarian cancer cells (18-20).

The aim of this work was to identify the effect of the essential oil obtained from *C. boreale* on the cell proliferation and induction of apoptosis in KB (a cell line derived from a human epidermal carcinoma of the mouth) cells.

Materials and Methods

Plant material and isolation of the essential oil The aerial parts of *C. boreale* were collected in September, 1998 from the area of Mt. Mireuk in Korea. The identity was confirmed by Dr. Bong-Seop Kil, College of Natural Science, Wonkwang University. The voucher specimen (no. 98-53) was kept at the Herbarium of the College of Natural Science, Wonkwang University. The crushed materials of *C. boreale* (1 kg) were subjected to steam distillation for 3 hr, using a modified Clevenger-type apparatus in order to obtain the essential oil. The essential oil was stored in a deep freezer (-70°C) to minimize the loss of volatile compounds. The antibacterial activity and

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chemical composition of the essential oil from *C. boreale* were reported previously (13).

Cell culture The KB cells, in the form of 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, 10 µg/mL streptomycin and 0.25 µg/mL fungizone. KB cells were maintained as monolayers in a plastic culture plate at 37°C in a humidified atmosphere containing 5% CO₂.

Cell viability Cell viability was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO, USA) staining according to the method of Kim *et al.* (21). Briefly, KB cells were plated at a density of 10⁵ cells/well into 24-well plates and treated with 0.05, 0.10, 0.20, 0.40, 0.80, and 1.60 mg/mL of the essential oil dissolved in 10% dimethyl sulfoxide (DMSO) for 12 hr. After the 12-hr incubation, the medium was replaced by one containing 100 µg/mL MTT, with phosphate buffered saline (PBS) washing in between, and the cells were incubated for a further 4 hr. 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.

Cell cycle analysis Flow cytometric analysis was performed according to the method of Lee *et al.* (22). KB cells that had been treated with different concentrations of the essential oil for 12 hr were collected and fixed with 70 % ethanol at 4°C for 1 hr. After washing with PBS, the cells were treated with RNase A (1 mg/mL) and stained in the dark with propidium iodide (Sigma, 50 µg/mL) for 30 min at 4°C. The stained cells were quantitatively analyzed by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NH, USA).

Nuclear morphology analysis Morphological changes of the apoptotic cells were determined by fluorescence microscopy according to the method of Li *et al.* (23). KB cells that had been treated with different concentrations of the essential oil for 12 hr were collected and fixed in 100% ethanol, and stained with Hoechst-33258 (Sigma, 0.5 µg/mL) for 15 min at 37°C, 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.

DNA extraction and DNA gel electrophoresis The characteristic ladder pattern of DNA break was analysed by agarose gel electrophoresis. KB cells that had been treated with different concentrations of the essential oil for 12 hr were collected, and washed with PBS twice, DNA from the KB cells was isolated by a Wizard Genomic

DNA purification kit (Promega Co., Madison, WI, USA). Isolated genomic DNA was subjected to 2.0% agarose gel electrophoresis at 100 V for 1 hr. DNA was visualized by staining with ethidium bromide under UV light.

Western blotting Western blotting was performed according to the method of Lee *et al.* (24). Briefly, KB cells that had been treated with different concentrations of the essential oil for 12 hr were collected, and the proteins in the cell lysates were separated on 8% SDS-polyacrylamide minigels for PARP detection and 12.5% SDS-polyacrylamide minigels for caspase-3 detection, 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 hr at room temperature, and incubated with anti-PARP and anti-caspase-3 monoclonal antibodies (Oncogene, Darmstadt, Germany). 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), with the chemiluminescent visualization being carried out by exposing blots to Hyperfilm ECL (Amersham Pharmacia biotech, Little Chalfont Buckinghamshire, England) for 15 s to 10 min.

Statistical analysis Data are presented as the mean and S.E.M. for the indicated number of separate experiments. Statistical analysis of data was performed using Duncan's multiple range test with one-way analysis of variance. The level of significance was set at p-values less than 0.001.

Results and Discussion

The cell viability determined by MTT assay is shown in Fig. 1. The essential oil inhibited the growth of KB cells in a dose-dependent manner. Cell viability was obviously inhibited at the essential oil concentration of 0.05 mg/mL ($p < 0.001$).

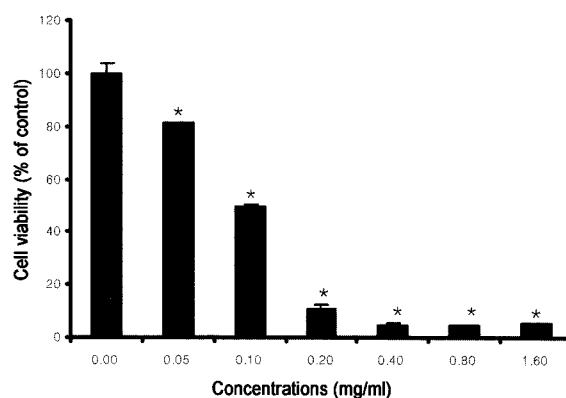


Fig. 1. Effect of essential oil obtained from *Chrysanthemum boreale* Makino on the cell proliferation in KB cells. KB cells were plated into 24-well plates and treated with different concentrations of the essential oil for 12 hr. Cell proliferation was determined by MTT assay and expressed as a percentage of the absorbance value obtained without essential oil. The results are expressed as the mean \pm S.E.M. from three different experiments with triplicate cultures. * $p < 0.001$ compared with control.

The redistribution of cell cycle phases was analyzed after the treatment with various concentrations of the essential oil for 12 hr (Fig. 2A). The proportions of cells in the G0/G1 and S-phases increased while that in the G2/M-phase decreased in essential oil-treated cells when compared with control (0 mg/mL). Cells with a sub-G1 DNA content, a hallmark of apoptosis, were seen in the essential oil-treated groups following 12-hr exposure at concentrations of 0.1 and 0.2 mg/mL in KB cells. Nucleic acid staining with Hoechst 33258 revealed the typical apoptotic nuclei, which exhibited highly fluorescent condensed chromatin in the essential oil-treated cells (Fig. 2B). The morphological changes and cell death of the KB cells were significantly increased in a dose-dependent manner. Most cells were detached from the dishes and cell rounding and shrinking occurred at essential oil concentrations of 0.1 and 0.2 mg/mL. Moteki *et al.* (25) showed that the morphological changes showing apoptotic bodies were observed in the human leukemia HL-60 cells

treated with 1,8-cineole, which is the main component of essential oil prepared from bay-leaves (*Laurus nobilis* L.). To determine whether the essential oil induces apoptosis in KB cells, we investigated the DNA fragmentation, which is a biochemical hallmark for apoptosis (Fig. 2C). The essential oil induced endonucleolytic DNA cleavage in a dose-dependent manner. The efficient induction of apoptosis was observed in the KB cells treated with essential oil at concentrations of 0.1 and 0.2 mg/mL for 12 hr.

The expression and activation of caspase-3 by cleavage was examined, and, to further demonstrate caspase-3 activity, the cleavage kinetics of one of its natural substrates, PARP, was examined by Western blot analysis (Fig. 3). Treatment with the essential oil caused a dose-dependent activation of caspase-3, as indicated by a progressive decrease of cytosolic levels of pro-caspase-3, with an early and complete disappearance of the inactive form of this effector protease, 12 hr after treatment.

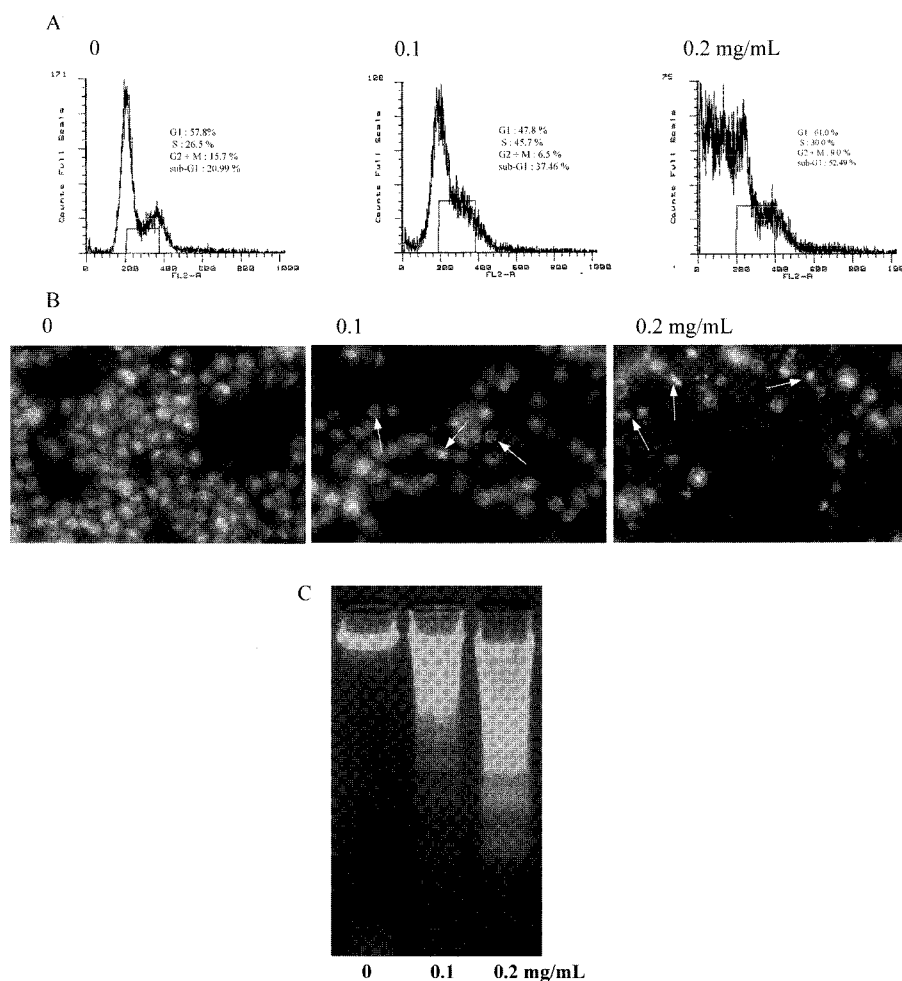


Fig. 2. *Chrysanthemum boreale* Makino essential oil-induced apoptosis in KB cells. KB cells were treated with the essential oil (0.1 and 0.2 mg/mL) for 12 hr. Non-treated cells were used as a negative control (0 mg/mL). Following the treatment, cells were collected for the following three kinds of experiment for apoptosis induction. (A) Flow cytometric analysis. The cell percentages in each phase are shown. The percentages of apoptotic cells were determined by propidium iodide staining followed by flow cytometric analysis. (B) The analysis of morphological change under fluorescence microscopy after staining with Hoechst 33258. The apoptotic cells are indicated with arrows. Normal nuclear morphology was observed in untreated cells, whereas small, fragmented, and condensed nuclei with typical apoptotic morphology were observed in treated cells. (C) DNA fragmentation analysis. Intracellular DNAs were isolated and analyzed by agarose gel (2.0%) electrophoresis. Data are representatives of at least three independent experiments.

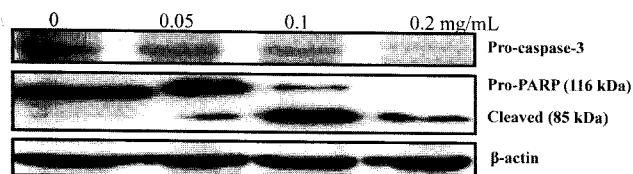


Fig. 3. Western blot analysis of the caspase-3 and poly-ADP ribose polymerase (PARP) cleavage in KB cells treated with the essential oil of *Chrysanthemum boreale* Makino for 12 hr. Proteins in whole cellular lysates with untreated control were electrophoresed in SDS-PAGE gels and transferred to nitrocellulose membranes. Caspase-3 and PARP were identified using specific antibodies. Pro-caspase-3 was decreased in a dose-dependent manner and the uncleaved 116-kDa and 85-kDa cleavage products of PARP were detected. This immunoblot is a representative of three independent assays performed at different time points.

Soldani *et al.* (26) reported that after caspase-3 activation, some specific substrates for caspase-3 which were important for the occurrence of apoptosis, such as PARP, were cleaved. Ray *et al.* (27) reported that PARP was required for DNA repair and that activated caspase-3 cleaved PARP at Asp 216 to generate a 31 kDa apoptotic fragmentation during apoptosis. Caspase-3 activation is essential for DNA fragmentation to occur in the apoptosis induced by a variety of stimuli. Decker *et al.* (10) reported that PARP is one of the earliest proteins targeted for a specific cleavage to the signature 85-kDa fragment during apoptosis. The present result showed that cleavage of the 116-kDa proform of PARP into the 85-kDa signature fragment was detected in KB cells after they had undergone 12 hr of treatment with the essential oil.

In conclusion, the essential oil of *C. boreale* Makino induced apoptosis in KB cells. The induction of cancer cell apoptosis or death, without affecting healthy cells or producing side-effects, is a major goal for the development of new therapeutic agents. The normal essential oil level of *C. boreale* which will guarantee safety when used in foods has already been established. Even though more precise efficiency and safety data are required to accurately evaluate the amount of essential oil that could be used for preventive or therapeutic purposes in the treatment of some kinds of cancer, our results suggest that the essential oil of *C. boreale* is a strong candidate as an anticancer agent.

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