Thesis for the Degree of Master of Science

Induction of apoptosis by the extract of marine algae symbiotic fungus *Chaetomium* sp. in A431 human epidermoid carcinoma cells

by
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(해조류 공생 곰팡이 *Chaetomium* sp. 추출물에 의한 인간 피부암 세포주 A431에서의 자가세포사멸 유도)

Advisor: Prof. Gun Do Kim

by

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ABSTARCT

Fungus *Chaetomium* sp. is encountered as a causative agent of infections in human and it is ubiquitous in nature. Its secondary metabolite is known for having many biological activities including anti-fungal activity and toxicity in mitochondria but still remains unknown for its anti-cancer efficacy. In this study, I found that the extract of fungus *Chaetomium* sp. has a potentiality to inhibit A431 cell growth in a dose- and time-dependent manner. Its cytotoxicity was examined by using cell viability assay and the mechanism of growth inhibition and cell death were elucidated by either Western blot analysis or flow cytometry. The extract induces apoptotic cell death through type I death receptor pathway including the activation of Fas, caspase-3, -6, -7 and -8, and other target proteins of caspases. However, the extract did not show any effect on the mitochondria and there was no cleavage of Bid and cytochrome c release. Furthermore, the extract inhibits cell cycle progression by arresting G_1/S phase and up-regulating Cdks inhibitory proteins. It also inhibits migration of the cells which was confirmed by wound-healing assay. The results suggested that the extract has a great potentiality to induce apoptosis in A431 human epidermoid carcinoma cells via both cell cycle arrest and inhibition of cell migration.

INTRODUCTION

Marine flora have been used for medicinal purposes in India, China and Europe. The people of China and Japan have been consuming seaweeds since ancient times [Boopathy and Kathiresan, 2011]. Marine fungi, having a rich profile of biologically active metabolites, were described as filamentous fungi of terrestrial origin, specifically those from three genera namely *Penicillium, Aspergillus* and *Fusarium* [Lange, 1996]. Although terrestrial fungi have represented as a major biomedical resource (penicillin from Penicillium, for example), studies to develop the biomedical potential of marine fungi are very few [Fenical and Jensen, 1993].

Chaetomium sp. is a large genus of the fungal family, Chaetomiaceae, which comprises over one hundred species. Members of this filamentous fungal genus are commonly found in soil, air and plant debris, and are encountered as causative agents of infections in humans [Wijeratne et al., 2006]. In addition, it is noted for their secondary metabolite content with significant biological activities and seems to be a generous producer of anti-tumor alkaloid products such as cytochalasins. To date, more than 120 compounds have been reported from this genus including chaetochromins B, C, and D, chaetocins B and C, chetracin A, isocochliodinol, neocochliodinol, musanahol and globosuxanthone [Abdel-Lateff, 2008; Ding et al., 2006].

Cancer is characterized by an uncontrolled cell cycle progression and deregulation of apoptosis. Therefore, induction of apoptotic cell death and

inhibition of cell cycle progression is a promising emerging strategy for the prevention and treatment of cancer [Lee and Lim, 2011]. Apoptosis is the best characterized form of programmed cell death. It is an important process in maintaining homeostasis which can be triggered by many factors such as radiation, chemotherapeutic drugs and apoptotic signaling [Mahmood and Shukla, 2010]. Apoptotic signaling can proceed via two different pathways, (i) via death receptors expressed on the plasma membranes of cells, or (ii) alternatively via mitochondria, which contain several proteins that regulate apoptosis [Cho et al., 2009]. Significantly, the death receptor pathway is initiated by the ligation of membrane bound tumor necrosis factor (TNF) or Fas receptors, which result in a caspase-8-dependent cascade, followed subsequent by the occurrence of cell death [Kruidering and Evan, 2000]. Death receptors of TNF family such as Fas and TNFR1 are well studied death pathways that recruit FADD and procaspase-8 to the receptor [Liu and Chang, 2011]. Procaspase-8 recruitment through FADD leads to auto-cleavage and activation of effector caspases such as caspase-3, eventually cause cell death [Thorburn, 2004]. Among them, the Fas/FasL system is a key signal pathway involved in apoptosis regulation in several different cell types [Walczak and Krammer, 2000], and two types of Fas signaling pathways were proposed in different cell lines [Guicciardi and Gores, 2009]. In the Fas type I pathway, procaspase-8 binds to the death-inducing signaling complex (DISC) in response to Fas ligation. The internalized Fas/FADD/procaspase-8 complex further elicits downstream protease activation and apoptosis execution [Liu and Chang, 2011]. In type II pathway, mitochondria

are essential for apoptotic program execution. Whereas, in type I cells, mitochondrial dysfunction likely functions as an amplifier for the apoptotic signal [Guicciardi and Gores, 2009].

In terms of mechanism(s) of chemopreventive agents and in addition to the induction of apoptosis, studies in recent years have focused on the modulation of cell survival pathways such as cell cycle arrest, since the normal regulation of cell cycle progression and division are important events in the development of cancer [Meeran and Katiyar, 2008]. Many cytotoxic and DNA damaging agents have been reported to arrest a normal cell cycle in G_0/G_1, S, or G_2/M phase [Orren et al., 1997]. Progression of cell cycle is mediated by a phylogenetically conserved family of protein kinases known as cyclin dependent kinases (Cdks) and many aspects of cell cycle control requires negative regulation of Cdks [Niculescu III et al., 1998]. The negative regulation of Cdk activity is achieved either by the phosphorylation of their catalytic subunit or via the binding of Cdk inhibitory proteins known as CKIs [Morgan, 1995]. Increases in the levels of CKIs binds to cyclin-Cdk complexes render these complexes inactive. Two families of mammalian CKIs have been described as INK4 and Cip/Kip inhibitors [Sherr and Roberts, 1995]. INK4 inhibitors contain an ankyrin repeat motif and are specific for Cdk4 and Cdk6, the most divergent among cell cycle-associated Cdks. There are four known members of the family: p15, p16, p18 and p19. Cip/Kip inhibitors target a broader spectrum of Cdks, including Cdk2, Cdk4 and Cdk6, and possibly, Cdk1. Their family consists of three members: $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ [Niculescu III et al., 1998].

The aim of this study is to determine the effects of the extract from the fungus *Chaetomium* sp. on apoptosis and cell migration inhibition in A431 human epidermoid carcinoma cells. It was confirmed that the extract profoundly induces cell cycle arrest along with the activation of CKIs and type I death receptor apoptosis and also inhibits migration of A431 cells.

MATERIALS AND METHODS

Fungal isolation and extraction

The fungal strain, *Chaetomium* sp. was isolated from the edible marine brown alga *Undaria peterseniana* collected in GeoMun Island, Yeosu, Korea and identified based on morphological evaluation and 18S rRNA analyses (SolGent Co., Ltd., Daejeon, Korea). A voucher specimen is deposited at Pukyong National University with the code MFC295. The fungus was cultured in 3.0 L round flask (1 L) for three weeks (static) at 29°C in SWS medium consisted of soytone (0.1%), soluble starch (1.0%), and seawater (100%). The mycelium and broth were separated by filtration using cheesecloth. The mycelium was extracted with CH_2Cl_2–MeOH (methyl alcohol) (1:1) to afford mycelium extract (26 mg). Following further purification steps, I found molecular weight of the extract approximately (M.W. 646) but still under the verification of its structure. Stock solutions (50 mM) were prepared in DMSO and then stored at 4°C. The extract was diluted in fresh media before each experiment; the final DMSO concentration was < 0.1%.

Antibodies and reagents

All antibodies (AIF, BID, cytochrome c, cleaved caspase-3 (Asp175), cleaved caspase-6 (Asp162), cleaved caspase-7 (Asp198), cleaved caspase-8 (Asp387), cleaved PARP (Asp214), DFF45/35 (ICAD), cleaved DFF45 (Asp224), DcR3, DR5,

DR3, Fas, FADD, phosphor-FADD (Ser194), FLIP, Lamin A/C, $p15^{INK4B}$, $p16^{INK4A}$, $p21^{waf1/cip1}$, $p27^{kip1}$, TNFR1, TNFR2, TRADD, RIP, GAPDH and β-actin), anti-rabbit IgG, anti-mouse IgG (horseradish peroxidase-coupled), anti-mouse IgG (H+L), F(ab′)2 Fragment (Alexa Fluor® 555 Conjugate) and anti-rabbit IgG (H+L), F(ab′)2 Fragment (Alexa Fluor® 488 Conjugate) were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Mouse and rabbit normal serum were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Amersham™ ECL Advance™ Western Blotting Detection Kit and Hyperfilm™ ECL were purchased from GE healthcare (Buckinghamshire, UK). DAPI (4′, 6-diamidine-2′-phenylindole dihydrochloride) and Prolong Gold Antifade Reagent were obtained from Roche Applied Science (Indianapolis, IN, USA) and Invitrogen (Grand Island, NY, USA), respectively. EZ-Cytox Cell Viability Assay Solution WST-1® was from Daeil Lab Service (Seoul, Korea), ribonuclease (RNase A) and 20X PBS buffer were from Biosesang Inc. (Gyeong-gi, Korea), and PRO-PREP™ protein extraction solution was from iNtRON biotechnology (Gyeonggi, Korea). Ethanol, methanol, dimethylsulfoxide, protease inhibitor cocktail, Tween® 20, Z-VAD-FMK and propidium iodide were purchased from Sigma-Aldrich (St. Louis, MO, USA), and 37% formaldehyde was from Junsei Chemical Co., Ltd, (Tokyo, Japan).

Cell culture

A431 (human epidermoid carcinoma) and HEK 293 (human embryonic kidney)

cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). A431 and HEK 293 cells were cultured in Dulbeccos Modified Eagles Medium (DMEM) containing 4.5 g/L glucose with L-glutamine (Cellgro by Mediatech, Inc., Manassas, VA, USA), heat-inactivated 10% fetal bovine serum (Cellgro), 1% 100 U/ml penicillin and 100 μg/ml streptomycin (PAA Laboratories GmbH, Pasching, Austria) in culture flask (SPL Lifesciences, Gyeonggi, Korea). The cells were cultured in humidified atmosphere of 95% air and 5% CO_2 at 37° C. 1X Trypsin-EDTA (Cellgro) was used for trypsinization.

Cell viability measurement

Cells in exponential growth phase were suspended at a final concentration of 1×10^5 cells/ml in 96-well cell culture plate in triplicates. The cells were then treated with various concentrations (0.1, 0.3, 0.5, 0.7, 0.9, 1.2 and 1.5 μM) of the extract and incubated for 12 h. After the treatment, 10 μl of WST-1® was added onto each well and the cells were incubated further for 3 h at 37°C and then O. D. 460 nm was measured with ELISA reader (Molecular Devices, Sunnyvale, CA, USA). To investigate caspase-dependent inhibition, A431 cells were cultured in 96-well cell culture plate and treated with a pan-caspase inhibitor, Z-VAD-FMK (50 μM) before treatment of the extract (0.3, 0.5 and 1 μ M). Then the effect of Z-VAD-FMK was measured by WST-1® assay.

Wound migration assay

The wound migration assay was performed according to standard methods [Bellyei et al., 2010]. A431 cells were grown on 6-well cell culture plate to 100% confluence. The confluent cells were carefully wounded with sterile polished pasteur pipette tips and any cellular debris was removed by washing with 1X PBS. The wounded monolayer was then incubated in the presence of 0.3, 05 and 0.7 μM of the extract for 12 h.

Immunofluorescence

Cells grown on coverglass-bottom dishes were incubated with 0.1, 0.5 and 1 μM of the extract for 12 h. For this, cells were pre-treated with DAPI solution for 30 min at 37°C and then fixed with 4% formaldehyde for 15 min at room temperature, and blocked for 1 h in 5% mouse and rabbit normal serum of host of primary antibodies with 0.3% Triton X-100. Fixed and blocked cells were incubated with 0.1 μg/ml of each primary antibody (cleaved caspase-3, -8, PARP, $p21^{waf1/cip1}$ and β-actin) and secondary antibody (anti-mouse IgG (H+L), F(ab′)2 Fragment (Alexa Fluor® 555 Conjugate) and anti-rabbit IgG (H+L), F(ab′)2 Fragment (Alexa Fluor® 488 Conjugate)) according to the manufacturer's instruction. Stained cells were mounted on Prolong Gold Antifade Reagent followed by observation under a Nikon ECLIPS 50i microscope equipped with CDD camera (Nikon, Tokyo, Japan). Images were captured and processed with High-Content Analysis Software (Cambridge Healthtech Ins., Needham, MA, USA).

FACS analysis

The cells were incubated with 0.5 and 1 μM of the extract for 12 h and harvested by trypsinization. Before staining, the cells were fixed in 70% ethanol for 6 h at 4°C. Then the cells were resuspended in 1X PBS containing RNase A (10 μg/ml) and incubated for 1 h at 37°C, and DNA was stained with propidium iodide (40 μg/ml) for 10 min. Distribution of cell cycle was examined using FACS Calibur (Becton Dickinson, San Jose, CA, USA).

Western blot analysis

Cultured A431 cells in 100 mm dish were treated with 0.1, 0.5 and 1 μM of the extract for 12 h and then washed with ice-cold 1X PBS and harvested by scrapping. The harvested cells were collected by centrifugation, and then lysed in ice-cold protein extraction solution with protease inhibitor cocktail. After incubation on ice for 30 min, the insoluble materials were removed by centrifugation at 14,000 rpm for 20 min at 4°C. Each protein sample was resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and was electro-transferred to a nitrocellulose membrane (PALL Life Sciences, Pensacola, FL, USA) and then blocked in 1X PBST buffer containing 5% skim milk for 1 h at room temperature. The blots were probed with the primary antibodies for overnight at 4°C and then washed in PBST, followed by incubation for 1 h with secondary antibodies. Finally the blots were washed in PBST and visualized by an ECL detection solution (Pierce, Rockford, IL, USA).

Statistical analysis

The Prism 5.0 software (GraphPad, San Diego, CA, USA) for window was used to determine the statistical significance of differences between values for various experimental and control group. Determinations were performed in triplicate and the results are presented as means±standard error of the mean (S.E.M.). ANOVA post hoc and subsequently Dunnett's multiple comparison tests were used for statistical analysis.

RESULTS

Growth inhibition by *Chaetomium* sp. extract against A431 cells

The effect of the *Chaetomium* sp. extract on the growth of cells were evaluated by cell viability assay. As shown in Fig. 1A, the treatment of the extract (0.1-1.5 μM for 12 h) on A431 cells resulted in the inhibition of cell growth in a dose-dependent manner, and the viability values were 34.56 and 21.66% at 0.5 and 0.9 μM, respectively. On the other hand, the extract has no significant effect on non cancerous human embryonic kidney HEK293 cells (Fig. 1B).

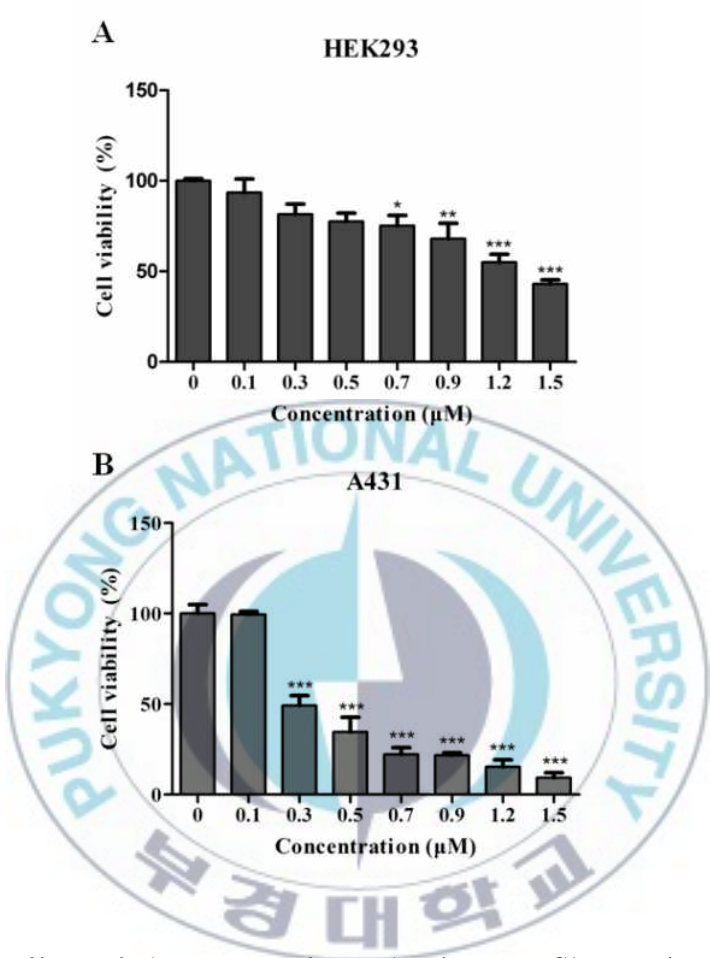

Figure 1. The effect of the extract from the fungus *Chaetomium* sp. on the cell growth and cell viability of HEK293 human embryonic kidney cells (A) and A431 human epidermoid carcinoma cells (B). The data were expressed as the percentage of cell viability and represented the means ± SE of three experiments in which each treatment was performed in multiple wells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Induction of apoptosis by the extract

To determine whether the extract isolated from the fungus *Chaetomium* sp. may induces apoptosis on A431 cells, the activities of various apoptotic proteins were analyzed using Western blot analysis. As shown in Fig. 2A, when A431 cells were treated with the extract (0.1, 0.5 and 1 μM) for 12 h, the expression of active effector caspases (caspase-3, -6 and -7) were elevated, compared to the control, in a dose-dependent manner. As apoptosis is usually associated with the activation of the caspase cascade, it is interesting to check the activation of caspase-8, the initiator caspase that plays an extensive role in the death receptor pathway. I found that the caspase-8 was activated after 12 h treatment of the extract. An increased level of Bid indicated that the level of the truncated Bid was down-regulated. Also, cytochrome c and AIF, in relation to mitochondria, were decreased in the extract treated cell. Increased expression of active caspases targeted major cellular proteins such as poly ADP-ribose polymerase (PARP), lamin A/C and DFF45/35. I found that the cleaved form of PARP, lamin A/C and DFF45/35 were increased in *Chaetomium* sp. extract treated cells.

As shown in Fig. 2B, the treatment of the extract caused the slight activation of Fas, DR3 and the phosphorylated Fas associated death domain (FADD). Unlike FADD, TNFR associated factor (TRAF) decreased with TNFR1 and TNFR2, it indicated that the extract activated DR3 but could not induce DR3 pathway. The effects of the extract on the expression of different anti-apoptotic

proteins, FLICE inhibitory protein (FLIP) and receptor-interacting protein (RIP) on A431 cells were also examined. Results showed that the extract down-regulated the expression of FLIP and RIP as compared to control. In addition, the involvement of other receptors in the death receptor pathway also have been investigated. However, the extract did not affect DR5 and decoy receptor 3 (DcR3).

In Fig. 3, the morphological observation also supported that control was seen uniformly green in normal morphology, while the treated cells were bright green with condensed nucleus, indicates apoptosis induction. The expression levels of cleaved capsase-3 and -8 were up-regulated in the cytoplasm after the treatment of the extract. It indicates that the death receptor mediated apoptosis was induced by the extract.

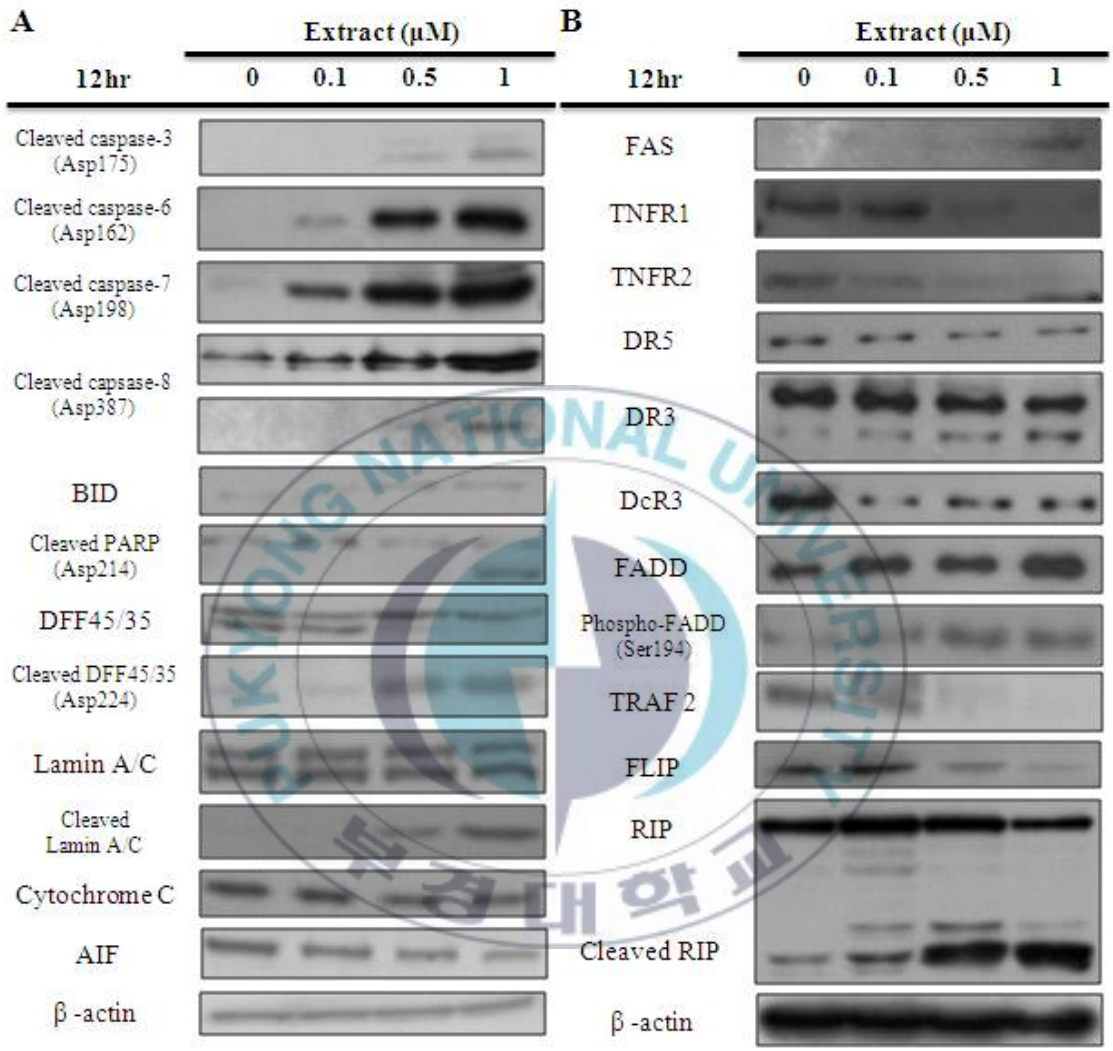

Figure 2. The effect of the extract (0.1, 0.5 and 1 μM) on the expression of protein related in apoptosis for 12 h. The total protein extracts derived from A431 cells were analyzed by Western blot using various antibodies.

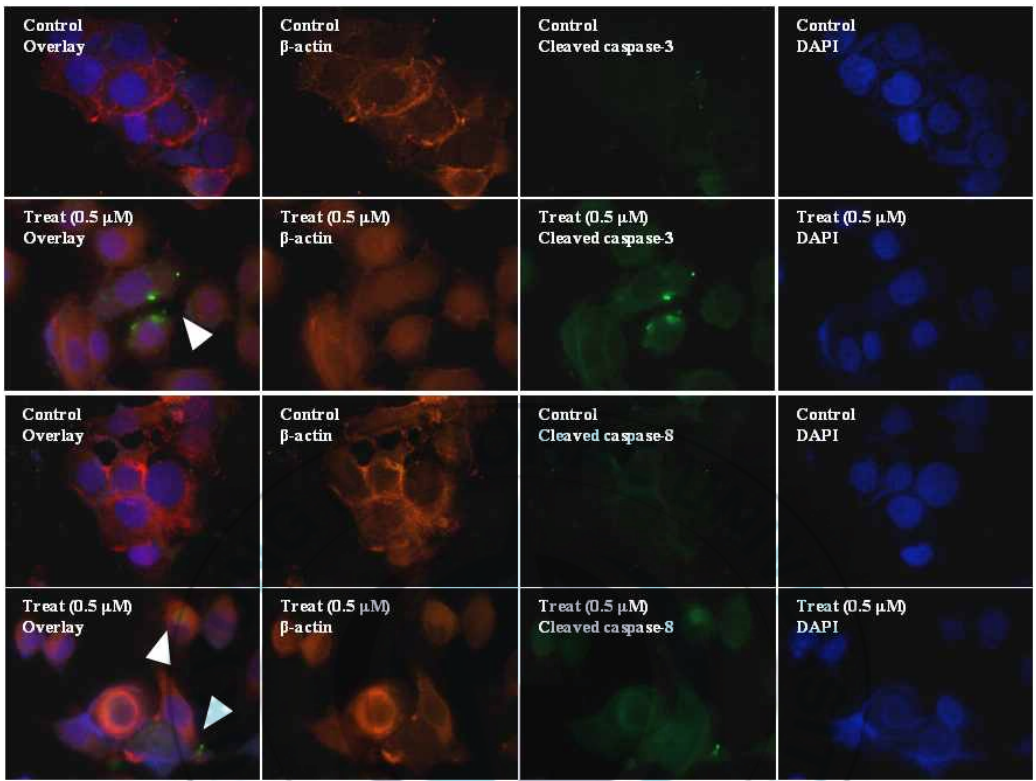

Figure 3. The effect of the extract on the expressions of proteins involved in apoptosis. Changes in the morphology of A431 cells were observed by staining with indicated antibody after 12 h treatment of the extract. Green stained cells indicate cleaved caspase-3 and -8, red stained cells indicate β-actin and blue stained cells indicate DAPI staining for the nuclear morphology after 12 h. The arrows indicated elevated expression of cleaved caspase-3 and -8. Photographs were taken with a fluorescence microscope under the 1,000x magnification.

Effect of the extract on caspase-dependent apoptosis

To investigate the mechanism of the extract induced apoptosis, the activation of caspase-3, -8, the cleavage of PARP and the phosphorylation of FADD were also investigated. As shown in Fig. 4, the extract increased expression of active form of cleaved caspase-8, -3, the cleavage of PARP and the phosphorylation of FADD in a time-dependent manner. Additionally, Fig. 5 showed that Z-VAD-FMK (a broad caspase inhibitor) remarkably attenuated the extract-induced cell death.

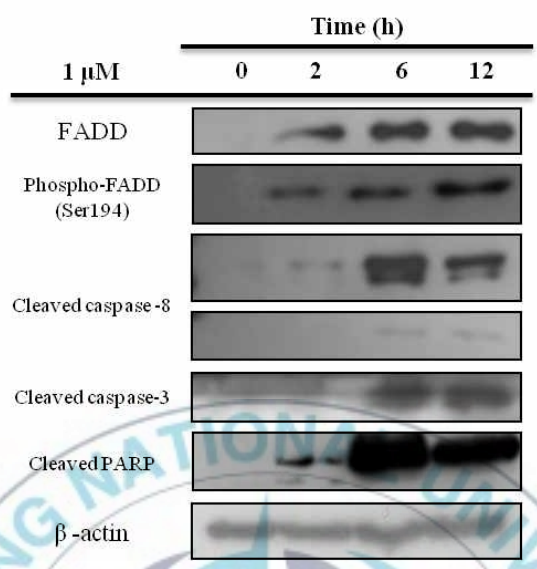

Figure 4. The effect of the extract (1 μM) on the expression of apoptosis related proteins.

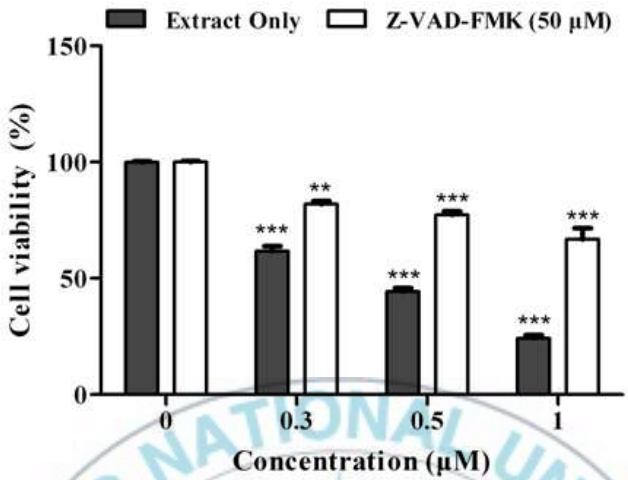

Figure 5. The effect of the extract in relation with caspase-dependent apoptosis. A431 cells were pre-treated with Z-VAD-FMK (50 μM) for 1 h and, were subjected to the extract combined treatment for 12 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Inhibitory effect of the extract on cell migration

To determine whether the extract affects the motile ability of A431 cells, cell migration was investigated using wound migration assays. Cell migration is the one of important standard point and major process underlying cell metastasis. As shown in Fig. 6, non-treated the control cells migrated into the wound area after 12 h to an extent where in the wound edges were already undistinguishable. However, the treated cells displayed significantly slower wound closure compared to the control cells.

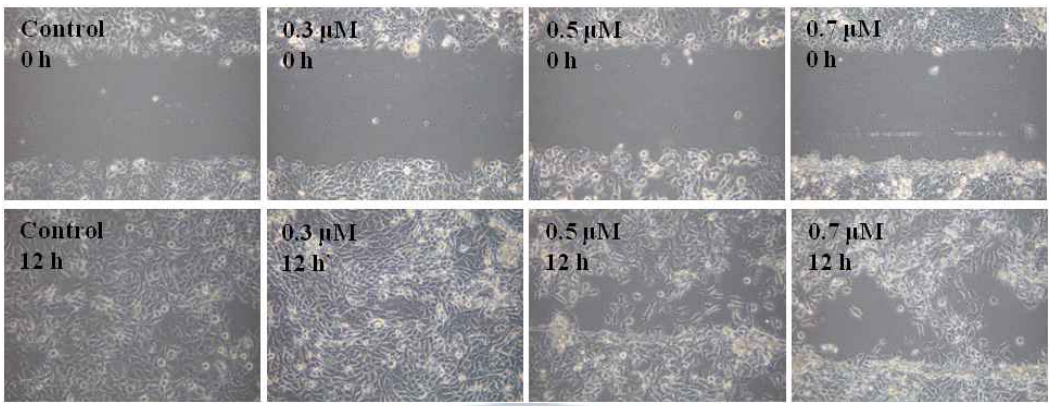

Figure 6. The effect of the extract on the wound healing activity. Confluent cells cultured in 6-well dishes were wounded with a sterile pipette tip and were incubated with or without 0.3, 0.5 and 0.7 μM for 0 h and 12 h. Photographs were taken with an inverted microscope under the 40x magnification.

Chaetomium sp. extract induces cell cycle arrest

In order to understand the mechanism of growth inhibition, the effects of extract on A431 cells was investigated through both flow cytometry and Western blot. As shown in Fig. 7A and 7B, the extract slightly changed distribution of DNA in a dose-dependent manner. When the cells were treated with the extract, its G_0/G_1 phase gradually increased (from 6.69% to 21.77%) and the S phase decreased as compared to the control. The changes in the expression of cell cycle protein levels after the treatment of the extract were also investigated. Remarkable changes in the expression of proteins that are known to play a role in cell cycle progression were observed in the presence of the extract in A431 cells. As shown in Fig. 7C, inhibitory proteins related to cell cycle, $p15^{INK4B}$, $p16^{INK4A}$, $p21^{waf1/cip1}$ and $p27^{kip1}$ were up-regulated after the treatment of the extract. The expression level of $p21^{waf1/cip1}$ was also examined using immunofluorescence (Fig. 7D). It showed that the extract increases cellular expression on $p21^{waf1/cip1}$ as compared to the control.

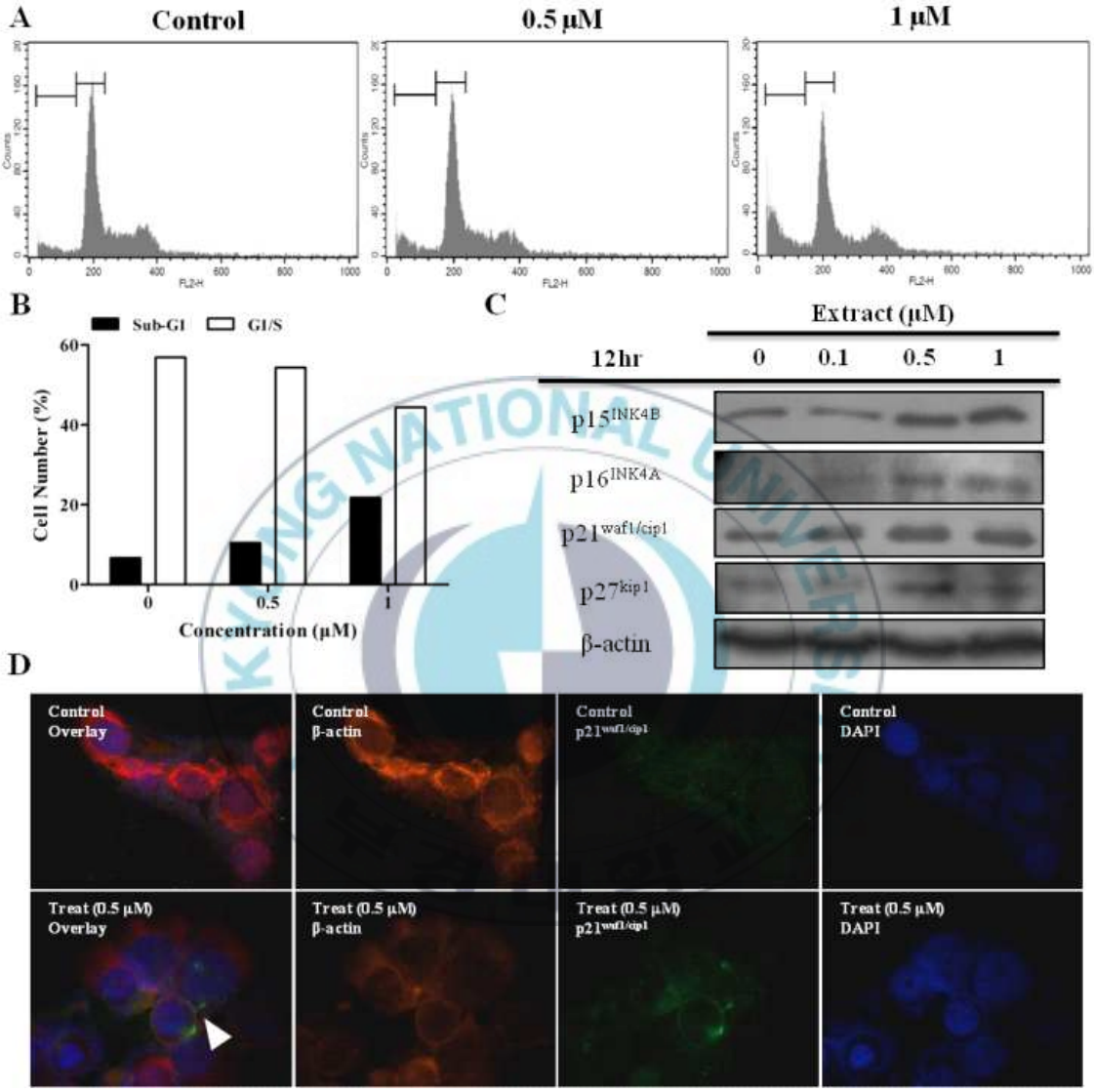
A
Control
0.5 μM
1 μM
B
Sub-G1
G1/S
Cell Number (%)
Concentration (μM)
C
Extract (μM)
12hr
0
0.1
0.5
1
p15INK4B
p16INK4A
p21waf1/cip1
p27kip1
β-actin
D
Control Overlay
Control β-actin
Control p21waf1/cip1
Control DAPI
Treat (0.5 μM) Overlay
Treat (0.5 μM) β-actin
Treat (0.5 μM) p21waf1/cip1
Treat (0.5 μM) DAPI

Figure 7. The effect of the extract on cell cycle arrest. A431 cells treated with the extract were analyzed by FACS analysis staining with propidium iodide, the distribution histogram of the nuclear DNA detected by flow cytometry was indicated according to the cell cycle phase (A, B). The total proteins from the cells treated with the extract were examined using antibodies indicating the panel (C). Changes in the expression of A431 cells were observed by staining antibody after 12 h treatment of the extract. Green stained cells indicate $p21^{waf1/cip1}$, red stained cells indicate β-actin and blue stained cells indicate DAPI staining (D). Photographs were taken with a fluorescence microscope under the 1,000x magnification.

DISCUSSION

Cancer causes significant morbidity and mortality in human and is a major public health problem worldwide in which the main treatments involve surgery, chemotherapy, and/or radiotherapy [Abdiryim et al., 2011]. The rising incidence of skin cancer in humans makes it equivalent to malignancies of organs. Therefore, it is necessary to intensify our efforts for better understanding and development of novel treatment and preventive approaches for skin cancer [Prasad et al., 2009]. Natural products and their derivatives represent more than 50% of all the drugs in clinical use and plays an important role to prevent the cancer incidences as synthetic drug formulations that cause various harmful side effects [Boopathy and Kathiresan, 2011]. The present study evaluated the potent anti-cancer activity of *Chaetomium* sp. extract on A431 human epidermoid carcinoma cells. Regulation of apoptosis and cell cycle is an important process to preserve cell homeostasis between cell death and cell proliferation, which means that the induction of apoptosis and suppression of cell cycle is an advantageous strategy for cancer therapy [Choi et al., 2011]. Recent evidences have suggested that apoptosis should be an underlying mechanism by which various anti-cancer and chemopreventive agents exert anti-cancer effect, especially from natural compound [Lee and Lim, 2011]. So, A431 human epidermoid carcinoma cells were used as an *in vitro* model. First, the cytotoxicity of the extract from fungus *Chaetomium* sp. in A431 cells was determined by WST-1® assay. The number of viable A431 cells was evaluated in the absence or in the presence of

various concentration of the extract by WST-1®, and the IC_{50} of the extract was estimated. As shown in Fig. 1, the IC_{50} value in A431 cells was about 0.3 μM whereas its value in HEK293 cells was about 1.5 μM. Under microscopic examination, as increasingly higher concentrations of the extract on A431 cells cause subsequent lose of contact with neighboring cells, and finally floated into a medium (data not shown). The results indicate that the extract have more efficient inhibitory effect on the growth of A431 cells as well as exhibits the morphological features of apoptosis.

Apoptosis is a fundamental cellular activity and provides protection against cancer progression by eliminating genetically altered cells and hyper-proliferative cells [Cho et al., 2009]. In mammalian cells, caspase cascade has been shown to be involved in chemical induced apoptosis [Lee and Lim, 2011]. Therefore, the expression level of caspase-3, -6, -7 and -8 was investigated using Western blot analysis (Fig. 2A). Results clearly showed that the extract induces the activation of each caspase in a dose-dependent manner. Furthermore, the cellular activation of caspase-3 and -8 (Fig. 3) and FADD (Fig. 4) were shown to increase in a time-dependent manner. Activation of caspases cleaves many cellular substrates such as DFF, lamin A, PARP and Bid and they were examined by western. Cleavage of DFF45/35 (homologous in ICAD) by caspase-3 leads to the activation of CAD, which, in turn, cleaves genomic DNA within internucleosomal regions and generates multimers of nucleosomal domainsized fragments [Hamsa et al., 2011]. Nuclear lamin is the cellular substrate of caspase-6, its cleavage indicates nuclear condensation [Koo et al.,

2011]. PARP is cleaved by caspase-3 during early apoptosis in many different cell lines. The cleavage of PARP results in preventing the recruitment of the catalytic domain to the sites of DNA damage and has been suggested to occur in order to prevent depletion of energy (NAD and ATP) that is thought to be required for later stages of apoptosis [Boulares et al., 1999]. Unlike caspases activation, the Bid, cytochrome *c* and the AIF were not induced. The cleavage of Bid to tBid, a substrate for caspase-8, is essential for linking the death receptor mediated death receptor pathway to the mitochondrial pathway [Gahlot et al., 2010]. Several studies have shown that chemotherapeutic-agent-induced apoptosis involves cleavage of cytosolic Bid into truncated form of Bid (tBid) by caspase-8. Notably, tBid can induce loss of mitochondrial membrane potential and promotes cytochrome *c* and AIF release [Liang et al., 2012]. In addition, p53 tumor-suppressor gene exerts its anti-tumor effect through the induction of either cell-growth arrest or apoptosis [Evan et al., 1995]. p53 protein stimulates the release of cytochrome *c* from mitochondria and procaspase-3 activation [Marchenko et al., 2000]. There was no expression of p53 protein in A431 cells. So, these results indicate that the extract could not induce mitochondrial apoptosis. Moreover, the extract stimulates activation of Fas and FADD in a dose-dependent manner, which indicates that the extract-induced caspase-8 activation is probably due to the stimulation of death receptor (Fig. 2B). But the extract could not cause activation of TNFR and DR. A decoy receptor for FasL termed DcR3, acts as a competitor of Fas such as anti-apoptotic molecule and decreased after treatment of the extract [Özören and El-Deiry, 2003].

Interestingly, the treatment of the extract down-regulate the expression of FLIP. FLIP is the major protein that prevents caspase-8 activation at the DISC [Gahlot et al., 2010]. Thus, A431 cells undergo as Fas-mediated mitochondria independent apoptosis by *Chaetomium* sp. extract.

Activation of the death receptor apoptosis by caspases, which are responsible, either directly or indirectly, for the cleavages of cellular proteins [Cho et al., 2009]. In order to verify the involvement of caspases in the extract induced cell death, the cell viability was examined using pan-caspase inhibitor. The broad-spectrum caspase inhibitor Z-VAD-FMK is commonly used to study the role of caspases in cell signaling. As shown in Fig. 5, one set was treated with different concentration of the extract and another set with the extract and Z-VAD-FMK (50 μM). The result showed that the extract could not promote cell death in presence with Z-VAD-FMK, and this indicates that the extract induces caspase-dependent apoptosis.

The process of tumor cell metastasis is a complex cascade of events, which involves numerous steps such as proliferation, separation of cells from the primary tumor, adherence of the cells to a new location, angiogenesis, the migration of cancer cells into the stroma and the proteolysis of the matrix [Larkins et al., 2006]. It has been reported, that the impediment of local cell proliferation is the critical step in the control of metastasis [Bellyei et al., 2010]. Accordingly, the inhibitory effect of the extract on cell migration in A431 cells was investigated. Wound-healing assays showed a slower wound closure following the extract treatment, indicating decreased cancer cell motility (Fig. 6).

Inhibition of cell cycle progression is an important step in cancer therapy because abnormal cell proliferation is observed in cancer cells [Lopez-Saez et al., 1998]. In order to elucidate the mechanism for the effects of the extract on A431 cells, the cell cycle pattern was examined using FACS analysis. When A431 cells were treated with the extract, the number of treated cells in G_0/G_1 phase was higher than the control cells with the decrease of S phase population (Fig. 7A, B). These results suggest that the extract arrests the cell cycle progression of A431 cells at the G_0/G_1 phase in a dose-dependent manner. In addition, the cell cycle regulatory proteins were examined by western blot analysis (Fig. 7C). Regulation of proteins that mediate critical events of the cell cycle may be a useful anti-tumor target [Stewart et al., 2003]. Cyclins and cyclin-dependent kinases (Cdks) complexes are regulators of cell cycle progression where cyclins are regulatory units and Cdks are catalytic units, and these proteins are regulated by inhibitory proteins [Chilampalli et al., 2011]. Cdks inhibitory proteins, $p15^{INK4B}$, $p16^{INK4A}$, $p21^{waf1/cip1}$ and $p27^{kip1}$, negatively regulate kinase activity of the cylin and Cdks complexes which are up-regulated in cancer cells in response to anti-proliferative signals, and well known for arresting G_0/G_1 and S phase [Toyoshima and Hunter, 1994]. With *in vitro* data, up-regulated expression of Cdks inhibitory protein p21 by the extract was found through the immunofluorescence (Fig. 7D). After DNA damage signaling, the "p53 → $p21^{waf1/cip1}$ pathway" is activated. Activated p53 induces the expression of its downstream target, including $p21^{waf1/cip1}$, and then arrest cell cycle at the G_1/S or G_2/M phases [Kalimutho et al., 2011]. Considering p53 mutated A431 cells, the

up-regulated expression of $p21^{waf1/cip1}$ by the extract might be p53 independent.

In conclusion, the extract from *Chaetomium* sp. has potent anti-cancer effects on induction of type I death receptor apoptosis and G_1/S cell cycle arrest in A431 cells (Fig. 9). The present results provide new possibility of chemotherapy for epidermoid cancer. Further studies focus on the effects of the extract on other signaling pathways and the detailed mechanisms of apoptosis *in vivo* and p53-p21 relation in cell cycle arrest will have to be elucidated.

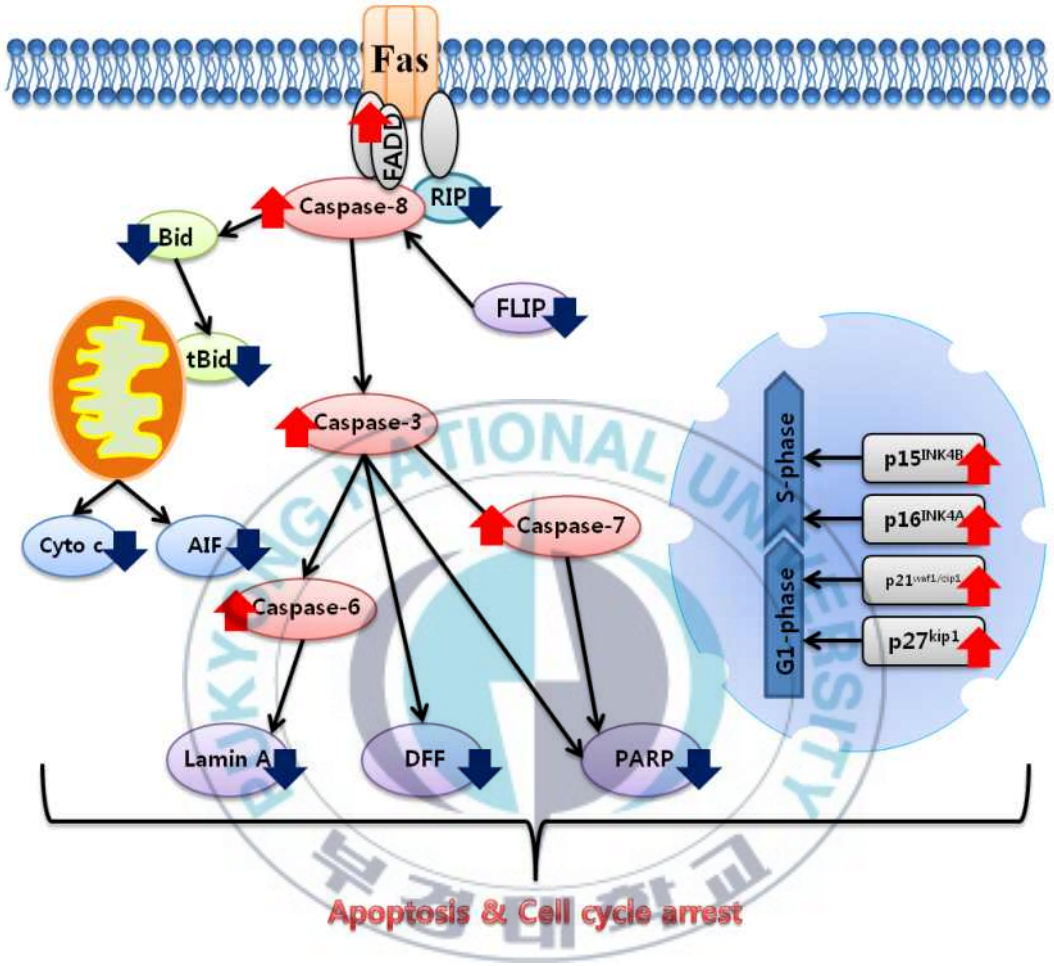

Figure 8. Proposed the extract-induced apoptosis and cell cycle arrest signaling in A431 cells.

국문 초록

세포의 죽음은 necrosis와 apoptosis로 구분되며, 이것은 세포의 형태학적 및 생화학적인 특성에 의하여 구분될 수 있다. 그 중 apoptosis는 개체의 발생단계나 DNA 손상, 바이러스 감염 등에 따른 유전적 조절 하에서 일어나는 개체보존 수준에서 손상된 세포들의 제거를 위한 중요한 방어기전이라는 점에서 생리적 또는 화학적 외상에 의한 세포의 죽음인 necrosis와 구별된다.

Chaetomium sp.는 Chaetomiaceae에 속하는 곰팡이로써 흙, 공기나 식물 잔해에서 흔히 발견된다. 자연 상태에서 이 균류는 인간에게 감염을 유발하기도하고, *Chaetomium* sp.의 추출물 및 2차 대사산물은 다양한 생리활성 효과를 가지고 있다고 보고되어 있다. 그러나 이 균류의 대사산물에 대한 항암효과 및 그 기작에 관해서는 아직 알려져 있지 않다. 따라서 본 연구에서는 *Chaetomium* sp.의 추출물에 민감하게 반응하는 A431 피부암세포를 대상으로 *Chaetomium* sp.의 항암작용 기전을 해석하고자 하였다.

A431 피부암세포의 apoptosis 유도에 미치는 *Chaetomium* sp. 추출물의 영향을 조사하였으며, apoptosis 조절작용에 관여하는 주요한 유전자들의 발현 및 활성도 변화를 조사하였다. DAPI 염색을 통하여, 추출물 처리에 의한 A431 세포의 증식억제 과정이 형태적 변형을 동반한 apoptosis 유도와 연관성이 있음 확인하였고 apoptotic body 출현이 증가함을 관찰할 수 있었다. 또한, flow cytometry 분석으로 sub-G1 기에 속하는 세포의 빈도가 추출물의 처리에 비례하여 증가한다는 것을 확인하였다. 추출물 처리에 따른 apopotosis의 유도에서 Bcl-2 family에 속하는 인자들의 발현에는 큰 변화가 없었으나, caspase-3 및 -8의 활성이 매우 높게 증가되는 것을 관찰할 수 있었으며, 이는 PARP, lamin A/C 및 DFF45/35 단백질의 분해와

연관성이 있었다. 또한 caspase의 저해제인 Z-VAD-FMK로 caspase의 활성을 인위적으로 차단시켰을 경우, 추출물에 의한 apoptois 유도 현상이 유의적으로 감소되어 추출물에 의한 A431 세포의 apoptosis가 caspase 의존적이라는 것을 증명할 수 있었다. 그리고 caspase-8의 활성에 따른 death receptor의 발현을 조사한 결과, Fas의 발현이 추출물에 의해 증가하였음을 확인하였다.

본 연구의 결과만으로 *Chaetomium* sp.에 의한 피부암세포의 증식억제 기전을 명확하게 제시할 수는 없으나, 이상의 결과들은 *Chaetomium* sp.의 생화학적 항암 기전을 이해하는데 중요한 기초자료로서 활용될 수 있을 것으로 생각된다.

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