The mechanism of acacetin-induced apoptosis on Oral squamous cell carcinoma cell line

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The mechanism of acacetin-induced apoptosis on Oral squamous cell carcinoma cell line

Directed by Professor Cha, In-Ho

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This certifies that the Dissertation

of Kim, Chae Doo is approved.

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ABSTRACT.

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Acacetin (5, 7-dihydroxy-40-methoxyflavone) – present in safflower seeds, plants, flowers, and Cirisium rhinoceros Nakai – has been reported to exhibit anti-peroxidative, anti-inflammatory, anti-plasmodial, and anti-proliferative activities. The objective of this study is to investigate the mechanism of acacetin-induced apoptosis of the oral squamous cell carcinoma cell line. Acacetin caused 50% growth inhibition (IC_{50}) in HSC-3 cells at 25μg/ml over 24 hours in the MTT assay. Apoptosis was characterized by DNA fragmentation and an increase in sub-G1 cells and involved the activation of caspase-3 and PARP (poly-ADP-ribose) polymerase. Maximum caspase-3 activity was observed with 100μg/ml of acacetin treated for 24 hours. Caspase-8 and -9 activation, which mediate the activation of caspase-3, were confirmed. Acacetin caused a reduction in Bcl-2 expression leading to an increase in the Bax: Bcl-2 ratio. In addition, it caused a loss of mitochondrial membrane potential that induced the release of cytochrome c into the cytoplasm. Pretreatment with the casapse-3 (Z-DEVD-FMK), -8 (Z-IETD-FMK), and -9 inhibitor (z-LEHD-fmk) inhibited the acacetin-induced loss of mitochondrial membrane potential and release of cytochrome *c*. The mitogen-activated protein kinases (MAPKs) were activated by acacetin and pre-treating the cells with each of the MAPKs specific inhibitors apparently inhibited the acacetin-induced cytotoxicity of the HSC-3 cells. Moreover, when the cells were treated with the MAPKs inhibitor, the PARP cleavage, increase in sub G1 cells, increase in the Bax: Bcl-2 ratio, and release into cytoplasm of

cytochrome c – which were induced by acacetin – were inhibited. In conclusion, acacetin induces the apoptosis of the oral squamous cell carcinoma cell line, which has a close relation to its ability to activate the MAPK-mediated signaling pathways with the subsequent induction of a mitochondria- and caspase-dependent mechanism. These results strongly suggest that acacetin might have an effect on HSC-3 cells growth inhibition and therapeutic potential in oral squamous cell carcinoma.

Keywords: Acacetin, Apoptosis, Oral squamous cell carcinoma

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I. INTRODUCTION

Oral squamous cell carcinoma is the most common cancer in the oral and maxillofacial region, accounting for more than 90% of all oral cancers (Chen et al., 1999). Although great progress has been made in treatment modalities and technologies, the 5-year survival rate of oral squamous cell carcinoma patients has remained steady at 50–60 % (Kim et al., 2002).

Varieties of treatments have been used to treat oral squamous cell carcinoma, including various forms of surgery, radiotherapy, cytotoxic chemotherapy, molecularly targeted therapy and immunotherapy. However, protocols for management of oral squamous cell carcinoma converge, and may include primary surgery and post-operative radiotherapy, and sometimes chemo-radiotherapy in selected cases (Shaw et al., 2011). Chemotherapy drugs in oral squamous cell carcinoma have historically been most frequently used including cisplatin, fluorouracil and methotrexate (Vermorken et al., 2005).

Because conventional chemotherapeutic agents have been associated with numerous significant clinical complications, alternative or less toxic chemical treatments for oral cancer are required (Yamachika et al., 2004). Therefore, several efforts to develop new drugs that are substitutes for the existing drug, with the same efficacy but fewer side effects, from natural products are in progress (Lin et al., 2012; King et al., 2007; Chen et al., 2006; Myoung et al., 2003).

Unlike normal cells, cancer cells have an s transition process in which they leave the primary site to move to other parts through the circulatory system but grow abnormally due to paralysis of the control

of apoptosis, which means the process of programmed cell death. Apoptosis occurs through the regulation of several proteins and gene expression according to the cell signals, accompanied by morphological changes such as cell shrinkage, condensed chromosomes, DNA fragmentation, and apoptotic body formation (Orrenius et al., 2004). Cell death by apoptosis is an intrinsic cell suicide mechanism of the cells and regulated by the signal transduction pathway in cells (Daniel et al., 2004).

The Bcl-2 family is the representative protein involved in the regulation of apoptotic cell death, consisting of anti-apoptotic and pro-apoptotic members. Bcl-2 and Bcl-xL, which are the anti-apoptotic members of this family, prevent apoptosis either by sequestering proforms of caspases or by preventing the release of mitochondrial apoptogenic factors such as cytochrome c and AIF (apoptosis-inducing factor) into the cytoplasm. On the other hand, Bax and Bak, which are pro-apoptotic members of this family, trigger the release of caspases from death antagonists via heterodimerization. Caspases activation is initiated by inducing the release of mitochondrial apoptogenic factors into the cytoplasm via acting on the mitochondrial permeability of the transition pore (Tsujimoto et al., 1998).

Caspase is known as the most important executor on apoptosis, and the two main caspase cascades have been described. The first pathway links caspase-8 to death receptors expressed at the cell surface including Fas, TNFR1, and DR3. In the second pathway, caspase-9 is activated by a variety of death stimuli from both outside and inside the cell. Active caspase-8or caspase-9 can cleave and activate an overlapping set of effector caspases including caspase-3, -6, and -7, resulting in enhanced expression of protease activity in the cell (Nuñez G et al., 1998).

MAPKs, such as JNK, ERK, and p38 kinase, are activated by various extracellular stimuli, and mediate the signal transduction cascades and play an important role in regulating apoptosis (Cargnello et al., 2011). The MAPKs signaling pathways modulate gene expression, mitosis, proliferation, motility, metabolism, and programmed death apoptosis. ERK1 and ERK2 are well-characterized MAPKs activated in response to growth stimuli. Both JNKs and p38-MAPK are simultaneously activated in response to a variety of cellular and environmental stresses (Wada et al., 2004).

Flavonoids, predominant components of fruits and vegetables, have been widely investigated for their role in human disease and health. These compounds have been shown to influence cell cycle

progression and alter protein expression (Duthie et al., 1999; Plaumann et al., 1996; Gerritsen, 1998). Many flavonoids such as soy protein extract, luteolin, green tea polyphenol, and silibin also have been shown to possess anti-cancer effects against oral cancer cell lines, suggesting potentially important resources for the discovery of tumor suppressive agents (Chen et al., 2006; Kingsley et al., 2011; Yang et al., 2008; Srinivasan et al., 2008).

Acacetin (5, 7-dihydroxy-40-methoxyflavone) is present in many plants – such as thistle, safflower seed, and acacia – and induces apoptosis. It has been reported to have an anti-peroxidative, anti-inflammatory, anti-plasmodial and anti-proliferative effect by blocking cell cycle progression (Yim et al., 2003; Kraft et al., 2003; Pan et al., 2006). Several recent studies have shown that acacetin inhibits the proliferation of A549 lung cancer cells and HepG2 human liver cancer by blocking the cell cycle progression (Hsu et al., 2003; Hsu et al., 2004). Others have reported that acacetin inhibits the growth of human breast cancer MCF-7 cells and induces apoptosis in human prostate cancer cells (Shim et al., 2007). In addition, acacetin inhibits ovarian cancer cells by induced angiogenesis and tumor growth in vivo through inhibiting HIF-1a and VEGF expression (Liu et al., 2011).

As outlined above, the anti- cancer effect of acacetin has been reported in several types of cancer, but the effect of acacetin on oral squamous cell carcinoma has not been reported. Therefore, this author studied the mechanisms involved in acacetin-induced apoptosis of the oral squamous cell carcinoma cell line (HSC-3).

II. Materials and methods

1. Materials

The Japanese Cancer Research Resources Bank (JCRB) provided HSC-3 established from a squamous cell carcinoma located on the tongue. Unless specified otherwise, all the chemicals and laboratory apparatus used in this study were obtained from the Sigma Chemical Co. (St. Louis, MO) and Falcon Lab ware (Becton-Dickinson, NJ), respectively. Caspase inhibitors (z-DEVE-fmk, FK009; z-IETD-fmk, FK023; z-LEHD-fmk, FK022) were purchased from ICN (Ohio) and dissolved

in dimethylsulfoxide (DMSO) immediately before use. MAPK inhibitors (SP600125, SB203580, and PD98059) were supplied by TOCRIS (MI). The final DMSO concentration did not exceed 0.5% (v/v) in any of the experiments. Fetal bovine serum (FBS), penicillin G, and streptomycin were obtained from GIBCOBRL (Gaithersburg, MD). ELISA kits were obtained from Roche Diagnostics GmbH (Germany).

2. Cell culture and treatment

HSC-3 was established from squamous cell carcinoma located on the tongue. The Japanese Cancer Research Resources Bank (JCRB) provided the cells utilized in this study. They were routinely maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 U/ml of penicillin, and 100 μg/ml of streptomycin, in an atmosphere of 5% CO_2 in air at 37℃. One million cells per milliliter were re-suspended in either 2 ml or 100 μl of the media and spread onto either 6-well or 96-well flat-bottomed plates, respectively. When the cells reached 90% confluence, a fresh batch of serum-free DMEM was added to the cultures, and the HSC-3 cells were then exposed to different concentrations (0-100 μg/ml medium) of the acacetin in the presence or absence of MAPK inhibitors or caspase inhibitors. At various times after the treatment, the cells were examined for any signs of cytotoxicity and apoptosis.

3. Measurement of cell viability

3-(4, 5-dimethylthiazol-2yl-)-2, 5-diphenyl tetrazolium bromide (MTT) was used to examine the cell viability. Briefly, the cultured HSC-3 cells were exposed to the acacetin with and without MAPK or caspase inhibitors. At various exposure times, 100 μl of an MTT solution (5 mg/ml in PBS as stock solution) was added to each well, and the cells were incubated for a further 4 h at 37 °C. Seventy micro liters of DMSO was then added to each well, and the absorbance of the plates was read at 560 nm using a Spectra Count TM (Packard Instrument Co., USA) ELISA reader.

4. Cell cycle analysis

The progression of the cell cycle was determined using flow cytometric analysis after staining with

propidium iodide (PI). Initially, the suspension (2 x 10^6 cells) of acacetin with and without MAPK or caspase inhibitors treated HSC-3 cells was fixed with 80% ethanol at 4 °C for 24 h, and incubated overnight at 4 °C with 1 ml of a PI staining mixture (250 μl of PBS, 250 μl of 1 mg/ml RNase in 1.12% sodium citrate, and 500 μl of 50 μg/ml PI in 1.12% sodium citrate). After staining, 1 x 10^4 cells were analyzed using the FACS Caliber® system (Becton Dickinson, USA).

5. Hoechst 33258 staining

Morphological changes of the apoptotic cells were also examined using fluorescence microscopy. At various concentrations after treating HSC-3 cells with acacetin, the cells were harvested, fixed with absolute ethanol, and stained with Hoechst 33258 for 15 min at 37 °C. The cells were then visualized using fluorescence microscopy (Olympus BX50, Japan) with UV excitation at 300 - 500 nm. Cells containing condensed and/or fragmented nuclei were considered apoptotic cells.

6. Assessments of Apoptosis by TUNEL Assay and DNAGel Electrophoresis

A terminal deoxy-nucleotidyl-transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed according to the manufacturer's protocols (In Situ Cell Death Detection Kit, Roche Diagnostics Corp., USA). Cells (1×10^6/well) were plated onto six-well plates and exposed to several concentrations of acacetin with and without MAPK or caspase inhibitors for 24 h. After treatment, cells were collected and determined as previously described. TUNEL-positive cells were analyzed and quantified using a FACS Caliber instrument (BD Biosciences, San Jose, CA, USA) equipped with BD Cell Quest Pro software. Approximately 1×10^6 cells per well were incubated without (control) or with several concentrations of acacetin for 24-hour exposure. Cells from each sample were collected and the DNA was isolated for agarose gel electrophoresis as previously described. After electrophoresis in a 1.5% agarose gel containing ethidium bromide (EtBr, Invitrogen) in a 0.5x TBE buffer (AMRESCO Inc., USA), the DNA in gel was resolved with UV light and photographed.

7. Preparation of mitochondrial and cytosolic fractions

Cells grown in the presence of acacetin (100 μM) or no acacetin in 6-well plates for 24 h were

washed twice with ice-cold PBS and lysed in 100 μl of ice-cold buffer A [20 mM HEPES (pH 7.6), 10 mM KCl, 1.5mM MgCl2, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 250 mM sucrose, protease inhibitor cocktail] for 30 min at 4°C. Unbroken cells and nuclei were removed by centrifugation at 1,000-× *g* for 10min at 4°C. The supernatants were further centrifuged at 16,000-× *g* for 30 min at 4°C and the resulting supernatants were used as cytosolic protein fractions. The pellets were dissolved in lysis buffer B containing 1% NP-40 for 30 min at 4°C. After centrifugation at 16,000-× *g* for 30 min at 4°C, the supernatants were collected and considered as mitochondrial protein fractions.

8. Preparation of cell lysates

At various times after treating the HSC-3 cells with the acacetin, the cells were collected and resuspended in a lysis buffer (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 μg/ml aprotinin, 100 go/ml PMSF, and 250 mM sucrose). After centrifugation at 700-× *g* for 10 min, the supernatant was transferred into a new tube and further centrifuged at 10,000-× *g* for 30 min to isolate the cytosolic fraction. The pellets were then used as the mitochondrial fraction. In addition, the nuclear extracts were prepared according to the manufacturer's instruction using a NE-PERTM nuclear and cytoplasmic extraction reagents kit (Pierce Rockford, USA). Protein content of the cell lysates was quantified using the Bradford method.

9. Western blot analysis

An equal amount of protein (20-40 μg/sample) was separated electrophoretically by 6-12% SDS-PAGE and blotted onto PVDF membranes. The blots were probed with the primary antibodies and incubated with a horseradish peroxidase-conjugated anti-IgG in blocking buffer for 1 h. After washing, the blots were developed with enhanced chemiluminescence (ECL) (Santa Cruz Biotechnology, USA) and exposed to X-ray film (Eastman-Kodak, USA). The polyclonal antibodies specific to Bcl-2 (SC-783,1:200), caspase-3 (SC-7148, 1:200), and caspase-8 (SC-6134, 1:100), extracellular signal-regulated kinase (ERK; SC-94, 1:200)and poly (ADP ribose) polymerase (PARP; SC-7150, 1:200), and the monoclonal antibodies specific to Bax (SC-7480, 1:100), cytochrome *c* (SC-13156, 1:500), caspase-9(SC-17784, 1:100), phosphorylated-ERK (p-ERK; SC-7383, 1:200), p-c-Jun

N-terminal kinase (p-JNK; SC-6254, 1:100), and p-p38 (SC-7973, 1:200)were purchased from Santa Cruz Biotechnology. The polyclonal antibody specific to β-actin (1:500) was purchased from Sigma Chemical Co. The monoclonal antibody specific to p-p38 (4631, 1:200), p38 (9212, 1:2000) and the polyclonal antibody specific to JNK (9252, 1:1000) were purchased from Cell Signaling (MA, USA).

10. Statistical analysis

All the data are expressed as a mean ± standard error (SE). One-way ANOVA using SPSS ver. 19.0 software was used for multiple comparisons. A $P < 0.05$ was considered significant.

III. RESULTS

Acacetin induces cytotoxicity in a dose- and time-dependent manner. Initially, an MTT assay was used to determine if acacetin had any cytotoxic effect on HSC-3 cells (Fig. 1). As shown in the figure, the addition of the acacetin significantly reduced the viability of HSC-3 cells in a dose- (Fig. 1A) and time-dependent manner (Fig. 1B). After incubating the cells with 25μg/ml acacetin for 24-h, cell viability was found to be 46.95 ± 1.23%. However, when 50μg/ml of the acacetin was added, only approximately 32% of the cells were viable.

Fig. 1A

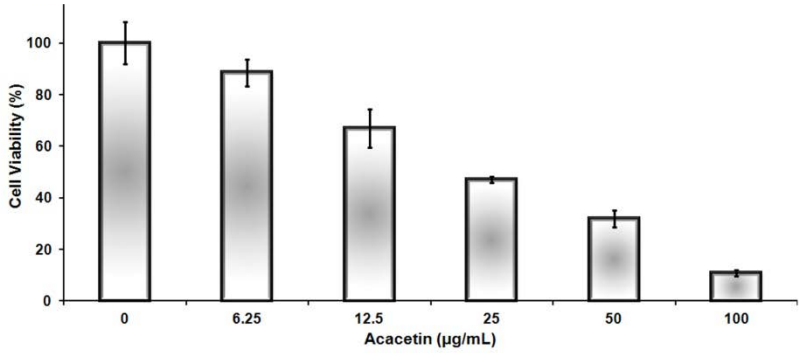


Fig. 1B

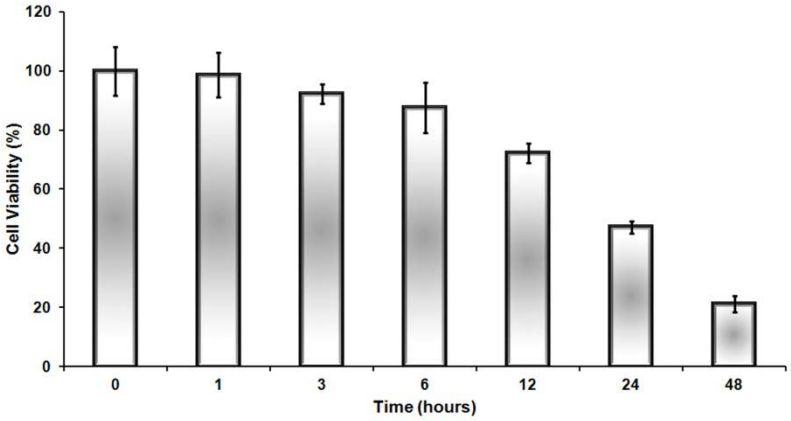


Fig. 1 Acacetin causes cytotoxicity to HSC-3 cells in a dose- and time-dependent manner. HSC-3 cells were treated with the indicated concentrations of the acacetin for 24 h (A) or with 25μg/ml of the acacetin for various times (B) and then processed for the MTT assay. The results are reported as a mean ± SE of the triplicate experiments, and the different superscripts represent the significant differences ($P < 0.05$) between the groups using the Scheffe's multiple range test.

The effect of the acacetin on the determination of morphological changes in HSC-3 cells. Nucleic acid staining with Hoechst 33342 revealed typical apoptotic nuclei, which exhibited highly fluorescent condensed chromatin in cells treated with the acacetin 25μg/ml for 24 h (Fig. 2A). The morphological changes and cell death of HSC-3 cells were significantly increased at 25 μg/ml acacetin, and most cells were detached from the dishes and cell rounding and shrinking occurred at the acacetin. To determine whether the acacetin induces apoptosis in HSC-3 cells, we investigated the DNA fragmentation, which is a biochemical hallmark for apoptosis (Fig. 2B). The results

demonstrated that the acacetin induced endonucleolytic DNA cleavage in a dose-dependent manner.

Fig. 2A **Fig. 2B**

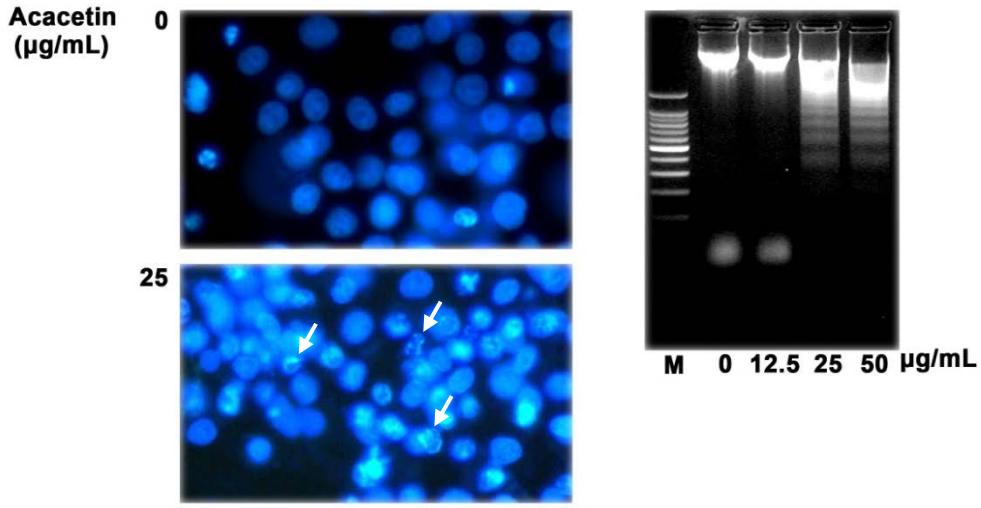
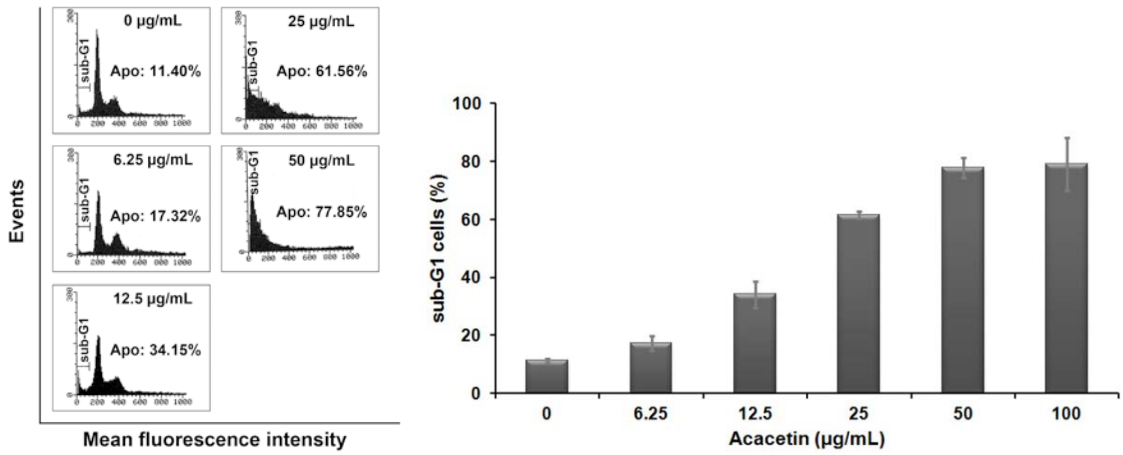


Fig. 2Acacetin induces apoptotic death of HSC-3 cells. The cells were treated with the indicated concentrations of acacetin for 24 h. After incubation, the cells were processed for nucleic staining of Hoechst 33258 (A) and for DNA fragmentation (B). Arrows indicate that the apoptotic cells have fragmented and/or condensed nuclei.

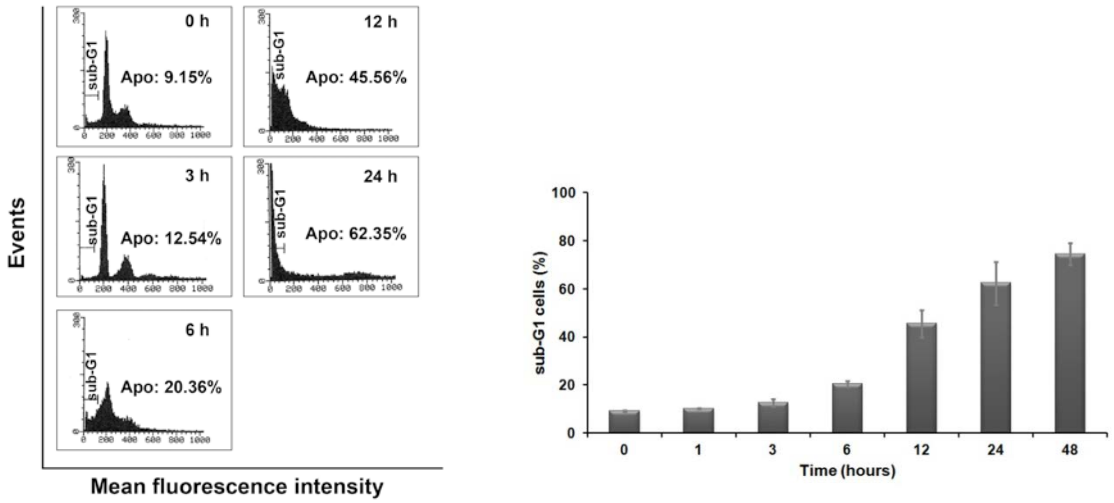
Acacetin induces apoptotic death of HSC-3 cells. The acacetin-treated HSC-3 cells were subjected to apoptosis assays to determine if the acacetin induces apoptosis (Fig. 3). Initially, PI staining revealed a dose- (Fig. 3A) and time-dependent (Fig. 3B) increase in the cell populations in the sub-G1 phase of the cell cycles after the acacetin treatment. In the absence of acacetin, only 11.4% of the total cell population was apoptotic. However, 61.56 ± 1.21% and 77.85 ± 3.32% of the cell population were apoptotic, when the cells were treated with 25 and 50μg/ml of the acacetin, respectively. This suggests that the acacetin induces cell death via apoptosis in HSC-3 cells.

Fig. 3A



	Concentration (μg/ml)						
	0	**3.125**	**6.25**	**12.5**	**25**	**50**	**100**
sub-G1 cells (%)	11.40± 0.56	12.55± 1.21	17.32± 2.54	34.15± 4.62	61.56± 1.21	77.85± 3.32	79.11± 9.12

Fig. 3B



	Time (hours)						
	0	**1**	**3**	**6**	**12**	**24**	**48**
Sub-G1 cells (%)	9.15± 0.41	10.10± 0.14	12.54± 1.48	20.36± 1.24	45.56± 5.61	62.35± 8.84	74.51± 4.65

Fig. 3 Flow cytometric analysis of Acacetin-treated HSC-3 cells. The cells were treated with the indicated concentrations of acacetin for 24 h (A) or with 25 μg/ml of the acacetin for various times (B). After incubation,

the cells were processed for flow cytometric analyses after PI staining. Each bar shows the mean ± SE of three separate experiments.

To evaluate whether the impact of acacetin on HSC-3 cells' viability involves the process of cell apoptosis, HSC-3 cells were assessed by TUNEL assays. In TUNEL results, acacetin treatment significantly increased the percentage of positively stained (i.e., apoptotic) cells in a dose- (Fig. 4A) and time- (Fig. 4B) dependent manner. The indicated concentration of acacetin-treated cells ranged from 19.1 to 87.3% for 24 h (Fig. 4).

Fig. 4A

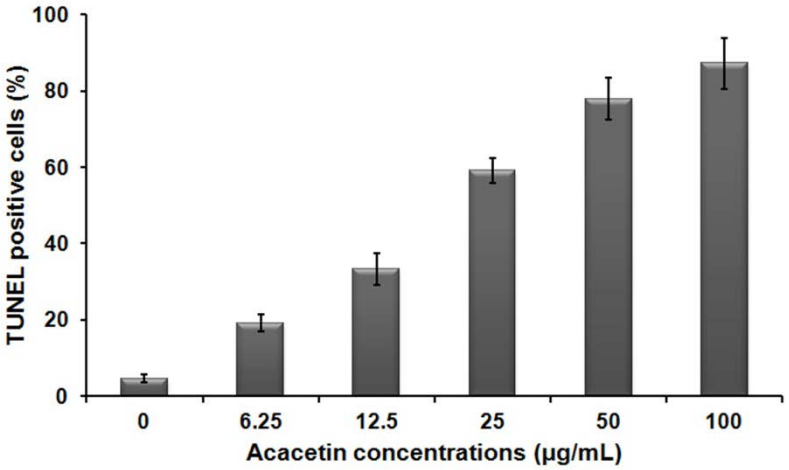


Fig. 4B

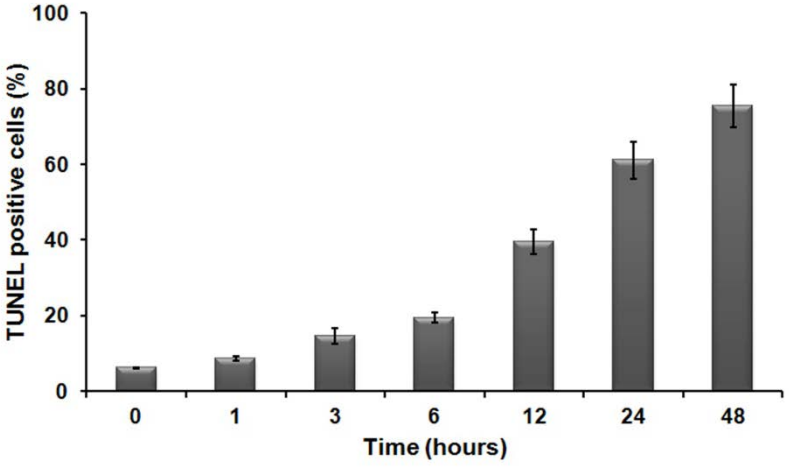


Fig.4 Effects of acacetin on the apoptosis of HSC-3 cells. Quantitative analysis of TdT-mediated dUTP-biotin nick end labeling results. The TdT-mediated dUTP-biotin nick end labeling apoptotic index (TUNEL-positive to DAPI-stainable cell ratio) was significantly higher in acacetin-treated HSC-3 cells in comparison with the

controls in a dose- (A) and time- (B) dependent manner.

Acacetin induces apoptosis by the activation of Caspase-3, -8, and -9. The acacetin-treated HSC-3 cells were investigated for caspase-3, caspase-8, and caspase-9 activities by colorimetric enzymatic assay and/or western blotting. As shown in Fig. 5, both caspase-3 and -9 activities increased in a dose- (Fig. 5A) and time- (Fig. 5B) dependent manner after acacetin treatment. Our results suggested that the acacetin-induced apoptosis was mediated through the activation of caspase-3 and -9.

Fig. 5A

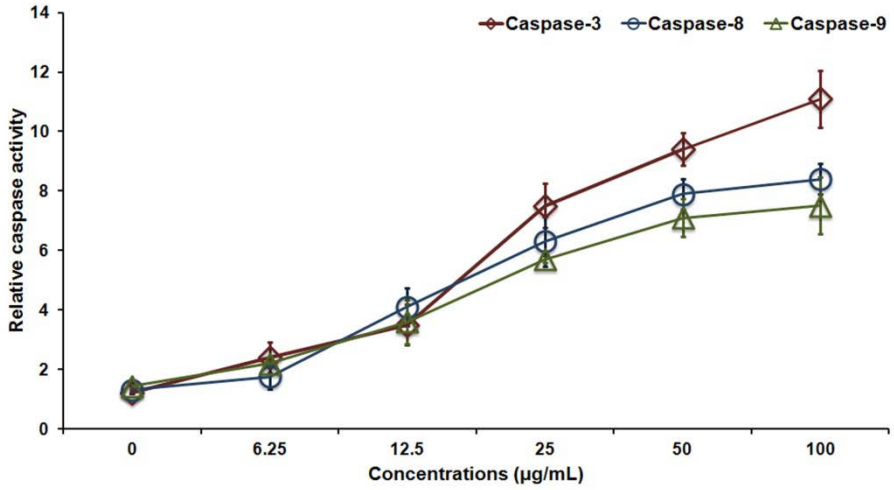


Fig. 5B

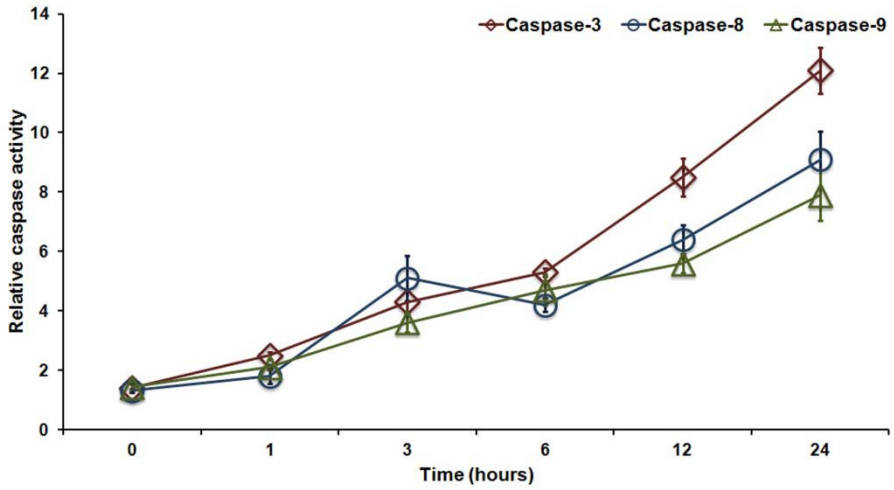


Fig. 5. Activity of caspase-3, -8 and -9 in HSC-3 cells after treatment with indicated concentration of acacetin

for 24 h (A) and with acacetin 25μg/ml for various times (B).

As shown in Fig. 6, the dose-dependent activation of caspase-3 and -9 was observed after treating HSC-3 cells with acacetin. In particular, approximately 50% and 100% degradation of procaspase-3 was observed when the cells were exposed to 12.5 and 25 μg/ml of acacetin for 24 h, respectively. In contrast, the acacetin-mediated degradation of procaspase-3 or procaspase-9 was more apparent than that of procaspase-8. These results strongly suggest the involvement of a caspase-dependent pathway in the acacetin-mediated apoptosis of HSC-3 cells.

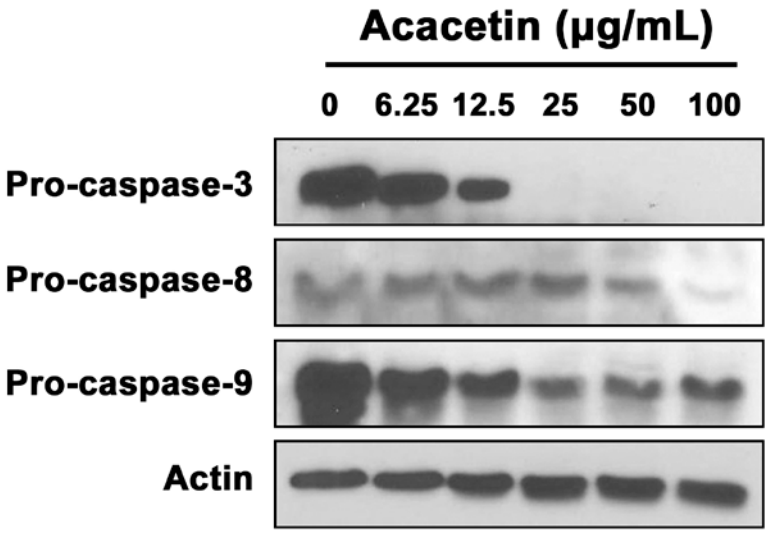


Fig. 6. HSC-3 cells were exposed to the indicated concentrations of acacetin for 24 h, and the expression of several pro-caspases was determined by Western blot analysis.

To further verify the involvement of caspases-3 and caspases-9 in the acacetin induced apoptosis of HSC-3 cells, the caspase-3 inhibitor (Z-DEVE-FMK), caspase-8 inhibitor (Z-IETD-FMK), and caspase-9 inhibitor (Z-LEHD- FMK) were pretreated (Fig. 7). The results demonstrated that the caspase-3 inhibitor (Z-DEVE-FMK) and caspase-9 inhibitor (Z-LEHD-FMK) almost completely suppressed the acacetin-induced caspase-3 and -9 activity in a dose- (Fig. 7A) and time-(Fig. 7B) dependent manner, and they also increased the viable HSC-3 cells.

HSC-3 cells were treated with several concentrations of acacetin for 24 h or various times in the

presence or absence of 50 μM of z-DEVE-fmk, z-IETD-fmk or z-LEHD-fmk to confirm that caspase activation plays a critical role in the acacetin-mediated apoptotic process. These inhibitors significantly inhibited the acacetin-induced cytotoxicity in the cells in a dose- and time-dependent manner (Fig. 8). In particular, the inhibitory effect of z-DEVE-fmk or z-LEHD-fmk was higher than z-IETD-fmk. This suggests that the caspase-dependent pathway is closely related to the acacetin-induced cell death of HSC-3 cells.

Fig. 7A **Fig. 7B**

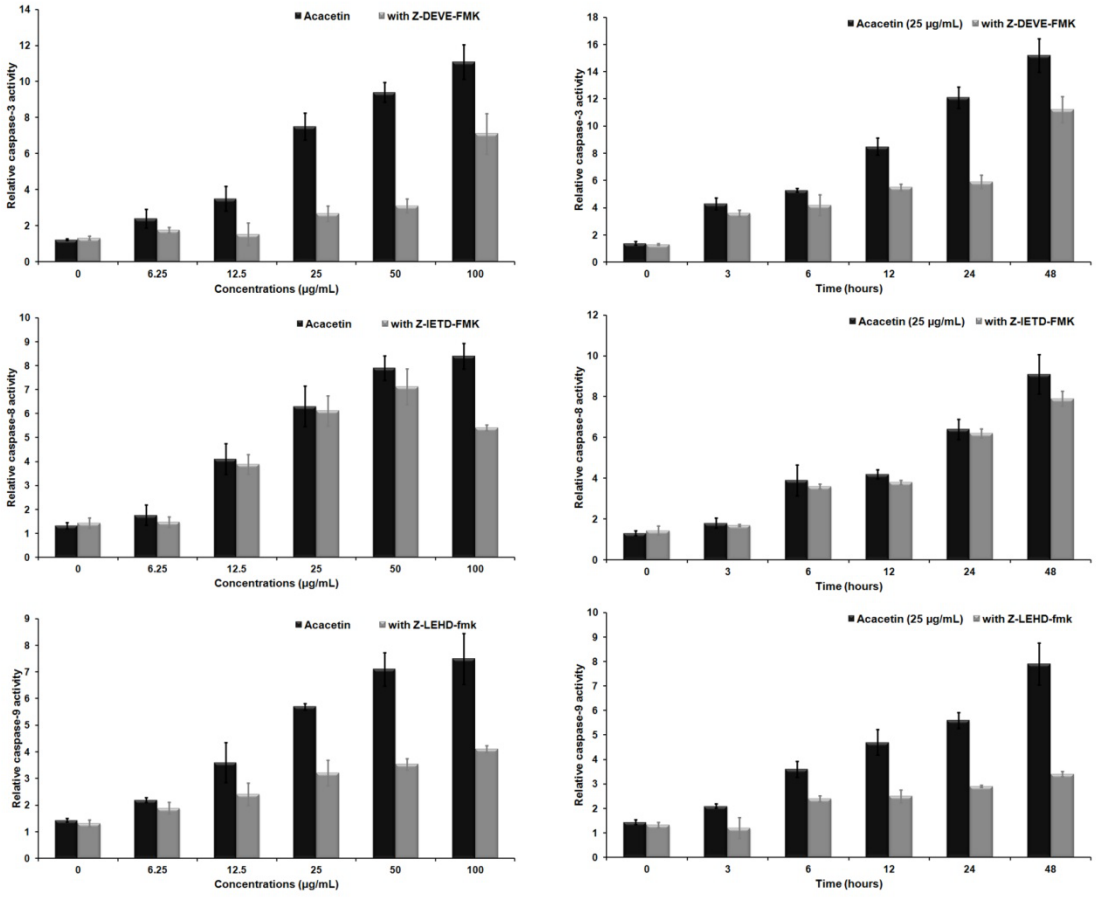


Fig. 7. Acacetin stimulates caspases-3 and -9 activities in HSC-3 cells. Cells were pretreated with the caspase-3 inhibitor (Z-DEVE-FMK), caspase-8 inhibitor (Z-IETD-FMK), and caspase-9inhibitor (Z-LEHD-FMK) for 1 hand then treated with different concentrations (A) of acacetin incubated for various time periods (B).

Fig. 8A

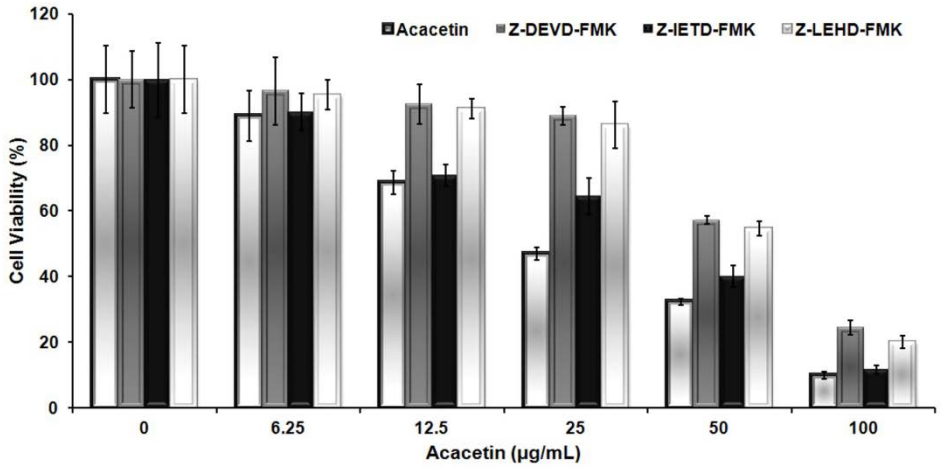


Fig. 8B

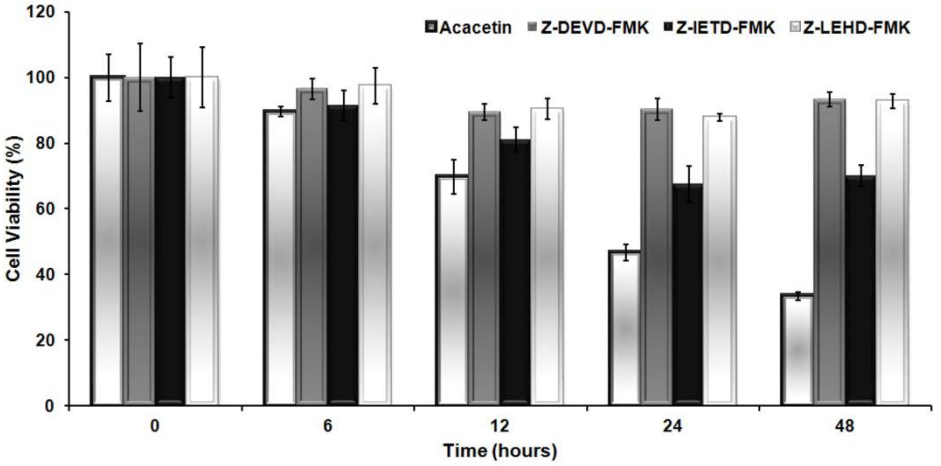


Fig. 8. The acacetin-mediated apoptosis of HSC-3 cells involves a caspase-dependent mechanism. The cells were also pretreated with 50 μM of caspase inhibitors, z-DEVD-fmk, z-IETD-fmk, or z-LEHD-fmk 1 h before exposing them to several concentrations of acacetin for 24 h and acacetin 25 μg/ml for various times. MTT-reducing activity was then determined. Each bar represents the mean ± SE of three separate experiments. Different superscripts represent the significant differences ($p < 0.05$) between the groups using the Scheffe's multiple range tests.

Effect of Acacetin on the cleavage of PARP. As PARP is a downstream substrate of caspase-3, the cleavage of PARP is an indicator of apoptosis. Treatment with acacetin in HSC-3 cells resulted in

the cleavage of PARP to yield an 85-kDa cleaved fragment in a dose- (Fig. 9A) and time- (Fig. 9B) dependent manner.

Fig. 9A

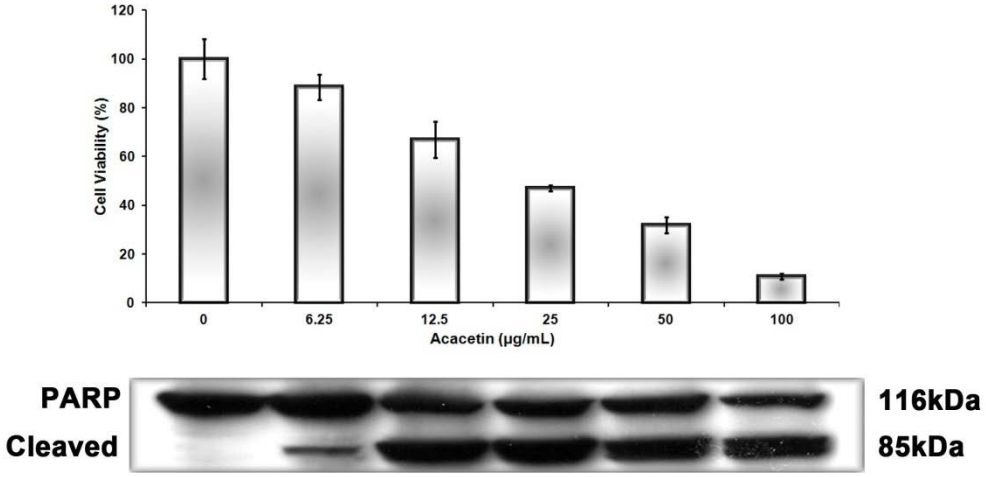


Fig. 9B

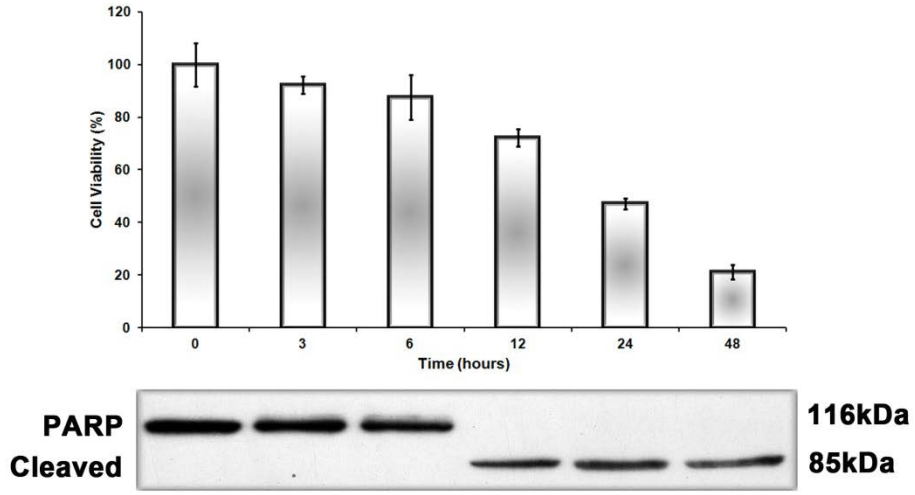


Fig. 9. The effect of acacetin on PARP in a treatment with acacetin in HSC-3 cells resulted in the cleavage of PARP to yield an 85-kDa cleaved fragment in a dose- (Fig. 9A) and time- (Fig. 9B) dependent manner.

Mitochondrial stress is an important event in acacetin-mediated apoptosis of HSC-3 Cells. To evaluate the effect of acacetin on the mitochondrial membrane potential, Bax and Bcl-2, respectively, we performed Western blot analysis for Bax and Bcl-2. Acacetin-treatment increased the intensity

of the bands corresponding to the Bax protein in mitochondrial fractions but significantly reduced the intensity of the band of Bcl-2 protein (Fig. 10). In addition, the amount of cytosolic cytochrome *c* was apparently higher in the cells treated with 25μg/ml of acacetin for 24 hours. Therefore, mitochondrial stress might play a role in the acacetin-mediated apoptosis ofHSC-3 cells.

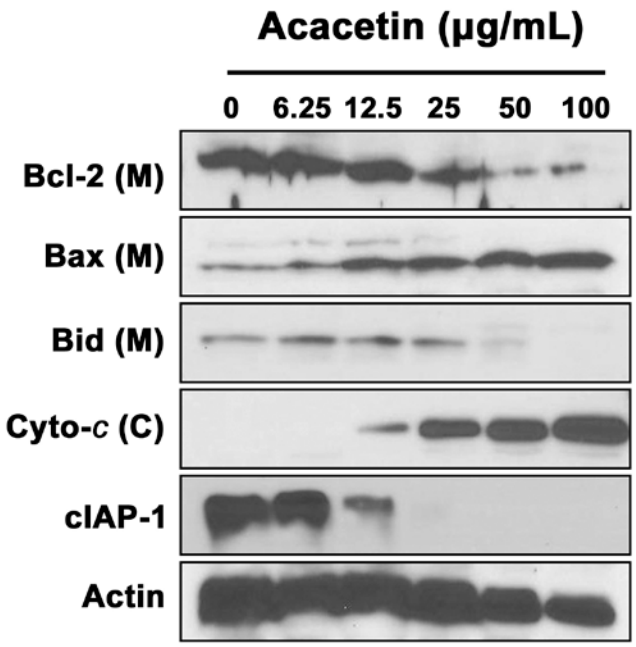


Fig.10. Involvement of mitochondrial stress in the acacetin-mediated apoptosis of HSC-3 cells. The cells were treated with the indicated doses of acacetin for 24 h. Cell lysates were analyzed by 12% SDS-PAGE followed by immunoblot analysis. A representative result from three independent experiments is shown. M and C represent the mitochondria and cytosolic fractions, respectively.

MAPK activation is a key step in the apoptotic process in acacetin-treated HSC-3 cells. To assess the involvement of MAPK in acacetin-treated HSC-3 cells, the level of phosphorylated MAPKs were measured using Western blot analysis (Fig. 11). A dose-dependent increase in the phosphorylated forms of MAPKs was observed by treating the HSC-3 cells with acacetin (Fig. 11). The pattern of ERK and p38 phosphorylation after the acacetin treatment was quite different from the cases in the JNK. The p-ERK and p-p38 level returned to the basal level after being treated with 12.5μg/ml of acacetin for 6h, and increased 10-fold after 25μg/ml of the treatment compared with the untreated control cells.

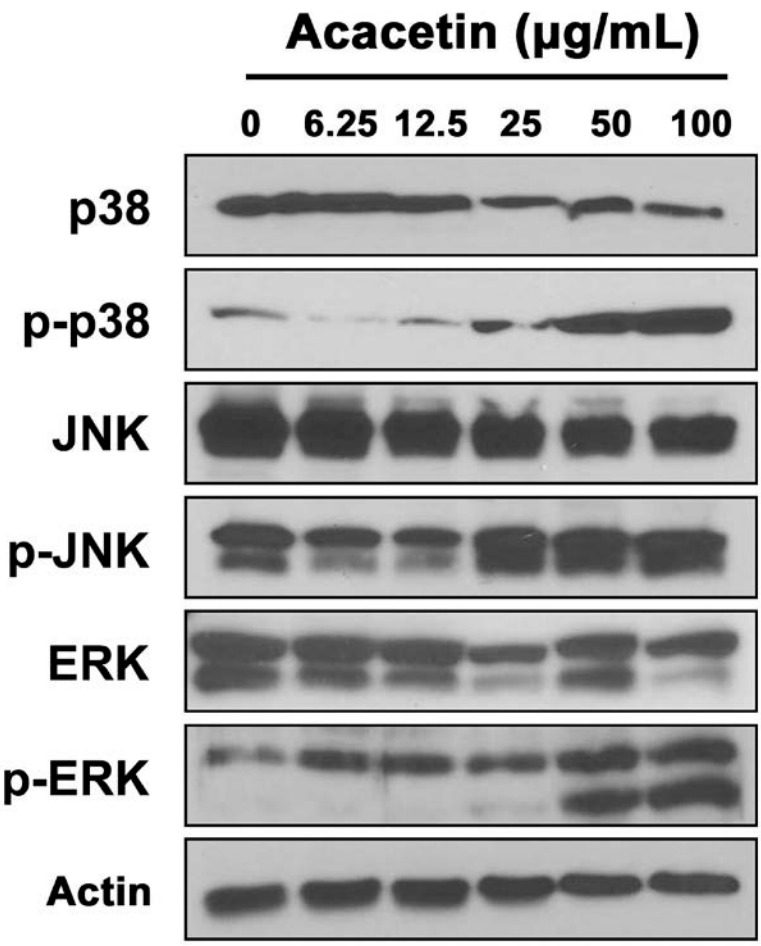


Fig. 11. The effect of acacetin on the phosphorylation of MAPKs in HSC-3 cells. The cells were exposed to indicate concentrations of acacetin for 6 h and examined by Western blot analysis. The results from three independent experiments were quantified through densitometry and the representative data is shown.

To determine if MAPK phosphorylation plays an important role in regulating acacetin-mediated apoptosis, we treated the HSC-3 cells with various concentrations of acacetin and MAPK-specific inhibitors. In the cells treated with 25μg/ml of acacetin for 24 hours, the cells stained positively for trypan blue, which was increased significantly by treating the cells with the MAPK inhibitor (Fig. 12). The most significant inhibition of acacetin-induced cytotoxicity was observed when the cells were treated with SB203580 or PD98059, rather than SP600125. This suggests that p38 and/or ERK are a key regulator in the acacetin-induced apoptosis of HSC-3 cells.

Fig. 12A

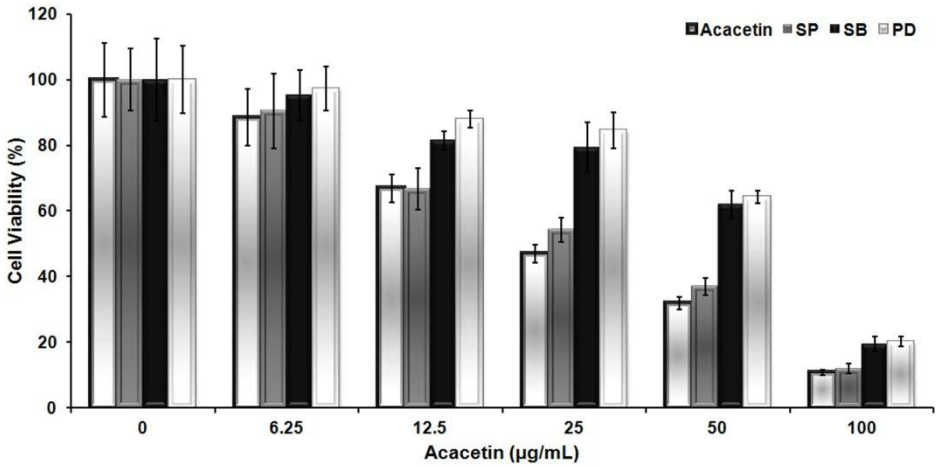


Fig. 12B

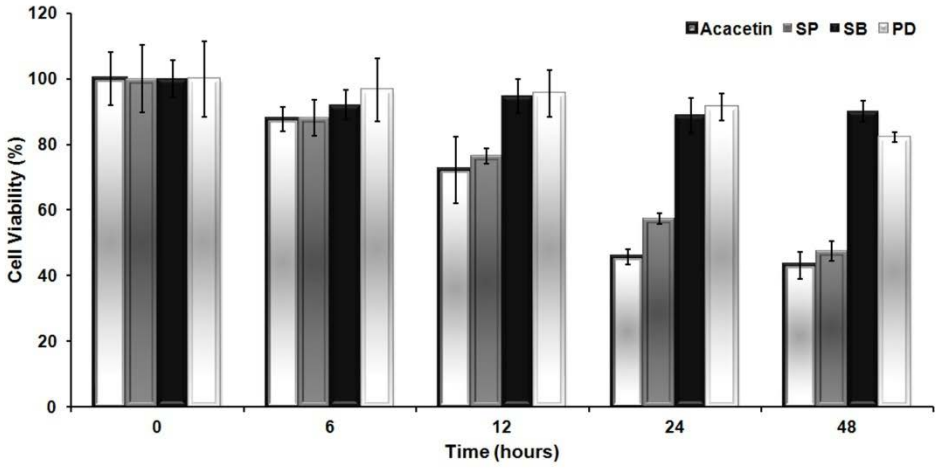


Fig. 12. The effect of MAPKs inhibitor on cell viability. The cells were exposed to several concentrations of acacetin for 24 h or 25μg/ml of acacetin for various times in the presence or absence of MAPK inhibitors (10 μM). The viability reducing activity of the cells were determined using an MTT assay, as described in Materials and methods. Each bar shows the mean ± SE of three separate experiments.

Consistent with the results of trypan blue staining, the induction of the PARP 85 kDa cleaved from the 116 kDa form original protein after the acacetin treatment and its suppression by the pretreatment with the MAPK inhibitor (PD98059) was also observed by Western blot analysis. In parallel with the results shown in Fig. 13, flow cytometric analysis showed that a pretreatment with the MAPK

inhibitors (SP600125, SB203580, PD98059) prevented the migration of the cells into the sub-G_1 phase of the cell cycles (Fig. 14). The results suggest that the MAPKs mediate an important signal that regulates apoptosis in acacetin-exposed cells.

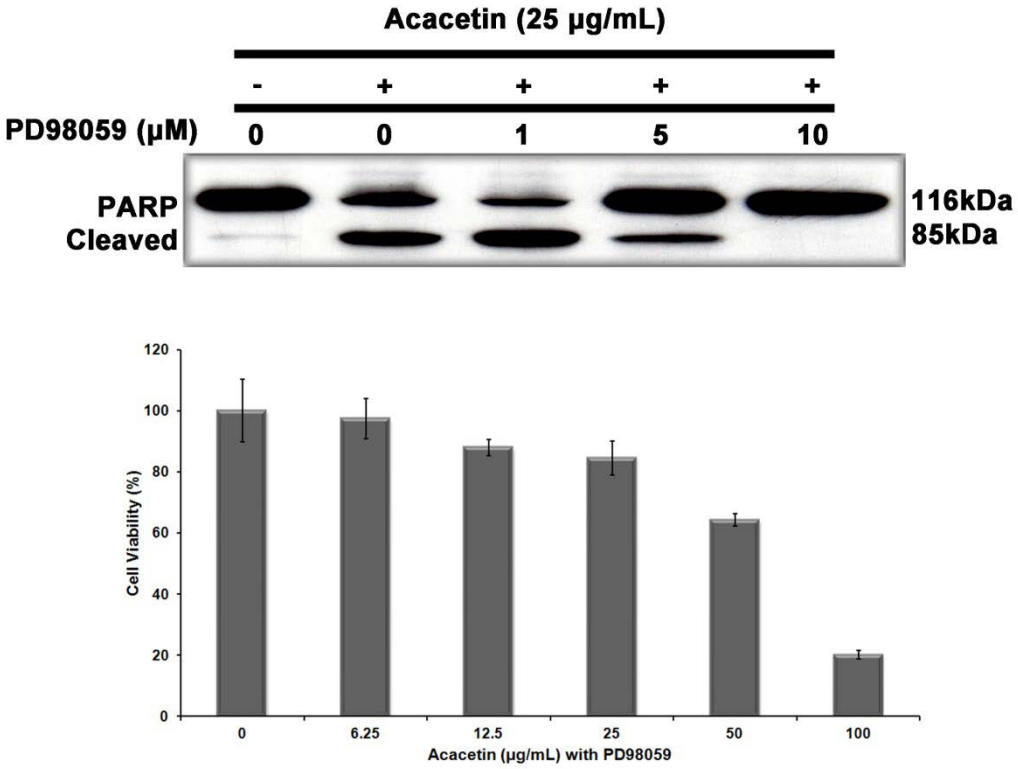


Fig. 13. The effect of the MAPKs inhibitor on the PARP cleavage in the acacetin-treated HSC-3 cells. The HSC-3 cells were pretreated with the indicated doses of each of the MAPK inhibitors for 1 h before adding 25μg/ml acacetin into the cultures for another 24 h. The cells were then processed for trypan blue staining and Western blot analysis to determine the cytotoxicity and PARP cleavage, respectively. Different superscripts represent the significant differences ($p < 0.05$) between the groups using the Scheffe's multiple range tests.

Fig. 14A

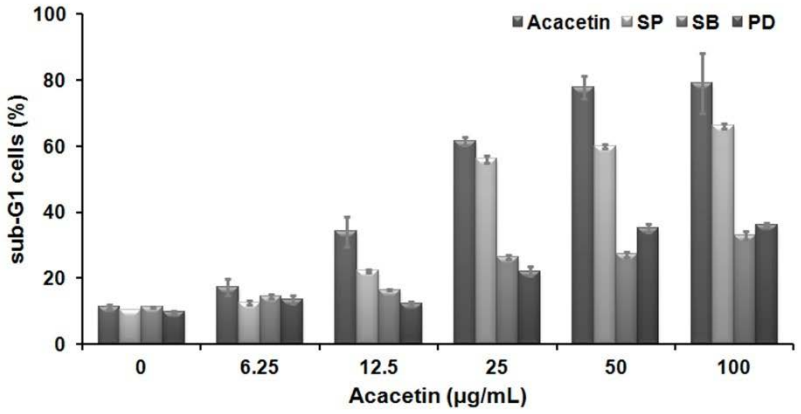


Fig. 14B

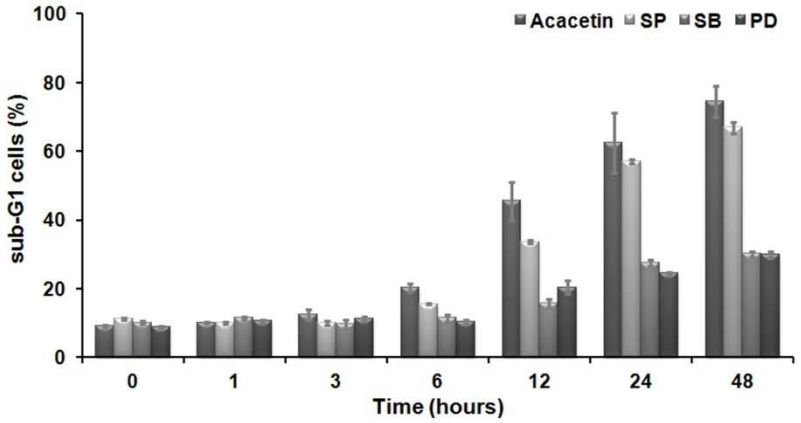


Fig. 14. The effect of MAPKs inhibitors on the cell cycle of acacetin-treated HSC-3cells. HSC-3 cells were pretreated with MAPK inhibitors (10 μM) for 1 h before adding the indicated dose of acacetin for 24 h (A) and/or 25 μg/ml acacetin for various times (B). The cells were stained with PI, and the cell numbers in the sub-G_1 phase, which means the apoptotic cells, were calculated using the WinMDI 2.8 program.

Mitochondrial stress is a key event in the acacetin-induced apoptosis mediated by MAPK pathways. The acacetin treatment increased the intensity of the bands corresponding to the Bax protein in the mitochondrial fractions but reduced the level of the Bcl-2 protein (Fig. 10). In addition, acacetin induced the release of cytochrome *c* from the mitochondria into the cytosol. However, the ERK-specific inhibitor prevented these acacetin-mediated mitochondrial changes (Fig. 15).

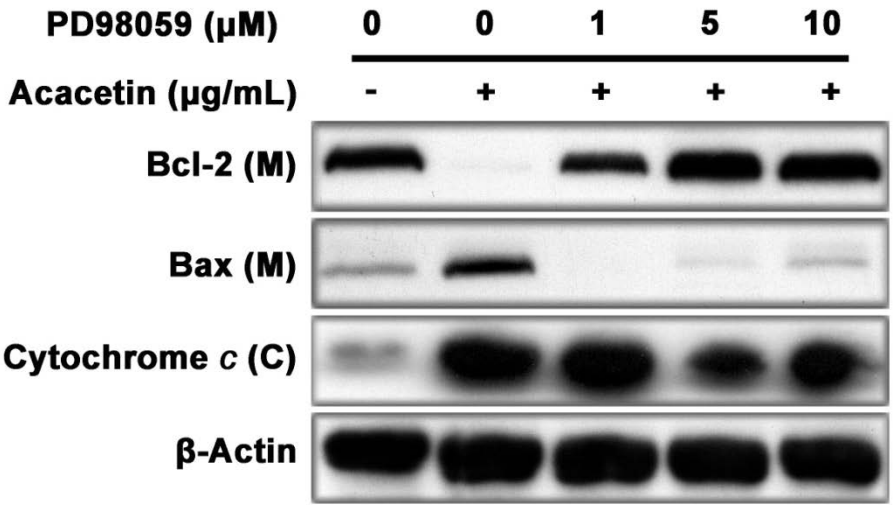


Fig. 15. The effect of MAPKs inhibitor on the involvement of mitochondrial stress in the acacetin-induced apoptosis of HSC-3 cells. The cells were pretreated with the indicated concentrations of PD98059 for 1 h before exposing the cells to 25 μg/ml acacetin for a further 24 h. Cell lysates were analyzed by immunoblotting to determine the level of mitochondrial apoptogenic factors. M and C represent the mitochondria and cytosolic fractions, respectively.

IV. Discussion

This author has confirmed that acacetin induced the apoptosis of the oral squamous cell carcinoma cell line (HSC-3) and the mechanism is involved in apoptosis.

In the present study, an MTT assay was used to determine if acacetin had any cytotoxic effect on HSC-3 cells. The result was that the acacetin significantly reduced the viability of HSC-3 cells in a dose-and time-dependent manner. Significantly, acacetin caused 50% growth inhibition (IC_{50}) of HSC-3 cells at 25μg/ml over 24 hours. Nucleic acid staining with Hoechst 33342 revealed typical apoptotic nuclei, with highly fluorescent condensed chromatin in cells treated with the acacetin 25μg/ml for 24 hours. DNA fragmentation also took place at a time- and dose-dependent manner. To evaluate whether the impact of acacetin on HSC-3 cell viability involves the process of cell apoptosis, HSC-3 cells were assessed by TUNEL assays. In the TUNEL results, acacetin treatment significantly increased the percentage of positively stained cells in a dose- and time-dependent manner. These results suggest that the acacetin inhibits the growth of the oral squamous cell carcinoma cell line (HSC-3) via the cell apoptosis process.

The acacetin-treated HSC-3 cells were investigated to determine the cell cycle observed during apoptosis. In human prostate cancer and non-small cell lung cancer A549 cells, acacetin blocks cell cycle progression in G1 and/or G2-M and induces apoptosis as a result of increased p53 and p21/WAF1 expression (Singh et al., 2005; Hsu et al., 2004). Acacetin also inhibits the growth of human breast cancer MCF-7 cells, and causes apoptotic DNA fragmentation and an increase in the sub-G1 population (Shim et al, 2007). The result of this study showed an increase in the sub-G1 phase and decrease in the S/M phase in a dose-and time-dependent manner. This result suggests that acacetin inhibits cell growth via blocking the cell cycle progression.

The caspase-3 activation plays a central role in several apoptotic mechanisms (Gianinazzi et al., 2003; Matsura et al., 1999; Zheng et al., 1998). Caspases are activated by a variety of apoptotic stimuli, and cell death proteases have been divided into upstream (initiator) and downstream (effector) caspases based on the sites of action in the proteolytic caspase cascade.

Two main caspase cascades have been described in mammalian cells, which are intrinsic and extrinsic pathways. The extrinsic pathway links caspase-8 to death receptors expressed at the cell surface including Fas, TNFR1 and DR3. In the intrinsic pathway, caspase-9 is activated by a variety of death stimuli from both outside and inside the cell (Ola et al., 2011). Active caspase-8 or caspase-9 can cleave and activate an overlapping set of effector caspases including caspase-3, -6, and -7, resulting in enhanced expression of protease activity in the cell. Therefore, caspase is known as the most important executor of apoptosis, and could be used to determine the degree of cell death through the activation of this enzyme (Takahashi et al., 1996; Faleiro et al., 1997; Li et al., 1997; Stennicke et al., 1998).

Therefore, this author demonstrated acacetin-induced activation of caspase-3, -8, and -9 and PARP in the oral squamous cell carcinoma cell line (HSC-3). In particular, approximately 50% and 100% degradation of procaspase-3 was observed when the cells were exposed to 12.5 and 25 μg/ml of acacetin for 24 hours, respectively. However, the acacetin-mediated degradation of procaspase-3 or procaspase-9 was more apparent than that of procaspase-8 in this study. Other study reported that cell growth inhibition and apoptotic induction of acacetin decreased in the presence of caspase-8

inhibitor treating in Hep G2 cells (Hsu et al., 2004). Another study demonstrated that the acacetin-induced caspase-7 activation involved the activation of caspase-8 and -9 in the MCF-7 cells (Shim et al., 2007). Compared with other studies, the activity of caspase-8 in this study was low, but the reason for the difference was not clarified in this study. PARP is a key signaling nuclear protein involved in triggering DNA repair. This enzyme can catalyze poly (ADP-ribose) ligation to an acceptor protein, including itself. During apoptosis, PARP is cleaved by the activation of caspase-3, resulting in DNA damage and apoptosis (Kaufmann et al., 1993). In this study, treatment with acacetin in HSC-3 cells resulted in the cleavage of PARP in a dose- and time- dependent manner. This author found that acacetin-induced apoptosis of HSC-3 cells was involved in caspase cascades

Apoptotic cell death is divided into the external pathway associated with the receptor and the internal pathway associated with the mitochondria. In the representative mechanism of apoptosis associated with mitochondria, the Bcl-2 inhibits apoptosis and Bax induces apoptosis in the Bcl-2 family (Adams & Cory, 1998).

Mitochondrial outer-membrane permeabilization is an important process in apoptosis and is under the control of the Bcl-2 family proteins (Jourdain et al, 2009). In particular, the release of cytochrome c into the cytosol is considered an important initiative step in the apoptotic process, which is tightly regulated by the equilibrium between the anti-apoptotic Bcl-2 and pro-apoptotic Bad and Bax (Gogvadze et al., 2006). In this study, the level of Bax protein in the mitochondrial fractions was increased and the level of Bcl-2 protein was reduced after acacetin treatment of the HSC-3 cells. Moreover, the amount of cytosolic cytochrome *c* was increased in the cells treated with 25μg/ml of acacetin for 24 hours. Therefore, mitochondrial stress might play a role in the acacetin-mediated apoptosis of HSC-3 cells.

The family of MAPKs plays a central role in the signaling pathway of cell proliferation, survival, and apoptosis, including extracellular-regulated kinase 1/2(Erk 1/2), stress-activated protein kinase/c-Jun NH4-terminal kinase (SAPK/JNK1/2) and p38 MAPK (Dhillon et al., 2007). The extracellular signal–regulated kinases (ERKs) control cell division, and inhibitors of these enzymes are being explored as anti cancer agents. The c-Jun amino-terminal kinases (JNKs) are critical regulators of

transcription, and JNK inhibitors may be effective in the control of rheumatoid arthritis. The p38 MAPKs are activated by inflammatory cytokines and environmental stresses and may contribute to diseases like asthma and autoimmunity (Johnson et al., 2002).

Acacetin has been shown to inhibit lipo-polysaccharide (LPS)-induced activation of phosphatidylinositol 3-kinase (PI3K/Akt) and Erk 1/2, but not p38 MAPK in murine macrophage Raw 264.7 cells (Pan et al., 2006). Another study reported that acacetin treatment resulted in the activation of SAPK/JNK1/2 and c-Jun, and a slight decrease on Erk1/2 and p38 MAPK activation in MCF-7 cells (Shim et al., 2007). Others demonstrated that acacetin inhibits the invasion and migration of human non-small cell lung cancer A549 cells and human prostate cancer DU145 cells by suppressing the p38 MAPK signaling pathway (Chien et al., 2011; Shen et al., 2010). This author found that acacetin treatment resulted in the activation of Erk and p38 MAPK, and a relatively decreased JNK activation. In addition, when MAPKs inhibitors were treated on the HSC-3 cells, acacetin-induced apoptosis including cell growth inhibition, increased Sub-G1 cells, PARP cleavage and mitochondrial potential change were prevented. These results suggest that acacetin has a close relation to its ability to activate the MAPKs-mediated signaling pathways with the subsequent induction of a mitochondria- and caspase-dependent mechanism.

In summary, this author investigated the mechanisms involved in acacetin-induced apoptosis of the oral squamous cell carcinoma cell line (HSC-3). This study showed that acacetin-induced apoptosis involves the activation of mainly caspase-3 and caspase-9. This study also demonstrated that acacetin induced loss of mitochondrial membrane potential to cause the release of cytochrome c from mitochondria. Acacetin induced activation of the Erk and p38 MAPK signaling pathways.

CONCLUSION

This study confirmed that acacetin induces the apoptosis of the oral squamous cell carcinoma cell line (HSC-3) and the apoptosis is involved in caspase signal transduction and mitochondrial stress, and this process in regulated by MAPKs (ERK/p38). These results revealed the anti-cancer effect of acacetin in the oral squamous cell carcinoma cell line, which may act as a promising therapeutic agent for the treatment of oral squamous cell carcinoma. In the future, acacetin could be further examined to justify its effectiveness in the prevention of oral squamous cell carcinoma.

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ABSTRACT (IN KOREAN)

Acacetin이 구강편평상피세포암종의 세포사멸에 미치는 영향과 기전

<지도교수 차 인 호>

연세대학교 대학원 치의학과

김 채 두

Acacetin (5,7-dihydroxy-40-methoxyflavone)은 엉겅퀴, 홍화씨, 아카시아 등 여러 식물에 존재하며 apoptosis를 유도하고 세포주기의 진행을 차단 함으로써 anti-peroxidative, anti-inflammatory, anti-plasmodial 및 anti-proliferative 효과를 나타내는 것으로 보고 되었다.

본 연구의 목적은 acacetin이 구강편평상피암 세포주(HSC-3)의 세포사멸에 미치는 영향과 그 기전에 대해 알아보고자 함이다. MTT assay에서 25 μg/ml의 acacetin을 24시간 동안 처리하였을 때 세포주의 50%이상의 성장 억제를 보였다. 세포사멸은 DNA 분절과, Sub-G1 cells의 증가, caspase-3와 PARP의 발현을 통해 세포사멸을 확인할 수 있었다. 100 μg/ml의 acacetin을 24시간 동안 처리하였을 때 caspase3의 최대 활성을 확인하였고, caspasc-3의 활성을 매개하는 caspase-8과 caspase-9의 활성을 확인하였다. Acacetin은 Bcl-2의 발현을 감소시킴에 따라 Bax와 Bcl-2의 비율을 증가시켰으며, 이는 mitochondrial membrane potential의 손실을 일으켜 세포질 내로 cytochrome c의 방출을 유도하였다. Caspase-3, (Z-DEVD-FMK), -8 (Z-IETD-FMK), and 9 inhibitor (z-LEHD-fmk) 를 처리하였을 때 mitochondrial membrane potential의 손실과 세포질 내로 cytochrome c의 방출을 억제하였다. Acacetin에 의해 The mitogen-activated protein kinases (MAPKs)의 활성이 발생하였으며, MAPKs의 inhibitor를 처리하였을 때 acacetin으로 유도되는 구강암

세포주의 세포독성이 억제 되었다. 또한 MAPKs inhibitor 처리시 acacetin으로 유도되는 PARP의 분절, sub-G1기 세포수의 증가, Bax와 Bcl-2 비율의 증가, cytochrome c의 세포질 내로의 방출 등이 억제 되었다. 결과적으로 acacetin의 구강편평상피암 세포주의 세포사멸을 유도하며, acacetin 에 의한 세포사멸은 caspase 활성 경로와 mitochondria 에 매개 되는 apoptosis 신호 전달 과정과 활성화된 MAPKs의 신호전달과정이 관여되어 발생함을 보여주었다. 이러한 acacetin의 구강암 세포주의 성장 억제 효과 및 기전의 연구 결과는 향후 구강편평상피암의 치료제로서의 가능성을 가지며, 이러한 효과를 명확히 규명하기 위한 연구들의 진행에 도움이 될 것이다.