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박사학위논문

사람 인두 편평암세포에서 무화과 나무 latex의 caspase와 Bcl-2 family 의존적 세포사멸 유도

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ABSTRACT

Latex of *Ficus carica* L. induces apoptosis through caspase and Bcl-2 family in FaDu human hypopharynx squamous carcinoma cells.

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Ficus carica L. (common Fig), which is one of the first plants cultivated by humans, originated in the Mediterranean basin and grows currently worldwide including southwest Asia and South Korea. This plant have been used as traditional medicine for treatment disease as hemorrhoids, metabolic, cardiovascular, respiratory, and skin infections. Its pharmacological properties have recently been studied in detail, but research on anti-cancer effect of latex has been limitedly studied on several cell lines, prostate cancer, breast cancer, and leukemia. In this study, we investigated the anti-cancer activity of latex and the underlying mechanism by latex on FaDu cells. We confirmed through SDS-PAGE analysis that latex of *Ficus carica* contains cysteine protease ficin. Our data showed that latex inhibited cell growth in a dose-dependent manner. In addition, latex treatment markedly induced apoptosis in FaDu cells as determined by FACS analysis. Latex also elevated expression level of cleaved caspase-9, -3 and PARP (poly(ADP-ribose) polymerase). Furthermore, latex increased the expression of Bax (pro-apoptotic factor) whereas decreased the expression of Bcl-2 (anti-apoptotic factor). Taken together, these results suggested that latex containing the ficin inhibited the cell growth and induced apoptosis by caspase and Bcl-2 family signaling pathway in FaDu cells. Therefore, latex can be provided as an novel chemotherapeutic drugs due to its growth inhibition effects and induction of apoptosis in human oral cancer cells.

I. Introduction

Oral squamous cell carcinoma (OSCC) is the representative form of oral cancer with a high potential for local invasion and lymph node metastasis (1). The development of OSCC are closely related with accumulation of genetic alterations induced by chronic exposure to carcinogens such as tabacco and alcohol (1,2). OSCC patient account for ~3% of the newly diagnosed cancer cases per year, but the five-year survival rate has not significantly changed during the past 30 years, still below 50% (3,4). Current OSCC patients receive care from advanced therapies, such as surgery, radiotherapy, and chemotherapy, but many side effects have been reported (5,6). Therefore, interest in natural products that have relatively few side effects and have pharmacological effect is increasing. As a representative example, Taxol® (paclitaxel), which was extracted and separated from Taxus brevifolia L, currently being used to treat cancer patients in clinical practice (7).

Ficus carica L. (common Fig, family Moraceae), which is one of the first plants cultivated by humans, originated in the Mediterranean basin and grows currently worldwide including southwest Asia and South Korea (8,9). Traditionally, leaves, fruits, and roots of Fig have been used for treatment as hemorrhoids, metabolic, cardiovascular, respiratory, and anti-inflammatory remedy (10,11). In addition, Fig latex has been used to treat skin infections such as warts and viruses (12,13). Despite the fact that Fig latex has been used in ancient traditional medicine, its pharmacological properties have recently been studied in detail. Latex released when immature fruits or leaves of Fig are harvested, and it contains many proteases including ficin, which is cysteine protease (14). However, why latex contains high amounts of such protease still remain enigmatic and, according to several reports, they participate as a defense mechanisms to protect yourself against plant pathogens like fungi and insects (15,16). A few recent studies have shown Fig latex exhibits antioxidant, antibacterial, anti-Herpes Simplex Virus (HSV), and anthelmintic activities (17-21). Furthermore, Rubnov et al, reported that a mixture of 6-O-acyl-β-d-glucosyl-β-sitosterols has been isolated from Fig has inhibitory effects on proliferation of various cancer cell lines, lymphoma, leukemia, prostate

cancer, and breast cancer (22). However, research on anti-cancer effect of latex has been limitedly studied on several cell lines, and the mechanism is not well-known.

Therefore, to aid understanding of the latex anti-cancer activity, we investigated the effect of latex on human FaDu hypopharynx squamous carcinoma cells.

II. Materials and Methods

1. Preparation of latex from *Ficus carica* L

Fresh latex was collected from the fig (*Ficus carcia*) tree growing in private orchard in Hwasun-gun (Korea) by separation of immature green fruit from tree shoots in July. The collected latex fluid was lyophilized, and dried powder was dissolved in distilled water (100 mg/mL), and the solution filtered through a 0.22-μm syringe filter. The extract was stored at −20°C until use.

2. Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and coomassie brilliant blue R-250 were purchased from Sigma - Aldrich (St. Louis, MO, USA). Primary and secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), except anti-β-actin (AB Frontier, Seoul, Korea). Minimum essential medium Eagle (MEM) and a penicillin/streptomycin solution were purchased from WelGene (Daegu, Korea). Fetal bovine serum (FBS) was purchased from Corning (Corning, NY, USA). PE-Annexin V Apoptosis Detection Kit was purchased from BD Bioscience (San Diego, CA, USA).

3. Cell culture

Human hypopharynx squamous carcinoma FaDu cells were purchased from American Type Culture Collection (ATCC, Rockville, USA). The cell were cultured in MEM medium containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified 5% CO_2 incubator at 37°C.

4. SDS-PAGE

The total protein concentration in latex samples was determined using the BCA protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as a standard. Protein of latex (10 and 20 ug) were mixed five times concentrated reducing sample buffer (Elpis Biotech, Daejeon, Korea) and heated at 95 °C for 10 min. Prepared protein were separated with 10% SDS-PAGE gel

using Mini-protean III cell (Bio-Rad Laboratories, Hercules, CA, USA) at 100 V. The gels were stained with coomassie Brilliant Blue R-250 for 30 min. The molecular mass of the protein band was estimated by comparison with standard molecular weight markers (6-240 kDa).

5. Cytotoxicity

Cytotoxicity was determined by measuring formazan generated by mitochondrial-dependent redox reaction of viable cells using MTT solution (5 mg/mL). Cells were seeded at a density 2 × 10^5 cells/mL in 12-well plates, incubated for 15h, and then treated with latex (0, 5, 10, 25, 50, and 100 μg/mL) for 24 h. After 24 h. MTT assay was performed, generated formazan was dissolved in DMSO, and then measured absorbance at 590 nm in a microplate reader (Epoch; BioTek Instruments, Winooski, VT, USA). The results was expressed as the cell viability rate by setting the absorbance of untreated control cells to 100% and the latex-treated cells was calculated as the surviving percentages. The percentage of cell viability was calculated using the following formula:

$$Cell\,viability\,(\%) = \frac{Means\,Abs.\,of\,the\,sample - Means\,Abs.\,of\,the\,blank}{Means\,Abs.\,of\,the\,control - Means\,Abs.\,of\,the\,control} X100$$

6. Flow cytometry analysis

FaDu cells were treated with latex (0, 10, 25, and 50 μg/mL) for 24 h, after which they were harvested using 0.25% trypsin and washed twice with pre-chilled phosphate buffered saline (PBS). The cell pellet were re-suspended in a 300 μL binding buffer, and then stained with Annexin V and 7-AAD (BD Biosciences) for 10 min at room temperature. Subsequently, the suspension was analyzed using a FACScalibur (BD Biosciences). The quantitatively data was expressed as density plots using BD Cell quest pro software (BD Biosciences). Non-stained cells were viable cell, and cells stained with Annexin V only were considered as early apoptosis cells, and those that stained for both Annexin V and 7-AAD were considered as late apoptosis. Also, cells stained with 7-AAD

only were considered as necrosis cells.

7. Western blot analysis

FaDu cells were treated with latex (0, 10, 25, and 50 μg/mL) for 24 h and cells were lysed with protein extraction reagent (iNtRON Biotechnology, Seongnam, Korea) for 20 min on ice, and then centrifuge at 12,000 rpm for 15 min at 4°C. The supernatant was quantified for protein concentration using the BCA protein assay (Pierce, Rockford, IL, USA) method. Denatured protein (20 μg/lane) were separated with 8% or 15% SDS-PAGE gels and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). After blocking with 5% BSA for 1 h, membranes were incubated with the specific primary antibodies, Bax (1:1000), Bcl-2 (1:1000), Cleaved caspase-3 (1:500), Cleaved capsase-9 (1:500), PARP (1:1000) and β-actin (1:2000), at 4°C overnight. The membranes were washed with TBST containing 0.1% (v/v) Tween-20 for 30 min, followed by incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Protein were detected by Immobilon Western Chemiluminescent HRP Substrate (ECL, Millipore, Bedford, MA, USA) and visualized on a MicroChemi 4.2 device (DNR Bioimaging Systems, Jerusalem, Israel). The density of each band was quantified using Image J software and expression levels of proteins were quantitatively analyzed through comparisons with β-actin as internal control, respectively.

8. Statistical analysis

All data are expressed as the means ± standard deviation (SD). All data were derived from at least three independent experiments. Statistical significance was determined using One-way ANOVA followed by Turkey's analyses in GraphPad Prism 5 (GraphPad Software Inc., CA, USA). Statistical significance was set to $* p < 0.05$.

Ⅲ. Results

1. Electrophoresis profiles of latex samples

To analysis protein composition in our crude latex sample, SDS-PAGE electrophoresis was performed. The results are shown in Fig. 1. SDS-PAGE experiments detected two major protein bands at around 28 kDa and 25 kDa as well as several protein/peptide bands of molecular weight below 17 kDa (Fig. 1). A protein band at around 28 kDa exhibited cysteine protease ficin, while low molecular weight proteins, far below the 28 kDa characterizing native ficin, explained by some proteolysis/autolysis occurs upon latex collection.

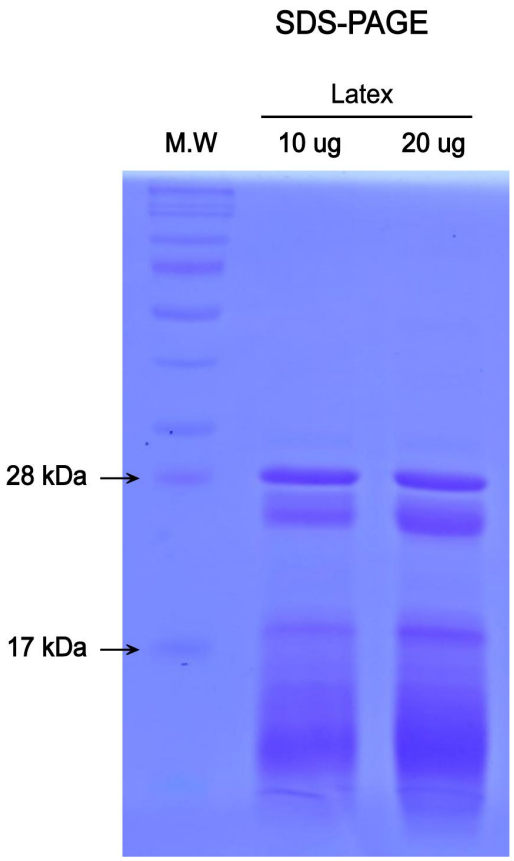

Figure 1. SDS-PAGE analysis of latex proteins. Samples of latex collected from Ficus carica L and kept frozen at -20°C before use. M.W., molecular mass standards.

2. Effect of Latex on viability of FaDu cells

To evaluate the effect of latex on viability of FaDu cells, FaDu cells were incubated with varying concentration of latex (0, 5, 10, 25, 50, and 100 μg/mL) for 24 h. Cell viability was determined using MTT assay. As shown in Fig. 2, from 10 μg/mL of latex, the FaDu cell viability began to decrease and cell viability rate for each concentration was 90%, 54%, 38% and 29% at 10, 25, 50 and 100 μg/mL, respectively. The 50% inhibitory concentration of the cell viability (IC50) was at 25 μg/mL at 24 h.

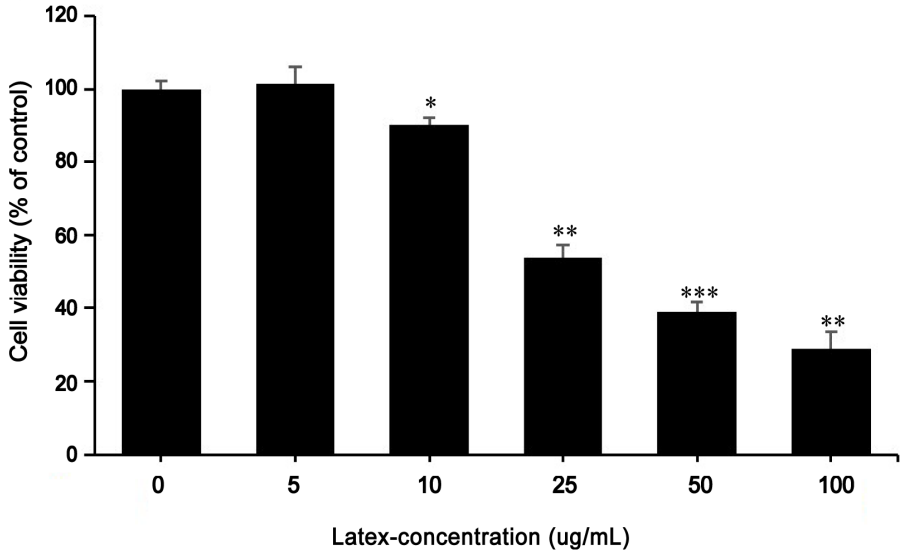

Figure 2. Effect of latex on FaDu cell viability. Cells were treated with various concentration of latex (0, 5, 10, 25, 50, and 100 μg/mL) for 24 h. Cell viability was determined using MTT assay. Results were expressed as a percentage of the control and expressed as means ± SD of three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001 compared with the control group.

3. Induction of apoptosis by latex in FaDu cells

To understand the mechanisms of inhibition of cell growth by latex, FACS cytometry was performed using Annexin V/7-AAD to assess apoptosis. The FaDu cells were treated with 10, 25, and 50 μg/mL latex for 24 h, after which they were double stained with Annexin V-PE and 7-AAD. As shown in Fig. 3, the early apoptosis rates were 3.47%, 11.02%, and 35.02% at 10, 25, and 50 μg/mL latex, respectively, and at late apoptosis rate in each group were 0.49%, 2.88%, and 9.87%, respectively. The population of total apoptotic cells was increased to 3.86%, 13.9%, and 44.89% at 10, 25, and 50 μg/mL latex, respectively, compared with control (1.83%). These results suggest that suppression of cell growth by latex was related in apoptosis in FaDu cells.

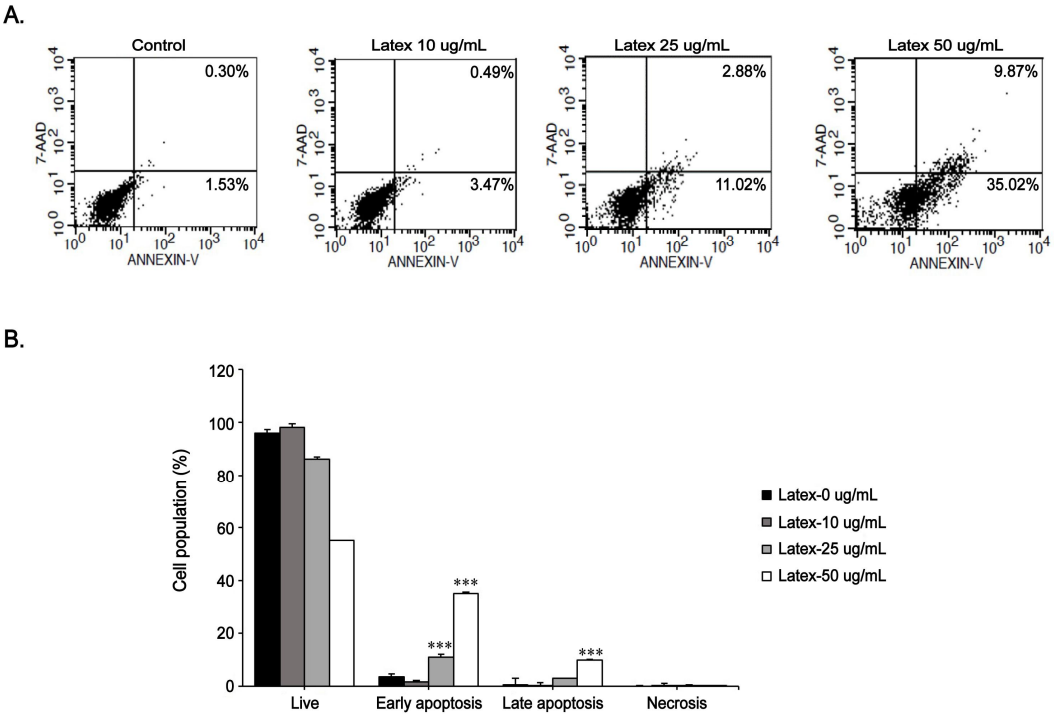

Figure 3. Induction of apoptosis by latex in FaDu cells. Cells were treated with various concentrations of latex (0, 10, 25, and 50 μg/mL) for 24 h. (A) Annexin-V/7-AAD double-staining showed the percentage of apoptotic cells after latex treatment. The proportion of cells in each quadrant are marked on the figures. The percentage of each sections are shown in (B). Data are expressed as means ± SD of three independent experiments. ***p < 0.001 compared with the control group.

4. Activation of caspase by latex in FaDu cells

Because most apoptosis was induced by the serial activation of caspase, we evaluated whether latex-induced apoptosis was related caspase activation by western blot analysis. FaDu cells were treated with latex as previously indicated, and then lysed. Figure 4A and B shows that cleaved caspase-9 and -3 were notably increased by latex in dose-dependent manner, and as a result, cleavage of PARP, which downstream apoptotic indicator, significantly increased. Bax, as pro-apoptosis protein, and Bcl-2, as anti-apoptosis protein, play critical roles in regulating intrinsic apoptosis. In Figure 4C,D, and E, the latex significantly increased the Bax protein, while decreased the Bcl-2 protein in a dose-dependent manner. These results corroborated FACS cytometry analysis that the latex was increased apoptosis in FaDu cells. Collectively, the results suggest that latex-induced apoptosis can be induced by mitochondria-caspase-dependent apoptotic signaling in FaDu cells.

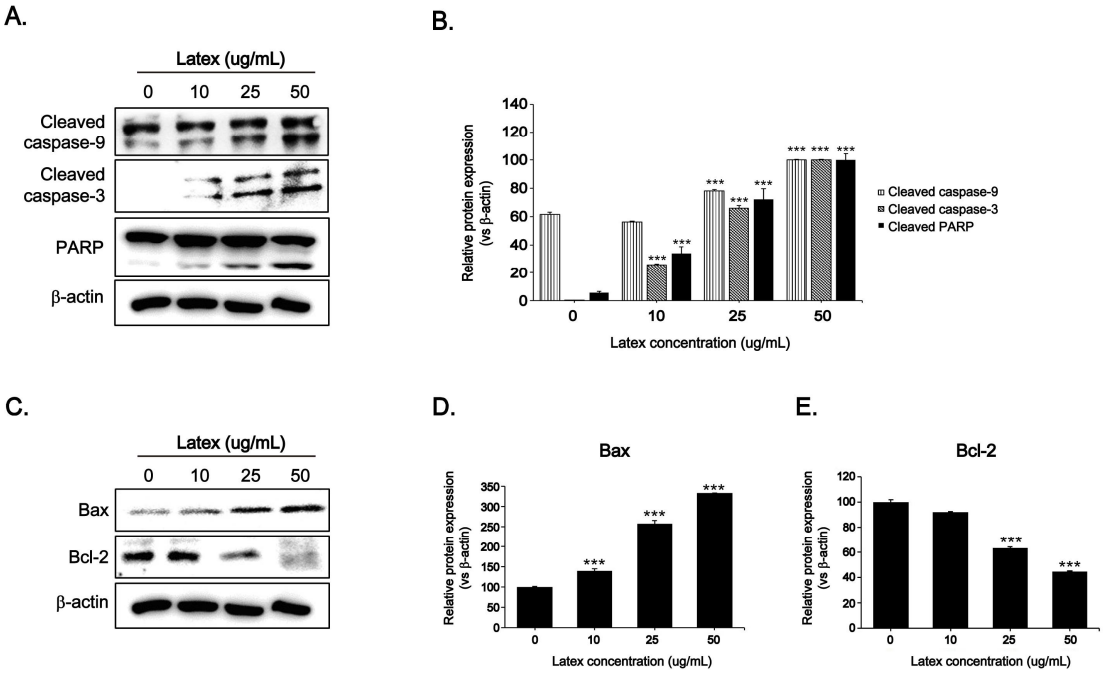

Figure 4. The expression levels of apoptosis-related proteins in FaDu cells treated with latex. Cells were treated with various concentrations of latex (0, 10, 25, and 50 μg/mL) for 24 h. (A-E) After latex treatment, the expression of apoptotic-related protein (cleaved caspase-9, cleaved caspase-3, PARP, Bax, and Bcl-2) was assessed by western blotting, and β-actin was used as the loading control. Data are expressed as means ± SD of three independent experiments. ***$p < 0.001$ compared with the control group.

Ⅳ. Discussion

Ficus carica has been used in traditionally medicine as hemorrhoids, metabolic, cardiovascular, respiratory, and anti-inflammatory remedy (10,11). A few recent studies have shown Fig latex has many bioactivities such as antioxidant, antibacterial, anti-Herpes Simplex Virus (HSV), and anthelmintic activities (17-21). However, research on the anti-cancer effect of latex is limited to several cancers such as glioblastoma multiforme, lymphoma, leukemia, prostate cancer, breast cancer, and stomach cancer cells, and the mechanism is not known (21-23). Therefore, this study was investigated the effect of latex in FaDu human oral squamous cancer cells in order to broaden the understanding of anti-cancer effect of latex.

First, we performed the SDS-PAGE analysis to determine the protein composition in our crude latex sample. SDS-PAGE experiments showed that it contains the cysteine protease ficin, which has 28 kDa molecular weight. Recently, Raskovic et al. reported that ficin included in Fig latex was a novel protease with gelatinolytic activity (24). Gelatinases such as MMP2 and MMP9 are highly expressed in various cancer cells, and these helps cancer cells penetration the surrounding tissues cancer cells (25). Therefore, a good anti-cancer effect can be expected for latex having gelatinolytic activity, which suppresses the expression of these. The scientific research on cytotoxicity of Fig latex was firstly performed by Ullman et al. in the 1940s (26). Ullman et al. revealed that small doses of Fig latex was inhibited the growth of the tumor in mice bearing a benzpyrene-induced sarcoma and even disappearance of small tumors, but high doses was lethal in albino rats (26). Recently, Khodarahmi et al. reported that crude Fig latex exhibited cytotoxicity at 10 μg/mL in 48 h, and IC50 was at 17 μg/mL in HeLa cervical cancer cells (27). In addition, in stomach cancer cells, IC50 of Fig latex was at 5 mg/ml (23). Our results showed that crude Fig latex exhibited cytotoxicity at 10 μg/mL in 24 h, and IC50 was at 25 μg/mL in FaDu cells; suggested that inhibition of growth of cancer cells by Fig latex is more effective in FaDu cells than those in HeLa cells and stomach cells. The molecular mechanism of the action of Fig latex on

cell viability was investigated using Annexin-V/7-AAD assays to assess apoptosis. FACS results showed that inhibition of growth of cancer cells by latex is due to cell apoptosis. As a consistent with our data, Tezcan et al proved through the FACS analysis that 0.25 mg/ml Ficus carica latex (FCL) induced apoptosis in GBM cell lines, T98G, U-138MG and U-87MG (21). Therefore, we evaluated the caspase activity using western blotting to verify the cell apoptosis by latex. The caspase family consists of cysteine proteases that are indispensable in the process of apoptosis (28). Caspases-3 is a main executing factor in process of apoptosis and caspase-9 is activated in the mitochondria-mediated intrinsic apoptosis pathway (28). Our results showed that cleaved caspase-3 and -9 significantly increased in dose-dependent manner and the cleavage of native PARP (116 kDa) into its small fragment PARP (89 kDa) increased accordingly. In addition, the Bcl-2 family is one of the best studied gene for apoptosis (29). Among of the Bcl-2 family, Bax is apoptosis-promoting protein and Bcl-2 is anti-apoptotic protein (29). In our experiment, anti-apoptotic Bcl-2 protein levels decreased and anti-survival Bax protein levels increased by Fig latex in FaDu cells. Collectively, these results suggest that apoptosis of FaDu cells by Fig latex may be mediated by the activation of caspase and Bcl-2 family signaling pathways.

In conclusion, our study suggest that the anti-cancer effect of Fig latex inhibits the cell growth and induced cell apoptosis in FaDu cells through extrinsic death receptor and intrinsic mitochondrial-dependent apoptotic signaling pathway. Nevertheless a more detailed study of the apoptosis mechanism by Fig latex remain to be researched further.

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