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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), one of the first plants cultivated by humans, originated in the Mediterranean basin and currently grows worldwide, including southwest Asia and South Korea. It has been used as a traditional medicine for treatment of metabolic, cardiovascular, and respiratory diseases as well as hemorrhoids and skin infections. Its pharmacological properties have recently been studied in detail, but research on the anti-cancer effect of its latex has been only been studied on a limited basis on several cell lines, such prostate cancer, breast cancer, and leukemia. In this study, we investigated the anti-cancer activity of the latex of *Ficus carica L.*and its underlying mechanism in FaDu human hypopharynx squamous carcinoma cells. (*See Ed. note above*) We confirmed through SDS-PAGE analysis and gelatinolytic activity analysis that the latex of *Ficus carica* contains cysteine protease ficin. Our data showed that the latex inhibited cell growth in a dose-dependent manner. In addition, the latex treatment markedly induced apoptosis in FaDu cells as determined by FACS analysis, elevated expression level of cleaved caspase-9, -3 and PARP (poly (ADP-ribose) polymerase), and. increased the expression of Bax (pro-apoptotic factor) while decreasing the expression of Bcl-2 (anti-apoptotic factor). Taken together, these results suggested that latex containing the ficin inhibited cell growth and induced apoptosis by caspase and the Bcl-2 family signaling pathway in FaDu human hypopharynx squamous carcinoma cells. These findings point to the potential of latex of *Ficus carica* to provide a novel chemotherapeutic drug due to its growth inhibition effects and induction of apoptosis in human oral cancer cells.**

Key words: *Ficus carica* L. Latex, FaDu human hypopharynx squamous cancer cells, Apoptosis

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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 tobacco 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, such as gastrointestinal complications, impaired immune function and decreased bone marrow function, have been reported [5,6,7]. 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 [8].

Ficus carica L. (common fig or 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 [9,10]. Traditionally, leaves, fruits, and roots of fig have been used for treatment as hemorrhoids, metabolic disorders, cardiovascular diseases, respiratory (sore throats, cough, and bronchial problems) disorders, and anti-inflammatory remedy [11,12]. In addition, fig latex has been used to treat skin infections such as warts and viruses [13,14]. 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 [15]. 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 [16,17]. A few recent studies have shown fig latex exhibits antioxidant, antibacterial, anti-Herpes Simplex Virus (HSV), and anthelmintic activities [18-22]. 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 [23]. 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 FaDu human hypopharynx squamous carcinoma cells.

Materials and Methods

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.

Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), coomassie brilliant blue R-250, IAA (Iodoacetic acid), PMSF (Phenylmethanesulfonyl fluoride), EDTA (Ethylenediaminetetraacetic acid), and DTT (Dithiothreitol) 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).

Cell culture

Human hypopharynx squamous carcinoma FaDu cells were purchased from American Type Culture Collection (ATCC® HTB-43™, 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.

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).

Gelatinolytic activity

The gelatinolytic activity was measured using the ninhydrin method as described by Raskovic et al. [24]. The IAA (cystein protease inhibitor), PMSF (serine protease inhibitor), EDTA (metalloprotease inhibitor), DTT (cystein protease inhibitor and reducing agents) at the concentration of 2 mM were pre-incubated with latex sample for 1 h at room temperature (25°C). To measurement of gelatinolytic activity, 100 μL of gelatin (5 mg/mL) dissolved in 50 mM Tris-Hcl (pH 8.1) was mixed with 10 μL of sample or inhibitor mixture and incubated at 37°C for 5 h. After addition of 110 μL of 20% (w/v) PEG 6000 solution, reaction mixture was incubated for 1 h at 4°C. The solution was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant (20 μL) was mixed with 200 μL of 5% (w/v) ninhydrin solution, incubated at 100°C for 10 min. Absorbance was measured at 570 nm, and expressed as percentage (%) of maximal activity.

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 15 h, 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:

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

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.

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.

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 Tukey's analyses in GraphPad Prism 5 (GraphPad Software Inc., CA, USA). Statistical significance was set to * $p < 0.05$.

Results

Electrophoresis profiles and gelatinolytic activity of latex samples.

To analysis protein composition in our crude latex sample, SDS-PAGE electrophoresis was performed. The results are shown in Fig. 1A. 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. 1A). Many papers have been reported that fig latex has proteolytic activity, so we analyzed the gelatinolytic activity of latex using ninhydrin method. As the result, in Fig. 1B, the gelatinolytic activity of latex pretreated with the IAA or boiling completely disappeared, whereas that of the latex only was 100%. However, there was no change in activity by other protease inhibitors; suggesting that this activity was associated essentially with cysteine protease.

Effect of Latex on viability of FaDu cells.

To evaluate the effect of latex on viability of FaDu cells, FaDu cells were treated 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 (IC_{50}) was at 25 μg/mL in 24 h.

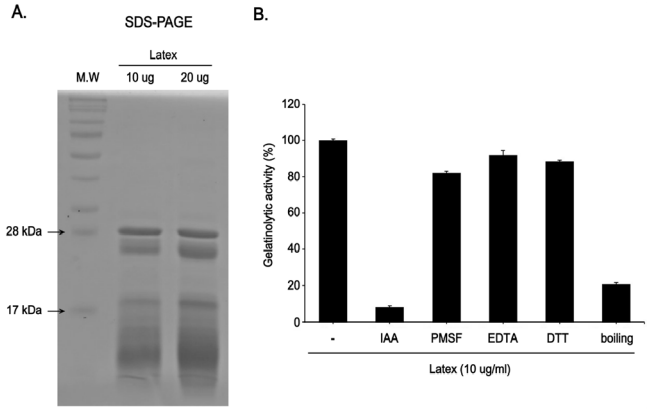

Fig. 1. SDS-PAGE analysis and gelatinolytic activity of latex proteins. (A) M.W, Gendepot molecular weight standards. Samples of latex collected from *Ficus carica* L. and kept frozen at -20°C before use. (B) gelatinolytic activity of latex samples was measured by ninhydrin methods (described by "Material and Methods" part). After latex pretreatment with selected inhibitor for 1 h, residual activity was determined as the percentage of the enzymatic activity in latex only sample.

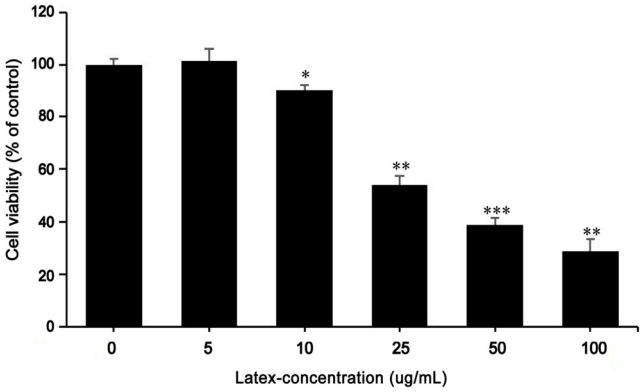

Fig. 2. Effect of latex on FaDu cell viability. Cells were treated with various concentration of latex (0, 5, 10, 25, 50, and 100 μg/mL) for 24 h. Cell viability was determined using MTT assay. Results were expressed as a percentage of the control. Data are expressed as means ± SD of three independent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

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.

Activation of caspase and Bcl-2 family 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. Fig. 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

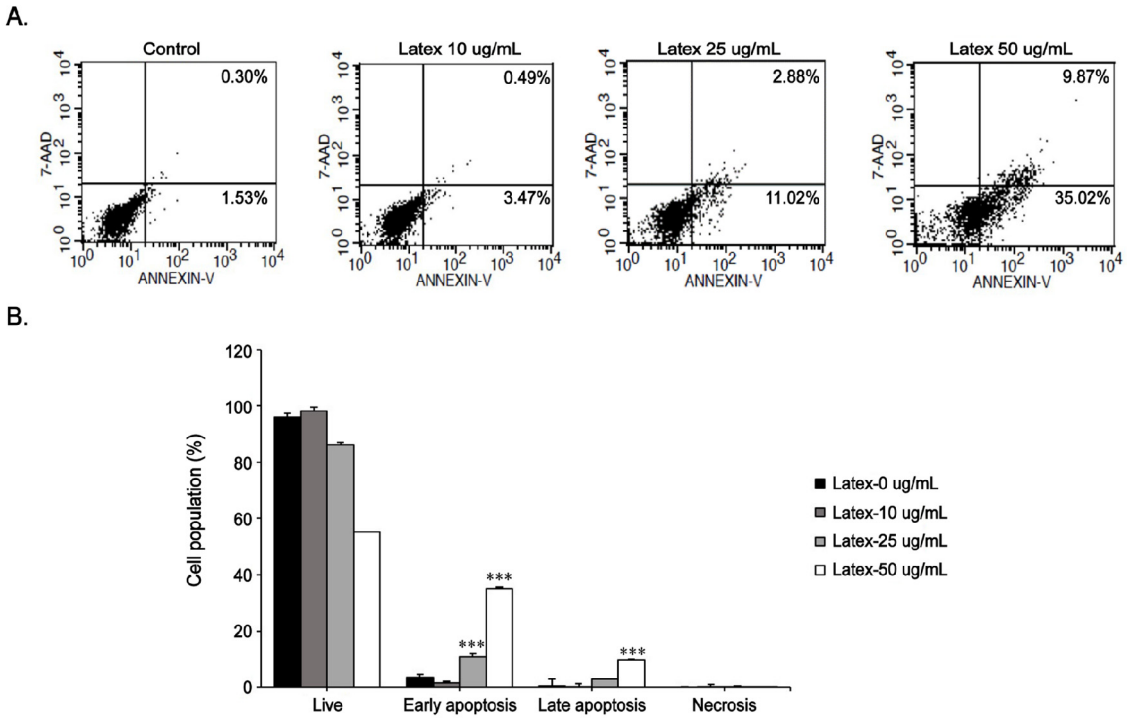

Fig. 3. Induction of apoptosis by latex in FaDu cells. Cells were treated with various concentrations of latex (0, 10, 25, and 50 μg/mL) for 24 h. (A) Annexin-V/7-AAD double-staining showed the percentage of apoptotic cells after latex treatment. The proportion of cells in each quadrant are marked on 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.

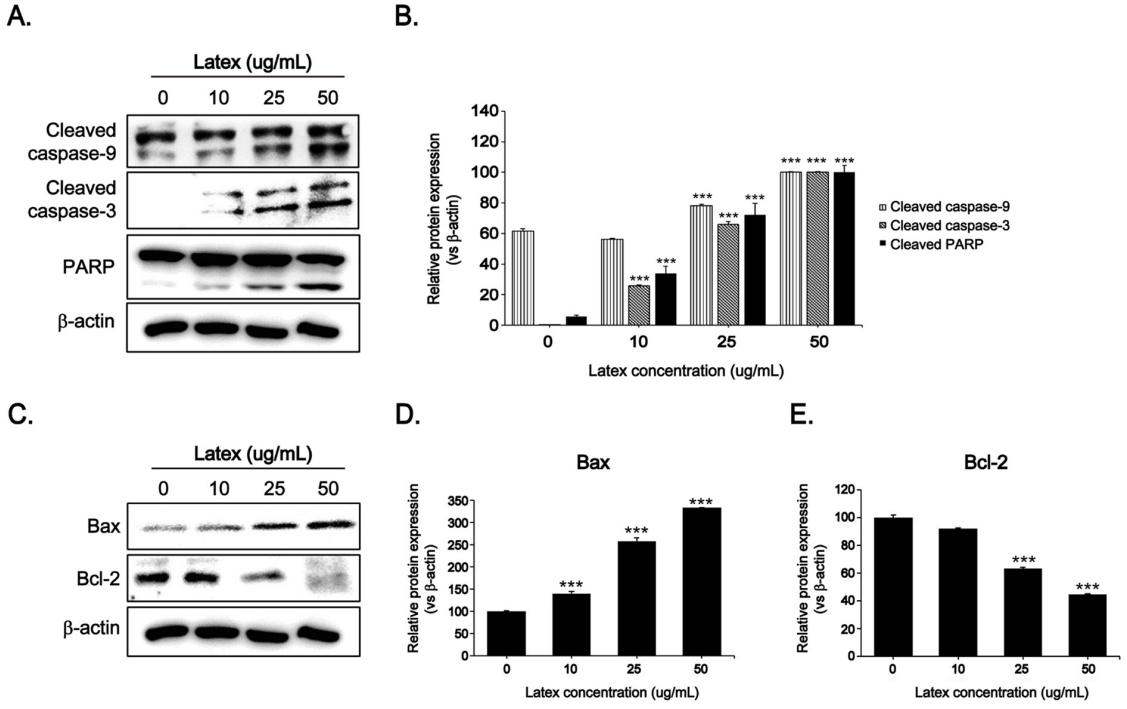

Fig. 4. The expression levels of apoptosis-related proteins in FaDu cells treated with latex. Cells were treated with various concentrations of latex (0, 10, 25, and 50 μg/mL) for 24 h. (A) After latex treatment, the expression of apoptotic-related protein (cleaved caspase-9, cleaved caspase-3, PARP, 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.

intrinsic apoptosis. In Fig. 4 C, 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.

Discussion

Ficus carica L. has been used in traditionally medicine as hemorrhoids, metabolic, cardiovascular, respiratory, and anti-inflammatory remedy [11,12]. A few recent studies have shown fig latex has many bioactivities such as antioxidant, antibacterial, anti-Herpes Simplex Virus (HSV), and anthelmintic activities [18-22]. 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 [22,23,25]. Therefore, this study was investigated the effect of latex in FaDu human hypopharynx squamous carcinoma 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, as a results, two major protein bands at around 28 kDa and 25 kDa detected. Raskovic et al., reported that protein bands at around 48 kDa, 25 kDa and 14 kDa detected in SDS-PAGE analysis of fig latex, and protein bands at above 24 kDa were ficin and at below 24 kDa were the result of autolysis/proteolysis in the sample collecting [24,26,27]. In addition, Baeyens-Volant et al. identified protein bands at 27, 24 and below 18 kDa in SDS-PAGE analysis of fig latex [15]. According to their chromatographic results, protein band at above 20 kDa were isoform of ficin (A-E, cysteine protease) and at below 20 kDa were some proteolyzed protein/peptide [15]. Therefore, in our result of SDS-PAGE, it can be known that 28 kDa or 25 kDa are protein band of ficin or isofrom ficin and band below 25 kDa is a proteolyzed protein/peptide. Futhermore, many studies reported that fig latex has proteolytic activity, such as collagenolytic, caseinolytic, and gelatinolytic activity [15,24, 28]. Recently, Raskovic et al. reported that gelatinolytic activity of fig latex was inhibited by PMSF and caseinloytic activity was inhibited by IAA and E-64 (cystein protease inhibitor) [24]. However, in our result, gelatinolytic activity of fig latex was inhibited by IAA, not change by PMSF. These different results are considered to be due to differences in preparation process of samples. They collected samples in August and pre-treated with benzene, but we collected the samples in July and immediately used them for the experiment without pretreatment. In addition, since the proteolytic activity of each sample was different [28].

The scientific research on cytotoxicity of fig latex was firstly performed by Ullman et al. in the 1940s [29]. 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 [29]. Recently, Khodarahmi et al. reported that crude fig latex exhibited cytotoxicity at 10 μg/mL in 48 h, and IC_{50} (48 h) was at 17 μg/mL in HeLa cervical cancer cells [30]. In addition, in stomach cancer cells, IC_{50} of fig latex was at 5 mg/ml [25]. Our results showed that crude fig latex exhibited cytotoxicity at 10 μg/mL in 24 h, and IC_{50} (24 h) 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 cancer cells. The molecular mechanism of the action of fig latex on inhibition of 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 [22]. 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 [31]. Caspases-3 is a main executing factor in process of apoptosis and caspase-9 is activated in the mitochondria-mediated intrinsic apoptosis pathway [31]. 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, anti-apoptotic Bcl-2 protein levels decreased and anti-survival Bax protein levels increased by fig latex in FaDu cells. Bcl-2 family is one of the best studied gene for apoptosis [32]. 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.

Conflict of interest

The authors declare that they have no conflicting interests.

Acknowledgments

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