J. Korean Soc. Appl. Biol. Chem. **53**(4), 470-477 (2010)

Article

Cytotoxicity of Dioscin and Biotransformed Fenugreek

Chae Hyun Yum[1], Hyun Ju You[1], and Geun Eog Ji[1,2]*

[1]*Department of Food and Nutrition, Seoul National University, Seoul 151-742, Republic of Korea*
[2]*Research Institute, BIFIDO Co., Ltd., Gangwon-do 250-804, Republic of Korea*

Received March 26, 2010; Accepted April 12, 2010

***Trigonella foenum-graecum*, commonly known as fenugreek, is widely used as a spice in India, Pakistan, and China for centuries. It has been suggested that biotransformation of the glycosides by various microorganisms increase the biological activity and bioavailability. This study aims to characterize the biotransformation of the fenugreek saponins by food microorganisms and to assess the cytotoxicity of the biotransformed fenugreek extract and dioscin which is a compound produced during biotransformation against various cell lines. The production of dioscin was confirmed by TLC and LC-MS analysis. The cytotoxicities against HT-29 colon cell line were increased after biotransformation by *Aspergillus usamii*, *Bifidobacterium infantis*, *Bifidobacterium* sp. Int57, and *Leuconostoc paramesenteroides* but decreased by *Aspergillus niger*. The IC_{50} values of dioscin against five different tumor cell line cells ranged 0.72-3.22 and 2.88-8.53 μM in 2% and 10%(v/v) FBS containing media, respectively. Real time cell analysis showed that HT-29 cells died immediately after treatment with dioscin above 7.5 μM. Dioscin at 5 μM increased sub G1 phase cells when assessed with flow cytometric analysis. Since caspase-3 activity was increased by dioscin, cytotoxicity of dioscin might be related to apoptosis. Taken together, the structure and the level of saponin components in fenugreek were differentially affected by various food microorganisms. The safety and the cytotoxicity of dioscin produced during biotransformation need to be further evaluated *in vivo*.**

Key words: biotransformation, cancer cells, cytotoxicity, dioscin, *Trigonella foenum-graceum*

Trigonella foenum-graecum, commonly known as fenugreek, is a widely used spice with a long history in China, India, Egypt, and Mediterranean areas [Basch *et al*., 2003]. Fenugreek seeds, which are used for medicinal purposes, contain a large amount of dietary fibre (48%) in dry weight. In addition, they also contain high content of saponins (4.8%) and the alkaloid trigonelline (0.37%), which may have a role in hypocholesterolemic activity [Srinivasan, 2006]. An unusual amino acid isolated from fenugreek, 4-hydroxyisoleucine, was shown to exert significant insulin secretagogue properties [Narender *et al*., 2006]. In traditional Chinese medicine fenugreek seeds were used as a tonic as well as a cure for weakness and edema [Yoshikawa *et al*., 1997]. In recent research, fenugreek seeds were experimentally shown to protect against breast [Amin *et al*., 2005] and colon cancers [Raju and Bird., 2006]. The hepatoprotective properties of fenugreek seeds were also reported in experimental models [Thirunavukkarasu *et al*., 2003; Kaviarasan and Anuradha, 2007]. Also, fenugreek seeds protect against experimental cataract by virtue of antioxidant properties [Gupta *et al*., 2009].

Phytochemical components in the plants usually exist in the form of glycosides, which after entering the body may be converted to aglycones through biotransformation by microbial enzymes in the intestine. The biotransformation of glycosides can affect their bioavailability and absorption patterns. Use of microorganisms that are safely applied in food processing such as lactobacilli, yeast, algae, and bacilli allows a very safe method of biotransformation [He *et al*., 2006]. Moreover, biological production processes are more environmentally friendly than chemical transformations [Fernandes *et al*., 2003].

Fenugreek contains saponins such as diosgenin, dioscin (diosgenin-3-yl-α-L-rhamnopyranosyl-(1-2)-[α-L-rhamnopyranosyl-(1-4)]-β-D-glucopyranoside), and their derivatives. Diosgenin, the aglycone of dioscin, is produced using chemical hydrolysis of fenugreeks or yellow ginger tubers and used as the main precursor of steroid

*Corresponding author
Phone: +82-2-880-8749; Fax: +82-2-884-0305
E-mail: geji@snu.ac.kr

doi:10.3839/jksabc.2010.072

hormones in the pharmaceutical industry [Raju and Mehta, 2009]. The bioactivities of dioscin were shown to include antitumor [Liu *et al*., 2004], antifungal [Sautour *et al*., 2004], and antivirus activities [Ikeda *et al*., 2000]. In cell culture studies dioscin exerted apoptosis-inducing effects against human myeloblast leukemia HL-60 cells [Liu *et al*., 2004] and human cervical cancer Hela cells [Cai *et al*., 2002].

Although various researches were performed on the effects of fenugreek extracts on diabetes, cancer, and allergy, no research was performed regarding the biotransformation of fenugreek and the effect of biotransformed fenugreek extracts (BFE) on cytotoxicity. In this research, the production of dioscin during biotransformation by various food microorganisms and the cytotoxicity of BFE were assessed. The cytotoxicity of dioscin on colon cancer cell line was analyzed by using MTT assay, real time cell analysis, flow cytometric analysis, and caspase-3 assay.

Materials and Methods

Chemicals and reagents. Dioscin (98% by HPLC) was purchased from Aktin Chemicals, Inc. (Chengdu, China). The materials used for cell culture such as media, fetal bovine serum (FBS), antibiotic-antimycotic (AA) solution and related reagents were obtained from GIBCO® products (Invitrogen Life Technologies, Carlsbad, CA). Trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), RNase, propidium iodide (PI), and caspase-3 Fluorimetri Assay Kit were purchased from Sigma-aldrich chemical (St. Louis, MO).

Preparation of microbial enzyme extracts. Five *Bifidobacterium* strains-*Bifidobacterium* sp. Int 57 (Int57), *B. infantis* (Infantis), *Bifidobacterium breve* YC2 (BRE), *B. pseducatenulatum* SJ32 (SJ32), and *Bifidobacterium* sp. SH5 (SH5)-were isolated from the human feces and *Leuconostoc paramesenteroides* PR (PR) were from the root of *Puerariae* radix as previously described [Park *et al.*, 1999; Choi and Ji, 2005]. *Aspergillus niger* KCTC 6906 (*A. niger*) and *Aspergillus usamii* KCTC 6956 (*A. usamii*) were purchased from Korean Collection for Type Cultures (KCTC; Daejeon, Korea). All the bacterial strains were inoculated and cultured in MRS medium containing 0.05%(w/v) L-cysteine·HCl at 37°C anaerobically. The incubated bacteria were collected by centrifugation (3000×g for 30 min at 4°C) and the harvested pellet was washed twice with 100 mM sodium phosphate buffer (pH 6.8). The pellet was resuspended in 100 mM phosphate buffer and disrupted in a cell disrupter (Stansted Fluid Power, Essex, UK). *A. niger* and *A. usamii* were incubated in Potato Dextrose broth (Difco, Sparks, MD) and Malt Extract broth (Difco), respectively, under aerobic condition with shaking at 24°C for 7 days. The mycelia of *A. niger* and *A. usamii* were removed by filtration and the filtrate was treated with 80% (w/v) ammonium sulfate. The precipitate was harvested by centrifugation (15,000×g for 30 min at 4°C), dissolved in 100 mM phosphate buffer, and then was dialyzed. The cytoplasm extracts from bacterial cells and the concentrated media extracts from mold were used as crude enzyme sources and were stored at −80°C before use.

Preparation of fenugreek extract. Fenugreek seeds were obtained from Kyung-dong Market in Seoul, Korea. Fifty grams of fenugreek were powdered and extracted with 10 volumes of 25%(v/v) ethyl alcohol at 60°C for 24 h on a shaking water bath (Jeiotech, Kimpo, Korea), and then filtered with filter paper (541 Whatman, Kent, UK). Fenugreek extract was distilled in a vacuum rotary evaporator at 50°C to remove the ethyl alcohol solvent. The non-transformed fenugreek extract (NFE) was freeze-dried and stored at −80°C before use. For the biotransformation, the lyophilized powder was dissolved in 100 mM phosphate buffer (pH 6.8) at 20 mg/mL concentration.

Biotransformation of fenugreek extract by crude microbial enzymes. Five hundred μL of fenugreek extract and 500 μL of crude microbial enzymes were mixed and incubated at 45°C for 24 h. After incubation, the mixture was extracted with 1 mL *n*-BuOH. The *n*-BuOH layer was analyzed by thin layer chromatography (TLC) and LC-MS.

Cell Culture. Human colorectal carcinoma HCT 116 (KCLB 10247), colorectal adenocarcinoma HT-29 (KCLB 30038), and lung adenocarcinoma A-549 (KCLB 10185) cell lines were purchased from the Korean Cell Line Bank (Seoul, Korea). Human colorectal adenocarcinoma Caco-2 (ATCC, HTB-37), human liver hepatocelluar carcinoma Hep G2 (ATCC, HB-8065) and the normal colonic epithelial FHC cells were obtained from American Type Culture Collection (Manassas, VA). HT-29, Caco-2, and Hep G2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10%(v/v) of FBS and 1%(v/v) of AA solution. HCT 116 and A-549 cells were incubated in RPMI-1640 media containing 10%(v/v) of FBS and 1%(v/v) of AA solution. FHC cells were cultured in DMEM/F12 media containing 25 mM HEPES, 10 ng/mL cholera toxin, 5 μg/mL insulin, 5 μg/mL transferring, 100 ng/mL hydrocortisone, 10%(v/v) of FBS, and 1%(v/v) of AA solution. The cell culture was maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Cell viability assay. Five different kinds of cells were seeded in 96-well plates. Cells were incubated in the

presence of BFE (500 μg/mL) and NFE (500 μg/mL), and various concentrations of dioscin. Dioscin was dissolved in dimethyl sulfoxide (DMSO) the concentration of which was not greater than 0.1%(v/v) in all experiments. At the end of incubation, MTT solution (5 mg/mL) was added to every well, followed by incubation for 1.5 h. The precipitated formazan product was dissolved with 200 μL DMSO and the absorption was measured at 570 nm on a microplate reader (Bio-Rad Laboratories, Philadelphia, PA). The optical density of the control cells was taken as 100% viability. Each reaction was performed in 6 replicates.

Analysis of fenugreek by TLC. TLC was used in TLC aluminium sheets (20×20 cm, precoated with silica gel 60 ($60F_{254}$, Merck Co., Darmstadt, Germany). The developing solvent for TLC was BuOH-Acetic acid-Water (4:1:5, v/v/v, higher phase). Visualization was carried out by 10% H_2SO_4 sprayings. After spraying, a TLC plate was heated at 110°C for 15 min. Fenugreek saponins were identified by spots and color shown on the plate.

Analysis of fenugreek saponins by LC-MS. LC-MS analysis was conducted using a LC pump (Binarypump Agilent Technologies 1200series (Bin pump SL)), a reversed-phase column (Zorbax Eclipse XDB-C18 (4.6×50 mm, 1.8 μm), an detector (PDA (DAD SL, Agilent Technologies 1200 series)), and software (Data Acqusition Version B.02.01). Elution was carried out at a flow rate of 1.0 mL/min using a solvent gradient consisting of A (10 mM Ammonium acetate) and B (100% acetonitrile) with following temporal profile : 0-3 min, linear gradient from 80% A and 20% B to 20% A and 80% B; 3-5 min, from 20% A and 80% B to 5% A and 95% B; 5-6 min, from 5% A and 95% B to 5% A and 95% B; 6-7 min, from 5% A and 95% B to 100% B; 7-8 min, from 100% B to 50% A and 50% B; 8-9 min, from 50% A and 50% B to 80% A and 20% B.

Real time cell analysis (RTCA). RTCA is an impedance sensor technology which monitors the simultaneous changes in cellular status and growth pattern using xCELLigence RTCA system (Roche Diagnotics, Mannheim, Germany) and 96-well E-plate™ (ACEA Biosciences, San Diego, CA). Cells were seeded to a concentration of 1.2×10^4 cells/well and were incubated for 24 h. Then seeded cells were serum-starved by incubation in FBS-deprived DMEM media for another day. After 48 h of seeding, various concentrations (2.5, 5, 7.5, 10, and 25 μM) of dioscin were treated in DMEM containing 2% FBS and were monitored every hour for 4 days. Each reaction was performed in 3 replicates. Normalized cell index (CI) was analyzed by integrated software and the values of IC_{50} were determined at 24 and 72 h after treatment, respectively.

Flow cytometric analysis (FACS). FACS analysis was performed for the assessment of the cell cycle. HT-29 colon cancer cells were seeded in 6-well plate to a concentration 5×10^5 cells/well. Twenty-four h after seeding, medium was replaced with DMEM without FBS with 1% antibiotics. After another 24 h, dioscin, dissolved in 0.1% DMSO, was treated at 0 and 5 μM concentrations. Cells were collected 0, 3, 6, 9, 12, 18, and 24 h after treatment of dioscin. Cells were harvested and fixed by 75% ethanol at −20°C for at least 24 h. Thereafter, the suspension was centrifuged and the pellet was washed with phosphate buffer saline (pH 7.4). Then cells were reacted with RNase (1 mg/mL) solution in distilled water for 30 min. After another centrifugation, cells were stained by PI and were incubated for 1 h at room temperature in the dark. Cell cycle analysis was performed using a FACSCalibur™ flow cytometry system (BD Biosciences, San Jose, CA, USA) within 1 h. and the CellQuest software (BD Biosciences, San Jose, CA) for data analysis.

Caspase-3 activity. HT-29 cells were seeded with 2×10^6 cells in 100 mm culture dishes. After 24 h, medium was replaced with DMEM without FBS. After 24 h, medium was changed with DMEM with 10% FBS and control (0.1% DMSO). Then 2.5 μM of dioscin were treated. After 48 and 72 h, cells were harvested by trypsin-EDTA and pellet was used in next step.

Caspase-3 activity was measured using a Caspase-3 assay kit according to the manufacturer's protocol. After harvesting the cell lysates, plates were measured using a microplate reader (FLx800 microplate fluorescence reader BIO TEK Instruments, INC) at 405 nm. All measurements were performed in quadruplicate.

Statistical analysis. Data are expressed as the mean± SD and were analyzed by Student's t-test, using SPSS software (version 17.0; SPSS Inc., Chicago, IL). P values <0.05 was considered statistically significant.

Results

Changes in cytotoxicity and composition of fenugreek extracts after biotransformation by microbial enzymes. NFE at 500 μg/mL showed cytotoxic effect up to 65.8± 5.1% inhibition on the growth of HT-29 colon cancer cells in comparison to the control. After biotransformation by crude enzyme extracts from *A. usamii*, BRE, Int57, *L. paramesenteroides,* and SH5, the cytotxicities were increased (Fig. 2). Interestingly, the growth of HT-29 cells were rather promoted than inhibited when treated with fenugreek extract biotransformed by *A. niger* (*A. niger*-BFE). As shown in TLC profile (Fig. 3A), spots A-D shown in NFE were considerably decreased, instead two novel spots E and F appeared as major products after

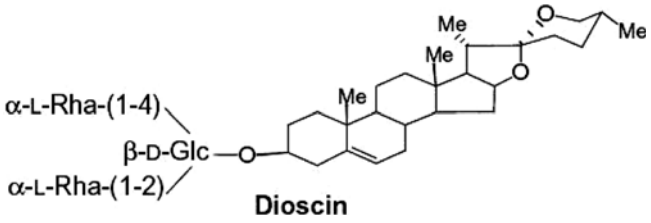

Fig. 1. Chemical structure of dioscin. (Rha, Rhamnose; Glc, Glucose; Me, Methyl group)

biotransformation by experimental microbial enzymes except SJ32-BFE and *A. niger*-BFE. Among the two newly appeared spots, spot F had same Rf value as standard dioscin (Fig. 3B). The identity of dioscin was further confirmed using LC-MS analysis. SJ32 did not show any noticeable biotransformation, whereas *A.niger* showed a most distinct biotransformation pattern resulting in the production of spot X in the upper region in TLC.

Composition changes of fenugreek saponins by biotransformation with LC-MS. The electrospray ionization mass spectrometry (LC-ESI-MS) displayed an intense ion at 721 *m/z*. Dioscin standard precursor has 869 *m/z* and product ion is 721 *m/z*. and this is detected in LC-ESI-MS. Fig. 4A. showed dioscin standard and it was detected by 239 intensity. Fig. 4B and Fig. 4C are profiles of NFE and BFE by Infantis and they were detected by 5 and 131 intensity at the same concentration (10 mg/mL), respectively. NFE had a low level of dioscin whereas Infantis-BFE contained dioscin as a major product.

Cytotoxic effect of dioscin on various cancer cell lines. The cytotoxicities of dioscin against five tumor

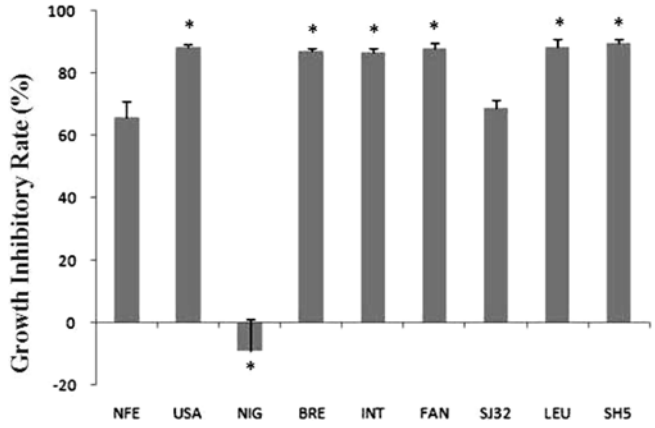

Fig. 2. Growth inhibitory rate of NFE (500 μg/mL) and various BFEs (500 μg/mL) on HT-29 colon cancer line. (NFE, non-biotransformed fenugreek extract; USA, *A. usamii*-BFE; NIG, *A. niger*-BFE; BRE, BRE-BFE; INT, Int57-BFE; FAN, Infantis-BFE; SJ32, SJ32-BFE; LEU, PR-BFE; SH5, SH5-BFE) Cells were exposed to fenugreek extracts for 72 h and the cell viability was measured by MTT assay. $p<0.05$ compared to NFE.

cell lines occurred in a dose-dependent manner in MTT assay. Table 1 represents the values of half maximal inhibitory concentration (IC_{50}) of dioscin in each cell line. The susceptibility of cells to dioscin was different depending on the type of cell lines and the initial number of seeded cells. As the number of cells was increased, the value of IC_{50} tended to be increased. The concentration of FBS in media was also related to the cytotoxic effect of dioscin. The IC_{50} values of dioscin against five different tumor cell line cells ranged 0.72-3.22 and 2.88-8.53 μM

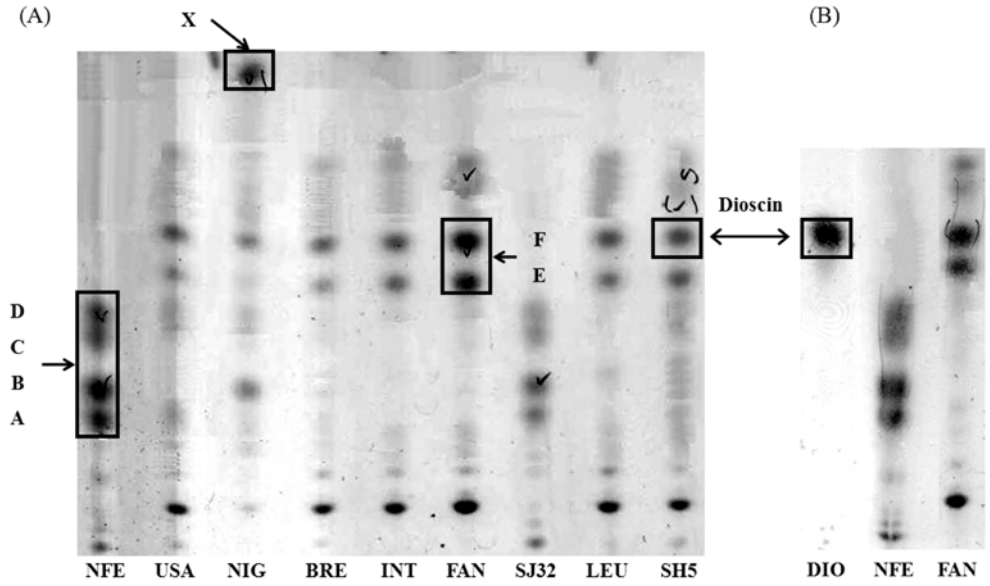

Fig. 3. Composition changes of fenugreek saponins by biotransformation with TLC assay. The profile of TLC analysis of NFE and BFEs (A) and of dioscin, NFE, and FAN (B). (abbreviations are the same as in Fig. 2; DIO, dioscin standard). A-D: compounds of NFE. E, F: compounds produced after biotransformation. X: a major product produced by *A. niger*.

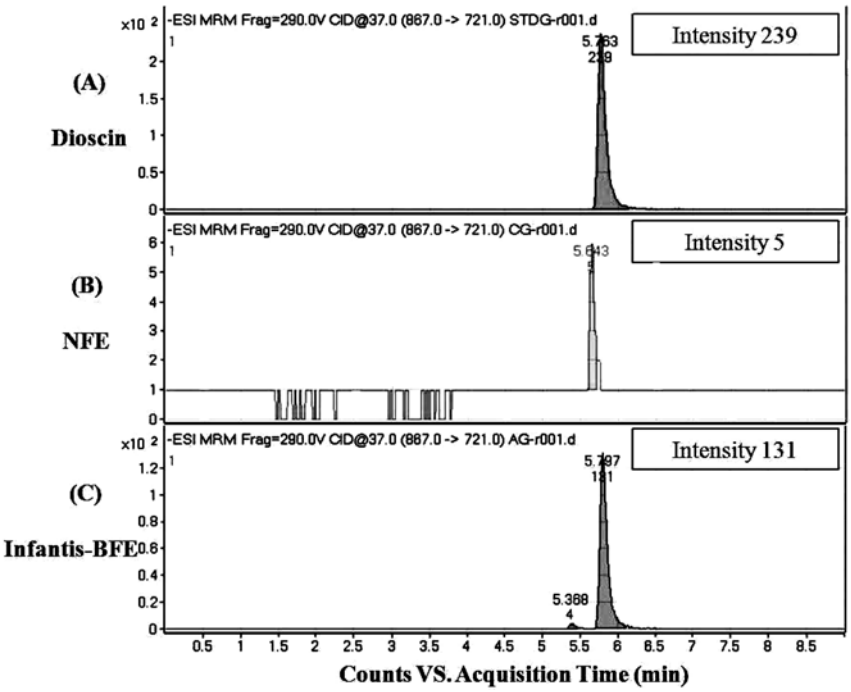

Fig. 4. The electrospray ionization mass spectrometry (LC-ESI-MS) chromatograms of dioscin present in standard (A), NFE (B), and Infantis-BFE (C).

in 2% and 10%(v/v) FBS containing media, respectively. The cell viability was decreased in all of the cancer cell lines when the concentration of FBS contained in media was lowered. Among the three colon cancer cell lines (HT-29, Caco-2, and HCT 116) used in this study, HT-29 was the least susceptible one.

Real time cell analysis (RTCA). From the seeding the cells to the termination of experiments, the electronic readout is displayed as an arbitrary unit called Normalized CI. Increases in CI suggest the increasing cell numbers and/or increased cell adhesion to contact area. Fig. 5 showed the changes of cell status after the treatment of various concentrations of dioscin on HT-29 cells. Dioscin significantly decreased the proliferation of the cells in time- and dose- dependent manner. Above 7.5 μM, HT-29 cells died immediately after the treatment with dioscin. When the cell viability was quantified by the end-point assay such as MTT assay, this immediate cytotoxic effect could not be distinguished from the cytostatic or anti-proliferating effects shown in the profiles of 2.5 μM and 5 μM treated groups. The growth pattern of cells at 5 μM dioscin showed that the proliferation of cells began to decrease after 12 h. The time-dependent IC_{50} values were lower than that of MTT assay, which were 2.7 μM at 24 h and 1.2 μM at 48 h, respectively. During prolonged incubation, IC_{50} values were further decreased.

Table 1. IC_{50} values of dioscin on various cell lines (μM)

Type of cancer cell line	Number of cells (/well)	IC_{50} (mM)	
		FBS 10%	FBS 2%
HT-29	6×10^3	5.21	0.72
Caco-2	1×10^4	4.55	0.85
	2.5×10^4	8.53	3.22
HCT-116	4×10^3	2.88	1.40
	6×10^3	3.54	1.74
HepG2	3.5×10^3	6.97	0.79
A-549	3×10^3	4.01	1.06

Dioscin was treated with various cell lines with different seeded numbers and different concentration of FBS for 72 h.

Changes in the distribution of cell cycle and caspase-3 activity. To investigate the growth inhibitory effect on the cell cycle, cells were treated with 5 μM of dioscin for 24 h and each phase of cell cycle was determined by flow cytometric analysis with PI stainging. The percentage of cell population in sub-G1 phase (2.0, 27.6, 59.2, 72.9, and 94.0% at 0, 6, 12, 18, and 24 h, respectively) was continuously increased during incubation (Fig. 6). Cell lysates were evaluated for the protease activity of caspase-3 in order to assess the effect of dioscin on apoptosis. At 2.5 μM dioscin caspase-3 activity was increased 1.6 and 1.9 fold after 48 and 72 h, respectively (Fig. 7).

Discussion

Fenugreek is widely used as a spice in India, Pakistan,

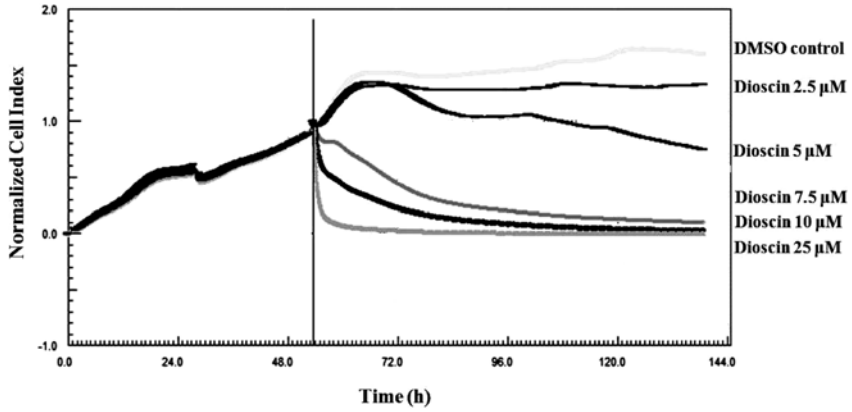

Fig. 5. Cell growth pattern assessed by real time cell analyzer. This figure showed real time cell analysis after treatment of various concentrations of dioscin. The center line indicates the time of treatment of dioscin. Increases in CI suggest the increasing cell numbers and/or increased cell adhesion to contact area. HT-29 cells were seeded to a concentration of 1.2×10^4 cells/well.

and China for centuries. The seeds containing high amount of saponins (4.8%) were suggested to play a role in the hypocholesterolemic [Srinivasan, 2006], antidiabetic activities [Narender *et al*., 2006] and cytotoxic activity on breast cancer cell line [Amin *et al*., 2005].

Deglycosylation of saponins were suggested to enhance their biological activity and bioavailability [Tawab *et al*., 2003]. In the present study, the saponin fractions of fenugreek extract were converted to deglycosylated forms with various food microorganisms to improve the bioactivity of fenugreek saponins. The differential production of dioscin by various microbial enzymes was newly confirmed in this study. The increased cytotoxic effects of BFEs through the biotransformation process were related to the production of dioscin. This was further supported by the result that the cell cytotoxicity was not affected by SJ32-BFE which did not show noticeable biotransformation. Interestingly, *A. niger*-BFE showed low level of dioscin but produced compound X as a major product. Since dioscin was confirmed as a cytotoxic compound, disappearance of cytotoxicity in *A. niger*-BFE might be due to the further transformation of cytotoxic dioscin into non-cytotoxic compound X. When relationships between structure and cytotoxicity of dioscin derivatives against HL-60 leukemia cells were assessed, diosgenin and diosgenin-β-D-glucoside showed very weak cytotoxicity (IC_{50}>20 μg/mL) [Mimaki *et al*., 2001]. The attachment of α-L-rhamnosyl residue at C-2 of the glucosyl moiety led to the increase in cytotoxicity (IC_{50} 1.8 μg/mL) whereas dioscin possessing additional α-L-rhamnosyl residue at C-4 of the inner glucosyl moiety showed slightly decreased activity (IC_{50}=3.3 μg/mL). In our previous study, the crude enzyme extract of *A. niger* contained extracellular α-L-rhamnosidases and was carried out with derhamnosylation on saponin compounds [Wie *et al*., 2007]. Further studies to identify the chemical structure and biological activities of the compound X need to be conducted. However, the loss of cytotoxic effect of *A. niger*-BFE might be attributed to the detachment of rhamnosyl residues and/or following modifications of glycosyl moieties through biotransformation process.

Dioscin represents a typical example of spirostane-type saponin. Its inhibitory activity against tumor cells were reported in several experiments using Hela cells [Cai *et al*., 2002], K562 cells [Liu *et al*., 2004], and HL-60 cells [Mimaki *et al*., 2001]. However, analysis on the cytotoxicities of dioscin against other carcinoma cell lines was newly performed in the present study. In all of the five different experimental cancer cell lines including colon, lung, and hepatocarcinoma cells, the IC_{50} ranged 0.72-3.22 μM in 2%(v/v) FBS containing media, and 2.88-8.53 μM in 10%(v/v) FBS containing media. Compared to the results from other studies, the susceptibility of the cell lines to dioscin used in our study were similar to Hela and HL-60 cells and greater than K562 cells. Noticeably, the cytotoxicity against the normal cells was also observed in this study suggesting that dioscin may act as a toxic compound *in vivo*. The cytotoxicity of dioscin was also affected by the seeded cell numbers and the concentrations of FBS in culture media. For example, IC_{50} of dioscin was much lower in the presence of 2% FBS than 10% FBS.

RTCA data showed distinguishable patterns of cytostatic, anti-proliferating, and immediate cytotoxic effects depending on the dioscin concentrations, which was not detected by the end-point MTT assay. Dioscin over 7.5 μM showed a very potent cytotoxic effect on HT-29 cells. The cells tended to be detached from the surface of culture well and died immediately after dioscin treatment. Cells treated with 2.5 μM dioscin showed cytostatic profile after 12 h

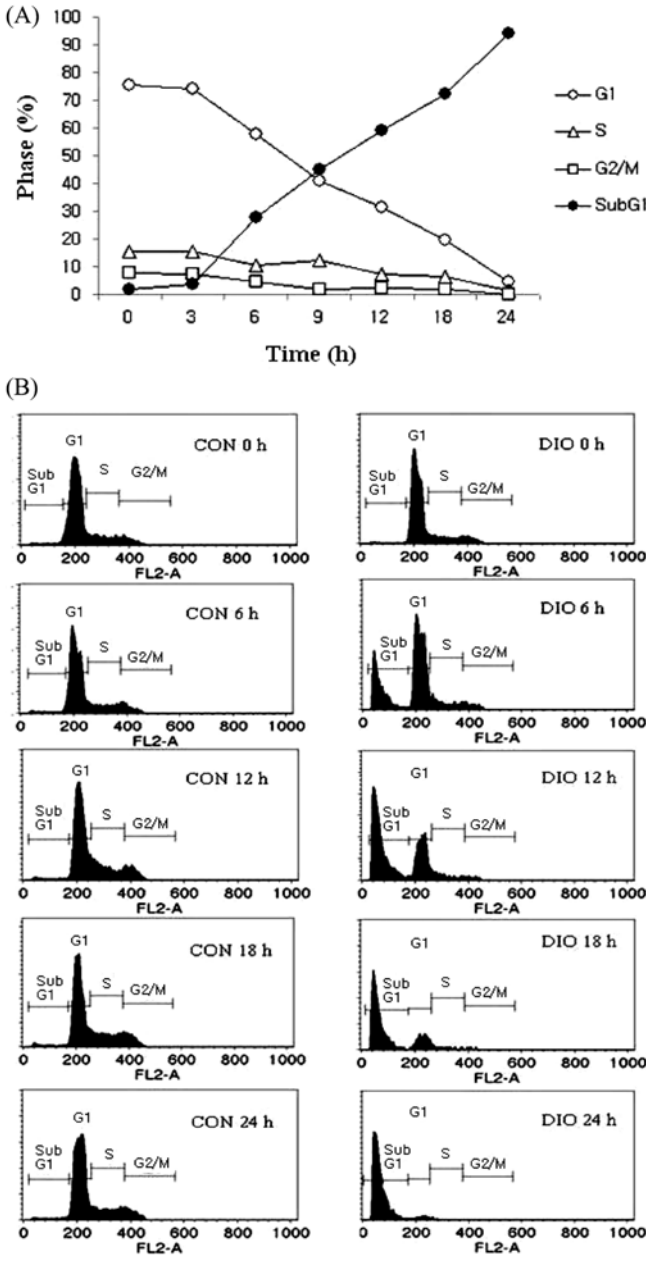

Fig. 6. Representative histograms of flow cytometric analysis (A) and cell cycle distribution of flow cytometric analysis (B) of the cellular DNA content in 5 μM dioscin-treated and control HT-29 colon cancer cells for 24 h. HT-29 colon cancer cells were seeded in 6-well plate to a concentration 5×10^5 cells/well.

whereas the cells treated with 5 μM gradually lost viability after 12 h. In this regard, the increase in sub-G1 cells after treatment with 5 μM dioscin might be related to the time-dependent increase of caspase-3 activity, a well known indicator of apoptosis in mammalian cells [Earnshaw *et al*., 1999; Ren *et al*., 2009].

This study assessed the differential biotransformation of the fenugreek saponins by various food microorganisms and the mechanisms involved in the cytotoxicity of

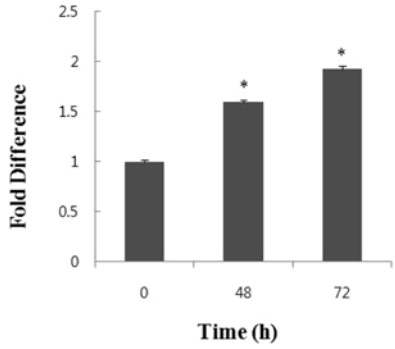

Fig. 7. Activation of caspase-3 in HT-29 colon cancer cell line treated with 2.5 μM of dioscin for 0, 48, and 72 h. Data are expressed as means±SD of quadruplicate determinations and indicated as fold difference compared to 0 h control. HT-29 colon cancer cells were seeded in 100 mm culture dishes to a concentration 2×10^6 cells/well.

dioscin, one of the major compounds produced during biotransformation.

Acknowledgments. This work was supported by the Gangwon Regional Innovation Agency, Ministry of Knowledge Economy and 21C Frontier Microbial Genomics and Applications Center Program, Ministry of Science and Technology (MG08-0303-4-0), Republic of Korea.

References

Amin A, Alkaabi A, Al-Falasi S, and Daoud SA (2005) Chemopreventive activities of *Trigonella foenum graecum* (Fenugreek) against breast cancer. *Cell Biol Int* **29**, 687-694.

Basch E, Ulbricht C, Kuo G, Szapary P, and Smith M (2003) Therapeutic applications of fenugreek. *Altern Med Rev* **8**, 20-27.

Cai J, Liu M, Wang Z, and Ju Y (2002) Apoptosis induced by dioscin in Hela cells. *Biol Pharm Bull* **25**, 193-196.

Choi EK and Ji GE (2005) Food microorganisms that effectively hydrolyze O-glycoside but not C-glycoside isoflavones in *Puerariae radix*. *J. Food Sci* **70**, C25-C28.

Earnshaw WC, Martins LM, and Kaufmann SH (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem* **68**, 383-424.

Fernandes P, Cruz A, Angelova B, Pinheiro HM, and Cabral JMS (2003) Microbial conversion of steroid compounds: recent developments. *Enzyme Microb Technol* **32**, 688-705.

Gupta SK, Kalaiselvan V, Srivastava S, Saxena R, and Agrawal SS (2009) *Trigonella foenum-graecum* (Fenugreek) protects against selenite-induced oxidative stress in experimental cataractogenesis. *Biol Trace Elem Res* (in press)

He X, Liu B, Wang G, Wang X, Su L, Qu G, and Yao X (2006)

Microbial metabolism of methyl protodioscin by *Aspergillus niger* culture-a new androstenedione producing way from steroid. *J Steroid Biochem Mol Biol* **100**, 87-94.

Ikeda T, Ando J, Miyazono A, Zhu XH, Tsumagari H, Nohara T, Yokomizo K, and Uyeda M (2000) Anti-herpes virus activity of Solanum steroidal glycosides. *Biol Pharm Bull* **23**, 363-364.

Kaviarasan S and Anuradha CV (2007) Fenugreek (*Trigonella foenum graecum*) seed polyphenols protect liver from alcohol toxicity: a role on hepatic detoxification system and apoptosis. *Pharmazie* **62**, 299-304.

Liu MJ, Wang Z, Ju Y, Zhou JB, Wang Y, and Wong RN (2004) The mitotic-arresting and apoptosis-inducing effects of diosgenyl saponins on hunan leukemia cell lines. *Biol Pharm Bull* **27**, 1059-1065.

Mimaki Y, Yokosuka A, Kuroda M, and Sashida Y (2001) Cytotoxic activities and structure-cytotoxic relationships of steroidal saponins. *Biol Pharm Bull* **24**, 1286-1289.

Narender T, Puri A, Shweta S, Khaliq T, Saxena R, Bhatia G, and Chandra R (2006) 4-hydroxyisoleucine an unusual amino acid as antidyslipidemic and antihyperglycemic agent. *Bioorg Med Chem Lett* **16**, 293-296.

Park SY, Ji GE, Ko YT, Jung HK, Ustunol Z, and Pestka JJ (1999) Potentiation of hydrogen peroxide, nitric oxide, and cytokine production in RAW 264.7 macrophage cells exposed to human and commercial isolates of *Bifidobacterium*. *Int J Food Microbiol* **46**, 231-241.

Raju J and Bird RP (2006) Alleviation of hepatic steatosis accompanied by modulation of plasma and liver TNF-alpha levels by *Trigonella foenum graecum* (fenugreek) seeds in Zucker obese (fa/fa) rats.*Int J Obes* **30**, 1298-1307.

Raju J and Mehta R (2009) Cancer chemopreventive and therapeutic effects of diosgenin, a food saponin. *Nutr Cancer* **61**, 27-35.

Ren MR, Hur JS, Kim JY, Park KW, Park SC, Seong CN, Jeong IY, Byun MW, Lee MK, Seo KI (2009) Anti-proliferative effects of Lethariella zahlbruckneri extracts in human HT-29 human colon cancer cells. *Food Chem Toxicol* **47**, 2157-2162.

Sautour M, Mitaine-Offer AC, Miyamoto T, Dongmo A, and Lacaille-Dubois MA (2004) Antifungal steroid saponins from *Dioscorea cayenensis*. *Chem Pharm Bull* **52**, 1353-1355.

Srinivasan K (2006) Fenugreek (*Trigonella foenum-graecum*) : a review of health beneficial physiological effects. *Food Rev. Int* **22**, 203-224.

Tawab MA, Bahr U, Karas M, Wurglics M, and Schubert-Zsilavecz M (2003) Degradation of ginsenosides in humans after oral administration. *Drug Metab Dispos* **8**, 1065-1071.

Thirunavukkarasu V, Anuradha CV, and Viswanathan P (2003) Protective effect of fenugreek (*Trigonella foenum graecum*) seeds in experimental ethanol toxicity. *Phytother Res* **17**, 737-743.

Wie HJ, Zhao HL, Chang JH, Kim YS, Hwang IK, and Ji GE (2007) Enzymatic modification of saponins from Platycodon grandiflorum with *Aspergillus niger*. *J Agric Food Chem* **55**, 8908-8913.

Yoshikawa M, Murakami T, and Komatsu H (1997) Medicinal foodstuffs. IV. Fenugreek seed. (1): structures of trigoneosides Ia, Ib, IIa, IIb, IIIa, and IIIb, new furostanol saponins from the seeds of Indian *Trigonella foenum-graecum L*. *Chem Pharm Bull* **45**, 81-87.