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Effect of Sulfur Enriched *Young Radish Kimchi* on the Induction of Apoptosis in HT-29 Human Colon Cancer Cells

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Abstract

Young radishes (YR, *yeolmu* in Korean) were cultivated in soil with and without sulfur. Control *YR-kimchi* and sulfur *YR-kimchi* were prepared using the young radishes cultivated in the soil without and with 1,818 g/m^3 sulfur, respectively. Fermentation of the *YR-kimchis* were conducted at 5°C for 6 weeks. The control and sulfur *YR-kimchis* were reached pH 4.39 and pH 4.31 with 0.98% and 1.04% acidity at 5 weeks, respectively. At a higher concentration of 20 μL/assay, the sulfur *YR-kimchi* juice exhibited higher inhibitory effects (84%) on the growth of HT-29 human colon cancer cells than the control *YR-kimchi* (57%). Methanol extract from the *YR-kimchis* also led to similar results to those of the juices. In the inhibition study by hematocytometer, *YR-kimchis* inhibited the growth of cells in a time-dependent manner. Sulfur *YR-kimchi* induced apoptosis as determined by 4,6-diamidino-2-phenylindole (DAPI) staining and decreased Bcl-2 expression of active anticancer compounds, when compared to the control *YR-kimchi*. These results suggested that preparing *kimchi* using YR cultivated in the presence of sulfur, which can help to synthesize active compounds, could increase the anti-cancer activity of sulfur *YR-kimchi*.

Key words: *young radish kimchi*, sulfur, HT-29 human colon adenocarcinoma cell, apoptosis

INTRODUCTION

Colon cancer is a major cause of cancer-associated mortality in the US (1) and the chance of a colon cancer attack has been increasing for Koreans. Epidemiologic studies indicate that the incidence of colon cancer is inversely correlated with the consumption of fruits and vegetables (2). The consumption of cruciferous vegetables such as broccoli, Brussels sprouts and cabbage has been associated with a decreased risk for many cancers (3). Young radish, one of these cruciferous vegetables, has a large amount of vitamin A, ascorbic acid and essential minerals (4,5). In addition, there are various kinds of phytochemicals, such as isothiocyanates and fiber in young radishes (6). Isothiocyanates are released upon chewing or maceration of certain cruciferous vegetables. Sulfuraphane is an isothiocyanate that present naturally in many widely consumed vegetables (7).

Previous study (8) and Kim et al. (9), reported that young radishes grown in soil containing sulfur increased quinine reductase (QR) activity in Hepa 1c1c cells and an isothiocyanate-like compound was analyzed using high performance liquid chromatograph (HPLC). Kim et al. (10) also suggested that the administration of extracts of young radish cultivated with sulfur suppressed pulmonary tumorigenesis, possibly due to increased activity of detoxification enzymes in the liver and lungs, and partly due to cell cytotoxcity.

Kimchi (*baechu kimchi*) contains higher levels of vitamins (vitamin C, β-carotene, vitamin B complex, etc.), minerals(Na, Ca, K, Fe, and P), dietary fibers (24% on a dry basis; 7.7% soluble dietary fiber; 16.2% insoluble dietary fiber), and other functional components (11). Phytochemicals such as benzyl isothiocyanate, indole compounds, thiocyanate, and sitosterol are the active compounds found in kimchi, which have shown antimicrobial, anticancer, and antiatherosclerotic functions (12). However, little is currently known about the functional properties of *young radish kimchi* (*YR-kimchi*).

In the present study, we investigated the effects and the enhanced functional properties of *YR-kimchi* which contains naturally occurring sulfur, on the growth inhibition, the apototic effects and the expression of Bax and Bcl-2 genes in HT-29, human colon cancer cells.

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MATERIALS AND METHODS

Ingredients and preparation of the *young radish kimchis*

Young radishes (YR, *yeolmu* in Korean), YR-control and YR-sulfur samples were cultivated in the soil without sulfur and with 1,818 g/m^3 sulfur, respectively. *YR kimchis* were prepared using the YR-control and YR-sulfur. Young radishes were offered by Gyoungnam Agricultural Research and Extension Services (Korea). Garlic, ginger, green onion, red pepper, red pepper powder, anchovy juice were purchased at a Bujun market in Busan, Korea.

Young radish kimchis were prepared based on the standardization of manufacturing method (13), following composition: preserved young radish 100 g, crushed garlic 2.9 g, crushed ginger 1.6 g, green onion 8.0 g, red pepper 7.0 g, red pepper powder 4.2 g and anchovy juice 3.7 g. Fermentation properties of the *YR-kimchis* were investigated during fermentation at 5°C for 6 weeks.

Measurement of fermentation properties

The young radish tissue and juice were homogenized together by stomacher 400 Lab Blender (Seward Medical London SEI IPP, UK). pH and acidity were determined by pH meter (Corning 220, USA) and AOAC method (14), respectively. Plate count techniques were used for count lactic acid bacteria (*Leuconostoc* sp. and *Lactobacillus* sp.). Phenylethyl alcohol sucrose agar medium (PES medium) was used for the determination of *Leuconostoc* sp. and plates was incubated at 20°C for 5 days. Modified LBS agar medium (m-LBS medium) with acetic acid and sodium acetate was used for the determination of *Lactobacillus* sp. and the plates were incubated at 37°C for 3~4 days. Viable cell numbers were determined as colony forming units per mL (15).

Preparation of juices and methanol extracts of samples

YR-kimchis were fermented at 5°C for optimally fermented point (pH 4.3). The juices were made as follows. *YR-kimchis* homogenized by Homogenizer (Anjel juicer, Korea) were centrifuged at 9,000 rpm for 20 min and following by filtering using a filter (0.45 μm).

For preparing of methanol extracts, the *YR-kimchis* were freezing-dried and powdered. Twenty folds of methanol were added to the powdered samples and extracted twice with shaking. The methanol extracts were evaporated using rotary evaporator, concentrated, then dissolved in dimethylsulfoxide (DMSO, Amresco, Solon, Ohio, USA).

Cell culture

HT-29 human colon adenocarcinoma cells were obtained from American Type Culture Collection (ATCC, Rockbille, MD, USA) and cultured in RPMI-1640 medium (Gibco Co., Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco Co.) at 37°C in a humidified atmosphere containing with 5% CO_2. The medium was changed twice or three times each week.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay

The anti-proliferative effect of *young radish kimchis* was assessed by MTT (Sigma) assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzyme. The cells were dissociated with 0.05% trypsin-0.02% EDTA and 180 μL of cell suspensions (1×10^4 cells/mL) were seeded in each well of 96-well microtitre plates. Treated with control or *YR-kimchis* supplemented medium (1.5 mg/mL, 3 mg/mL) for 48 hr and 20 μL of MTT (5 mg/mL in PBS) solution was added. After an additional incubation for 4 hr, the supernatant medium was carefully removed from the wells. Formazan crystals were dissolved by adding 150 μL DMSO. The optical densities were recorded on a ELISA reader at 540 nm (16).

Growth inhibition study

For growth inhibition analysis, 1 mL of cancer cells were plated at density of 1×10^5 cells/plate (6-well microtitre plates, Nunc) and treated with control or *YR-kimchis* supplemented medium (1.5 mg/mL) for desired time (1~4 days). The cells were trypanized, washed with phosphate-buffered saline (PBS), and scored using the Trypan Blue method (17).

Nuclear staining with 4,6-diamidino-2-phenylindole (DAPI)

Untreated control and *young radish kimchis* treated cancer cells were harvested, washed with PBS, and fixed with 3.7% paraformaldehyde (Sigma) in PBS for 10 min at room temperature. Fixed cells were washed with PBS and stained with DAPI (Sigma) solution for 10 min at a room temperature. The cells were washed two more times with PBS and analyzed via a fluorescence microscope (Olympus BX50, Japan) (18).

RNA extraction and reverse transcription-polymerase chain reaction

Total RNA was isolated using a Trizol reagent (Invitrogen Co., CA, USA) following the manufacture's recommendations. Total RNA was digested with RNase-

free DNase (Roche, IN, USA) for 15 min at 37°C and repurified by the RNeasy kit according to the manufacture's protocol (Quiagen, CA, USA). cDNA was synthesized from 2 μg total RNA. By incubation at 37°C for 1 hr with AMV reverse transcriptase (Amersham) with random hexanucleotide according to the manufacture's instruction. Primers to specifically amplify the genes interested were as follows; forward, 5′-ATG-GA C-GGG-TCC-GGG-GAG-3′ and reverse, 5′-TGG-AA G-AAG-ATG-GGC-TGA-3′ for Bax gene; forward 5′-CAG-CTG-CAC-CTG-ACG-3′ and reverse, 5′-GCT-G GG-TAG-GTG-CAT-3′ for Bcl-2 gene. For the internal control gene GAPDH, forward, 5′-CGG-AGT-CAA-CGG-ATT-TGG-TCG-TAT-3′ and reverse, 5′-AGC-CT T-CTC-CAT-GGT-GGT-GAA-GAC-3′ were used. Amplification was performed in a master-cycler (Eppendorf, Hamburg, Germany) with cycles of denaturation at 94°C, annealing at 58°C, and extension at 72°C for 30 sec, respectively. The amplified PCR products were run in 1.0% agarose gels and visualized by ethidium bromide (EtBr).

Statistical analysis

The data were presented as mean±SD. Differences between the means of the individual groups were assessed by one-way ANOVA with Duncan's multiple range tests. Differences were considered significant at $p<0.05$. The statistical software package, SAS v9.1 (SAS Institute Inc., NC, USA), was used for these analyses.

RESULTS AND DISCUSSION

Fermentation properties of young radish kimchis

The control *YR-kimchi* and the sulfur *YR-kimchi* were prepared using young radishes cultivated without and with sulfur, respectively. Fermentation properties (pH, acidity, and the numbers of *Leuconostoc* sp. and *Lactobacillus* sp.) of the *YR-kimchis* were investigated during fermentation at 5°C for 6 weeks (Table 1). Control *YR-kimchi* and sulfur *YR-kimchi* showed pH 4.39 and pH 4.31 with 1.04% and 1.41% acidity at 5 weeks, respectively. In the control *YR-kimchi*, the number of *Leuconostoc* sp. increased rapidly up to the 4th week and arrived at the highest point of 4.4×10^8 CFU/mL, which was maintained until 6th week. The number of *Lactobacillus* sp. was maintained at a low level (10^6 CFU/mL) for 3 weeks, but increased rapidly up to the 4th week and arrived at 1.4×10^8 CFU/mL, which was maintained until 6th week. Sulfur *YR-kimchis* had higher levels of *Leuconostoc* sp. than the control *YR-kimchi* in the beginning of fermentation on 1~3 weeks and both *YR-kimchis* were similar in the latter period of fermentation during 4~6 weeks. The sulfur *YR-kimchi* had a lower content of *Lactobacillus* sp. than the control *YR-kimch* in the latter period of fermentation. These results were consistent with Kong et al. (19). The acidity was related to the lactic acid bacteria in *kimchi* and *Leuconostoc* sp. was predominate in the early stages of fermentation and was more responsible for the initial anaerobic state of *kimchi* than *Lactobacillus* sp. (20). That is, sulfur *YR-kimchi* has higher contents of *Leuconostoc* sp., which resulted in a refreshing taste and lower contents of *Lactobacillus* sp. during the fermentation.

Inhibitory effect and morphological changes of the *young radish kimchis*

To investigate whether the control *YR-kimchi* and sul-

Table 1. Changes in pH, acidity and *Leuconostoc* sp. and *Lactobacillus* sp. counts of the *YR-kimchis* during at fermentation at 5°C

	Week	pH	Acidity (%)	*Leuconostoc* sp.	*Lactobacillus* sp.
Control *YR-kimchi*[1)]	0	4.99	0.46	1.9×10^6	4.9×10^6
	1	4.95	0.47	6.7×10^6	6.5×10^6
	2	4.91	0.58	1.2×10^7	9.4×10^6
	3	4.87	0.73	6.6×10^7	1.6×10^7
	4	4.57	0.84	4.4×10^8	1.4×10^8
	5	4.39	0.98	3.6×10^8	9.8×10^7
	6	4.34	1.35	3.5×10^8	1.1×10^8
Sulfur *YR-kimchi*[2)]	0	5.10	0.49	4.4×10^6	5.6×10^6
	1	5.00	0.50	7.7×10^6	4.8×10^6
	2	4.91	0.56	6.0×10^7	1.3×10^7
	3	4.80	0.85	5.1×10^8	1.1×10^7
	4	4.71	0.96	4.6×10^8	1.2×10^8
	5	4.31	1.04	3.6×10^8	2.9×10^7
	6	4.28	1.41	1.9×10^8	2.4×10^7

[1)]*Young radish kimchi* prepared using young radish commonly cultivated in the soil without sulfur.
[2)]*Young radish kimchi* prepared using young radish cultivated in the soil with sulfur content of 1,818 g/m^3.

Table 2. Inhibitory effects of the *YR-kimchi* juices on the growth of HT-29 human colon cancer cells

	OD_{540} (Level of sample, μL/assay)	
	10	20
Control	0.523 ± 0.006[3)a4)]	
Control *YR-kimchi*[1)]	0.416 ± 0.010^{b} (21)[5)]	0.226 ± 0.006^{d} (57)
Sulfur *YR-kimchi*[2)]	0.291 ± 0.016^{c} (44)	0.086 ± 0.007^{e} (84)

[1)]*Young radish kimchi* prepared using young radish commonly cultivated in the soil without sulfur.
[2)]*Young radish kimchi* prepared using young radish cultivated in the soil with sulfur content of 1,818 g/m^3.
[3)]Values are mean ± SD.
[4)]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.
[5)]The values in parentheses are the inhibition rates (%).

Table 3. Inhibitory effects of methanol extracts from the *YR-kimchis* on the growth of HT-29 human colon cancer cells

	OD_{540} (Level of sample, mg/mL)	
	1.5	3.0
Control	0.521 ± 0.004[3)a4)]	
Control *YR-kimchi*[1)]	0.395 ± 0.043^{b} (24)[5)]	0.202 ± 0.020^{c} (61)
Sulfur *YR-kimchi*[2)]	0.378 ± 0.026^{b} (27)	0.108 ± 0.013^{d} (79)

[1)]*Young radish kimchi* prepared using young radish commonly cultivated in the soil without sulfur.
[2)]*Young radish kimchi* prepared using young radish cultivated in the soil with sulfur content of 1,818 g/m^3.
[3)]Values are mean ± SD.
[4)]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.
[5)]The values in parentheses are the inhibition rates (%).

fur *YR-kimchi* have anti-proliferative effects against HT-29 human colon cancer cells, cell counting and cytotoxicity assays were performed in cells treated *YR-kimchis* using an MTT assay and trypan blue staining. Treatment of the *YR-kimchi* juices showed a higher inhibitory effect on the growth of HT-29 human colon cancer cells using the MTT method, compared to the untreated controls (Table 2). In lower concentrations (10 μL/assay), the control *YR-kimchi* and the sulfur *YR-kimchi* juices showed growth inhibitory rates of 21% and 44%, respectively ($p>0.05$). In higher concentrations (20 μL/assay), the inhibitory rates were 57% and 84%, respectively ($p>0.05$). Methanol extracts from the *YR-kimchis* also led to similar results, compared to the *YR-kimchi* juices (Table 3). In the toxicity tests, methanol extracts from the *YR-kimchis* exhibited higher survival rates (about 95%) at the concentrations of 0~4.0 mg/mL using the 3T3-L1 fibroblast cells (data not shown). We used the safe range concentrations (1.5, 3.0 mg/mL) for inhibitory effect on the growth of HT-29 human colon cancer cells. In lower concentration (1.5 mg/mL), the growth inhibitory rates were 24% and 27%, respectively. The growth inhibitory rates of the control *YR-kimchi* and the sulfur *YR-kimchi* were 61% and 79% respectively, in the higher concentration (3 mg/mL) ($p>0.05$). These results suggested that preparation of *kimchi* using YR cultivated in the presence of sulfur could increase the inhibitory effect of sulfur *YR-kimchi*.

The anti-proliferative and anti-survival effects assessed by hematocytometer counts were also compared (Fig. 1). One day after seeding cells were treated with

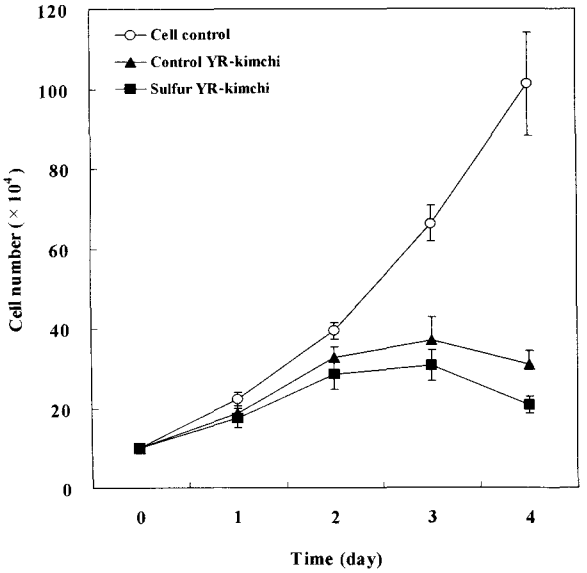

Fig. 1. Time-dependent growth inhibition by the *YR-kimchis* extract in HT-29 human colon cancer cells.
Cells were plated at 1×10^5 cells/60 mm plate, incubated for 24 hr and treated with 1.5 mg/mL of *young radish kimchis* for 4 days. The cells were trypsinized, washed with PBS and the viable cells were scored by hemocytometer counts. Each point represents the mean ± SD of three independent experiments.

1.5 mg/mL *YR-kimchi* extracts for up to 4 days. *YR-kimchis* treatment showed cell growth patterns in a time-dependent manner. At 1 day, the *YR-kimchis* showed similar cell growth numbers as the cell control; however, when time passed 2 days, there was a gradually different phase. These results demonstrated that the sulfur *YR-kimchi* was more effective on the anti-proliferative and anti-survival effects than the control *YR-kimchi*; these effects were found after 3 days. Kim et al. (9) demonstrated that YR cultivated in soil containing sulfur increased QR activity in Hepa 1c1c cells and isothiocyanate-like compounds were analyzed using HPLC. Therefore, the higher inhibitory effects of the sulfur *YR-kimchi* against the growth of HT-29 human colon cancer cells could be induced due to isothiocyanate-like compounds in YR cultivated with sulfur. Sulforaphane among isothiocyanates could be a major candidate compound occurring due to the cultivation with sulfur. Sulforaphane-like compounds were founded in the YR cultivated with sulfur (9). Sulforaphane inhibits the reinitiation of growth and decreases the cellular viability of HT-29 human colon carcinoma cells (21).

Apoptotic cell death from the *young radish kimchis*

To further characterize whether the growth inhibitory activity of *YR-kimchi* in the HT-29 cells was related to the induction of apoptosis, the presence of chromatin condensation was analyzed by fluorescent microscopy using the DNA-binding fluorescent dye DAPI (Fig. 2). In the absence of *YR-kimchis*, the HT-29 cells presented nuclei with homogeneous chromatin distribution. In the presence of 1.5 mg/mL *YR-kimchi*, no significant changes in chromatin distribution were observed in the nuclei of the HT-29 cells (data not shown). However, treatment with 3 mg/mL of *YR-kimchis* induced chromatin condensation and nuclear fragmentation, suggesting the presence of apoptotic cells. Condensation and formation of apoptotic bodies, a characteristic of apoptosis, were shown in the cells cultured with sulfur *YR-kimchi*, but very few were shown in the control *YR-kimchi*. These results suggested that the sulfur *YR-kimchi* was more effective in inducing the condensation and formation of apoptotic bodies, when compared to the control *YR-kimchi*. Treatment with sulfur *YR-kimchi* resulted in a growth inhibition coupled with the characteristic morphological features of apoptosis. It also could be induced due to isothiocyanate and sulforphane -like compounds in the young radishes cultivated with sulfur.

Induction of cancer cell death through an apoptotic pathway of sulforaphane is already known. The apoptotic pathway involves typical biochemical and ultrastructural modifications related to programmed cell death (22).

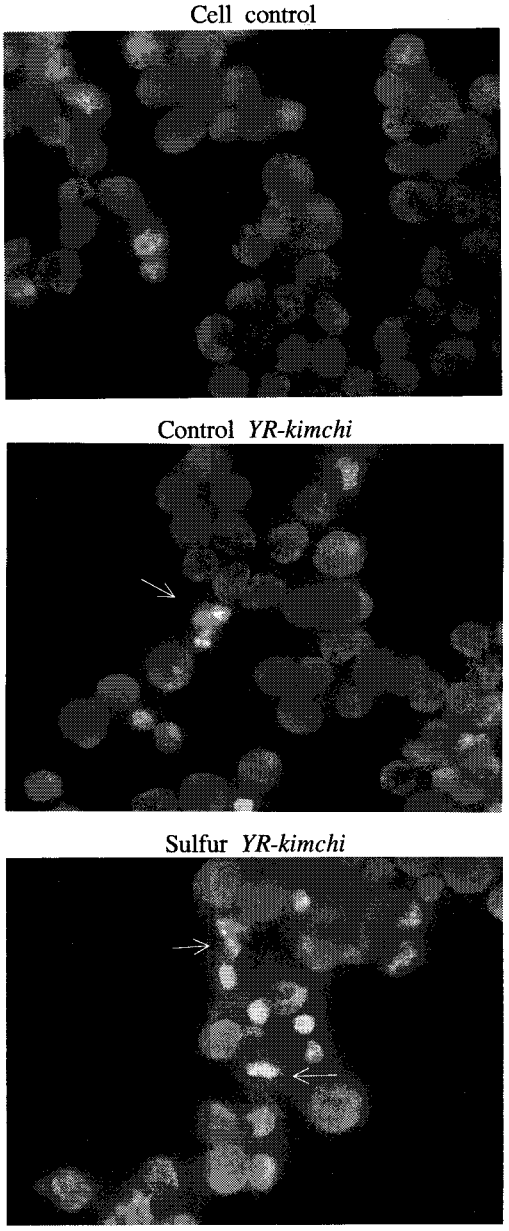

Fig. 2. Induction of apoptosis by *YR-kimchis* extract in HT-29 human colon cancer cells.
Cells were incubated with *young radish kimchi* for 48 hr and then stained with DAPI. After 10 min incubation at a room temperature, the cells were washed with PBS and photographed with a fluorescence microscope using blue filter. Magnification, ×400.

Expression of Bax and Bcl-2

To better determine which type of apoptotic pathway was induced by *YR-kimchis*, after 48 hr incubation with treatment of 1.5 mg/mL and 3 mg/mL of *YR-kimchi* methanol extracts and extracts from HT-29 cells were examined by RNA extraction and a reverse transcription-polymerase chain reaction (RT-PCR) using Bcl-2 and Bax primers (Fig. 3). In the presence of 1.5 mg/mL *YR-kimchis* extract, the expression level of anti-apoptotic

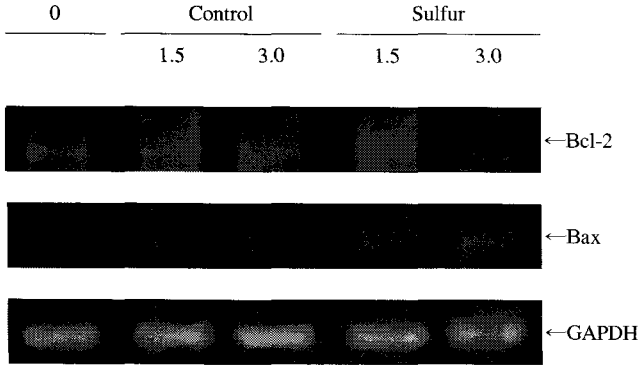

Fig. 3. Inhibition of Bcl-2 induction of Bax by *YR-kimchis* extract in HT-29 human colon cancer cells.
Cells were incubated with *young radish kimchis* for 48 hr and total RNA was isolated and RT-PCR was performed using indicated primers. The amplified PCR products were run in 1% agarose gel and visualized by EtBr staining. GAPDH was used as a house-keeping control gene.
Control: Control *YR-kimchi*, Sulfur: Sulfur *YR-kimchi.*

gene Bcl-2 did not significant change, but the treatment of 3 mg/mL of the sulfur *YR-kimchi* extract markedly downregulated the expression of Bcl-2 mRNA. This suggests that *YR-kimchis* extract induces apoptosis in HT-29 cells via a Bcl-2 dependent pathway. There was no difference in the expression level of apoptotic gene Bax in the cells treated with or without *YR-kimchi*. One of the best characterized regulators of apoptosis is the Bcl-2 family. Bcl-2 is an intracellular suppressor of apoptosis, which functions by heterodimerizing with its pro-apoptotic relative Bax (23-25). In our study, we observed that apoptosis induced by sulfur *YR-kimchi* in HT-29 human colon cancer cells was related to the decreased expression of the anti-apototic Bcl-2 protein and mRNA. These results suggested that *YR-kimchi* cultivated in the presence of sulfur, which contains abundant active compounds, could increase the anti-cancer activity of *YR-kimchi.*

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