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Antimutagenic and Anticancer Effects of Leaf Mustard and Leaf Mustard Kimchi

Yong-Taek Kim[1], Boh-Kyung Kim[2] and Kun-Young Park[2†]

[1]R&D Center, Daesun Distiling Co. Ltd., Busan 607-120, Korea
[2]Department of Food Science and Nutrition, and Kimchi Research Institute, Pusan National University, Busan 609-735, Korea

Abstract

In this study, we investigated antimutagenic and anticancer activities of leaf mustard (LM, *Brassica juncea*) and leaf mustard kimchi (LMK) during their fermentation period. Methanol extracts were prepared from raw mustard, brined leaf mustard in 10% Gueun salt solution for 2 hrs, leaf mustard fermented at 15°C for 5 days after brined in 10% Guenun salt solution for 2 hrs (Fr-LM), fresh leaf mustard kimchi (Fresh-LMK) and optimally ripened leaf mustard kimchi fermented at 5°C for 30 days (OR-LMK). OR-LMK showed the strongest inhibitory activities against the mutagenicities induced by aflatoxin B_1 in *Salmonella* Typhimurium TA100. LMs and LMKs inhibited the survival or growth of AGS human gastric adenocarcinoma cells and HT-29 human colon carcinoma cells in MTT assay and growth inhibition test. Among the extracts, OR-LMK and FR-LM exhibited strong antiproliferative effect against cancer cells, especially HT-29 cells. DAPI staining assay showed that OR-LMK induced apoptosis cell death of HT-29 cells in a dose-dependent manner. These results suggest that leaf mustards and leaf mustard kimchi have chemopreventive activities.

Key words: leaf mustard, leaf mustard kimchi, AGS human gastric adenocarcinoma cell, HT-29 human colon carcinoma cell, apoptosis

INTRODUCTION

Kimchi is a traditional Korean fermented dish made of vegetables seasoned with salt, spices and other condiments. The raw materials of kimchi are mainly green-yellow vegetables that are known to exhibit antimutagenic and anticancer activities (1-4). Kimchi and its ingredients have been investigated to determine chemopreventive properties.

Leaf mustard (*Brassica juncea*), a cruciferous cormophyte vegetable, is originated from China and is widely used as a food spice and folk medicines on a worldwide basis (5). In Korea, it is used for both food itself and the major ingredient of kimchi, a traditional fermented vegetable food. Kimchi including leaf mustard has recently attracted a lot of attention as a functional food for health maintenance and disease prevention. It contains a glucosinolate of allylisothiocyanate called sinigrin which gives its characteristic spicy flavor, and the active myrosinase leads to the creation of sulfurous substances and other related compounds. Of these materials, the lactic acid bacteria and other microoragnisms in the leaf mustard kimchi (LMK) have antibacterial effects, which delay the fermentation of LMK (6,7). Leaf mustard contains large quantities of dietary fiber, chlorophylls, β-carotene, and ascorbic acid which are known to have deoxidation characteristic. The antioxidative activities of crude chlorophylls and carotenoids of LMK against autoxidation of linoleic acid are much higher than those of α-tocopherol (8,9). Glucosinolates, thioglucosides isolated from leaf mustard, are biologically active compounds (10). Furthermore, leaf mustard has an antiatherogenic effect, reducing the level of plasma cholesterol and also increasing the level of high-density lipoprotein-cholesterol. In this study, we examined antimutagenic and anticancer activities of LMs and LMKs during their fermentation period.

MATERIALS AND METHODS

Preparations of mustard leaves and leaf mustard kimchis

Leaf mustard from Dolsan, Jeon-Nam, Korea were obtained. Purchased at Bujun market in Busan, Korea were garlic, ginger, green onion, red pepper, red pepper powder and anchovy juice. Raw leaf mustard (Raw-LM), leaf mustard brined in 10% Gueun salt (roasted salt) solution for 2 hrs (Br-LM), leaf mustard fermented at 15°C for 5 days after brined in 10% Guenun salt solution for 2 hrs (Fr-LM), fresh leaf mustard kimchi (Fresh-LMK)

†Corresponding author. E-mail: kunypark@pusan.ac.kr
Phone: +82-51-510-2839, Fax: +82-51-514-3138

and optimally ripened leaf mustard kimchi fermented at 5°C for 30 days (OR-LMK) were freeze-dried and powdered. LMKs were prepared based on the standardization of manufacturing method (11). Twenty folds of methanol was added to powdered samples and extracted three times by shaking. The methanol extracts were evaporated using rotary vacuum evaporator (Buchi 461, Switzerland) and dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Co., USA) for use.

Antimutagenicity test

A modified plate incorporation test (12) in which 30 mins liquid preincubation of *Salmonella* Typhimurium TA100 with the test compounds was employed to determine the antimutagenic effects of LMs and LMKs extracts on mutagenesis of AFB_1 (1). In the preincubation test, 0.5 mL of S9 mix (prepared from S9 fraction of the liver of Spargue-Dawley rats treated with Arclor 1254) was distributed into sterile capped tubes in an ice bath and then 0.1 mL of the testers from overnight culture ($1\sim2\times10^9$ cells/mL), 0.1 mL of the test compound (50 μL of mutagen and 50 μL of the LMs and LMKs extracts) were added. The tubes were gently vortexed and preincubated at 37°C for 30 min. Two mL of the top agar kept at 45°C was added to each tube and vortexed for 3 seconds. The resulting entire mixture was overlaid on the minimal agar plates. The plates were incubated at 37°C for 48 hrs and then the revertants bacterial colonies on each plate were counted (13).

Cell culture

AGS human gastric adenocarcinoma cells and HT-29 human colon carcinoma cells were procured from the Korea Cell Line Bank (Seoul, Korea) 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 two or three times each week.

MTT assay

The anti-proliferative effect of LMs and LMKs were assessed by MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide, Sigma] assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzyme. After treatment with LMs and LMKs for 48 hrs, MTT (5 mg/mL in PBS) solution was added to each well. After an additional incubation for 3 hrs, the supernatant medium was carefully removed from the wells. Formazan crystals were dissolved by adding dimethylsulfoxide (DMSO, Amresco, Solon, Ohio, USA). The optical densities were recorded on a ELISA reader at 540 nm (14).

Growth inhibition study

The cultured cancer cells (2×10^4 cells/mL) were plated in 24 well plates and incubated for 24 hrs. Growing cells in culture medium enriched with 10% FBS for 6 days were cultured on the presence or absence of LMs and LMKs. The cells were trypsinized, washed with phosphate-buffered saline (PBS) and counted by hemocytometer.

Fluorescent detection of apoptotic nucleus

Untreated control and LMK treated HT-29 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 4,6-diamidino-2-phenylindole (DAPI, Sigma) solution for 10 min at a room temperature. The cells were washed two more times with PBS and analyzed with a fluorescence microscope (Olympus BX50, Japan) (15). Nuclei were considered to have the normal phenotype when glowing brightly and homogenously. Apoptotic nuclei were identified by condensed chromatin gathering at the periphery of the nuclear membrane or total fragmented morphology of nuclear bodies.

Statistical analysis

The data were presented as mean±SEM. 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

Antimutagenic effects

The methanol extracts of Raw-LM, Br-LM, Fr-LM, Fresh-LMK and OR-LMK showed antimutagenic effect against AFB_1 in the *Salmonella* Typhimurium TA100 strain (Table 1). Most of the LMs and LMKs exhibited strong antimutagenic effects. At 1.25 mg/plate, the inhibition rates of LMs and LMKs for AFB_1 were 47~56%. The mutagenicity mediated by AFB_1 was inhibited by 59~73% in the presence of 1.25 mg/plate of LMs and LMKs extracts. Park et al. (16) reported that the extract from kimchi inhibited the mutagenicity against AFB_1 in *Samonella* Typhimurium TA100 and Cho (17) reported optimally rippened kimchi exhibited strong antimutagenic and anticancer effects. Leaf mustard kimchi against AFB_1 in *Samonella* Typhimurium TA100 also led to the similar results. Kim (18) isolated antioxidative active compound isorhamnetic diglucoside from leaf mustard and Kang (19) isolated isorhamnetin 3-O-β-glu-

Table 1. Inhibitory effect of methanol extract from making step of mustard leaf (ML) *kimchi* on the mutagenicity induced by AFB_1 (0.46 μg/plate) in *Salmonella* Typhimurium TA100

Sample	Revertants/plate	
	0.63 mg/plate	1.25 mg/plate
Spontaneous	127±18	127±18
Control(AFB_1)	1331±37[a]	1331±37[a]
AFB_1+Raw-LM[1)]	765±13[b] (47)[6)]	594±27[b] (61)
+Br-LM[2)]	776±22[b] (46)	617±26[b] (59)
+Fr-LM[3)]	670±12[d] (55)	510±17[c] (68)
+Fresh-LMK[4)]	724±31[c] (50)	578±26[bc] (63)
+OR-LMK[5)]	651±14[d] (56)	454±20[d] (73)

[1)]Raw leaf mustard.
[2)]Leaf mustard in brined 10% Gueun salt solution for 2 hrs.
[3)]Leaf mustard fermented at 15°C for 5 days after brined in Gueun salt for 2 hrs.
[4)]Fresh leaf mustard kimchi (pH 5.7, acidity 0.25%).
[5)]Optimally ripened leaf mustard kimchi fermented at 5°C for 30 days (pH 4.3, acidity 0.85%).
[6)]Inhibition rate (%). [a~d]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

copyronoside as an antimicrobial active compound. The high antimutagenic effects of LMs and LMKs extracts probably results from the leaf mustard, the major source of leaf mustard kimchi.

MTT assay

To investigate anti-proliferative effects of LMs and LMKs against AGS human gastric adenocarcinoma cell and HT-29 human colon cancer cell, cell counting and cytotoxicity assays were performed in cells treated LMs and LMKs using MTT assay. As the concentrations were increased, the growth of the AGS cells were increasingly retarded in a dose-dependent manner. In the higher concentration (100 μg/plate), Fr-LM and OR-LMK showed the growth inhibitory rates of 67% and 78% on the growth of AGS cells, respectively (Fig. 1, $p<0.05$). Treatment of LMs and LMKs also led to the similar results on the growth of HT-29 human colon adenocarcinoma cell using MTT method, compared to untreated controls (Fig. 2, $p<0.05$). Most of the LMs and LMKs exhibited stronger anti-proliferative effect against HT-29 cells than AGS cells. In the higher concentration (100 μg/plate), the growth inhibitory rates on HT-29 cells were 94% and 73%, respectively, by the treatment with Fr-LM and OR-LMK. These results indicate that leaf mustard and leaf mustard kimchi have strong anti-proliferative effects against colon cancer cells than gastric cancer cells. The antioxidative activities of crude chlorophylls and carotenoids of leaf mustard kimchi against autoxidation of linoleic acid are much higher than those of α-tocopherol. There is also a report vitamin

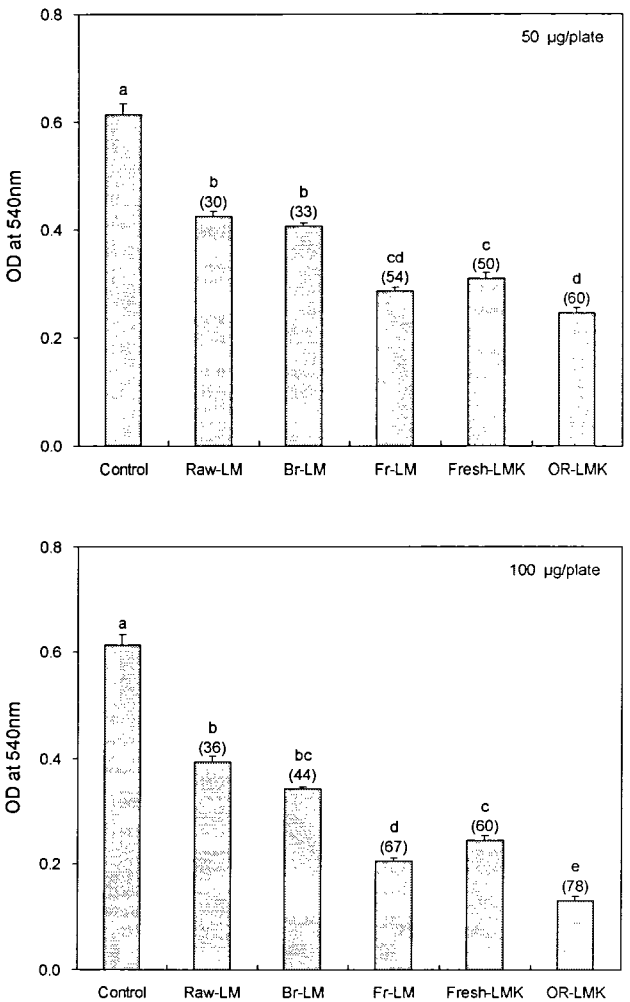


Fig. 1. Inhibitory effect of methanol extract of leaf mustard (LM) and leaf mustard kimchi on the growth of AGS human gastric adenocarcinoma cells in MTT assay.
Raw-LM: Raw leaf mustard, Br-LM: Leaf mustard brined in 10% Gueun salt solution for 2 hrs, Fr-LM: Leaf mustard fermented at 15°C for 5 days after brined in Gueun salt for 2 hrs, Fresh-LMK: Fresh leaf mustard kimchi (pH 5.7, acidity 0.25%), OR-LMK: Optimally ripened leaf mustard kimchi fermented at 5°C for 30 days (pH 4.3, acidity 0.85%). The values in parentheses are the inhibition rates (%). [a~e]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

C and β-carotene levels in this species are notably high among various chinese vegetables. In addition, thioglucosides isolated from leaf mustard, known as glucosinolates, are reported to be biologically active compounds. Accordingly, the higher inhibitory effects of OR-LMK against the growth of HT-29 human colon cancer cells can be induced by due to active compounds in leaf mustard. The anti-proliferative and anti-survival effects assessed by hematocytometer counts were also compared (Fig. 3). One day after seeding cells were treated with LMs and LMKs extracts for up to 6 days.

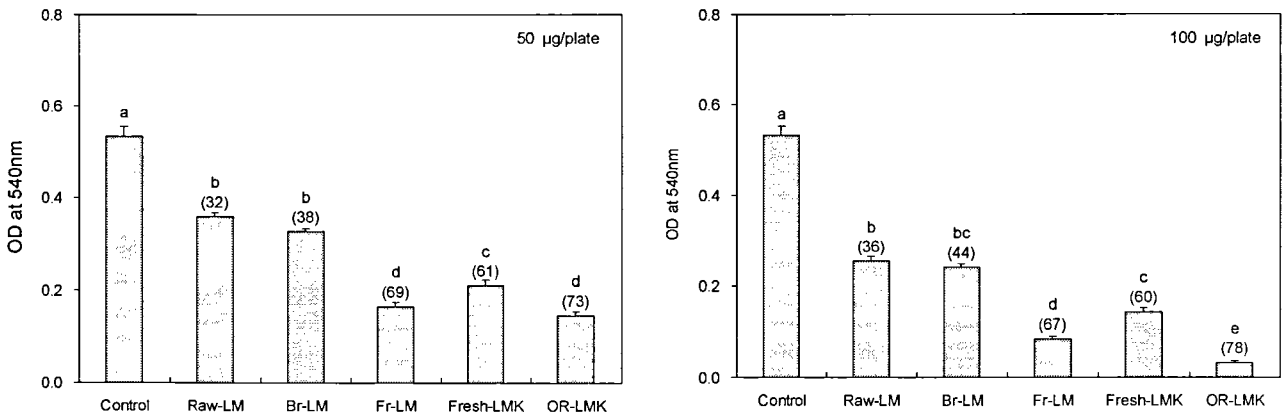


Fig. 2. Inhibitory effect of methanol extract of leaf mustard and leaf mustard kimchi on the growth of HT-29 human colon carcinoma cells in MTT assay. The abbreviated names are same the footnote of Fig. 1. The values in parentheses are the inhibition rates (%). $^{a\sim e}$Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

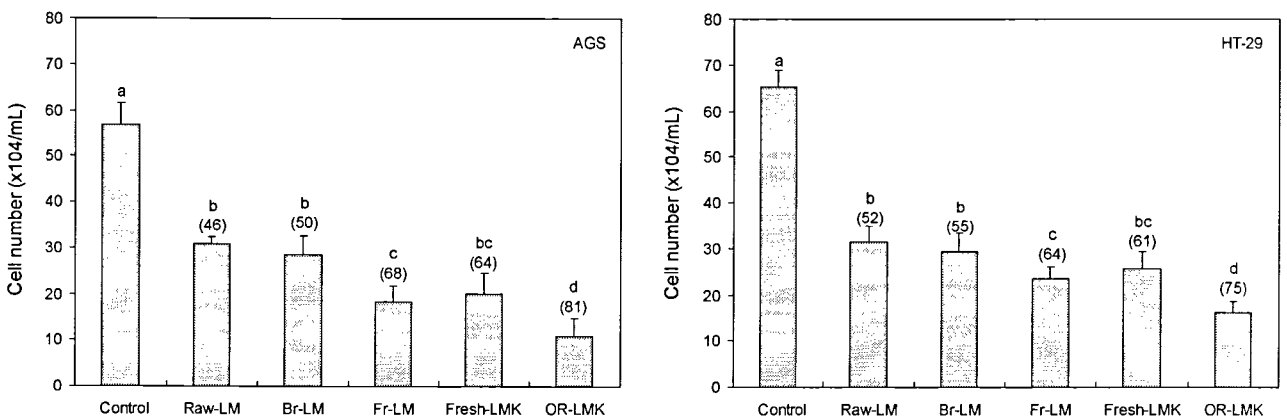
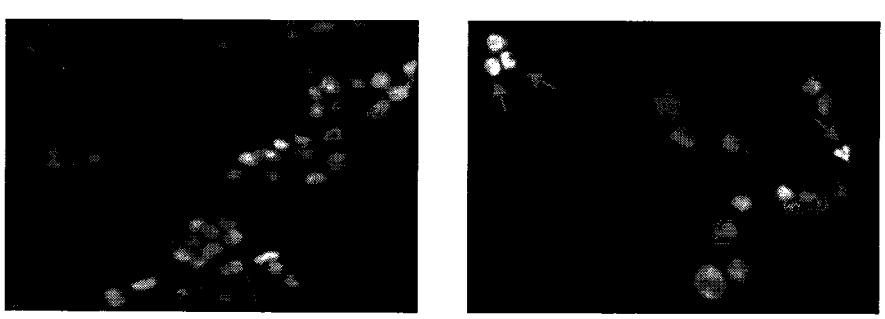


Fig. 3. Growth inhibitory effect of methanol extract from leaf mustard and leaf mustard kimchi against AGS human gastric adenocarcinoma cells and HT-29 human colon carcinoma cells after 6 days of incubation at 37°C. The abbreviated names are same the footnote of Fig. 1. The values in parentheses are the inhibition rates (%). $^{a\sim d}$Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.



Control Leaf mustard kimchi

Fig. 4. Appearance of apoptotic body in HT-29 human colon carcinoma cells treated with methanol extract from optimally ripened leaf mustard kimchi (fermented at 15°C for 6 days) after 24 hrs of incubation at 37°C. ×400, 500 μg/mL.

Growth inhibition showed similar results. These results demonstrated that the OR-LMK was more effective on the anti-proliferative and anti-survival effects than Fresh-LMK.

DAPI staining

Apoptosis has been shown to be important in a number of physiological processess such as embryonal development, immune regulation, and tissue homeostasis. It is characterized by chromatin condensation, cell shrinkage, nuclear fragmentation and formation of membrane bound apoptotic bodies. Hallmark changes of chromatin condensation and nuclear fragmentation are readily visible by DAPI staining. To further characterize whether the

growth inhibitory activity of OR-LMK in 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. 4). In the absence of OR-LMK, HT-29 cells presented nuclei with homogeneous chromatin distribution. In the presence of 500 μg/mL OR-LMK induced chromatin condensation and nuclear fragmentation, suggesting the presence of apoptotic cells. The results suggested that the OR-LMK was more effective on inducing the condensation and formation of apoptotic bodies, which means LMs and LMKs have anti-cancer activity. In brief, fermented leaf mustard and leaf mustard kimchi have more anti-proliferative, anti-survival effects than fresh leaf mustard and leaf mustard kimchi.

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