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# The Inhibitory Effects of Leek (Buchu) *Kimchi* Extracts on MCA-induced Cytotoxicity and Transformation in C3H/10T1/2 Cells

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## Abstract

**The anticarcinogenic effects of the methanol extracts from leek (buchu in Korean) *kimchi* and Korean cabbage *kimchi* were evaluated using cytotoxicity and transformation tests in C3H/10T1/2 cells. Various fractions of the 6-day fermented leek *kimchi* at 15°C, hexane, methanol soluble, dichloromethane, ethyl acetate, butanol and aqueous fraction, were also studied in the same system. The inhibitory effect of the leek *kimchi* (6-day fermented at 15°C, pH 4.29) was higher than that of the Korean cabbage *kimchi* (4-day fermented at 15°C, pH 4.21) on the cytotoxicity induced by 3-methylcholanthrane (MCA) in the C3H/10T1/2 cell system. While the MCA-treated culture (control) formed 21.0 foci of type II plus III in C3H/10T1/2 cells, 100 μg/ml of the methanol extract of the leek *kimchi* and that of the 4-day fermented Korean cabbage *kimchi* treated cultures reduced the formation of type II plus III foci to 7.4 and 11.3, respectively. Among the fractions of the leek *kimchi*, the dichloromethane fraction showed the highest inhibitory effect on MCA-induced cytotoxicity in C3H/10T1/2 cells. Fifty μg/ml of dichloromethane fraction from the leek *kimchi* suppressed the MCA-induced cytotoxicity by 77%. On the transformation test using MCA, the dichloromethane fraction considerably reduced the formation of type II plus III foci, especially type III foci. When 50 μg/ml of dichloromethane fraction from the leek *kimchi* was treated, the numbers of type III foci mediated by MCA were decreased to 1.7 compared to 10 for the control. These results indicate that leek *kimchi* has stronger anticarcinogenic effects than Korean cabbage *kimchi* and that the dichloromethane fraction of the leek *kimchi* may contain the major compound(s) that suppress the carcinogenesis in the eukaryotic cells.**

**Key words:** leek *kimchi*, C3H/10T1/2 cells, cytotoxicity, transformation

## INTRODUCTION

*Kimchi* is a Korean traditional fermented vegetable food with salt, spices and other condiments. *Kimchi* has many beneficial effects, including the prevention of colon cancer, the improvement of bowl movement, constipation and the maintenance of healthy diets for Koreans (1,2). Almost all vegetables cultivated in Korea are used to make *kimchi*. There is about 180 kinds of *kimchi* depending on the main vegetables and the proportion of the ingredients used (1). In these various *kimchi*, leek *kimchi* is a major traditional, special *kimchi* in Kyungsang province, Korea. Leek (Buchu in Korean, *Allium tuberosum* L.) *kimchi* is prepared with large quantities of red pepper powder and pickled anchovy; it is known as a good side dish because of the unique flavor of leeks and its hot taste (3-5).

Leeks, the major ingredient in leek *kimchi*, have been used as a food or drug for the treatment of abdominal pain, diarrhea, hematemesis, snakebite and asthma in traditional folk remedies in Korea (6). Leeks belonging to the *Allium* genus contain large amounts of potentially chemopreventive compounds such as vitamins (7), flavonoids (8-11), and organosulfur compounds (12-17). In addition, several studies indicated that high consumption of leek was associated with a reduced risk for colorectal cancer (18-20). In Japan, a decreased risk for colorectal cancer as well as for the subset of cancers in the lower part of the rectum (less than 5 cm from the anus) was observed with high consumption of Japanese leeks. In a preceeding study in Hawaii, a lower risk was found for colorectal cancer in Hawaiian Japanese with high consumption of leeks (18,19).

The most popular *kimchi* is Korean cabbage *kimchi*, which has already been demonstrated to have antimutagenic and anticancer activities (20-33). It has been known that vitamin C, β-carotene, dietary fibers, lactic acid bacteria, β-sitosterol, allyl compounds, and other phytochemicals seem to be responsible for such putative activities (7-17,27,31,34,35). In particular, the phytochemical in main vegetables may play a significant role in the antimutagenic and anticancer activities of *kimchi*. Although leek *kimchi* has abundant phytochemicals such as vitamins, flavonoids, and organosulfur compounds, the protective action of leek *kimchi* against cancer has been poorly elucidated until now. In this study, we investigated the inhibitory effects of leek *kimchi* extracts on MCA-induced cytotoxicity and transformation in C3H/10T1/2 cells as compared to optimally ripened Korean cabbage *kimchi*. Various fractions of the optimally ripened leek *kimchi* (6-day fermented at 15°C) such as hexane, methanol soluble, dichloromethane, ethyl acetate, butanol and aqueous fraction, were also evaluated in the same system.

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## MATERIALS AND METHODS

### Preparations of *kimchi* and extracts

In the preparation of leek *kimchi*, the leeks were cut into 2 pieces and soaked in 20% salt solution for 20 min at room temperature, then rinsed with water twice. For Korean cabbage *kimchi*, Korean cabbage was cut into 4 pieces and soaked in 10% brine for 12 hours at 10°C and then rinsed with tap water. The ingredient ratios of leek *kimchi* and Korean cabbage *kimchi* are shown in Table 1. The final salt concentrations of leek *kimchi* and Korean cabbage *kimchi* were 2.3% and 2.5%, respectively. Leek (from Kimhae, Korea), Korean cabbage (from Kimhae, Korea), garlic, radish, spring onion, ginger, red pepper powder, fermented anchovy juice (Miwon, Co.), salt (chunil salt for Korean cabbage *kimchi*, gueun salt from Sannaedle, Co. for leek *kimchi* ), sugar and glutinous rice powder were purchased from Bujun market in pusan, Korea. The prepared leek *kimchi* and Korean cabbage *kimchi* were put into pint jars and the lid closed tightly. Leek *kimchi* was fermented at 15°C for 0, 6 and 9 days, and Korean cabbage *kimchi* was fermented at 15°C for 4 days. The initial pH of leek *kimchi* was 5.19 and then decreased to 4.05 after 9 days fermentation. Optimally ripened *kimchi*, the 6 day-fermented leek *kimchi* and 4 day-fermented Korean cabbage *kimchi* showed pH of 4.29 and 4.21, respectively. After fermentation, the *kimchi* samples were freeze dried and powdered, and 20-folds of methanol was added to the powdered samples and extracted 3 times. The methanol extracts were evaporated using a rotary vacuum evaporator (Buchi 011 & 461, Switzerland), concentrated, then dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Co., USA) for experiment.

### Fractionation of leek *kimchi*

Freeze dried and powdered leek *kimchi* (4 kg, fermented for 6 days at 15°C, pH 4.3) was extracted with hexane (40 L), three times, by shaking for 8 hours and then taken as a hexane fraction (180 g). The residues were extracted with methanol by the same method as hexane extraction. After concentration, a methanol soluble fraction (MSF, 800 g) was taken. MSF was fractionated into dichloromethane fraction (96 g) and an aqueous layer using dichloromethane-methanol-$H_2O$ (10 : 1 : 9, v/v/v). Further fractionation of the aqueous phase with ethyl acetate resulted in an ethyl acetate fraction (16 g) and an aqueous layer fractionated into a butanol fraction (144 g) and an aqueous fraction (504 g). Each fraction was dried by a rotary vaccum evaporator and then dissolved in DMSO.

**Table 1.** Ingredient ratios of leek *kmchi* and Korean cabbage *kimchi*

| Ingredients | Leek *kimchi* (g) | Korean cabbage *kimchi* (g) |
|---|---|---|
| Leek | 100.0 | - |
| Korean cabbage | - | 100.0 |
| Red pepper powder | 9.0 | 3.5 |
| Crushed garlic | 5.0 | 1.4 |
| Crushed ginger | 2.0 | 0 6 |
| Anchovy juice | 13.0 | 2.2 |
| Sugar | 2.0 | 1.0 |
| Radish | - | 13.0 |
| Green onion | - | 2.0 |
| Glutinous rice paste | 13.0 | - |
| Final salt concentration | 2.3% | 2.5% |

### Carcinogens/Chemicals

3-Methylcholanthrene (MCA) and Giemsa stain were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Eagle's basal medium, fetal calf serum (FCS), 0.05% trysin-0.02 EDTA, penicillin-streptomycin were obtained from Gibco Chemical Co. (Grand Island, NY, USA).

### Cells

C3H/10T1/2 cells, mouse embryo fibroblast cells, were obtained from Japanese Cell Line Collection (Tokyo, Japan). The medium used for the cells was Eagle's basal medium supplemented with 10% fetal calf serum (FCS) and 100 unit/ml penicillin-streptomycin. Cultures were maintained in a humidified atmosphere of 5% $CO_2$ at 37°C. A medium change is made on the 5th day after seeding. The cells were transferred every 10 days, using phosphate buffered saline (PBS) and 0.05% trypsin-0.02% EDTA, and new flasks were seeded with $5\times10^4$ cells in 5 ml of medium each (36).

### Cytotoxicity assay

Cytotoxicity was determined by measuring the inhibition of colony formation (37,38). C3H/10T1/2 cells were plated at $2\times10^3$ cells/60mm dish. Twenty-four hours at 37°C under 5% $CO_2$ after seeding, the test compounds were added to serum-free Eagle's basal medium. The cells were treated with MCA (10 µg/ml), and in the absence or presence of *kimchi* samples. Following treatment for a period of 48 hours, the medium was changed, and the cells were allowed to grow an additional 7 days in the medium supplemented with 10% FCS. Surviving colonies were fixed with methanol, stained with Giemsa stain and counted. Cytotoxicity was expressed as the number of surviving colonies on the treated dishes divided by the number of surviving colonies on the control dishes.

### Transformation test

The transformation experiment was performed by a modified method of Reznikoff et al. (39). C3H/10T1/2 cells were seeded at the density of $2\times10^3$ cells in a 60 mm dish (10 dishes/group) Twenty-four hours after seeding, the cells were loaded with serum-free Eagle's basal medium containing MCA (10 µg/ml) and the *kimchi* samples or PBS (the control) for 48 hours. The medium was changed to a complete medium supplemented with 10% FCS. Subsequently, the medium was changed twice weekly until the cells reached confluence, then once a week. At the 6th week, cells in the dishes were fixed with methanol and stained by Giemsa, and the morphologically transformed foci were counted. Transformed foci were classified as three types (type Ⅰ, Ⅱ, and Ⅲ), by the morphological criteria initially established by Reznikoff et al. (39).

### Statistical analysis

Data analyses were performed using the statistical analysis system (SAS). Analysis of variance was used to determine possible differences among groups; Duncan's multiple range test was used for post hoc comparisons if significant group differences were found.

## RESULTS AND DISCUSSION

### Anticarcinogenic effects of leek *kimchi* extracts

C3H/10T1/2 cells have been widely used to study mechanisms of neoplastic transformation in mammalian cells and as a target indicator cell system to screen industrial chemicals for carcinogenicity (40). Since the cytotoxicity, mutation, and transformation of a chemical can be studied together in one cell system (39,41,42), the C3H/10T1/2 cell is believed to be very effective in investigating the mechanisms of genotoxicity of the chemicals. Using this C3H/10T1/2 cell system, we studied the effects of leek *kimchi* extracts on the cytotoxicity and cell transformation induced by the carcinogen.

Table 2 shows an inhibitory effect of the methanol extracts from leek *kimchi* and korean cabbage *kimchi* on MCA-induced cytotoxicity in C3H/10T1/2 cells. The inhibitory effect of leek *kimchi* was higher than that of Korean cabbage *kimchi*. 100 μg/ml of methanol extracts from 6 day-fermented leek *kimchi* (pH 4.29) and 4 day-fermented Korean cabbage *kimchi* (pH 4.21) reduced MCA-mediated cytotoxicity by 46% and 35%, respectively. Among the leek *kimchi* samples, the 6-day fermented (optimally ripened) leek *kimchi* exhibited a strong inhibitory activity of the cytotoxicity induced by MCA. Using the transformation test with C3H/10T1/2 cells, we investigated the protective effect of leek *kimchi* extracts against chemical carcinogenesis. No spontaneous morphological transformation has been observed in C3H/10T1/2 cells. In the MCA-treated cultures, discrete dense foci were seen at 6 weeks, after confluence had been attained. Foci are defined as an area of increased cell density and/or altered cell morphology in a confluent monolayer. Transformation foci formed by MCA in these cells were separated into type I, II and III foci (Fig. 1). Reznikoff et al. (39) reported that type I was not malignant altered foci, but type II and III were scored as malignantly transformed because a high percentage (50% for type II and over 85% for type III) of these foci produced tumors after these cells are inoculated subcataneously into irradiated C3H mice. As shown in Table 3, the number of transformed foci (type II and III) decreased after treatment of leek *kimchi* and Korean cabbage *kimchi* extracts. While the MCA-treated culture formed 21.0 foci of type II+III in C3H/10T1/2 cells, 100 μg/ ml of the methanol extract of 6-day fermented leek *kimchi* and that of the 4-day fermented Korean cabbage *kimchi* treated cultures produced 7.4 and 11.3, respectively ($p<0.05$) in the number of type II + III foci. The above data suggests that leek *kimchi* might have an anticarcinogenic effect by inhibiting the carcinogen-mediated cytotoxicity and neoplastic transformation in eukaryotic cells. We previously reported that leek *kimchi* had stronger antimutagenic and *in vitro* anticancer effects than Korean cabbage *kimchi* and the high inhibition rate of the leek *kimchi*

**Table 2.** Cytotoxicity of C3H/10T1/2 cells treated with 3-methylcholanthracene (MCA, 10 μg/ml) and MCA plus methanol extracts from leek *kimchi* (LK) and Korean cabbage *kimchi* (KCK) which were fermented at 15°C

| Sample | Number of cell colony | |
|---|---|---|
| | 50 μg/ml | 100 μg/ml |
| MCA (control) | $104\pm5^{a}$ (1.00)[1)] | |
| MCA+0-day fermented LK (pH 5.19) | $134\pm2^{c}$ (1.29) | $146\pm2^{cd}$ (1.40) |
| 6-day fermented LK (pH 4.29) | $136\pm2^{c}$ (1.29) | $151\pm2^{d}$ (1.46) |
| 9-day fermented LK (pH 4.05) | $131\pm1^{c}$ (1.26) | $144\pm3^{bc}$ (1.38) |
| 4-day fermented KCK (pH 4.21) | $123\pm3^{b}$ (1.18) | $140\pm2^{b}$ (1.35) |

[1)]2000 cells/60 mm dish were seeded and incubated for 24 hrs, and then MCA and kimchi fractions were treated for 48 hrs. Following treatment, the cells were allowed to grow an additional 7 days. Surviving colonies, containing more than 20 cells, were fixed with methanol, stained with Giemsa, and scored the colonies Cytotoxicity is expressed as the number of surviving colonies on the treated dishes divided by the number of surviving colonies on the control dishes.

[a~c]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

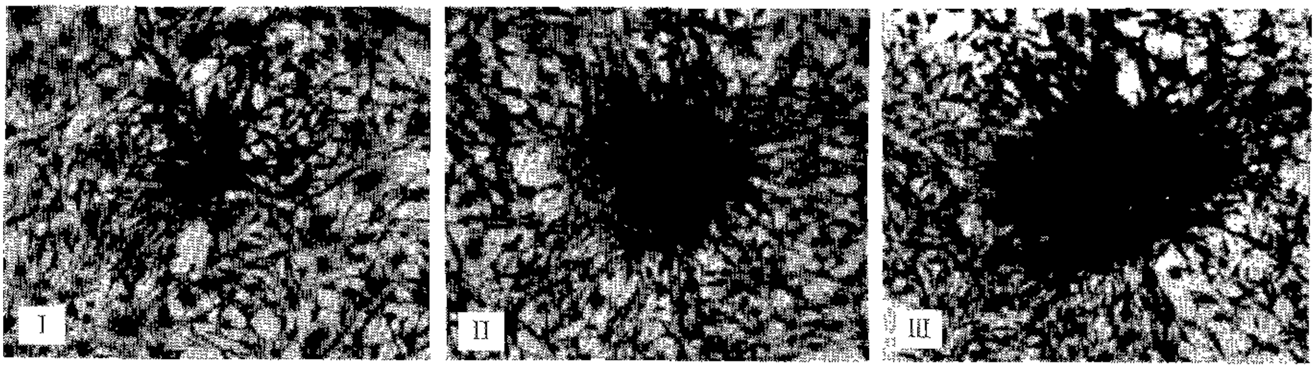


**Fig. 1.** Photomicrographs of 3 types of foci formed in the transformation test[1)] on C3H/10T1/2 cells treated with 3-methylcholanthrene (MCA, 10 μg/ml) (×40). I : Type I foci, II : Type II foci, III : Type III foci.

[1)]2000 cells were seeded in 60 mm/dishes and incubated for 24 hrs, and then MCA was treated for 48 hrs. After treatment, the medium was changed. Subsequently, the medium was changed at weekly intervals and, at 6 weeks, the dishes were fixed and stained. The foci were classified as type I, II and III using the morphological criteria.

**Table 3.** The inhibitory effect of methanol extracts from leek *kimchi* and korean cabbage *kimchi* which were fermented at 15°C for 6 days (pH 4.29) and 4 days (pH 4.21), respectively, on the transformation of C3H/10T1/2 cells treated with 3-methylcholanthracene (MCA, 10 μg/ml)[1)]

| Treatment | Conc. (μg/ml) | Total number | | | |
|---|---|---|---|---|---|
| | | Type I foci | Type II foci | Type III foci | Type II +III foci |
| MCA (control) | | 11.7 | 11.3 | 9.7 | 20.0 |
| MCA+Leek *kimchi* | 50 | 8.3 | 7.0 | 4.7 | 11.7 |
| | 100 | 4.7 | 4.7 | 2.7 | 7.4 |
| MCA+Korean cabbage *kimchi* | 50 | 8.3 | 8.7 | 8.0 | 16.7 |
| | 100 | 7.7 | 6.3 | 5.0 | 11.3 |

[1)]2000 cells were seeded in 60 mm/dishes, 10 dishes/group and incubated for 24 hrs, and then MCA and *kimchi* fractions were treated for 48 hrs. Following treatment, the medium was changed. Subsequently, the medium was changed at weekly intervals and, at 6 weeks, the dishes were fixed and stained, and then the type I, II, III foci were counted.

probably resulted from leek, the major ingredient of leek *kimchi* (43). These results support the fact that leek *kimchi* has stronger antimutagenic and anticarcinogenic effects than Korean cabbage *kimchi*.

**Anticarcinogenic effects of leek *kimchi* fractions**

In an effort to identify the active compounds of leek *kimchi*, the 6-day fermented leek *kimchi* was fractionated into 7 groups, methanol extract, hexane fraction (fr.), methanol soluble fr., dichloromethane fr., ethyl acetate fr., butanol fr. and aqueous fr., which were tested for anticarcinogenicity using C3H/10T1/2 cells. Leek *kimchi* fractions decreased significantly ($p<0.05$) the cytotoxicity mediated by MCA in C3H/10T1/2 cells, showing that leek *kimchi* plays a role in protecting cells against the toxic effect of the carcinogen *in vitro* (Table 4). In particular, the dichloromethane fr. of leek *kimchi* exhibited strong inhibitory activity of the cytotoxicity induced by MCA compared to other fractionated samples. 50 μg/ml of dichloromethane fr. from the leek *kimchi* suppressed the MCA-induced cytotoxicity by 77%. On the transformation test using MCA, the dichloromethane fraction considerably reduced the formation of type II and III foci (Table 5). When the dichloromethane fr. (50 μg/ml) was treated, the numbers of type II+III foci mediated by MCA were decreased to 5 compared to 25 of the control. These results indicate that the dichloromethane fraction of leek *kimchi* may contain the major compound(s) that suppress the carcinogenesis by MCA in the eukaryotic cells.

**Table 4.** The cytotoxicity of C3H/10T1/2 cells treated with 3-methylcholanthracene (MCA, 10 μg/ml) and MCA plus fractionated samples (50 μg/ml) from 6-day fermented leek *kimchi* (pH 4.3) at 15°C

| Sample | | Number of cell colony | Cytotoxicity[1)] |
|---|---|---|---|
| MCA (control) | | $108\pm4^{a}$ | 1.00 |
| MCA+ | MeOH ext. | $156\pm3^{de}$ | 1.44 |
| | Hexane fr. | $124\pm1^{e}$ | 1.15 |
| | Methanol soluble fr. | $164\pm4^{bc}$ | 1.52 |
| | Dichloromethane fr. | $191\pm4^{f}$ | 1.77 |
| | Ethyl acetate fr. | $154\pm2^{d}$ | 1.43 |
| | Butanol fr. | $129\pm5^{c}$ | 1.19 |
| | Aqueous fr. | $118\pm7^{b}$ | 1.09 |

[1)]The procedure is the same as the footnote of Table 2.
[a~f]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test

**Table 5.** The inhibitory effect of methanol extract and dichloromethane fraction ($CH_2Cl_2$ fr.) from 6-day fermented leek *kimchi* (pH 4.3) at 15°C on the transformation of C3H/10T1/2 cells treated with 3-methylcholanthracene (MCA, 10 μg/ml)[1)]

| Treatment | Conc. (μg/ml) | Total number | | | |
|---|---|---|---|---|---|
| | | Type I foci | Type II foci | Type III foci | Type II +III foci |
| MCA (control) | | 15.3 | 15.0 | 10.0 | 25.0 |
| MCA+MeOH ext. | 25 | 11.3 | 10.3 | 7.7 | 18.0 |
| | 50 | 8.3 | 8.7 | 4.7 | 13.0 |
| MCA+$CH_2Cl_2$ fr. | 25 | 8.7 | 4.3 | 3.7 | 8.0 |
| | 50 | 4.3 | 3.3 | 1.7 | 5.0 |

[1)]The procedure is the same as the footnote of Table 3.

Vitamin C, β-carotene, dietary fibers, lactic acid bacteria, β-sitosterol in Korean cabbage *kimchi* are already believed to be compounds which have antimutagenic and anticarcinogenic effects (7-17,27,31,34,35). It was previously reported that leeks contain sulfides (44), linalool (45), and flavonoid glycosides (8,9). β-Sitosterol and β-sitosterol-3-β-D-glucopyranoside were tentatively isolated from threechloromethane fraction of leeks (46). The anticarcinogenic effect of leek *kimchi* might be due to flavonoids, fatty acid, terpenoides and sterol, etc. in the dichloromethane fraction.

With these results, it can be concluded that leek *kimchi* samples have stronger anticarcinogenic effects than Korean cabbage *kimchi* and that the dichloromethane fraction of leek *kimchi* may contain the major compound(s) that suppress the carcinogenesis in the eukaryotic cells. Further studies on identifying active compounds of leek *kimchi* and their mechanisms are needed.

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