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# Apoptotic Effect of Proso Millet Grains on Human Breast Cancer MDA-MB-231 Cells Is Exerted by Activation of BAK and BAX, and Mitochondrial Damage-mediated Caspase Cascade Activation

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To examine the antitumor effect of proso millet grains, whether proso millet grains exert apoptotic activity against human cancer cells was investigated. When the cytotoxicity of 80% ethanol (EtOH) extract of proso millet grains was tested against various cancer cells using MTT assay, more potent cytotoxicity was observed against human breast cancer MDA-MB-231 cells than against other cancer cells. When the EtOH extract was evaporated to dryness, dissolved in water, and then further fractionated by sequential extraction using four organic solvents (n-hexane, methylene chloride, ethyl acetate, and n-butanol), the BuOH fraction exhibited the highest cytotoxicity against MDA-MB-231 cells. Along with the cytotoxicity, TUNEL-positive apoptotic nucleosomal DNA fragmentation and several apoptotic responses including BAK/BAX activation, mitochondria membrane potential ($\Delta\psi m$) loss, mitochondrial cytochrome *c* release into the cytosol, activation of caspase-8/-9/-3, and degradation of poly (ADP-ribose) polymerase (PARP) were detected. However, human normal mammary epithelial MCF-10A cells exhibited a significantly lesser extent of sensitivity compared to malignant MDA-MB-231 cells. Irrespective of Fas-associated death domain (FADD)-deficiency or caspase-8-deficiency, human T acute lymphoblastic leukemia Jurkat cells displayed similar sensitivities to the cytotoxicity of BuOH fraction, excluding an involvement of extrinsic apoptotic mechanism in the apoptosis induction. These results demonstrate that the cytotoxicity of BuOH fraction from proso millet grains against human breast cancer MDA-MB-231 cells is attributable to intrinsic apoptotic cell death resulting from BAK/BAX activation, and subsequent mediation of mitochondrial damage-dependent activation of caspase cascade.

**Key words :** Activation of BAK and BAX, breast cancer cells, butanol fraction, mitochondrial apoptosis, proso millet grains

## Introduction

Despite improved imaging and molecular diagnostic techniques, cancer is one of the serious diseases in humans. Particularly, breast cancer is the most common cancer among women in worldwide. There are many kinds of strategies for breast cancer treatment, for example, surgery, radiotherapy, chemotherapy, hormone therapy, and complementary therapies [30, 40]. However, these treatments are not satisfactory because of the limited efficacy of drugs and resistance to drugs have been reported [18, 33]. Therefore, it is necessary to find the more effective and less toxic anticancer compounds that can target multiple points in the apoptotic pathways to achieve synergistic actions [18].

Apoptosis, the process of programmed cell death, has been proposed as an efficient mechanism by which malignant tumor cells can be removed upon chemotherapeutic treatments, in that the induction of apoptotic cell death in tumor cells results in their own destruction into apoptotic bodies which can be cleared by surrounding cells without accompanying a local damaging inflammatory response [7]. In the literature, a high consumption of fruits and vegetables reduced risk of breast cancer [13, 21, 33]. Fruits and vegetables contain a diverse source of phytochemicals (carotenoids, tocopherols, and polyphenols) which possess chemopreventive effects at multiple stages of carcinogenesis [1, 5, 19, 23]. Chemoprevention is an active cancer preventive strategy. It is estimated that more than two-thirds of human breast cancers could be averted by lifestyle modifications including dietary changes

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[29].

Compared with the production of the major cereal grains such as rice, barley and wheat, the cultivation and harvesting of other miscellaneous cereals remained in a low level in Korea. Recently, due to increased demand for well-being foods, the interest in miscellaneous cereal grains as crude fibers and bioactive phytochemical sources that benefit human health and thus their consumption are also elevating in the country.

Although several studies were performed to extend our understanding on nutritional, antioxidant, antimicrobial, antimutagenic and anticarcinogenic, and antidiabetic effects of miscellaneous cereal grains harvested in Korea [8, 15, 20, 24-27], the study on their anticancer efficacy is still relatively rare. In a previous study, we evaluated in vitro anti-obese activities of 80% EtOH extracts of eight selected miscellaneous cereal grains (proso millet, hwanggeumchal sorghum, glutinous sorghum, yellow glutinous foxtail millet, green glutinous foxtail millet, golden foxtail millet, barnyard millet, and adlay) by employing murine 3T3-L1 murine preadipocytes, and found that the extract of proso millet grains possess the most remarkable anti-adipogenic efficacy [10]. Further, we found that the anti-adipogenic activity of the 80% EtOH of proso millet grains toward 3T3-L1 cells was due to inducing apoptotic cell death and inhibiting differentiation of 3T3-L1 preadipocytes into mature adipocytes. Since the EtOH extract of proso millet grains appeared to induce apoptosis in 3T3-L1 cells [10], it was likely that proso millet grains might exert inhibitory activity against human cancer cells through inducing apoptotic cell death.

To examine antitumor activity of proso millet (*Panicum miliaceum* L.) grains, in this study, the cytotoxicity of 80% EtOH extract of proso millet grains was examined against various human cancers cells. The EtOH extract exerted the highest cytotoxicity toward human breast cancer cell line MDA-MB-231 compared to other cancer cells tested. When the EtOH extract was further fractionated by sequential solvent extractions using n-hexane (Hex), methylene chloride (MC), ethyl acetate (EtOAc), and n-butanol (BuOH), the BuOH fraction exerted the highest cytotoxicity toward MDA-MB-231 cells, which was caused by apoptosis induction. Cytotoxicity of the BuOH fraction was compared between MDA-MB-231 and MCF-10A cells to examine whether malignant cancer cells are more sensitive than normal cells to the cytotoxicity of BuOH fraction. Additionally, to examine the involvement of the extrinsic pathway in the induced apoptosis, apoptotic responses were compared among the BuOH fraction-treated Jurkat A3 (wild-type), I2.1 (FADD-deficient), and I9.2 (caspase-8-deficient) cells. The data show that the BuOH fraction from proso millet grains exerted cytotoxicity against human breast cancer MDA-MB-132 cells via inducing intrinsic mitochondria-dependent apoptotic cell death pathway, and that proso millet grains may be applicable in developing functional foods with chemopreventive and chemotherapeutic potentials against breast cancer.

## Materials and Methods

### Reagents, antibodies, cells, and culture medium

The ECL Western blotting kit was purchased from Amersham (Arlington Heights, IL, USA), and Immobilon-P membrane was obtained from Millipore Corporation (Bedford, MA, USA). 3,3′-dihexyloxacarbocyanine iodide (DiOC6), 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), paraformaldehyde, and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, MO, USA). The FITC-Annexin V apoptosis kit was obtained from Clontech (Takara Bio Inc., Shiga, Japan), and the in situ Cell Death Detection Kit for terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick-end labeling (TUNEL) assay was from Roche Applied Science (Basel, Switzerland). The Qproteome Cell Compartment Kit was purchased from Qiagen (PL Venlo, Netherlands). Anti-cytochrome *c* was purchased from Pharmingen (San Diego, CA, USA). Anti-caspase-3 was purchased from BD Sciences (Chicago, IL, USA), anti-poly (ADP-ribose) polymerase (PARP), anti-β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-caspase-8, anti-BID and anti-caspase-9 were purchased from were from Cell Signaling Biotechnology (Beverly, MA, USA). Human breast cancer MDA-MB-231 cells, human colon cancer HCT-116 cells, human glioblastoma astrocytoma U87 cells, human osteogenic sarcoma Saos-2 cells, and human normal mammary epithelial MCF-10A cells were obtained from ATCC (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle's medium (DMEM, Hyclone, Gaithersburg, MD, USA) (Hyclone, Gaithersburg, MD, USA) containing 10% fetal bovine serum, and 100 mg/ml gentamycin. Human colon cancer RKO cells, human lymphobast Sup-T1 cells, human acute lymphoblastic leukemia Jurkat T cell clones A3 (wild-type), I2.1 (FAS-assoiated death domain [FADD]-deficient), and I9.2 (caspase-8-deficient) were obtained from ATCC and maintained in RPMI 1640 (Hyclone) containing 10% FBS, 20 mM HEPES (pH 7.0), 50 μM β

-mercaptoethanol, and 100 μg/ml gentamycin.

### Isolation of antitumor components from the grains of proso millet by 80% ethanol extraction and subsequent fractionation with organic solvents

Proso millet (*Panicum miliaceum*) grains were provided by National Institute of Crop Science, Rural Development Administration, Miryang, Gyeongnam 627-803, Korea. Extraction of proso millet grains with 80% ethanol (EtOH) and its sequential solvent fractionation using n-hexane (Hex), methylene chloride (MC), ethyl acetate (EtOAc), and n-butanol (BuOH) were performed as previously described [12]. In brief, dried proso millet grains were milled on a Blender 7012 (Dynamics Corporation, USA), and then extracted with 80% ethanol (EtOH) for 3 hr at 80℃. The EtOH extract was evaporated, dissolved in water, and then fractionated by sequential extraction using n-hexane (Hex), methylene chloride (MC), ethyl acetate (EtOAc), and n-butanol (BuOH). Each organic solvent extraction was repeated three times. Each organic solvent extract was repeated three times. The organic solvent extracts were concentrated by rotary vacuum evaporator (Heidolph LR 4000, Germany) and then lyophilized to dryness.

### Cytotoxicity assay

Cytotoxic effect of 80% EtOH extract and its organic solvent fractionations of proso millet grains on MDA-MB-231 cells was analyzed by an MTT assay, reflecting cell viability, as previously described [11]. In brief, MDA-MB-231 cells ($5\times10^3$/well) were added to a serial dilution of the sample in 96-well plates. After incubation for 48 hr or 72 hr, 50 μl of the MTT solution (1.1 mg/ml) was added to each well and incubated for an additional 4 hr. The colored formazan crystal produced from the MTT was dissolved in 150 μl of DMSO. The OD values of the solutions were measured at 540 nm using a plate reader.

### Flow cytometric analysis

Flow cytometric analysis for the cell cycle of MDA-MB-231 cells exposed to the BuOH fraction was performed as previously described [11]. The extent of necrosis was detected with an FITC-Annexin V apoptosis kit as previously described [9]. In brief, cells ($5\times10^5$) were washed with 1× binding buffer and then incubated with FITC-Annexin V-FITC and PI for 15 min before being analyzed by flow cytometry.

Changes in the mitochondrial membrane potential ($\Delta\psi m$) following treatment with the BuOH fraction were measured after staining with $DiOC_6$ [39]. After treatment with the BuOH fraction, the cells were harvested and incubated with PBS containing 50 nM $DiOC_6$ for 15 min at 37℃ prior to flow cytometric analysis.

Activation of BAK and BAX following treatment with the BuOH fraction was measured by flow cytometry as previously described [14]. Cells ($1\times10^6$) were washed with PBS and fixed in PBS/1.0% paraformaldehyde on ice for 30 min. Cells were then washed three times in PBS/1% FBS. Staining with conformation-specific antibodies against BAK (Ab-1) and BAX (6A7) was performed with a proper dilution of individual antibodies in 100 ml staining buffer (PBS, 500 mg/ml digitonin). Then, the cells were washed and resuspended in 100 ml staining buffer containing Alexafluor 488-labeled goat anti-mouse IgG. The conformational changes of BAK and BAX were measured by flow cytometry.

### Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

MDA-MB-231 cells were seeded at a density of $1.2\times10^5$ cells/well on 6-well plate with cover slip. After 12 hr incubation, the cells were treated with 100 μg/μl BuOH fraction for 48 hr. They were then subjected to fluorescence-TUNEL assay using an In Situ Cell Death Detection Kit as previously described [14]. Thereafter, the cells were mounted with PI on slides to label nuclei and then examined under a confocal laser scanning microscope.

### Preparation of cell lysates and Western blot analysis

The cellular lysates were prepared by suspending $5\times10^6$ MDA-MB-231 cells in 200 μl lysis buffer (137 mM NaCl, 15 mM EGTA, 1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS, 2.5 μg/ml proteinase inhibitor E-64, and pH 7.2). The cells were disrupted by sonication and extracted at 4℃ for 30 min. An equivalent amount of protein lysate (25 μg) was subjected to electrophoresis on a 4%-12% SDS gradient polyacrylamide gel with a MOPS buffer. The proteins were electrotransferred to Immobilon-P membranes, and then probed with individual antibodies. The detection of each protein was carried out using an ECL Western blotting kit, according to the manufacturer's instructions.

### Detection of mitochondrial cytochrome c release in cytosolic protein extract

To assess the mitochondrial cytochrome *c* release in MDA-MB-231 cells following treatment with BuOH extract of proso millet, cytosolic protein extracts were obtained, using an Qproteome Cell Compartment Kit. Briefly, approximately $5\times10^6$ cells were washed twice with cold PBS and then suspended in CE1 buffer. The cells were allowed to swell on ice for 10 min. The cells were centrifuged at 1,000 g for 10 min at 4℃. The supernatants were harvested as cytosolic extracts free of mitochondria, and analyzed for mitochondrial cytochrome *c* release.

### Statistical analysis

Unless otherwise indicated, each result in this paper is representative of at least three separate experiments. Statistical analysis was performed using Students t-test to evaluate the significance of differences between two groups and one-way ANOVA between three and more than three groups. In all graphs, * indicates $p<0.05$ between the untreated and treated cells. All data are expressed as the mean ± standard deviation (SD, for each group $n \geq 3$). One-way ANOVA followed by Dunnett's mulitple comparison test was also used for statistical analysis using IBM® SPSS® Statistics version 19.

## Results and Discussion

### Cytotoxicity of EtOH extract of proso millet grains against various human cancer cell lines

To examine antitumor activity of proso millet grains, whether its 80% EtOH extract can exert cytotoxic effect on various cancer cells such as human colon cancer HCT-116 cells, human glioblastoma astrocytoma U87 cells, human acute lymphobastic leukemia Sup-T1 cells, human osteogenic sarcoma Saos-2, and human breast cancer MDA-MB-231 cells was investigated. Results of MTT assay showed that the viabilities of HCT-116 cells, RKO cells, and U87 cells following exposure to the EtOH extract (200 μg/ml) for 72 hr were reduced to 87.5%, 89.3%, and 64.5% of control level, respectively, whereas those of Sup-T1 cells, Saos-2 cells and MDA-MB-231 cells were 62.4%, 56.0%, and 51.9%, respectively (Table 1). These results indicated that human acute lymphobastic leukemia Sup-T1 cells, human breast cancer MDA-MB-231 cells, and human osteogenic sarcoma Saos-2 cells were more sensitive to cytotoxicity of the EtOH extract compared to other cancer cells tested.

The tumor suppressor p53 plays a critical role in cellular events including cell cycle regulation, DNA repair, senescence, and apoptosis. However, the p53 gene is known to be the most frequently mutated in human cancers and thus mutations in the p53 gene have been identified in more than 50% of human cancers [34, 37]. The mutation of p53 in tumor cells results in production of mutant p53 proteins that alter wild-type p53 tumor suppression functions with concomitant acquisition of new oncogenic properties including deregulated cell proliferation, increased chemoresistance, and several other pro-oncogenic activities. In this context, the p53 alterations in several human tumors were reported to be associated to poor prognosis [6, 22]. It is of interest to note that human cancer cells with p53 mutations (MDA-MB-231, Saos-2, and Sup-T1) displayed higher sensitivities to the cytotoxicity of 80% EtOH extract of proso millet grains than did human cancer cells with wild-type p53 (HCT-116, RKO, and U87).

After 80% EtOH extract of proso millet grains was evaporated to dryness, dissolved in water, and subjected to fractionation by a series of solvent extractions using Hex, MC, EtOAc, and BuOH, the individual fractions were tested for cytotoxicity against MDA-MB-231 cells. As a result, the BuOH fraction appeared to contain the most cytotoxic activity, whereas the other extracts showed no such a significant cytotoxicity (Fig. 1A and 1B). In addition, when MDA-MB-231 cells were treated with the BuOH fraction at concentrations of 25-100 μg/ml for 72 hr, the half-maximal inhibitory concentration ($IC_{50}$) value of the BuOH fraction was

Table 1. Cytotoxic effect of ethanol extract on the viability of various human cell lines

| Cell line | Origin | Cell viability (%) | p53 status |
|---|---|---|---|
| HCT-116 | human colon cancer cells | 87.5 | wild type |
| RKO | human colon cancer cells | 89.3 | wild type |
| U87 | human glioblastoma astrocytoma cells | 64.5 | wild type |
| Sup-T1 | human lymphoblast cells | 62.4 | mutant |
| Saos-2 | human osteogenic cells | 56.0 | null |
| MDA-MB-231 | human breast cancer cells | 51.9 | mutant |

Individual cells ($5\times10^3 \sim 5\times10^4$/well) were incubated with vehicle (0.1% DMSO) or 80% EtOH extract of the grains of proso millet at a concentration of 200 μg/ml in 96-well plates for 72 hr and the final 4 hr was incubated with MTT. Each value is expressed as mean (n = 3 with three replicates per independent experiment).

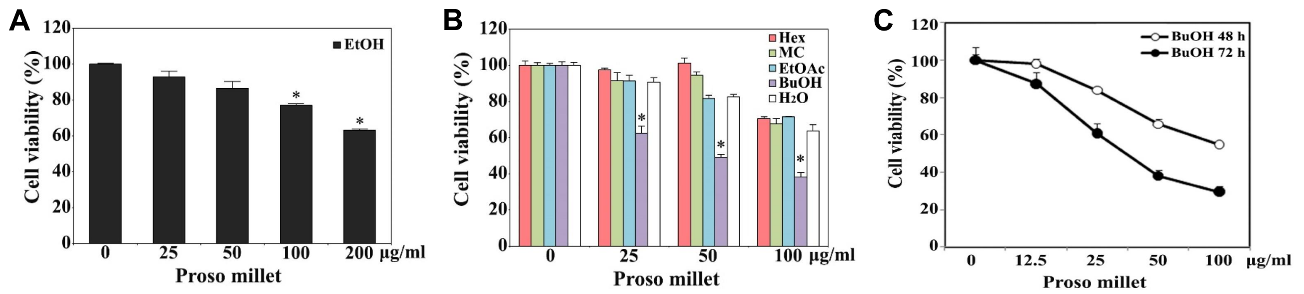


Fig. 1. Cytotoxic effect of 80% EtOH extract (A), individual organic solvent fractions (B), and kinetic analysis of the cytotoxicity of BuOH fraction (C) obtained from proso millet grains on human breast cancer MDA-MB-231 cells. The cells ($5\times10^3$/well) were incubated with 80% EtOH extract and its solvent fractions at indicated concentrations in 96-well plates for 48 hr and the final 4 hr was incubated with MTT to assess cell viability. For evaluating the cytotoxicity of the BuOH fraction, the cells were incubated in the presence of individual concentrations of BuOH fraction for either 48 hr or 72 hr, and the final for 4 hr was incubated with MTT to access cell viability. Data are mean ± SD, n=3 separate experiments.

approximately 35 μg/ml (Fig. 1C).

### Apoptotic activity of the BuOH fraction obtained from 80% EtOH extract toward MDA-MB-231 cells

To examine the molecular and cellular mechanism underlying the BuOH fraction-induced cytotoxicity against MDA-MB-231 cells, the cells accumulating in apoptotic sub-$G_1$ phase were analyzed by flow cytometry. After treatment of MDA-MB-231 cells with 25, 50, and 100 μg/ml of the BuOH faction for 48 hr, the percentage of sub-$G_1$ cells representing apoptotic cells was dose-dependently elevated from 1.3% to 25.5% (Fig. 2A). To understand the apoptotic signaling pathway involved in the BuOH fraction-induced apoptosis in MDA-MB-231 cells, the change in mitochondrial membrane potential ($\Delta\psi m$) of cells treated with the BuOH fraction was measured by flow cytometry using DiOC6 staining. Although exponentially growing (control) MDA-MB-231 cells showed negligible $\Delta\psi m$ loss, cells treated with 100 μg/ ml BuOH fraction showed 26.1% $\Delta\psi m$ loss (Fig. 2B). Since $\Delta\psi m$ loss is known to be one of the initial intracellular mitochondrial outer membrane damages that are accompanied by apoptotic cell death [36], these results suggested that mitochondrial damage, leading to $\Delta\psi m$ loss, was associated with the apoptosis induction in MDA-MB-231 cells treated with the BuOH fraction. The involvement of necrosis in the cytotoxicity of the BuOH fraction was further investigated by flow cytometry after FITC-conjugated Annexin V and PI staining. As shown in Fig. 2C, flow cytometric analysis using FITC-Annexin V and PI staining of MDA-MB-231 cells treated with 100 μg/ml BuOH fraction for 48 hr revealed that the rate of early apoptotic cells, which were stained only with FITC-Annexin V, increased to 22.1%, and the rate of late apoptotic cells, which were stained with both FITC-Annexin V and PI, increased to 10.4%, whereas the rate of necrotic cells, which were stained with only PI, appeared to be 8.3%. These results suggested that the cytotoxic effect of the BuOH fraction against MDA-MB-231 cells was mainly due to induction of apoptotic cell death rather than induction of necrotic cell death. To further confirm the involvement of apoptosis in the cytotoxicity of the BuOH fraction, induced apoptotic DNA fragmentation in MDA-MB-231 cells treated with 100 μg/ml BuOH fraction was examined using TUNEL assay. As shown in Fig. 2D, the BuOH fraction-treated cells mostly showed TUNEL-positive nuclei compared with control cells exhibiting no detectable TUNEL-positive nuclei.

Consequently, these results suggested that cytotoxic effect of the BuOH fraction from proso millet grains against MDA-MB-231 cells was lattributable to induction of mitochondria-dependent apoptotic cell death, resulting from mitochondrial outer membrane damages and resultant $\Delta\psi m$ loss.

### Involvement of BAK/BAX activation and mitochondrial cytochrome c-mediated caspase cascade activation in the BuOH fraction-induced apoptosis

To examine whether pro-apoptotic multidomain BCL-2 family members (BAK and BAX) were activated to cause mitochondrial outer membrane damages and $\Delta\psi m$ loss, the activation of BAK and BAX in MDA-MB-231 cells treated with 100 μg/ml BuOH fraction was investigated by Western blot analysis using active BAK- and active BAX-specific antibodies, respectively. As shown in Fig. 3A, the activation of both BAK and BAX was detected in the cells treated with 100 μg/ml BuOH fraction, demonstrating that the BuOH fraction-induced $\Delta\psi m$ loss was due to pro-apoptotic roles of the

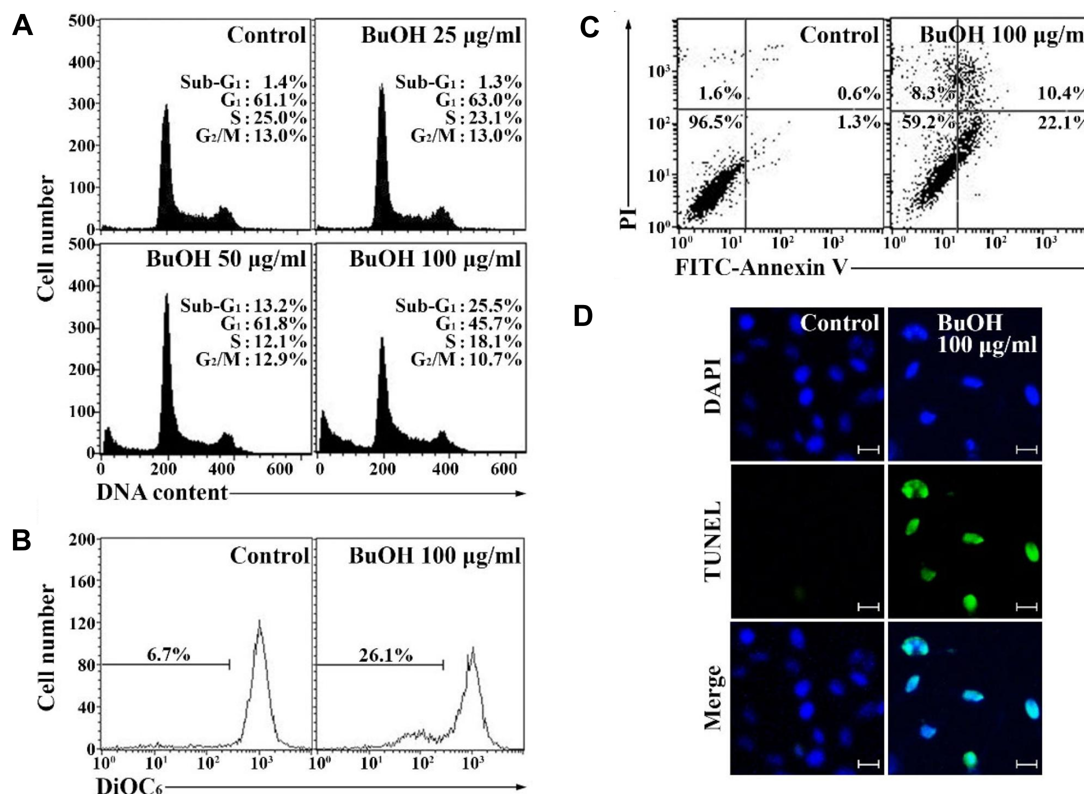


Fig. 2. Apoptotic change in the cell cycle distribution (A), mitochondrial membrane potential (Δψm) loss (B), apoptotic cell death (C), and TUNEL-positive apoptotic DNA fragmentation (D) in MDA-MB-231 cell after treatment with the BuOH fraction. The cells were incubated with the BuOH fraction at indicated concentrations for 48 hr and harvested to analyze cell cycle distribution, mitochondrial membrane potential (Δψm) loss, or TUNEL-positive apoptotic cells by flow cytometry, and apoptotic DNA fragmentation by TUNEL assay as described in Materials and Methods. A representative study is shown and two additional experiments yielded similar results.

active BAK and BAX molecules. With respect to the BuOH fraction-induced activation of BAK and BAX, leading to mitochondrial outer membrane damages and Δψm loss, the alteration in the expression levels of BCL-2 family proteins, including the pro-apoptotic BCL-2 proteins (BAD, BAK, and BAX) and the anti-apoptotic BCL-2 proteins (BCL-2 and BCL-XL), were investigated by Western blot analysis. As shown in Fig. 3B, there were no remarkable changes in the levels of pro-apoptotic and anti-apoptotic proteins in MDA-MB-231 cells treated with the BuOH fraction.

To further elucidate the BuOH fraction-induced mitochondria-dependent apoptotic signaling pathway in MDA-MB-231 cells, mitochondrial cytochrome c release into the cytoplasm, which is known to be involved in activation of the caspase cascade including capase-9, caspase-8, and capase-3, leading to and PARP degradation [17, 31], were investigated by Western blot analysis.

As a result, there was barely detectable cytochrome *c* in the cytosolic fraction of non-treated control MDA-MB-231 cells; however, the level of cytosolic release of mitochondrial cytochrome *c* became enhanced after treatment with 100 μg/ml BuOH fraction (Fig. 3C). In agreement with cytochrome c release, the caspase-9 activation that proceeded through proteolytic cleavage of inactive proenzyme (47 kDa) to active forms (37/35 kDa) was also detected (Fig. 3D). The cleavage of procaspase-3 (32 kDa) into active form (17 kDa) as well as the cleavage of PARP was also detected in the BuOH fraction-treated MDA-MB-231 cells. The caspase-8 through proteolytic cleavage of proenzyme (57 kDa) into active forms (43/41 kDa) was significantly enhanced. In addition, the level of BID protein (22 kDa), which is known to be degraded by active caspase-8 to generate the truncated BID (tBID, 15 kDa), causing mitochondrial cytochrome c release into the cytosol [4, 16], appeared to remain constant in MDA-MB-231 cells treated with the BuOH fraction. As a downstream target of the active caspase-3, the degradation of PARP was also detected along with the activation of caspase-3. Consequently, these results demonstrated that BuOH fraction-induced apop-

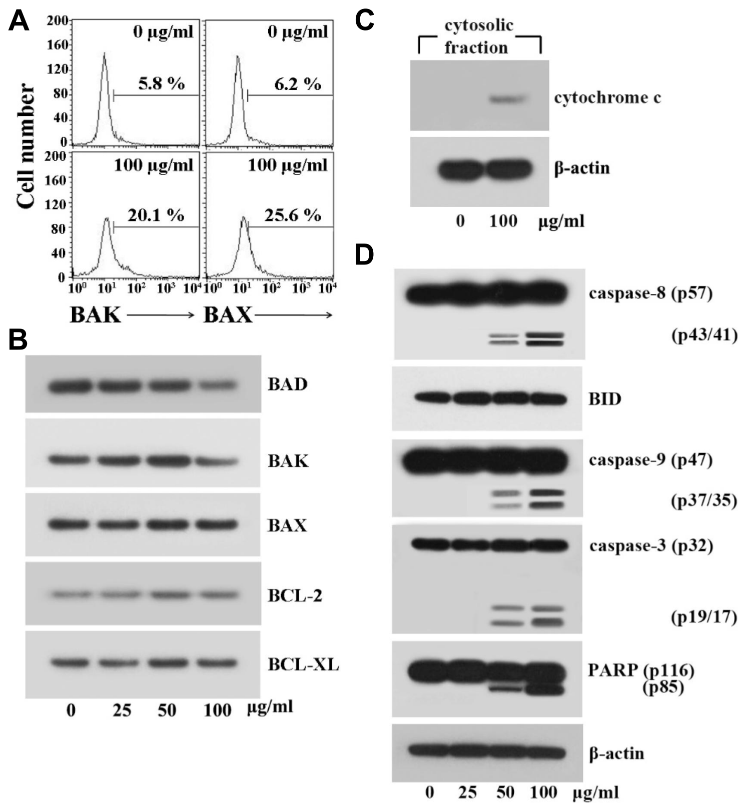


Fig. 3. Flow cytometric analysis of activation of pro-apoptotic BCL-2 family members BAK and BAX (A), and Western blot analysis of pro-apoptotic BCL-2 family proteins (BAD, BAK, and BAX) and anti-apoptotic BCL-2 family proteins (BCL-2 and BCL-XL) (B), mitochondrial cytochrome *c* release into cytoplasm (C), and Western blot analysis of caspase-9, -3, and -8, PARP, and b-actin (D). After MDA-MB-231 cells were incubated in the presence of BuOH fraction at various concentrations (vehicle 0.1% DMSO, 25, 50, and 100 mg/ml) for 48 hr, the cells were harvested. Flow cytometric analysis of activation of BAK and BAX, and Western blot analysis were performed as described in Material and Methods. A representative study is shown and two additional experiments yielded similar results.

tosis was caused by BAK/BAX activation, $\Delta\psi m$ loss, and subsequent activation of caspase-9/-3/-8.

### Comparison of cytotoxic effect of the BuOH fraction on wild-type (Clone A3), FADD-deficient (Clone I2.1), and caspase-8-deficient (Clone I9.2) Jurkat T cells.

During induction of apoptosis, caspase-8 activation can be provoked to act not only as the initiator caspase in the extrinsic death receptor (DR)-mediated apoptosis signaling pathway [28], but also as a downstream event of the intrinsic mitochondrial/caspase-9/caspase-3 apoptosis pathway to comprise a positive feedback loop involving tBID-mediated mitochondrial cytochrome c release in drug-induced apoptosis of tumor cells [32, 38]. To examine whether the death receptor (DR)/DR ligand system is involved in quercetin-induced apoptosis in Jurkat T cells, we sought to compare the apoptotic effect of the BuOH fraction on A3 cells with those on I2.1 and I9.2 cells, both of which are known to be refractory to the extrinsic DR-dependent apoptosis [38].

As shown in Fig. 4A, Western blot analysis showed that Jurkat A3 cells express both FADD and caspase-8, whereas I2.1 and I9.2 cells failed to express FADD and caspase-8, respectively. Under these conditions, MTT assay revealed that, following treatment with 100 μg BuOH fraction for 48 hr, all Jurkat T cell clones, irrespective of the presence of FADD and caspase-8 proteins, exhibited similar sensitivities to the cytotoxic effect of the BuOH fraction from proso millet grain (Fig. 4B).

These results confirmed that the activation of caspase-8 in the BuOH fraction-treated Jurkat T cells occurred as a consequence of the intrinsic mitochondria-dependent activation of caspase-9 and caspase-3, which occurred independently of the extrinsic apoptosis pathway.

### Cytotoxic effect of the BuOH fraction on human normal mammary epithelial MCF-10A cells

To examine whether the cytotoxic effect of the BuOH fraction is malignant cell specific, we compared its cytotoxic effect against human breast cancer MDA-MB-231 cells and normal mammary epithelial MCF-10A cells. The viabilities of MCF-10A cells following treatment with 50 μg/ml and 100 μg/ml BuOH fraction were sustained at levels of 87.6% and 75.3% of non-treated control, respectively, whereas those of MDA-MB-231 cells were reduced to the levels of 46.3% and 42.0%, respectively (Fig. 5). These results indicated that malignant MDA-MB-231 cells are significantly more sensitive than normal MCF-10A cells to the cytotoxicity of BuOH fraction.

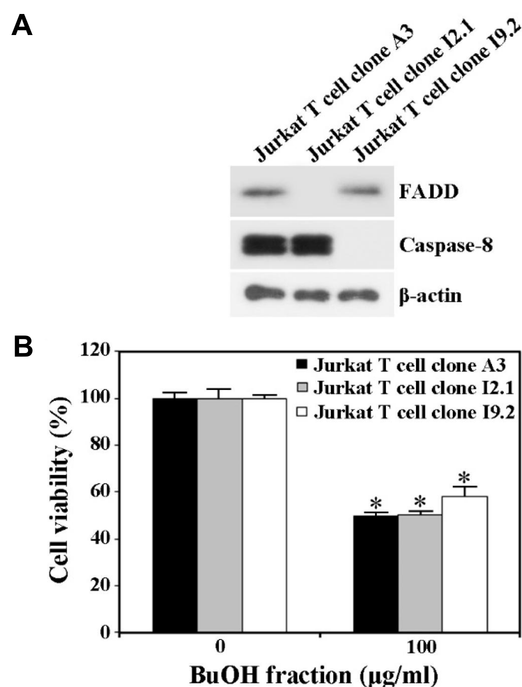


Fig. 4. Western blot analysis of caspase-8, FADD, and b-actin (A), and comparative effect of the BuOH fraction on cell viability (B) in wild-type Jurkat T cells (clone A3), FADD-deficient Jurkat T cells (clone I2.1) and caspase-8-deficient Jurkat T cells (clone I9.2). Western analysis was performed as described in Material and Methods. A3 cells, I2.1 cells, or I9.2 cells (5´$10^4$ cells/well) were incubated with vehicle (0.1% DMSO) or the BuOH fraction (100 mg/ml) in 96-well plates for 48 hr and the final 4 hr was incubated with MTT to assess cell viability. Each value is expressed as mean ± SD (n = 3 with three replicates per independent experiment). *$p$<0.05 compared to control.

Since the induction of apoptosis in tumor cells results in their own destruction into apoptotic bodies which can be cleared by surrounding cells without accompanying a local damaging inflammatory response, apoptosis induction is known as a highly efficient method in cancer chemotherapy [9]. In addition, there is an urgent need for development of anticancer drugs whose efficacy inducing apoptosis is confined to tumor cells and for further research on apoptosis inducing mechanisms which can specifically operate only in tumor cells rather than normal cells. Recently, consumption of whole grains was reported to exert chemopreventive effect on stomach, colorectal, and breast cancers; this set trends toward whole grain-based development of functional food with chemopreventive and anticancer activities [2, 3, 35]. In these contexts, the apoptotic activity of proso millet grains, which

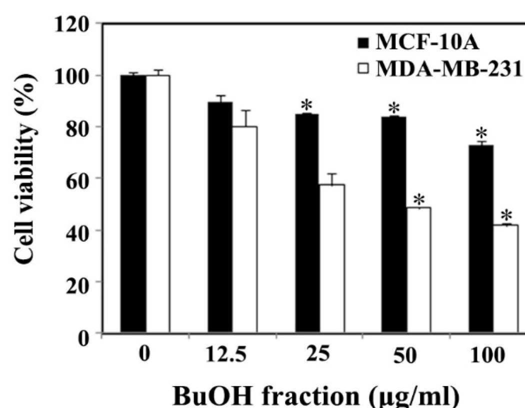


Fig. 5. Differential cytotoxic effect of the BuOH fraction on human normal mammary epithelial MCF-10A and human breast cancer MDA-MB-231 cells. Individual cells (5×$10^3$/well) were incubated with vehicle (0.1% DMSO) or the BuOH fraction at indicated concentrations in 96-well plates for 48 hr and the final 4 hr was incubated with MTT to assess the cell viability. Each value is expressed as mean ± SD (n = 3 with three replicates per independent experiment). *$p$<0.05 compared to control.

exerted predominantly on human breast cancer MDA-MB-231 cells rather than human normal mammary epithelial MCF-10A cells, confirmed that proso millet grains are a useful functional food source for chemoprevention and treatment of breast cancer.

In conclusion, the cytotoxicity of BuOH fraction from 80% EtOH extract of proso millet grains against human breast cancer MDA-MB-132 cells was predominantly attributed to intrinsic mitochondria-dependent apoptotic cell death, which proceeded through activation of pro-apoptotic multidomain BCL-2 family members (BAK and BAX), mitochondrial membrane potential (Δψm) loss, cytochrome c release into the cytosol, activation of caspase-9/-3/-8, PARP degradation, and apoptotic DNA fragmentation. The cytotoxic effect of BuOH fraction was much more significantly exerted against human breast cancer MDA-MB-231 cells than human normal mammary epithelial MCF-10A cells. These results are useful for evaluating proso millet grains as a functional food source with chemopreventive and chemotherapeutic potentials against breast cancer.

## The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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## 초록 : 기장 종자 유래 추출물의 인간 유방암 MDA-MB-231 세포에 대한 세포독성에 관련된 미토콘드리아 손상-의존적 아폽토시스 유도 효과

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잡곡 류에 속하는 기장(*Panicum miliaceum* L.)의 항암 효과를 알아보기 위해, 기장의 종자를 80% 에탄올(EtOH)로 추출하였으며, 이를 감압 농축하여 건조시키고 재차 물에 녹인 후 4가지 유기용매(헥산, 메틸렌 크로라이드, 에틸아세테이트 및 부탄올)로 순차적으로 추출 분획하였다. 다양한 인간 암세포에 대하여 80% 에탄올 추출물의 세포독성을 조사한 결과, 인간 유방암 세포주 MDA-MB-231에 대한 세포독성 효과가 가장 강하게 나타났다. 또한 에탄올 추출물 유래 각 유기용매 분획들 중에서 세포독성이 가장 높게 나타난 부탄올 분획을 사용하여, 유방암 세포주 MDA-MB-231에 대한 아폽토시스성 세포사멸 유도 효과를 조사하였다. 그 결과로서, BAK/BAX 활성화, 미토콘드리아 막 전위(Δψm) 손실, 미토콘드리아 시토크롬 c 방출, 카스파아제-8/-9/-3의 활성화, PARP의 분해, 그리고 TUNEL-양성 아폽토시스성 DNA 단편화와 같은 아폽토시스 반응들이 검출되었다. 한편, 인간 급성백혈병 Jurkat T 세포의 A3 클론(야생형), I2.1 클론(FADD-결손형) 및 I9.2 클론(카스파아제-8 결손형)은 부탄올 분획의 세포독성에 대해 유사한 감수성을 나타내었는데, 이는 부탄올 분획의 아폽토시스 유도 활성에는 외인성 아폽토시스 기전이 관련되지 않음을 시사한다. 흥미롭게도, 인간 정상 유방 상피세포 MCF-10A는 유방암 MDA-MB-231세포에 비해 부탄올 분획의 세포독성에 대하여 훨씬 낮은 감수성를 보였다. 이러한 연구결과는 기장 종자 유래 부탄올 분획의 인간 유방암 세포주 MDA-MB-231에 미치는 세포독성효과는 BAK/BAX 활성화에 따른 미토콘드리아 외막 손상 및 시토크롬 c 방출, 이에 수반되는 카스파아제 활성화에 의해 매개됨을 보여준다.