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Antiproliferative Constituents from the Vinegar Treated Small Black Soybean (*Glycine max* Merr.)

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Abstract – The chloroform-soluble fraction of the vinegar treated small black soybean [*Glycine max* (Leguminosae)] showed antiproliferative activity against human myeloid leukemia HL-60 cells, in terms of inhibition of proliferation and induction of apoptosis. Bioassay-guided chromatography of the chloroform-soluble fraction resulted in the isolation of two isoflavonoid compounds, genistein and daidzein, as active principles. Genistein showed more potent antiproliferative effects against HL-60 cells. Treatment of HL-60 cells with genistein induced apoptosis in a dose dependent manner. Apoptosis was judged by the detection of DNA fragmentation by a flow cytometry and the degree of apoptosis was assayed by RT-PCR.
Keywords – *Glycine max*, antiproliferative activity, genistein

Introduction

Small black soybean [*Glycine max* (Leguminosae)] is a cultivar in Korea. Black soybean has been used as a Chinese traditional medicine for detoxification, as an anti-inflammatory, and to improve the blood (Liao *et al.*, 2005). It has been reported that black soybean possesses higher antioxidative activity than soybean (Yang *et al.*, 1999). Lioa *et al.* (2005) reported that polysaccharide of black soybean promotes myelopoiesis and reconstitutes bone marrow after 5-flurouracil- and irradiation-induced myelosuppression. However, there are fewer reports specifically concerning small black soybean. In the course of searching for cell proliferation inhibitors from natural resources, a chloroform-soluble fraction of the vinegar treated small black soybean of *G. max* was found to show inhibitory effects on HL 60 cell line (human acute promyelocytic leukemia cell line). The active compounds were isolated from the methanolic extract of *G. max* and structures of these compounds were determined by physico-chemical and spectral evidences.

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Experimental

General procedure – ^{1}H- and ^{13}C-NMR spectra were determined on a JEOL JMN-EX 400 spectrometer. TLC was carried out on Merck precoated silica gel F_{254} plates, with Kiesel gel 60 (230 - 400 mesh, Merck) used as silica gel. Sephadex LH-20 was used for the column chromatography (Pharmacia, 25 - 100 μm). The column used for LPLC was Lobar-A (Merck Lichroprep Si 60, 240 - 10 mm). All other chemicals and solvents were analytical grade and used without further purification.

Materials – Small black soybean of *G. max* (the 2003 product) were purchased at Imsil, Jeonbuk, Korea. The soybeans were kept in un-milled rice vinegar in two weeks, and air-dried. A voucher specimen was deposited in the herbarium of the college of pharmacy, Woosuk University (WSU-04-024).

Extraction and isolation – The vinegar treated materials (300 g) were powdered and extracted (three times with MeOH at room temperature). The filtrate was evaporated *in vacuo* to give a dark brownish residue. The resultant methanolic extract (45 g) was subjected to successive solvent partitioning to give $CHCl_3$ (7 g), EtOAc (3 g), *n*-BuOH (6 g) and H_2O soluble fractions. Each fraction was tested for antiproliferative effects on HL 60 cell line (human acute promyelocytic leukemia cell line). Among

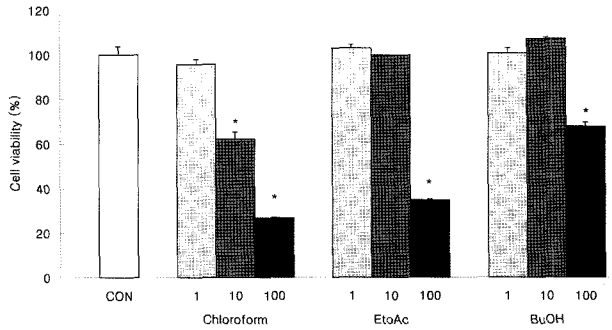

Fig. 1. Effects of the solvent fractions of the vinegar treated small black soybean (*G. max*) on HL60 leukemia cell viability (μg/ml).

these fractions, the $CHCl_3$ soluble fraction showed the most significant antiproliferative effects on HL 60 cell line (Fig. 1). Silica gel column chromatography of the $CHCl_3$ soluble fraction with *n*-hexane-$CHCl_3$-MeOH (7 : 7 : 1) gave nine fractions (fr.1-fr.9). The major fraction fr.6 was chromatographed on Lobar-A column (*n*-hexane-$CHCl_3$-MeOH, 4 : 10 : 1) and purified by Sephadex LH-20 column (MeOH) to yield compound **1** (25 mg). The fraction fr.7 was chromatographed on Lobar-A column ($CHCl_3$-MeOH, 10 : 1) and purified by Sephadex LH-20 column (MeOH) to yield compound **2** (15 mg).

Compound 1 – white powder; ^{1}H-NMR (600 MHz, DMSO-d_6) δ: 8.31 (1H, s, H-2), 7.38 (2H, d, J = 7.2 Hz, H-2', 6'), 6.82 (2H, d, J = 7.2 Hz, H-3', 5'), 6.36 (1H, d, J = 1.8 Hz, H-6), 6.21 (1H, d, J = 1.7 Hz, H-8); ^{13}C-NMR (125 MHz, DMSO-d_6): 180.1 (C-4), 164.8 (C-7), 162.0 (C-5), 157.6 (C-4'), 157.4 (C-9), 153.9 (C-2), 130.2 (C-2', 6'), 122.2 (C-3), 121.3 (C-1'), 115.0 (C-3', 5'), 104.2 (C-10), 99.1 (C-6), 93.8 (C-8).

Compound 2 – white powder; ^{1}H-NMR (600 MHz, DMSO-d_6) δ: 8.27 (1H, s, H-2), 7.95 (1H, d, J = 8.8 Hz, H-5), 7.42 (2H, d, J = 7.2 Hz, H-2', 6'), 6.96 (1H, dd, J = 8.8 Hz, H-6), 6.86 (1H, d, J = 1.8 Hz, H-8), 6.82 (2H, d, J = 7.2 Hz, H-3'); ^{13}C-NMR (125 MHz, DMSO-d_6): 174.5 (C-4), 162.4 (C-7), 157.4 (C-9), 157.1 (C-4'), 152.6 (C-2), 130.0 (C-2', 6'), 127.4 (C-5), 123.5 (C-3), 122.4 (C-1'), 116.6 (C-10), 115.0 (C-6), 114.8 (C-3', 5'), 102.1 (C-8).

Cell culture and *in vitro* cytotoxicity assay – Human acute promyelocytic (HL60) leukemia cells were maintained in a RPMI1640 medium that was supplemented with 10% fetal bovine serum. All of the cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. Cytotoxicity was measured by the microculture tetrazolium (MTT) method (Mosmann, 1983). The cells wers resuspended in a 100 μl RPMI1640 medium at 1×10^4 cells/ml after verifying cell viability by a trypan blue dye exclusion assay. One hundred μl of cell suspension were distributed into each well of a 96-well plate. After the treated cells were incubated for 44 hrs, 50 μl MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide} (1 mg/ml, Sigma) was added into each well. The plates were incubated for an additional 4 hrs. To dissolve formazan, 150 μl DMSO was added and the absorbance values of each well at 570 nm were measured using an ELISA reader (Molecular Devices, Sunnyville. CA).

Analysis of DNA fragmentation – Cells were lysed hypotonic solution (0.1% sodium citrate, 0.1% Triton-X100) containing 10 μg/ml of propidium iodide (PI), and DNA contenet of nuclei was analyzed by laser flow cytometer (FCM) on EPICS-XL (Coulter, Miami, FL). Both intact and fragmented nuclei were gated by forward light scatter (FSC) vs. side light scatter (SSC) for measurement of fluorescence intensity for PI. The percentage of fragmented nuclei (Sub G1 peak) was calculated as described by Ohkusu *et al.* (1995).

RT-PCR – HL60 leukemia cells were treated with the sample and incubated for 24 hrs at 37 °C. Total RNA was isolated from the samples using RNA extraction kit (Sigma, Saint Louis, USA). The sequences of the primers for RT-PCR were identical to the sense (from bp number 1 to 21, 5'-ATG GAC GGG TCC GGG GAG CAG-3') or antisense (from bp number 559 to 579, 3'-TCA GCC CAT CTT CTT CCA GAT G-5') sequences of Bax cDNA. RT-PCR was performed using the Two-Step RNA PCR kit from Takara (Japan) with the primers and 500ng of total RNA. The RT-PCR products were identified by analysis on a 1% (w/v) agarose gel (Yin *et al.*, 1997).

Statistical analysis – Data from an individual experiment were described as a mean ± standard error. All statistical analyses were performed on a statistical analysis system (SAS) program, and significant difference between mean values was determined by using Student's t-test that $p < 0.05$ was judged to be statistically significant (Dowdy *et al.*, 1983).

Results and Discussion

The chloroform-soluble fraction of the vinegar treated small black soybean showed antiproliferative activity against human myeloid leukemia HL-60 cells (Fig. 1). Bioassay-guided chromatography of the chloroform-soluble fraction resulted in the isolation of two isoflavonoid compounds, genistein (**1**) and daidzein (**2**), as active principles. Its spectral data including ^{1}H- and ^{13}C-NMR are consistent with those in literature (Kinjo *et al.*, 1987).

The HL-60 cells were incubated with various concen-

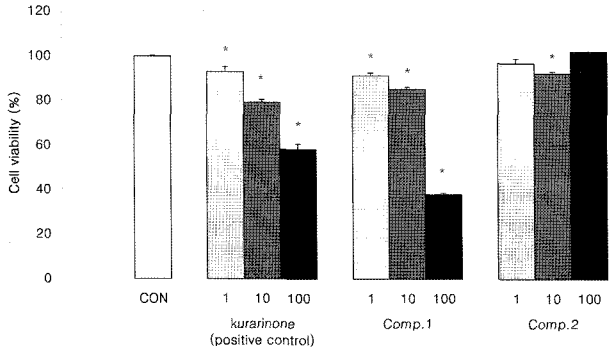

Fig. 2. Effects of the isolated compounds **1** and **2** on HL60 leukemia cell viability (μg/ml).

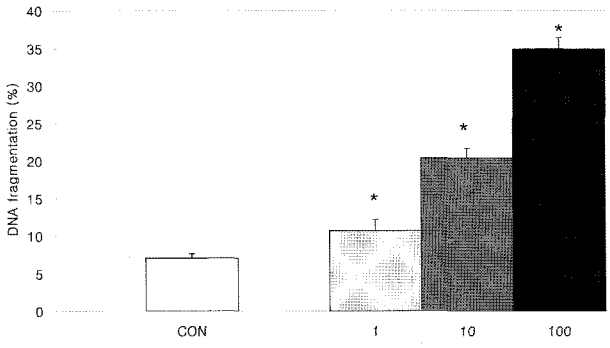

Fig. 3. Effects of compound **1** on the induction of apoptosis in HL60 leukemia cells.

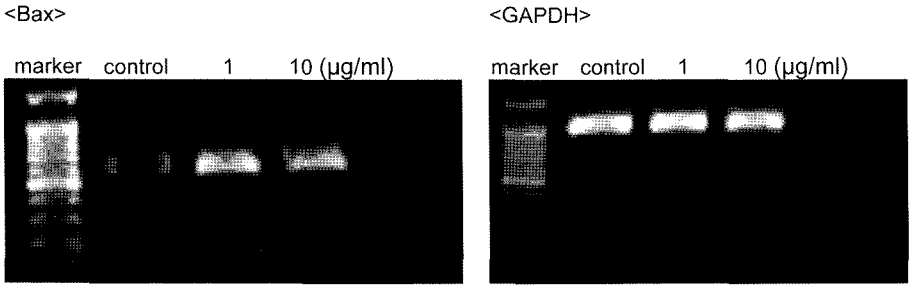

Fig. 4. Effects of compound **1** on the expression of Bax gene in HL60 leukemia cells. GAPDH : glyceraldehyde-3-phosphate dehydrogenase.

trations of isolated compounds, and the viability of the treated cells was compared with that of controls. Genistein showed the more potent growth inhibition than daidzein, as determined by a MTT assay (Fig. 2). To examine the effect of genistein on HL60 leukemia cells for inducing apoptotic cell death *in vitro*, HL60 cells were cultured with genistein (1-100 μg/ml) for 24 hrs. The level of DNA fragmentation (Sub G1 peak) was analyzed by flow-cytometric analysis. As shown in Fig. 3, DNA fragmentation in cultured HL60 leukemia cells was enhanced by the addition of genistein in a dose dependent manner.

To test the further effect of genistein on anticancer activity, apoptosis-related Bax gene expression was measured in the cultured HL60 leukemia cells treated with genistein. As shown in Fig. 4, genistein treated cells have an enhanced expression of Bax gene compared with untreated cells. However, the levels of control GAPDH gene were not changed in both cells. These results suggest that genistein effectively induce apoptosis-related Bax gene expression in the HL60 leukemia cells.

A literature survey revealed that various cellular effects of genistein, such as cell cycle arrest, inhibition of tyrosine kinases, downregulation of NF-k B, inhibition of Akt kinase, and mitochondrial damage may potentially contribute to apoptosis induction (Akiyama *et al.*, 1987; Traganos *et al.*, 1992; Spinozzi *et al.*, 1994; Davis *et al.*, 1999; Polkowski and Mazurek, 2000; Yoon *et al.*, 2000; Salvi *et al.*, 2002; Baxa and Yoshimura, 2003; Gong *et al.*, 2003). The induction of apoptosis via mitochondrial damage has been well-studied and is known to be initiated by the release of cytochrome c from the mitochondrial membrane into the cytoplasm (Martinou *et al.*, 2000). Besides these activities, additional studies have demonstrated that genistein can also inhibit DNA topoisomerase II, angiogenesis, metastasis, protein-histidine kinase, and 5α-reductase (Okura *et al.*, 1988; Huang *et al.*, 1992; Evans *et al.*, 1995; Fotsis *et al.*, 1995; Li *et al.*, 1999).

In conclusion, the vinegar treated small black soybean may be useful as a supplementary food for cancer, and it would be necessary to study the changed constituents of the vinegar treated small black soybean.

Acknowledgements

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