Natural Product Sciences
13(2) : 123-127 (2007)

Effect of Small Black Soybean Fraction on the T cell-mediated Immune Responses *in vivo* and Proliferation of Leukemia Cells *in vitro*

Chan-Ho Oh[1], Tae-Yong Shin, Byeong-Suk Chae, Kyu Hee Lee[1], Ju Sin Kim, Mi Kyeong Moon, Moon-Gu Cho[1], Jong-Hwa Kim[2], Suk-Heung Oh[1], Tae-Kyoo Lee[3], and Dae Keun Kim*

College of Pharmacy, Woosuk University, Samrye 565-701, Korea
[1]Dept. of Food Biotechnology, Woosuk University, Samrye 565-701, Korea
[2]Dept. of Pharmaceutical Engineering, Woosuk University, Samrye 565-701, Korea
[3]Dept. of Food Industry & Management, Woosuk University, Samrye 565-701, Korea

Abstract – We investigated effect of small black soybean fraction (SBSF) T cell-mediated responses for tumor surveillance and proliferation in leukemia cells *in vitro*. Each SBSF butanol fraction (SBSFBu) and SBSF chloroform fraction (SBSFCh) was administered *p.o.* once a day for 21 days in BALB/c mice and then levels of serum cytokines and subpopulation of lymphocytes were measured. Moreover, SBSF fraction was treated into the cultured various cell lines for proliferation in leukemia cell lines, NO production by RAW264.7 cells, and expression of p53 gene in U937 leukemia cells. These results showed that SBSFBu increased levels of serum IL-4 but not IL-2 and IFN-γ, and increased expression of $CD4^+$ T cells and $CD8^+$ T cells in splenocytes *in vivo*, while SBSFCh increased levels of serum IL-2 and IFN-γ but decreased IL-4, and increased $CD8^+$ T cells but not $CD4^+$ T cells. Moreover, both of SBSFBu and SBSFCh inhibited proliferation of HL60, U937, and L1210 leukemia cell lines in a dose-dependent manner, up-regulated NO production by RAW264.7 cells in a dose-dependent manner, and enhanced expression of p53 gene in U937 leukemia cells. Our findings indicate that SBSFBu and SBSFCh may enhance T cell-dependent immune responses, and that both of SBSFBu and SBSFCh may inhibit proliferation of leukemia cells by up-regulation of NO production and expression of p53 gene.
Keywords – *Glycine max*, lymphokines, $CD4^+$, $CD8^+$, cell proliferation, NO, p53

Introduction

Black soybean has been used as a health food in China. Black soybean has been reported to have antioxidant activity (Yang *et al.*, 1999; Furuta *et al.*, 2003) and to increase estrogenic effects making menopause related conditions ease (Zhao and Lou, 2006). Black soybean increased antiviral activity against a human respiratory illness virus (Yamai *et al.*, 2003) and polysaccharide of black soybean promotes myelopoiesis activity in the bone marrow, stimulates production of various hematopoietic growth factors from spleen cells, and reconstitutes bone marrow that has been myelosuppressed by irradiation and 5-FU (Liao *et al.*, 2005). A polysaccharide component from black soybean activated the immune response of mononuclear cells and inhibited proliferation in human leukemic U937 cells (Liao *et al.*, 2001).

The aim of this experiment is to study the effects of small black soybean solvent fractions on the T cell-mediated responses for tumor surveillance and antiproliferation in leukemia cells. Our results demonstrated that chloroform-soluble fraction increased IL-2 and IFN-γ leading to Th1 responses for tumor surveillance but butanol-soluble fraction increased IL-4 toward Th2 responses, and that both of butanol-and chloroform-soluble fractions induced inhibition of proliferation in leukemia cells, upregulation of NO production by RAW264.7 cells, and increased expression of p53 gene in leukemia cells.

Experimental

Animals – Male BALB/c mice at 5-6 weeks of age were purchased from Damul Science (Dajeon, Korea) and maintained with the standard rodent chow and water available *ad libitum*.

Cell lines – Human acute promyelocytic (HL60), human histiocytic (U937), and mouse lymphocytic (L1210) leukemia cell lines were provided by Korea Cell Line Bank (Seoul, Korea).

*Author for correspondence
Fax: +82-63-290-1812; E-mail: dkkim@mail.woosuk.ac.kr

Soybean material extraction and administration – The crude extract fractions were obtained from small black soybean of *G. max* (the 2003 product; Imsil, Jeonbuk, Korea) using chloroform and *n*-butanol. The extracts were dried and quantified for the total amount of crude extract. A stock solution was prepared at 100 mg of solid per mL in dimethyl sulphoxide (DMSO; Sigma) and was further diluted with RPMI 1640 immediately before treatment of the cells to achieve variously indicated concentrations (mg/mL). Each *n*-butanol-soluble fraction of small black soybean (SBSFBu) or chloroform-soluble fraction of small black soybean fraction (SBSFCh) was administered consecutively *p.o.* once a day for 21 days in BALB/c mice.

Serum preparation – Mice that administered SBSFBu or SBSFCh for 21 days were sacrificed and then serum was collected by heart puncture. The serum was stored at −70 °C for cytokine assay.

Preparation of lymphoid cells – Splenocyte suspensions were prepared from mice administered SBSFBu or SBSFCh for 21 days using Hanks' balanced salt solution (HBSS: Gibco Co., Grand Island, N.Y., USA.). Erythrocytes in the single cell suspensions were lysed by brief treatment with sterile red blood cell lysing buffer solution (Sigma). Subsequently, the cells were washed with HBSS and resuspended into a suspension of 1×10^7 cells/mL with RPMI 1640 complete medium supplemented with 10% fetal bovine serum (FBS) and penicillin (10 U/mL)-streptomycin (10 μg/mL).

Cytokine assay – The levels of serum IL-2, IL-4, and IFN-γ were determined using ELISA with cytokine monoclonal antibodies (BD Biosciences Pharmingen, U.S.A.). All measurements were carried out in duplicate. The results were measured in picograms per milliliter at 450 nm using an ELISA (enzyme-linked immunosorbent assay) microplate reader (Molecular Devices Co., Ltd., USA). The lower limit of sensitivity for each of the ELISA was equal to or smaller than 5 pg/mL.

Flow cytometry analysis – Splenocytes (1×10^6 cells/mL) from mice administered SBSFBu or SBSFCh were were preincubated with anti-Fc receptor monoclonal antibody (MAb) 2.4G2. The cells (1.0×10^6 cells/0.1 mL) were directly stained with phycoerythrin (PE)/fluorescein isothiocyanate (FITC) conjugated anti-B220/Thy1 or anti-CD4/CD8 antibody (Pharmingen, San Diego, CA, USA). The cells were incubated for 30 min in the dark, washed, and fixed with 1% paraformaldehyde until analysis. Cells were acquired (5,000 events per groupin the lymphocyte gate) and analyzed for two-parameter immunofluorescence using flow cytometry (Coulter, EPICS/ML).

Cell proliferation – The cultured various leukemia cell lines HL60, U937, and L1210 at various concentrations of SBSFBu or SBSFCh were plated into a 96-well plate at a density of 5×10^3 cells/well. Cell proliferation was measured by the microculture tetrazolium (MTT) method. The cells were resuspended in a 100 μL complete RPMI 1640 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 h, 50 μl MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} (1 mg/mL, Sigma) was added into each well. The plates were incubated for an additional 4 h. 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). Cell proliferation in non-stimulated cells from normal mice was used as a negative control. Cell proliferation rates (PR) were calculated as follows:

PR (%) = [(absorbance of the cell wells with/without mitogen-absorbance of the normal control cell wells without mitogen)/(absorbance of the normal control cell wells without mitogen)] × 100%.

NO production – RAW264.7 macrophage cell line (1×10^6 cells/mL) were treated by various concentrations of SBSFBu or SBSFCh and then cultured in complete RPMI 1640 medium for 24 h in the presence or absence of LPS 10 μg/mL (Sigma Chemical Co., St., Louse, MO) at 37 °C, 5% CO_2 incubation. The cell supernatants were harvested and NO production was measured at 570 nm using ELISA reader. Nitric oxide standard curve was measured with $NaNO_2$.

RT-PCR – U937 leukemia cells were treated by SBSFBu or SBSFCh fraction (each 1×10^{-5} mg/mL) and incubated for 24 h at 37 °C. Total RNA was isolated from the samples using RNA extract kit (Sigma, Saint Louis, USA). The sequences of the primers for RT-PCR were identical to the sense (from bp number 121 to 142, 5'-ATGGAGGAGCCGCAGTCAGATC-3') or antisense (from bp number 1,281 to 1,302, 3'-TCTTCCCGGACTGAGT CTGATT-5') sequences of p53 cDNA. RT-PCR was performed using the Two Step RNA PCR kit (Takara, Japan) with the primers and 100 ng of total RNA. RT-PCR products were identified by 1% (w/v) agarose gel electrophoresis.

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

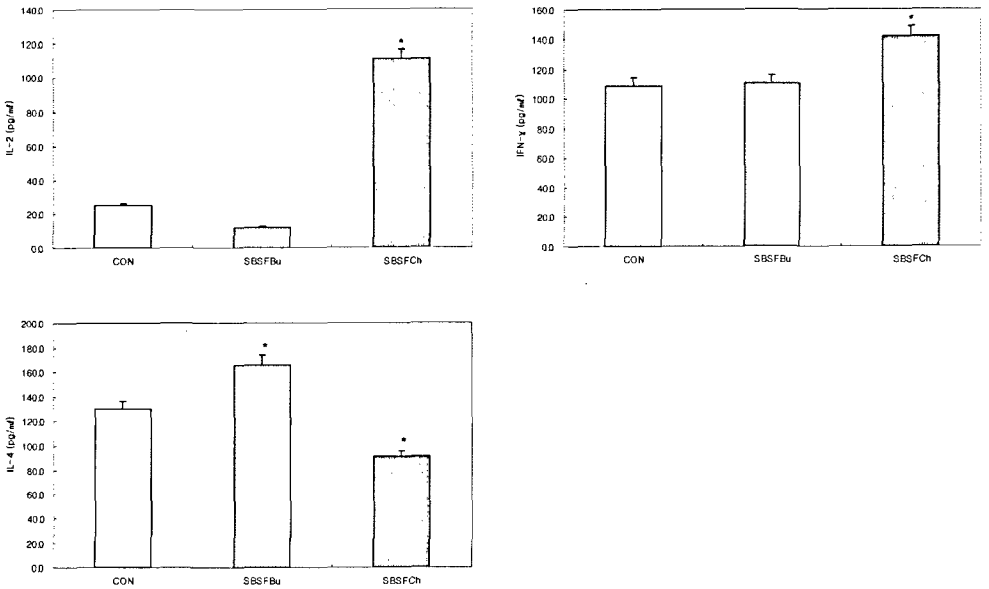

Fig. 1. Effect of the SBSF fraction on the production of lymphokines in mice serum. SBSFBu: SBSF butanol fraction. SBSFCh: SBSF chloroform fraction. Each SBSFBu and SBSFCh administered *p.o.* once a day for 21 days in BALB/c mice, and then serum was collected by heart puncture. Levels of serum cytokines, such as IL-2, IL-4, and IFN-γ, were measured using ELISA. All measurements were carried out in duplicate. Each value represents the mean ± S.E. * ($p < 0.05$): Significantly different from the value in each control.

values was determined by using Student's *t*-test that $p < 0.05$ was judged to be statistically significant.

Results and Discussion

Effect of the SBSF fraction on the production of lymphokines in mice serum – Th1 cells produce IL-2 and IFN-γ, which increase cell-mediated immune responses, while Th2 cells produce IL-4, IL-5 and IL-10, which stimulate antibody production by B cells and upregulate humoral immune responses (Murphy *et al.*, 2000). In this study, each SBSFBu and SBSFCh was administered *p.o.* once a day for 21 days in BALB/c mice and then serum was collected by heart puncture. Levels of serum cytokines, such as IL-2, IL-4, and IFN-γ, were measured using ELISA. Our results were observed that SBSFBu significantly increased levels of serum IL-4 but not IL-2, IFN-γ, while SBSFCh increased levels of serum IL-2 and IFN-γ but decreased IL-4 (Fig. 1). In addition, SBSFBu down-regulated Th1/Th2 ratio (IFN-γ/IL-4), while SBSFCh enhanced. Therefore, the results indicate that SBSFBu may induce humoral immune responses with a shift toward a Th2 response, whereas SBSFCh may induce cell-mediated immune responses with a shift toward a Th1 response effectively leading to tumor surveillance.

Effect of the SBSF fraction on the expression of subpopulation in splenocytes *in vivo* – It is known that

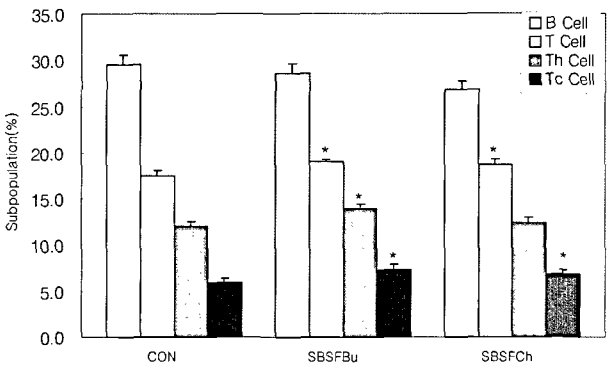

Fig. 2. Effect of the SBSF fraction on the subpopulation in splenocyte from mice. The suspensions of splenocyte from mice administered *p.o.* once a day for 21 days in BALB/c mice were prepared at 1×10^6 cells/well and subpopulation was measured by a laser flow cytometer staining with PE/FITC conjugated anti-B220/Thy1 or anti-CD4/CD8 antibody. Each value represents the mean ± S.E. Other legends and methods are the same as in Fig. 1. *($p < 0.05$): Significantly different from the value in each control.

$CD4^+$ T cells help B cell differentiate and mediate delayed-type hypersensitivity reactions and $CD8^+$ T cells participate in the host response against intracellular microorganisms and mediate cytotoxic and suppressor activities. We measured expression of subpopulation of splenocytes from mice that were administered SBSFBu or SBSFCh. These results demonstrated that SBSFBu significantly increased expression of $CD3^+$ T cells, $CD4^+$ T cells and $CD8^+$ T cells but not B220/Thy1 of B cells in splenocytes compared to controls, while SBSFCh increased

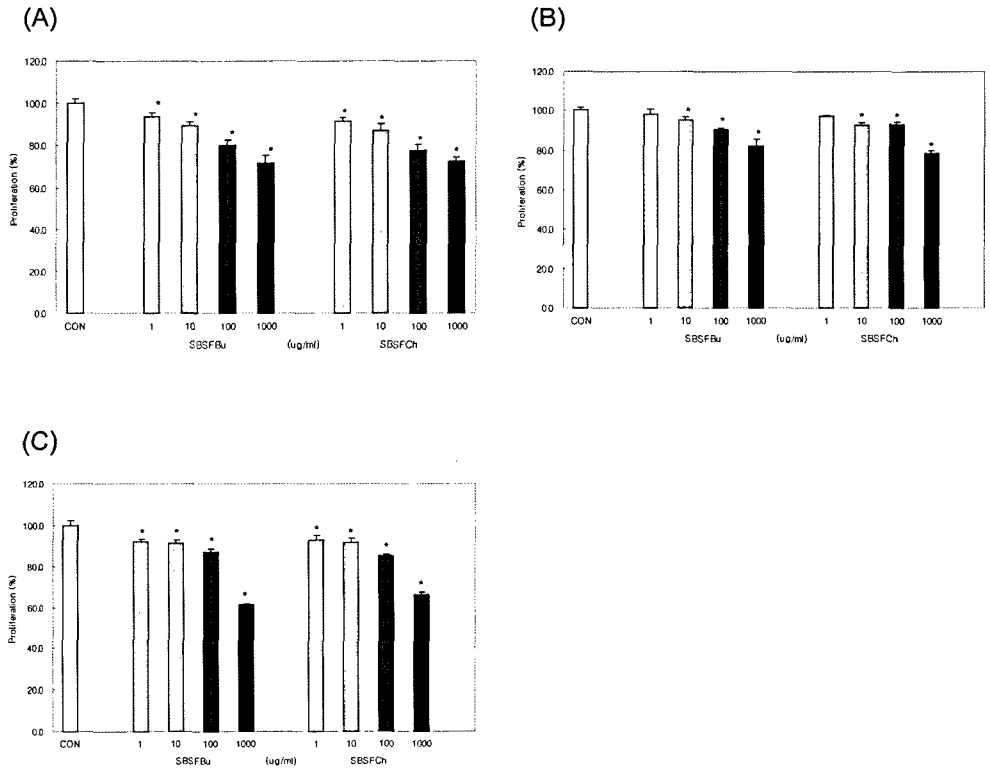

Fig. 3. Effect of the SBSF fraction on the *in vitro* proliferation of HL60 (human acute promyelocytic) (A), U937 (human histiocytic) (B), L1210 (mouse lymphocytic) (C) leukemia cell lines. Each SBSFBu or SBSFCh was treated into the cultured various cell lines at the indicated concentration and cultured for 48 h at 37 °C CO_2 incubation for proliferation in leukemia cells. The cell proliferation was assayed by MTT method. Each value represents the mean ± S.E. * ($p < 0.05$): Significantly different from the value in each control.

expression of $CD3^+$ T cells and $CD8^+$ T cells but not B220/Thy1 of B cells and $CD4^+$ T cells (Fig. 2). It is known that $CD4^+$ T cells differentiate into Th1 cells producing IL-2 and IFN-γ, Th2 cells producing IL-4, IL-5 and IL-10, and $CD8^+$ T cells produce IL-2 and IFN-γ (Murphy *et al.*, 2000). Therefore, our data suggest that SBSFBu may increase expression of $CD4^+$ T cells leading to differentiation into Th2 cells to produce IL-4, while SBSFCh may enhance expression of $CD8^+$ T cells leading to differentiation into Th1 cells to produce IL-2 and IFN-γ.

Effect of the SBSF fraction on the proliferation of leukemia cell *in vitro* – We investigated *in vitro* effect of SBSFBu or SBSFCh on the proliferation of HL60 (human acute promyelocytic) (A), U937 (human histiocytic) (B), L1210 (mouse lymphocytic) (C) leukemia cells. Each SBSFBu or SBSFCh was treated into the cultured various cell lines at the indicated concentration and cultured for 48 h at 37 °C CO_2 incubation for proliferation in leukemia cells. The cell proliferation was assayed by MTT method. Both of SBSFBu and SBSFCh significantly inhibited proliferation of HL60 (human acute promyelocytic), U937 (human histiocytic), and L1210 (mouse lymphocytic) leukemia cell in a dose-dependent manner (Fig. 3). These observation indicate that Both of SBSFBu and SBSFCh may have anti-tumour effect.

Effect of the SBSF fraction on the nitric oxide production in RAW264.7 cells – Nitric oxide (NO) has been reported to promote apoptotic cell death in the mouse macrophage cell line RAW264.7 and in the human promyelocytic leukaemia cell line U937 (Brockhaus and Brune, 1998). In the present study, RAW264.7 cells were cultured with various concentration of SBSFBu or SBSFCh for 24 h for NO production. The each OD was measured at 570 nm with a ELISA reader. Nitric oxide standard curve were measured with $NaNO_2$. The results demonstrated that both of SBSFBu and SBSFCh significantly upregulated NO production by RAW264.7 cells in a dose-dependent manner, indicating that SBSFBu and SBSFCh may increase production of NO by RAW 264.7 macrophage cell line and result in promotion of apoptotic cell death in leukaemia cell line, and that SBSFBu and SBSFCh may inhibit proliferation of leukemia cell lines through apoptosis induced by increased NO production.

Effect of the SBSF fraction on the expression of p53 gene in cultured U937 leukemia cells – p53 is well-known as a tumor suppressor gene. NO-induced apoptotic

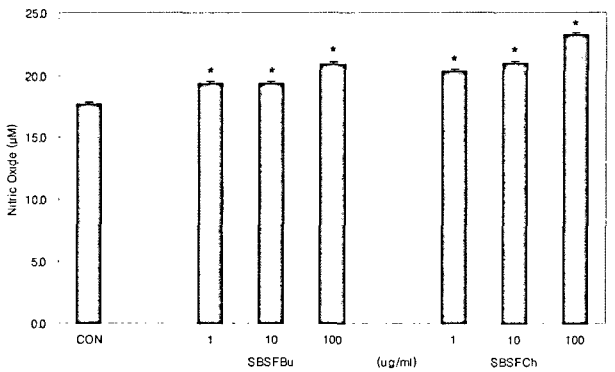

Fig. 4. Effect of the SBSF fraction on the nitric oxide production in RAW264.7 macrophage cell line. RAW264.7 cells were cultured with various concentration of SBSFBu or SBSFCh for 24 h at 37 °C, 5% CO_2 incubation in the presence or absence of LPS 10 μg/mL (Sigma Chemical Co., St., Louse, MO) at 37 °C, 5% CO_2 incubation for NO production. The each OD was measured at 570 nm with a ELISA reader. Each value represents the mean ± S.E. * ($p < 0.05$): Significantly different from the value in each control.

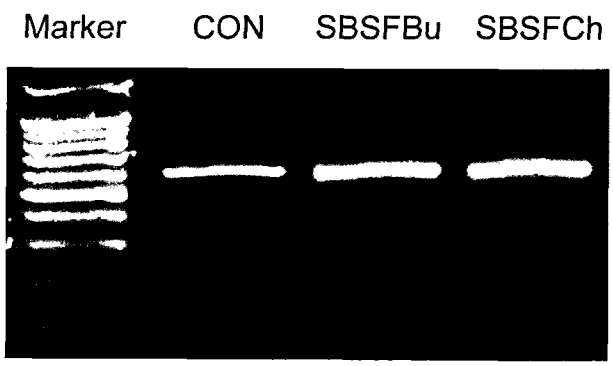

Fig. 5. Effect of the SBSF fraction on the expression of p53 gene in cultured U937 leukemia cells. U937 leukemia cells were treated by SBSFBu or SBSFCh fraction (each 1×10^{-5} mg/mL) and incubated for 24 h at 37 °C. Total RNA was isolated from the samples using RNA extract kit. RT-PCR was performed using the Two Step RNA PCR kit with the primers. RT-PCR products were identified by 1% (w/v) agarose gel electrophoresis.

cell death in the human promyelocytic leukaemia cell line U937 exemplifies p53-dependent and p53-independent executive death pathways. The observation shown in Fig. 3 and 4 expected SBSFBu and SBSFCh might induce increased expression of p53 gene in leukemia cells. Each SBSFBu and SBSFCh was treated and cultured for 24 h for expression of p53 gene in U937 leukemia cells. Both of SBSFBu and SBSFCh increased expression of p53 gene in U937 leukemia cell line, suggesting that SBSFBu and SBSFCh may induct apoptosis in leukemia cells via upregulation of p53 gene expression associated with NO-induced apoptotic cell death.

In conclusion, our findings indicate that SBSFCh may induce Th1 response to produce IL-2 and IFN-γ, which may lead to tumor surveillance but SBSFBu may induce Th2 response to produce IL-4, and that both of SBSFBu and SBSFCh inhibit cell proliferation in leukemia cells by induction of apoptosis via upregulation of NO production and expression of p53 gene.

Acknowledgements

This work was supported by the Jeonbuk Bioindustry Development Institute Grant funded by the Ministry of Commerce, Industry and Energy (MOCIE), Jeollabuk-do Provincial Government and Imsil-Gun.

References

Brockhaus, F. and Brune, B., U937 apoptotic cell death by nitric oxide: Bcl-2 downregulation and caspase activation. *Exp. Cell Res.* **238**, 33-41 (1998).

Furuta, S., Takahashi, M., Takahata, Y., Nishiba, Y., Oki, T., Masuda, M., Kobayashi, M., and Suda, I., Radical-scavenging activities of soybean cultivars with black seed coats. *Food Sci. Technol. Res.* **9**, 73-75 (2003).

Liao, H.F., Chou, C.J., Wu, S.H., Khoo, K.H., Chen, C.F., and Wang, S.Y., Isolation and characterization of an active compound from black soybean [*Glycine max* (L.) Merr.] and its effect on proliferation and differentiation of human leukemic U937 cells. *Anticancer Drugs* **12**, 841-846 (2001).

Liao, H.F., Chen, Y.J., and Yang, Y.C., A novel polysaccharide of black soybean promotes myelopoiesis and reconstitutes bone marrow after 5-flurouracil- and irradiation-induced myelosuppression. *Life Sciences* **77**, 400-413 (2005).

Murphy, K.M., Ouyang, W., Farrar, J.D., Yang, J., Ranganath, S., Asnagli, H., Afkarian, M., and Murphy, T.L., Signaling and transcription in T helper development. *Annu. Rev. Immunol.* **18**, 451-494 (2000).

Yamai M, Tsumura K, Kimura M, Fukuda S, Murakami T, Kimura Y. Antiviral activity of a hot water extract of black soybean against a human respiratory illness virus. *Biosci. Biotechnol. Biochem.* **67**, 1071-1079 (2003).

Yang, C.M., Yang, J.S., and Chao, P.Y., Gray prediction comparison on the antioxidative capacity of commercial black bean and soybean. *J. Nutrition Science* **24**, 201-214 (1999).

Zhao, Q.W. and Lou, Y.J., Estrogenic activity and its mechanism of ethanol extract from black soybean. *Zhongguo Zhong Yao Za Zhi* **31**, 825-828 (2006).

(Accepted March 29, 2007)