

***Ginseng radix* induces apoptosis in HL-60 cells and its mechanism as little relation with TNF- α production**

Hyun-Na Koo¹, Soon-Shik Shin², Jin-Han Park³, Na-Hyung Kim³, Seung-Heon Hong³ and Hyung-Min Kim^{1,*}

¹Department of Pharmacology, College of Oriental Medicine, Kyung Hee University, Seoul, 130-701, South Korea;

²Department of Prescriptionlogy, College of Oriental Medicine, Donggeui University, San 45-1, Yangjeong 2-dong, Busanjin-gu, Busan 614-052, South Korea; ³College of Pharmacy, Wonkwang University, Iksan, Jeonbuk, 570-749, South Korea

SUMMARY

Ginseng radix, the root of *Panax ginseng* C. A. Meyer (Araliaceae), is a medicinal plant used world-widely and has been reported to have various biological effects. To investigate the effects of *Ginseng radix* on HL-60 cell apoptosis, MTT assay, DNA fragmentation assay and flow cytometry were performed on HL-60 cells. Cells were treated with *Ginseng radix* at different concentrations (10^{-4} , 10^{-3} and 10^{-2} ; dilution rate). *Ginseng radix* significantly induced cells apoptosis with a time- and dose-dependent manner. To determine whether *Ginseng radix*-induced apoptosis is due to increase of tumor necrosis factor (TNF- α) secretion, enzyme-linked immunosorbent assay was performed on HL-60 cells. Unexpectedly, *Ginseng radix* (96 ± 5 pg/ml) significantly decreased the TNF- α secretion compared with control (174 ± 14 pg/ml). Furthermore, *Ginseng radix* with rIFN- γ synergistically increased nitric oxide production in mouse peritoneal macrophages. Taken together, our data indicate that *Ginseng radix* induce apoptosis on HL-60 cells without increase of TNF- α secretion and could be used for a supplementary remedy of cancer.

Key words: *Ginseng radix*; Apoptosis; Tumor necrosis factor- α ; Nitric oxide

INTRODUCTION

The genus *Panax* belongs to the family Araliaceae. Ginseng is an herb that is held in high regard for its regenerative properties. The principal dynamic constituents of ginseng are the ginsenosides. Certain combinations of these exist in various species of ginseng. *Panax ginseng* has been reported to have various biological effects such as antitumor, antioxidative,

antiallergic and antidiabetic activity (Baek *et al.*, 1995; Dey *et al.*, 2002; Choo *et al.*, 2003; Liu *et al.*, 2003). These activities might be due to a glycoside group called ginsenoside or panax saponin on the *Panax ginseng*, which was soluble in water.

For many years, the cytotoxic actions of chemotherapeutic drugs were ascribed solely to their ability to induce genotoxic damage. During the past decade, however, the evidence is gradually being shown that many cancer chemotherapeutic agents induce a cell death process known as programmed cell death or apoptosis. Apoptosis is characterized by distinct morphological features such as cell shrinkage,

*Correspondence: Prof Hyung-Min Kim, Department of Pharmacology, College of Oriental Medicine, Kyung Hee University, 1 Hoegi-Dong, Dongdaemun-Gu, Seoul, 130-701, South Korea. Tel: +82-2-961-9448; Fax: +82-2-968-1085; E-mail: hmkim@khu.ac.kr

chromatin condensation, plasma membrane blebbing, oligonucleosomal DNA fragmentation, and finally the breakdown of the cell into smaller units (apoptotic bodies) (Earnshaw *et al.*, 2002). It can be triggered by numerous stimuli, including antigens, carcinogens, ionizing radiation, growth factor withdrawal, and chemotherapeutic agents (Bortner *et al.*, 2002). Cytokines and their corresponding receptors are known to be important regulators of cell death (Ware *et al.*, 1996). Intracellular pathways that originate at the tumor necrosis factor (TNF)- α receptor are linked to apoptosis (Neuman *et al.*, 1999). Treatment of anti-TNF- α antibodies block apoptosis processes. TNF- α is a member of the cytokine family, and is released by leukocytes and occasionally other cells (Titus *et al.*, 1991; Keadle *et al.*, 2000; Weaver *et al.*, 2001). Recently we reported that *Taraxacum officinale* induces cell death through TNF- α secretion in Hep G2 cells (Koo *et al.*, 2004).

Macrophages play a significant role in host defense mechanisms. When activated, they inhibit the growth of a wide variety of tumor cells and microorganisms (Hajri *et al.*, 1998; Nascimento *et al.*, 1998). Nitric oxide (NO) was reported to participate in the cytolytic function of macrophages (Palmer *et al.*, 1988). Treatment of tumor cells with S-nitroso-N-acetylpenicillamine, a NO-generating compound, showed a similar tumoricidal effect. Stimulation of murine macrophages by LPS and IFN- γ results in the expression of an inducible NO synthase (iNOS), which catalyzes the production of large amounts of NO from L-arginine and molecular oxygen (Hibbs *et al.*, 1987).

Ginseng radix may act as an inducer of apoptosis in human leukemia cell line HL-60.

The aims of the present investigation are to explore whether *Ginseng radix* induces apoptotic cell-death in HL-60 cells and whether TNF- α secretion in *Ginseng radix*-induced cell death is

involved.

MATERIALS AND METHODS

Reagents

RPMI-1640, DMEM and fetal bovine serum (FBS) were purchased from Gibco/BRL (Burlington, Ontario, Canada). DMSO, LPS, propidium iodide (PI) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO, USA). Thioglycollate (TG) was purchased from Difco Laboratories (Detroit, MI). Murine rIFN- γ (1×10^6 U/ml) was purchased from Genzyme (Mnchen, Germany). Human anti-TNF- α antibodies and recombinant TNF- α were purchased from R & D system Inc. (Minneapolis, MN). Male C57BL/6 mice were purchased from Samtako BIO KOREA (Ohsan, South Korea).

Preparation of *Ginseng radix* extract

Ginseng radix was kindly provided as a gift by Dr. S.S. Shin of College of Oriental Medicine, Dong Eui University, South Korea. The roots were evaluated by 6 years old. No heavy metals were detected from *Ginseng radix* in the test by Korean Oriental Medicine Institute. The extract of *Ginseng radix* was prepared by filter pressing the grinded root using 0.45 m filter. The extract was diluted with saline by 10^{-4} – 10^{-2} rate.

Cell culture

HL-60 cells were obtained from the Korean Cell Line Bank (KCLB), Seoul, South Korea. They were maintained in RPMI 1640 medium containing 10 % heat-inactivated FBS supplemented with 100 unit/ml penicillin and 100 μ g/ml streptomycin at 37 °C under 5 % CO₂ in air. The cells were exposed to various concentration (10^{-4} , 10^{-3} and 10^{-2} ; dilution rate) with *Ginseng radix* for 24 h and 48 h.

Isolation of peritoneal macrophages

Peritoneal macrophages were isolated by following method. TG-elicited macrophages were harvested 3-4 days after i.p. injection of 2.5 ml TG to the mice and isolated, as reported previously (Chung *et al.*, 2003). Using 8 ml of HBSS containing 10 U/ml heparin, peritoneal lavage was performed. Then, the cells were distributed in DMEM, which was supplemented with 10 % FBS, in 4-well tissue culture plates (2.5×10^5 cells/well) incubated for 3 h, washed three times with HBSS to remove non-adherent cells, and equilibrated with DMEM that contained 10 % FBS before treatment.

MTT assay

Cells were seeded in 4-well plates (500 μ l/well at a density of 2×10^5 cells/ml) and exposed to various concentrations with *Ginseng radix* for 24 and 48 h. The cell survival fraction was determined with the MTT dye-reduction assay as described by Mosmann (Mosmann *et al.*, 1983), with some modifications. In brief, after incubation with *Ginseng radix*, MTT solution (5 mg/ml in PBS) was added (50 μ l/well). Plates were further incubated for 4 h at 37 $^{\circ}$ C, and the formazan crystals formed were centrifuged and the pellets dissolved by the addition of DMSO. Absorption was measured by spectrometer at 540 nm.

Cell morphology

Cells were plated in 12 well plates at a density of 1×10^5 cells/well. They were added to different concentrations of *Ginseng radix* and grown at 37 $^{\circ}$ C in a humidified 5 % CO₂ for 48 h. For the cell morphology experiment, the culture plates were examined under a phase microscope and photographed.

DNA extraction and electrophoresis

The characteristic ladder pattern of DNA

break was analyzed by agarose gel electrophoresis. Briefly, DNA from the HL-60 cells (2×10^6 cells/each group) was isolated by Genomic DNA purification kit (iNtRON BIOTECHNOLOGY Co., Ltd. Sungnam, South Korea). Isolated genomic DNA (10 μ g) was subjected on 1.5 % agarose electrophoresis at 100 V for 30 min. DNA was visualized by staining with ethidium bromide under UV light.

PI staining for flow cytometry

Proportions of cell cycle phase were analyzed by flow cytometry. In brief, the cells were fixed with 70 % ethanol at 4 $^{\circ}$ C for 60 min. After washing with PBS, the cells were treated with 0.5 ml of RNase and then with 1 ml of PI (100 μ g/ml in PBS) solution in dark at 4 $^{\circ}$ C for 60 min. After washing and passing through nylon mesh, the samples were kept on ice until measured. The DNA histogram was obtained with a flow cytometry cell sorter (Becton Dickinson).

Assay of TNF- α secretion

TNF- α concentration in the cells derived culture supernatants was measured by a modified ELISA, as described (Kim *et al.*, 1998). The ELISA was devised by coating 96-well plates of human monoclonal antibody with specificity for human TNF- α . Before use and between subsequent steps in the assay, coated plates were washed with PBS containing 0.05 % tween-20. All reagents used in this assay were incubated for 2 h at 37 $^{\circ}$ C. Recombinant human TNF- α were diluted and used as a standard. Serial dilutions starting from 1 pg/ml were used to establish the standard curve. Assay plates were exposed sequentially to rabbit anti-TNF- α antibody, and phosphatase-conjugated goat anti-rabbit IgG antibody and avidine peroxidase, and p-nitrophenyl phosphate and ABTS substrate solution containing 30 % H₂O₂.

The plates were read at 405 nm.

Measurement of nitrite concentration

To measure nitrite, 100 μ l aliquots were removed from a conditioned medium and incubated with an equal volume of Griess reagent (1 % sulfanilamide/0.1 % N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5 % H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined in a Titertek Multiskan (Flow Laboratories, North Ryde, Australia). NO_2^- was determined by using sodium nitrite as a standard. The cell-free medium alone contained 5 to 8 μ M of NO_2^- ; this value was determined in each experiment and subtracted from the value obtained from the medium with cells.

Statistical analysis

Results were expressed as the means \pm S.E. The significance of the mean difference was determined by Student's *t*-test for independent data. All statistical analyses were performed using SPSS v10.0 statistical analysis software. A value of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Ginseng radix induces apoptotic cell death in HL-60 cells

To examine whether *Ginseng radix* induces apoptotic cell death, we first assessed the effect of *Ginseng radix* on the viability of HL-60 cells using MTT dye assay. As shown Fig. 1, when the cells were treated for 24 h and 48 h with various concentration (10^4 , 10^3 and 10^2 d) of *Ginseng radix*, the cell viability significantly decreased in a dose-and time-dependent manner. We then addressed the issue of whether the loss of cell viability was due to apoptotic cell-death. Cells treated for 48 h with 10^4 , 10^3 and 10^2 d of *Ginseng radix* showed DNA

fragmentation (Fig. 2A). Apoptotic cell-death induced by *Ginseng radix* was also evidenced by morphological changes of the cells as shown Fig. 2B. *Ginseng radix* dose-dependently increased the plasma membrane blebbing. At concentration of 10^2 d *Ginseng radix*, the chromatin condensations and apoptotic bodies were widespread throughout the entire population. Moreover, as shown in Fig. 3, flow cytometric analyses determined that 33.7% of cells underwent apoptosis when the cells were treated for 48 h with the same dose of *Ginseng radix* as used for DNA fragmentation assays. Collectively, these data clearly demonstrate that *Ginseng radix* induces apoptosis in HL-60 cells.

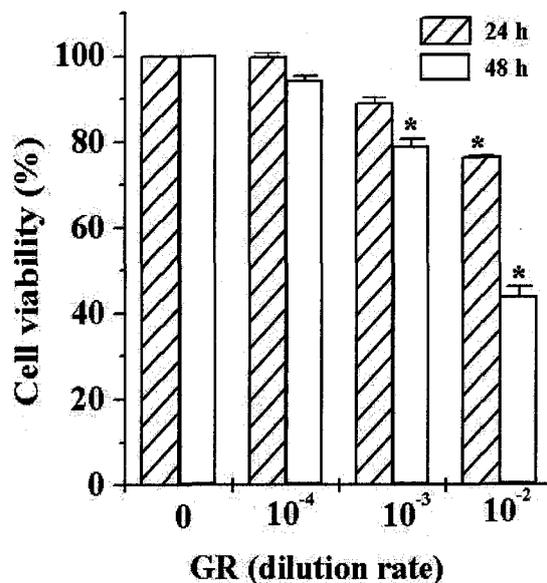


Fig. 1. Effect of *Ginseng radix* on cell viability of HL-60 cells. The cells (2×10^5 cells/ml) were incubated with various concentrations (10^4 , 10^3 and 10^2 ; dilution rate) of *Ginseng radix* for 24 and 48 h. Cell viability was determined by the MTT dye assay. Results represent three independent experiments in triplicate. Values from each treatment were expressed as a percent relative to the control (100%). Each data represents the mean \pm S. E. * $P < 0.05$ significantly different from the control value.

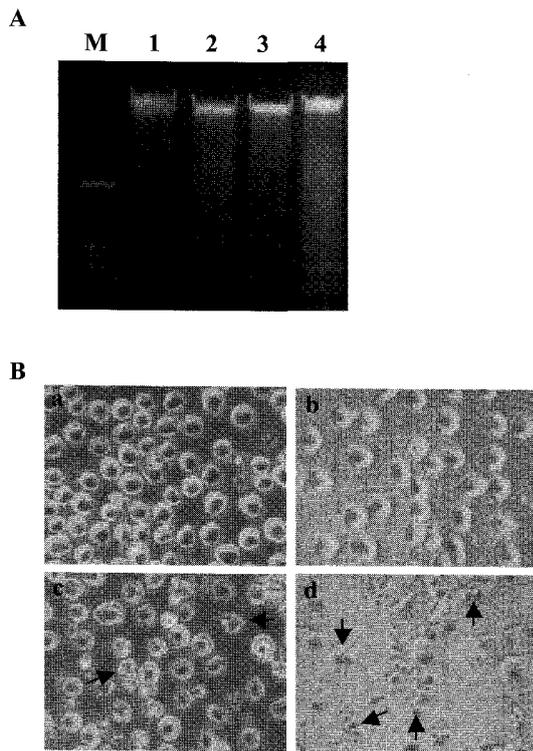


Fig. 2. Effect of *Ginseng radix* on apoptosis of HL-60 cells. The cells (2×10^6 cells/each group) were incubated with various concentrations of *Ginseng radix* for 48 h. Ten μ g of DNA were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide, and photographed under UV illumination. The standard DNA fragments (lane M) are shown on the left (panel A). Panel B, *Ginseng radix*-induced morphological changes of HL-60 cells. The cells were photographed under a microscope (magnification: $\times 100$). Arrows indicate multi-blebbing cells and apoptotic bodies. Panel a, control; panel b, 10^{-4} d of *Ginseng radix*; panel c, 10^{-3} d of *Ginseng radix* and panel d, 10^{-2} d of *Ginseng radix*.

***Ginseng radix* decrease TNF- α secretion**

To determine whether *Ginseng radix*-induced apoptosis is due to increase of TNF- α secretion, TNF- α secretion was quantified by ELISA method. TNF- α is a member of the cytokine family, and TNF- α receptors are linked to apoptosis. Unexpectedly, however *Ginseng radix* significantly decreased the TNF- α production compared with control (Table 1). These results

suggest that *Ginseng radix*-induced apoptosis have little concern with the secretion of TNF- α in HL-60 cells.

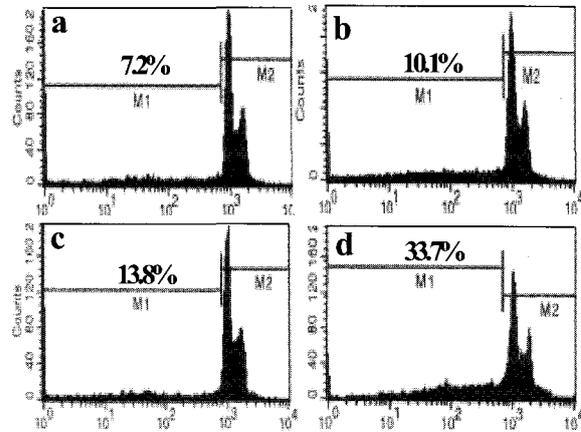


Fig. 3. Effect of *Ginseng radix* on cell cycle distribution of HL-60 cells. The cells (2×10^6 cells/each group) were incubated with various concentrations of *Ginseng radix* for 48 h. The cells were stained with PI solution for 1 h and analyzed for DNA content by flow cytometry. Data represent the result from one of three similar experiments. Panel a, control; panel b, 10^{-4} d of *Ginseng radix*; panel c, 10^{-3} d of *Ginseng radix* and panel d, 10^{-2} d of *Ginseng radix*.

Table 1. Effect of *Ginseng radix* on TNF- α secretion by HL-60 cells

<i>Ginseng radix</i>	TNF- α secretion (pg/ml)	
	24 h	48 h
0	174 \pm 14	291 \pm 13
10^{-4} d	126 \pm 14	123 \pm 11*
10^{-3} d	154 \pm 15	108 \pm 18*
10^{-2} d	96 \pm 5*	103 \pm 18*

The cells (1×10^6 cells/ml) were treated for 24 h and 48 h with various concentration (10^{-4} , 10^{-3} and 10^{-2} d) of *Ginseng radix*. The supernatants were collected and frozen at -80°C until assayed for TNF- α concentration. Each data value indicates the mean \pm S. E. of three separated experiments. * $P < 0.05$ versus medium alone control.

Ginseng radix with rIFN- γ synergistically increased NO production in mouse peritoneal macrophages

Finally, we investigated whether *Ginseng radix* induces NO production in macrophages. Macrophages play a significant role in host defense mechanisms. When activated, they inhibit the growth of a wide variety of tumor cells and microorganisms. Treatment of tumor cells with a NO-generating compound showed a similar tumoricidal effect. Peritoneal macrophages were cultured with IFN- γ (5 U/ml) and then stimulated with LPS (100 ng/ml) or 10^{-2} d of *Ginseng radix*. NO release was measured using the Griess method. As shown in Fig. 4, *Ginseng radix* alone had no effect, whereas *Ginseng radix* with IFN- γ significantly increased the NO production in macrophages.

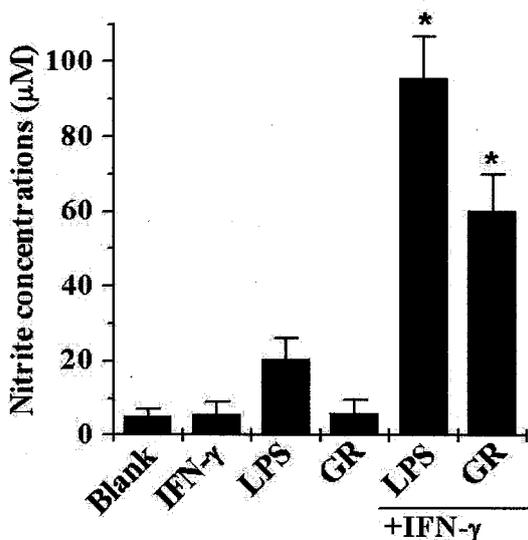


Fig. 4. Effect of *Ginseng radix* on NO production of mouse peritoneal macrophages. The cells (2.5×10^5 cells/each group) were cultured with IFN- γ (5 U/ml) and then stimulated with LPS (100 ng/ml) or 10^{-2} d of *Ginseng radix* at 6 h after incubation. After 48 h of culture, NO release was measured by the Griess method. Values are means \pm S. E. of duplicate determinations from three separate experiments (* $P < 0.05$).

DISCUSSION

The results reported here demonstrate that *Ginseng radix* induces apoptosis in HL-60 cells. The inductive effect has been characterized by three different methods, cell morphology, DNA fragmentation and flow cytometric assays. The results show that *Ginseng radix*-induced morphological changes in the cells are typically apoptotic, evidenced by multi-blebbing, chromosomal condensations and formation of apoptotic bodies. Similarly, both DNA fragmentation and flow cytometric assays show that *Ginseng radix*-induced DNA cleavages as well as formation of apoptotic bodies. Thus, we propose that *Ginseng radix* contains an inducing activity of apoptosis in HL-60 cells.

In our previous reports (Koo et al., 2004), we have shown that *Taraxacum officinale* induces cytotoxicity through TNF- α secretion in Hep G2 cells. TNF- α receptors are linked to apoptosis and treatment of anti-TNF- α antibodies block apoptosis processes. To assess whether *Ginseng radix*-induced apoptosis is due to increase of TNF- α secretion, we measured the TNF- α secretion in *Ginseng radix*-treated cells. Unexpectedly, however *Ginseng radix* significantly decreased the TNF- α production compared with control (Table 1). These results suggest that *Ginseng radix*-induced apoptosis have little relation with the secretion of TNF- α in HL-60 cells. Additionally, this fact indicate that other pathways are involved. In order to clarify the reason of this result, further experiments are necessary.

Macrophages play a significant role in host defense mechanisms. When activated, they produces NO as one of the defense mechanism. NO is beneficial in fighting against bacteria, fungi, viruses, and parasites (Nathan et al., 1992). NO generation also influences the cytotoxicity of macrophages and tumor-induced

immunosuppression. Treatment of tumor cells with S-nitroso-N-acetylpenicillamine, a NO-generating compound, showed a similar tumoricidal effect. We therefore expected that *Ginseng radix* could activate the macrophages. For this experiment, we used recombinant IFN- γ that primarily activate macrophages. Interestingly, *Ginseng radix* with IFN- γ significantly increased the NO production in macrophages. These results support that *Ginseng radix* is capable of inhibiting development of tumorigenesis.

In summary, our data provide that *Ginseng radix* induces apoptosis of HL-60 cells. Our data additionally show that *Ginseng radix*-induced apoptosis is not related TNF- α production. Although caution should be taken in extrapolating data obtained in an in vitro model to in vivo conditions, *Ginseng radix*-induced apoptosis of HL-60 cells may have a beneficial role in the treatment of cancer.

REFERENCES

- Baek NI, Kim DS, Lee YH, Park JD, Lee CB, Kim SI. (1995) Cytotoxicities of ginseng saponins and their degradation products against some cancer cell lines. *Arch. Pharm. Res.* **18**, 164-168.
- Bortner CD, Cidlowski JA. (2002) Cellular mechanisms for the repression of apoptosis. *Annu. Rev. Pharmacol. Toxicol.* **42**, 259-281.
- Choo MK, Park EK, Han MJ, Kim DH. (2003) Antiallergic activity of ginseng and its ginsenosides. *Planta Med.* **69**, 518-522.
- Chung HS, Shin CH, Lee EJ, Hong SH, Kim HM. (2003) Production of nitric oxide and tumor necrosis factor-alpha by *Smilacis rhizoma* in mouse peritoneal macrophages. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **135**, 197-203.
- Dey L, Zhang L, Yuan CS. (2002) Anti-diabetic and anti-obese effects of ginseng berry extract: comparison between intraperitoneal and oral administrations. *Am. J. Chin. Med.* **30**, 645-647.
- Earnshaw WC. (1995) Nuclear changes in apoptosis. *Curr. Opin. Cell Biol.* **7**, 337-343.
- Hajri A, Metzger E, Vallat F, Coffy S, Flatter E, Evrard S, Marescaux J, Arahamian M. (1998) Role of nitric oxide in pancreatic tumour growth: in vivo and in vitro studies. *Br. J. Cancer* **78**, 841-849.
- Hibbs JB Jr, Taintor RR, Vavrin Z. (1987) Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science* **235**, 473-476.
- Keadle TL, Usui N, Laycock KA, Miller JK, Pepose JS, Stuart PM. (2000) IL-1 and TNF-alpha are important factors in the pathogenesis of murine recurrent herpetic stromal keratitis. *Invest. Ophthalmol. Vis. Sci.* **41**, 96-102.
- Kim HM, Lee EH, Lim TG, Jung JA, Lyu YS. (1998) Inhibitory effect of *Asparagus cochinchinensis* on tumor necrosis factor-alpha secretion from astrocytes. *Int. J. Immunopharm.* **20**, 153-162.
- Koo HN, Hong SH, Song BK, Kim CH, Yoo YH, Kim HM. (2004) *Taraxacum officinale* induces cytotoxicity through TNF-alpha and IL-1alpha secretion in Hep G2 cells. *Life Sci.* **74**, 1149-1157.
- Liu ZQ, Luo XY, Liu GZ, Chen YP, Wang Z C, Sun YX. (2003) In vitro study of the relationship between the structure of ginsenoside and its antioxidative or prooxidative activity in free radical induced hemolysis of human erythrocytes. *J. Agric. Food Chem.* **51**, 2555-2558.
- Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55-63.
- Nascimento FR, Ribeiro-Dias F, Russo M. (1998) Cytotoxic activity of BCG-activated macrophages against L929 tumor cells is nitric oxide-dependent. *Braz. J. Med. Biol. Res.* **31**,

- 1593-1596.
- Nathan C. (1992) Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6**, 3051-3064.
- Neuman MG, Shear NH, Cameron RG, Katz G, Tiribelli C. (1999) Ethanol-induced apoptosis in vitro. *Clin. Biochem.* **32**, 547-555.
- Palmer RM, Ashton DS, Moncada S. (1988) Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* **333**, 664-666.
- Titus RG, Sherry B, Cerami A. (1991) The involvement of TNF, IL-1 and IL-6 in the immune response to protozoan parasites. *Immunology* **12**, A13-16.
- Ware CF, VanArsdale S, VanArsdale TL. (1996) Apoptosis mediated by the TNF-related cytokine and receptor families. *J. Cell Biochem.* **60**, 47-55.
- Weaver SA, Russo MP, Wright KL, Kolios G, Jobin C, Robertson DA, Ward SG. (2001) Regulatory role of phosphatidylinositol 3-kinase on TNF-alpha-induced cyclooxygenase 2 expression in colonic epithelial cells. *Gastroenterology* **120**, 1117-1127.