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Modulation of IL-12 and IFN-γ Secretions by Eleutheroside E, Tortoside A, and Syringaresinol from *Acanthopanax koreanum* Nakai

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Abstract − *Acanthopanax koreanum* Nakai (Araliaceae) is a medicinal plant indigenous to Korea. The root and stem barks of Acanthopanax species have been used as a tonic and sedative as well as in the treatment of rheumatism and diabetes. In our study, three lignans, eleutheroside E (EE), tortoside A (TA), and syringaresinol (SY), were isolated from the stem and root of *A. koreanum* in an effort to study the immunomodulating effect. We treated natural killer cells and dendritic cells with lignans (EE, TA, or SY), and analyzed their cytokine (IL-12 and IFN-γ) secretion. EE, TA, or SY markedly enhanced IL-12 secretion in mouse lymphoid (DC1) and myeloid type (DC2.4) dendritic cells after 48 hr of treatment. There were no significant differences in the cytokine stimulatory effects between EE, TA, or SY. Moreover, treatment of EE, TA, or SY significantly induced IFN-γ secretion by human NK cells (NK92MI) confirmed by ELISA assay. This study suggests that lignans from *A. koreanum* modulate cytokines, and that such modulation may provide the mechanism of action for many of their therapeutic effects.

Keywords: *Acanthopanax koreanum*, IL-12, IFN-γ, Dendritic cells, NK cells

INTRODUCTION

Acanthopanax koreanum Nakai (Araliaceae) is a medicinal plant indigenous to Korea (Kim and Chung, 1988). The root and stem barks of Acanthopanax species have been used as a tonic and sedative as well as in the treatment of rheumatism and diabetes (Perry and Metzger, 1980). However, little is known about its effects in the immune system and its mechanisms of action. Lignans and neolignans, found in a wide range of plants, display numerous pharmacological activities (Pool-Zobel *et al*., 2000; Arroo *et al*., 2002). In view of the important pharmacological properties, numerous studies have been carried out in an attempt to get a better knowledge of the biological events linked to lignans isolated from Acanthopanax species (Kim *et al*., 1995; Yook *et al*., 1998; Ban *et al*., 2002; Lee *et al*., 2004; Lin *et al*., 2008; Nhiem *et al*., 2009). It was reported that lupane triterpene glycosides from leaves of *A. koreanum* showed cytotoxic activities against leukemia, lung and breast cancer cell lines (Nhiem *et al*., 2009). Moreover, *A. senticosus* stems and their lignan components possessed antioxidant and hepatoprotective activities (Lee *et al*., 2004).

Modulation of cytokine secretion may offer novel approaches in the treatment of a variety of diseases (Spelman *et al*., 2006). For example, in the field of oncology, progress has been made in the therapeutic use of several interleukins, including interleukin (IL)-4, -6, -11 and -12. One strategy in the modulation of cytokine expression may be through the use of herbal medicines (Oleksowicz and Dutcher, 1994). In light of the adverse events experienced with cytokine-targeted therapy, it could prove useful to consider the use of phytotherapy in the modulation of cytokine expression (Spelman *et al*., 2006).

Dendritic cells (DCs) and cytotoxic T lymphocytes (CTLs) are the first-line protecting barrier against different pathogens (viruses, bacteria and neoplasms cells) (Krawczyk *et al*., 2006). Several different DC populations have been identified, each of which is differentiated via a unique pathway. Two DC subsets of myeloid origin have been described: Langerhans cells (LCs), present in the epidermis, which take up antigen and subsequently migrate to local lymph nodes to differentiate into DCs; and myeloid-line-

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age-derived DCs, located in the dermis, blood, and B-cell follicles, which lack LC markers. There is also evidence for the existence of a distinct lymphoid DC subset, which is represented by the $CD11c^-$ plasmacytoid DC (Banchereau and Steinman, 1998). Following antigen uptake in the periphery, immature DCs change and migrate into secondary lymphoid organs, where they mature and must interact in an antigen-specific manner with B cells and T cells to initiate an immune response (Banchereau and Steinman, 1998; Vissers *et al*., 2001). Immature myeloid- and lymphoid dendritic cells possess ability to phagocytose and present antigens to lymphocytes. They have also ability to produce IL-12, which is also known as natural killer cell stimulatory factor or cytotoxic lymphocyte maturation factor (Krawczyk *et al*., 2006).

The notion currently prevailing is that natural killer (NK) cells can be rapidly activated in the periphery by chemokines and/or inflammatory cytokines in conjunction with NK cell stimulatory factors such as IL-12, interferon (IFN) type 1, or IL-2 (Zitvogel, 2002). In particular, IFN-gamma (γ) is considered the prototypic NK cell cytokine, and its production by NK cells is known to shape the Th1 immune response (Mocikat *et al*., 2003), activate antigen presenting cells (APC) to further up-regulate MHC class I expression (Wallach *et al*., 1982), activate macrophage killing of obligate intracellular pathogens (Filipe-Santos *et al*., 2006), and have anti-proliferative effects on viral- and malignant-transformed cells (Maher *et al*., 2007).

In our study, three lignans, eleutheroside E (EE), tortoside A (TA), and syringaresinol (SY), were isolated from the stem and root of *A. koreanum* in an effort to study the immunomodulating effect. We treated natural killer cells (NK92MI) and dendritic cells (DC1 and DC2.4) with lignans, and analyzed their cytokine (IL-12 and IFN-γ) secretions.

MATERIALS AND METHODS

Preparation of eleutheroside E, tortoside A, and syringaresinol (EE, TA, or SY, respectively)

Dried branches and roots of *A. koreanum* were purchased from Susin Ogapy Co. (Chunahn, Chungnam, Korea). Three lignans, eleutheroside E (Fig. 1A), tortoside A (Fig. 1B), and syringaresinol (Fig. 1C), were isolated from the branch and root of *A. koreanum* and identified as reported previously (Kang *et al*., 2001; Cai *et al*., 2004) and provided by Dr. Jung-Jun Lee at Korea Research Institute of Bioscience & Biotechnology (KRIBB), Daejun, Korea. The resulting solutions were filtered step by step through various membrane filter sizes (0.8, 0.45, and 0.2 μm; Nippon Milipore Ltd., Tokyo, Japan). The samples were stored in -80^{o}C until used.

Cell culture

Mouse lymphoid type dendritic cell (DC1) and myeloid type dendritic cell (DC2.4) lines were kindly provided by Professor J. S. Kang (College of Medicine, Seoul National University, Seoul, Korea). DC1 and DC2.4 cells were resuspended in complete tissue culture medium (CTCM) consisting of RPMI-1640 medium (Sigma, Poole, UK) supplemented with 5% fetal calf serum (FCS; Sigma, Poole, UK), 100 u/ml penicillin/100 μg/ml streptomycin (Sigma, Poole, UK), 2 mM L-glutamine (Sigma, Poole, UK), and 5×10^{-5} M (2-ME, Sigma, Poole, UK). Cell cultures were placed in 25-cm^2 flasks (Corning, NY, USA) and incubated at 37^{o}C in a humidified, 5% CO_2 atmosphere. NK92MI cells, a human NK cell line transfected with human IL-2, were purchased from ATCC. Cells were maintained in alpha modification of minimum essential medium Eagle (alpha MEM; Gibco, NY, USA) supplemented with 12.5% fetal bovine serum (FBS; Sigma, Poole, UK), 100 u/ml

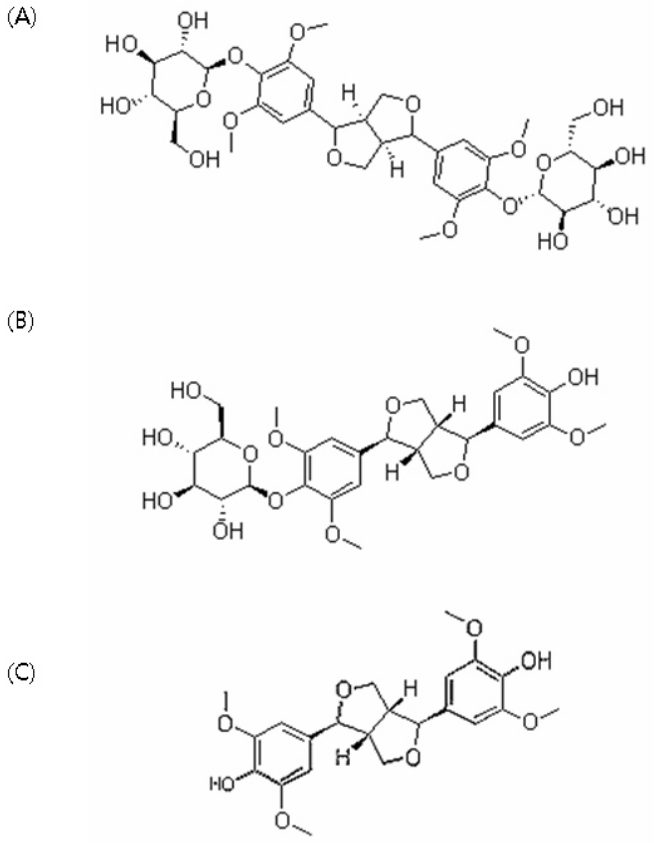

Fig. 1. Chemical structures of (A) elutheroside E, (B) tortoside A, and (C) syringaresinol.

penicillin/100 μg/ml streptomycin (Sigma, Poole, UK), 2 mM L-glutamine (Sigma, Poole, UK), 0.2 mM inositol (Sigma, Poole, UK), 20 mM folic acid (Sigma, Poole, UK), 12.5% horse serum (Sigma, Poole, UK), and 5×10^{-5} M 2-mercaptoethanol (2-ME, Sigma, Poole, UK).

Cell viability

In vitro cell viability was measured using MTS (Promega, Southampton, UK) assay as previously described (Lyu and Park, 2008). Briefly, cells (5×10^{-5} cells/ml) were added in 96-well flat bottomed tissue culture plates (NUNC, Life Technologies, Paisley, UK) in the absence or presence of EE, TA, or SY at various concentrations for 48 hr at 37°C in a humidified 5% CO_2 atmosphere. At the end of the incubation, 40 μl of combined MTS/phenazine methosulphate (PMS) solution was added into each well and further incubated for 4 hr at 37°C in a humidified, 5% CO_2 atmosphere. Formazan produced was determined by measuring the absorbance at 450 nm with an ELISA reader (Dynex Technologies, Chantiliy, VA, USA). The optical density of formazan formed in untreated control cells was taken as 100% of viability.

Enzyme-linked immunosorbent assay (ELISA) for cytokine secretions

Lignans were diluted with cell culture medium and tested in triplicate wells. Lignans were added to cells stimulated with 100 ng/ml of LPS and incubated at 37°C in 5% CO_2 atmosphere for 48 hr and 100 μl of culture supernantant was removed for the measurement of IL-12 and IFN-γ productions. For determination of cytokine secretions, 96-well MaxiSorp (NUNC, Life Technologies, Paisley, UK) plates were coated with purified anti-mouse IL-12 monoclonal antibody (BD Pharmingen, UK) or purified anti-human IFN-γ monoclonal antibody in binding buffer (0.1 M Na_2HPO_4, adjusted to pH 9.0 with 0.1 M NaH_2PO_4) overnight at 4°C. After washing the plates three times, which contained phosphate buffered saline (PBS) with 0.5% (*v/v*) Tween 20 (Sigma), the plates were blocked with 1% (*w/v*) bovine serum albumin (BSA; Sigma) at room temperature for 2 hr. Following three washes, 50 μl of cell culture supernatants were added and incubated overnight at 4°C; for standards, recombinant IL-12 and IFN-γ were included for each plate. After four washes, 100 μl of biotinylated anti-IFN-γ and IL-12 detection antibody which was diluted in 1% BSA in PBS- Tween were added and incublated at room temperature for 1 hr. Following four washes, the presence of biotinylated antibodies was detected with 1:1,000 dilution of streptavidin-peroxidase (BD Pharmingen). At the end of an hour incubation at room temperature, the plate were washed and developed using 0.1 mg/ml of 3,3',5,5'-tetramethylbenzidine substrate (TMB; Sigma) solution. The enzyme reaction was stopped with 1 M HCl and the colorimetric development was read at 450 nm with an ELISA reader (Dynex Technologies).

Statistical analysis

Student *t*-test was used for comparisons (MINITAB®, release 14.1, Minitab Inc., USA). Probability values (*p*-value) of <0.001, <0.01, or <0.05 were considered significant with 99.9, 99, or 95% of confidence, respectively.

RESULTS

Cytotoxic effects on DC1, DC2.4, and NK92MI cells

Mouse dendritic cells (DC1 and DC2.4) and human NK cells (NK92MI) were treated for 48 hours with *A. koreanum* lignans at the indicated dose and the viability was measured using the MTS assay. More than 95% of the cells survived at concentrations lower than10^{-4} g/ml (Table I-III). The result obtained showed that the selected concentration (lower than 1 μg/ml) of *A. koreanum* that was used for cytokine secretion assay had no major effect on the cells when compared to the controls.

Effect of EE, TA, or SY on IL-12 secretion in DC1 cells

Mouse lymphoid type DC1 cells were treated with EE, TA, or SY to investigate whether *A. koreanum* lignans can change IL-12 secretion, which is related to anti-cancer activities. When lipopolysaccharide (LPS)-stimulated DC1 cells were treated with 10^{-8}-10^{-11} g/ml, EE shifted the LPS-induced IL-12 secretion toward a more immunostimulative response. However, in the concentration range of 10^{-12}-10^{-17} g/ml of EE, there was no significant change compared with LPS control, with the exception of 10^{-13} g/ml which showed a significant increase of IL-12 (Fig. 2A). In the case of TA, a 30% increase of IL-12 secretion was observed at 5×10^{-10} g/ml and the most significant increase was observed at 10^{-8} mg/ml. In the concentration range of 10^{-10}-10^{-12} g/ml, there was no significant change (Fig. 2B). When cells were treated with SY, there was a 40% increase in IL-12 secretion at 10^{-15} g/ml and a 90% increase at 10^{-11} g/ml (Fig. 2C).

Effect of EE, TA, or SY on IL-12 secretion in DC2.4 cells

Mouse myeloid type DC2.4 cells were treated with EE, TA, or SY and their effects on IL-12 secretion were observed. When LPS-stimulated DC2.4 cells were treated with 10^{-9}-10^{-10} g/ml of EE, there was a 20-30% increase in IL-12 secretion but in the concentration range of 10^{-11}-

Table I. Cell viability (%) of DC1 when treated with *A. koreanum* lignans (EE, TA, or SY). DC1 was cultured in absence or presence of different concentrations of EE, TA, or SY and the viability was measured by MTS assay. Experiments were performed at least three times and each point represents the mean of triplicate ± SE

A. koreanum	Conc. (g/ml)					
	Untreated	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
EE	100 ± 0	102 ± 1	100 ± 2	99 ± 3	98 ± 2	97 ± 1
TA	100 ± 0	101 ± 2	100 ± 3	99 ± 1	98 ± 1	97 ± 1
SY	100 ± 0	101 ± 3	100 ± 2	99 ± 2	98 ± 3	98 ± 2

Table II. Cell viability (%) of DC2.4 when treated with *A. koreanum* lignans (EE, TA, or SY). DC2.4 was cultured in absence or presence of different concentrations of EE, TA, or SY and the viability was measured by MTS assay. Experiments were performed at least three times and each point represents the mean of triplicate ± SE

A. koreanum	Conc. (g/ml)					
	Untreated	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
EE	100 ± 0	100 ± 2	100 ± 4	99 ± 1	99 ± 2	99 ± 1
TA	100 ± 0	102 ± 2	99 ± 1	98 ± 2	97 ± 2	96 ± 3
SY	100 ± 0	101 ± 2	102 ± 1	99 ± 2	97 ± 1	95 ± 1

Table III. Cell viability (%) of NK92MI when treated with *A. koreanum* lignans (EE, TA, or SY). NK92MI was cultured in absence or presence of different concentrations of EE, TA, or SY and the viability was measured by MTS assay. Experiments were performed at least three times and each point represents the mean of triplicate ± SE

A. koreanum	Conc. (g/ml)					
	untreated	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
EE	100 ± 0	102 ± 1	100 ± 3	98 ± 1	97 ± 2	96 ± 1
TA	100 ± 0	100 ± 1	100 ± 2	99 ± 2	98 ± 3	97 ± 1
SY	100 ± 0	102 ± 2	102 ± 1	100 ± 2	99 ± 1	98 ± 2

10^{-17} g/ml, there was no significant change (Fig. 3A). When the cells were treated with SY, there was an increase at 10^{-11}-10^{-14} g/ml and the most significant IL-12 secretion was shown when treated with 10^{-13} g/ml (Fig. 3B). There was a less significant increase of IL-12 when DC2.4 cells were treated with SY. We could see an IL-12 secretion at 10^{-12}-10^{-13} g/ml, but there was no change at 10^{-14}-10^{-17} g/ml and concentrations above 10^{-11} g/ml (Fig. 3C).

Effect of EE, TA, or SY on IFN-γ secretion in NK92MI cells

Further studies were carried out to determine if *A. koreanum* lignans are able to effect the secretion of IFN-γ in human NK cells. IN LPS-stimulated NK-cells, EE significantly shifted the IFN-γ secretion toward a more immunostimulatory response. Particularly, an increase of IFN-γ was observed in the presence of EE (10^{-13}-10^{-19} g/ml) compared with LPS controls (Fig. 4A). When the cells were treated with TA, we could see a similar secretion pattern to EE. There was a 30% increase in IFN-γ secretion at 10^{-13} g/ml, and the most significant increase was observed at 10^{-7} mg/ml. However, IFN-γ secretion started to decrease when the cells were treated with the concentration higher than 10^{-6} g/ml (Fig. 4B). In the case of SY, we could see a gradual increase in IFN-γ secretion in a dose-dependant manner. There was a 40% increase of IFN-γ at 10^{-9} g/ml of SY. On the other hand, in the concentration range of 10^{-15}-10^{-17} g/ml, there was no significant change compared to the controls (Fig. 4C).

DISCUSSION

Immune-related illnesses have long been treated with herbal medicines. In regard to phytotherapy, immunomodulators may be defined as botanical medicines that alter the activities of the immune system via the dynamic regulation of informational molecules - cytokines, chemokines, and other peptides (Spelman *et al*., 2006). *Acanthopanax koreanum* Nakai is a native plant that grows on Jeju Island, located in the south of Korea (Nan *et al*., 2004). Previously, it was reported that acanthoic acid, a diterpene isolated from the root bark of *Acanthopanax koreanum*, inhibited IL-1 and TNF-α production by human monocyte/macrophages (Kang *et al*., 1996) and showed potent inhibitory

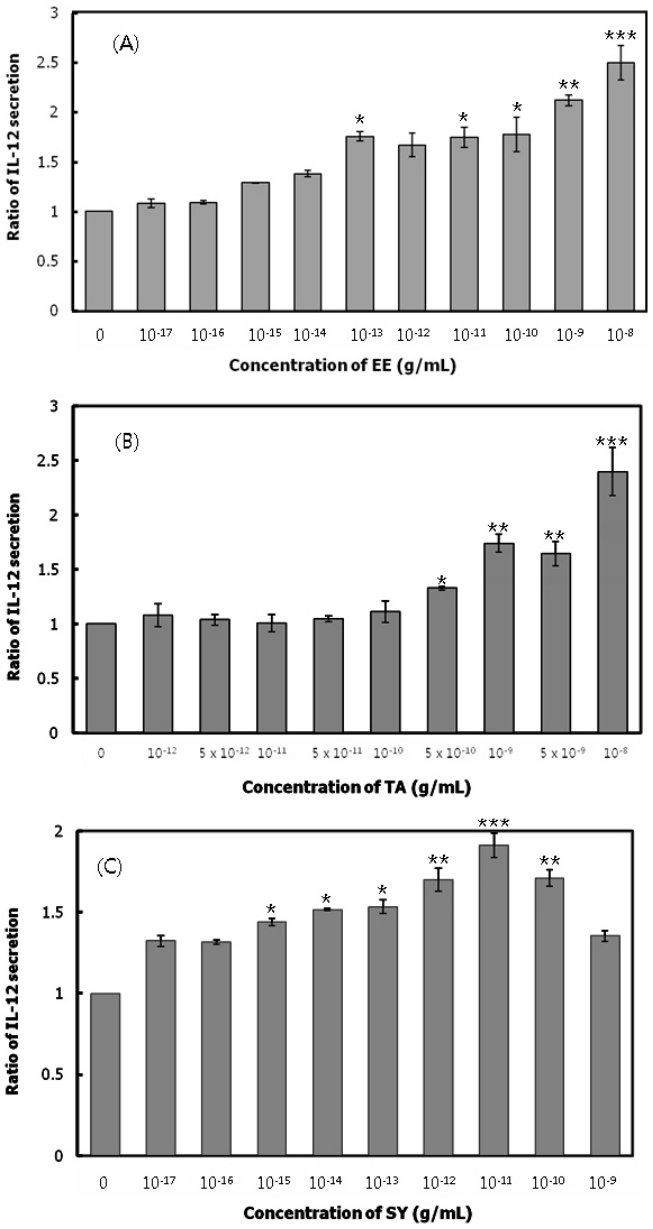

Fig. 2. Effect of lignans (A) EE, (B) TA, and (C) SY on the secretion of IL-12 in LPS (100 ng/ml)-stimulated DC1 cells. Values represent the mean ± standard deviation from at least three independent experiments with whole cells. Student's *t*-test was used to compare each of the treatment group with the control group, and the significance was determined. $*p<0.05$, $**p<0.01$, $***p<0.001$.

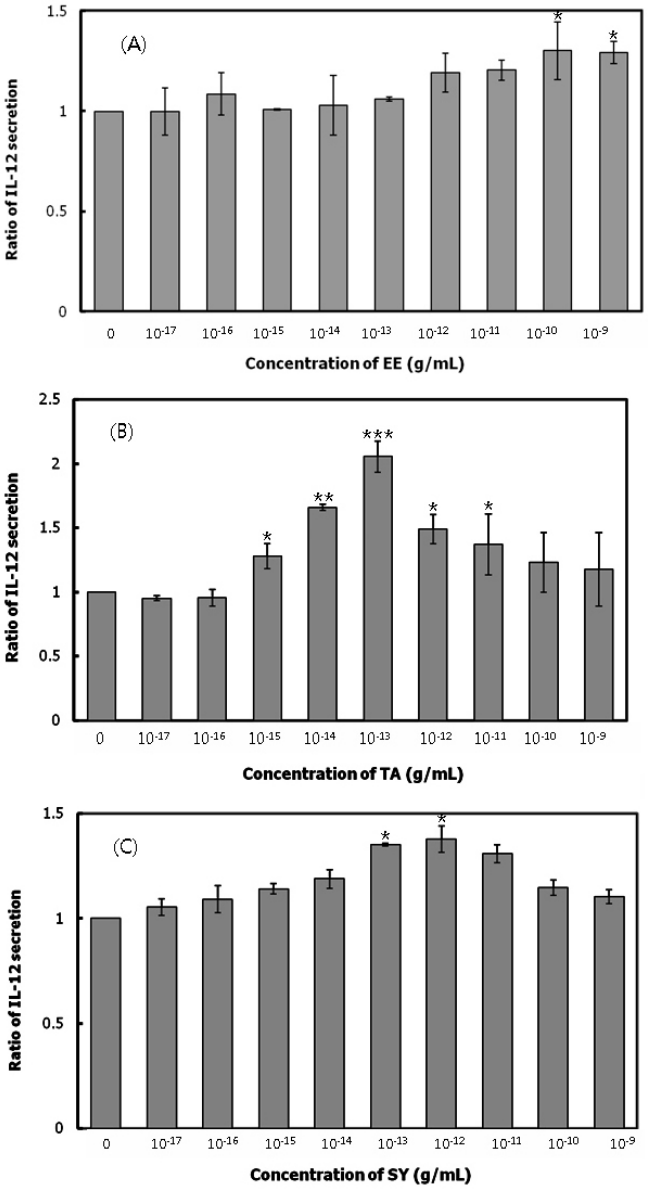

Fig. 3. Effect of lignans (A) EE, (B) TA, and (C) SY on the secretion of IL-12 in LPS (100 ng/ml)-stimulated DC2.4 cells. Values represent the mean ± standard deviation from at least three independent experiments with whole cells. Student's *t*-test was used to compare each of the treatment group with the control group, and the significance was determined. $*p<0.05$, $**p<0.01$, $***p<0.001$.

activity on IL-8 secretion by human colon adenocarcinoma cell line (Cai *et al*., 2003). Moreover, acanthoic acid inhibited TNF-α-medicated IL-8 production by blocking in both the MAPKs and NF-κB pathways in human colon epithelial cells (Kim *et al*., 2004).

The lignans are a group of chemical compounds found in plants. Lignans are one of the major classes of phytoestrogens, which are estrogen-like chemicals and also act as antioxidants (Messina and Barnes, 1991; Murkies *et al*., 1998). Plant lignans are polyphenolic substances derived from phenylalanine. Lignan-containing food include legumes, seeds, cereals/grain, berries, dried fruit, and vegetables (Thompson *et al*., 2006). Lignans are being studied

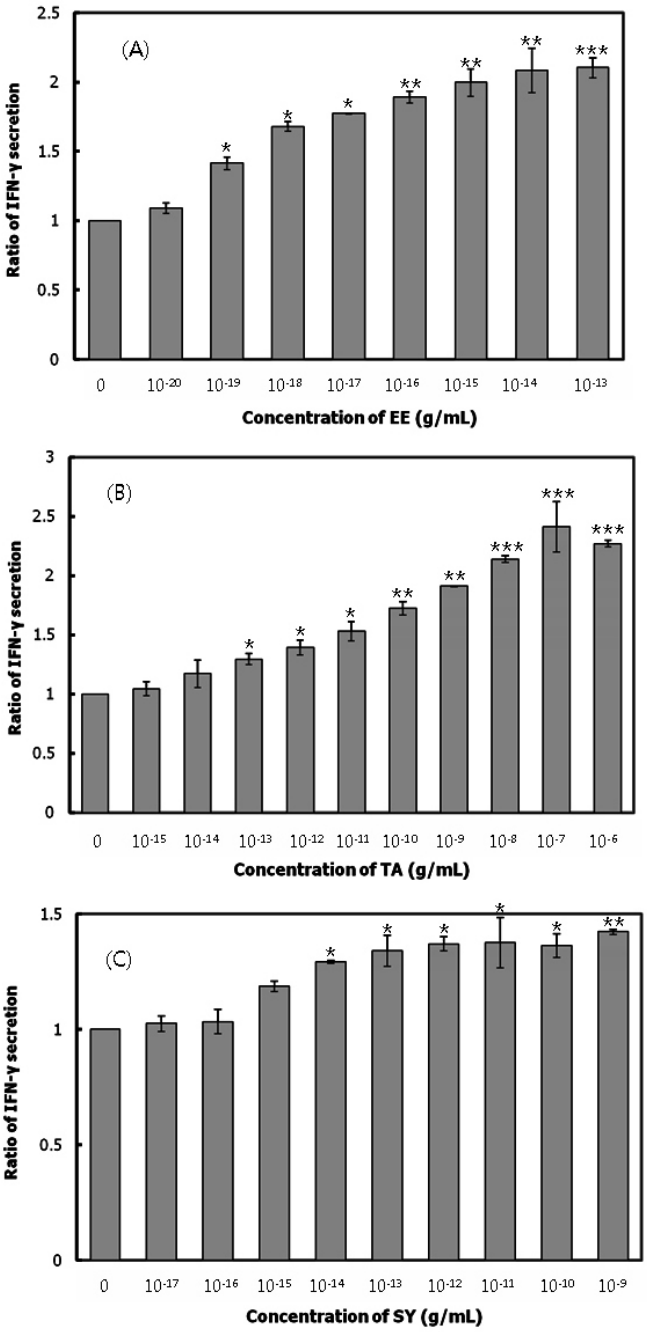

Fig. 4. Effect of lignans (A) EE, (B) TA, and (C) SY on the secretion of IFN-γ in LPS (100 ng/ml)-stimulated NK92MI cells. Values represent the mean ± standard deviation from at least three independent experiments with whole cells. Student's *t*-test was used to compare each of the treatment group with the control group, and the significance was determined. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

for possible use in cancer prevention. Lignans acts as anti-cancer compounds by blocking powerful growth factor receptors like epidermal growth factor (*EGF*), Her2, insulin like growth factor-1, and vascular endothelial growth factor (VEGF), the hormone responsible for stimulating blood vessels into tumors (Thompson *et al*., 2005; Wang *et al*., 2005; Cotterchio *et al*., 2006). Several studies on lignans from *A. koreanum* have been reported. Lupane-triterpene glycoside, acankoreoside I, showed growth inhibitory effect in lung cancer, breast cancer, and leukemia cell lines (Nhiem *et al*., 2009). Also, three lignans isolated from the root, namely eleutheroside E, tortoside A, and hemiariensin, exhibited potent inhibitory activity against NFAT transcription factor (Cai *et al*., 2004). However, there were no studies which confirmed its pharmacological effects regarding cytokine secretion and its relevance to cancer.

Since *A. koreanum* have been traditionally used to treat immune-related illnesses (Nan *et al*., 2004), we studied the Th1 cytokine secretion effect of lignans (EE, TA, or SY) isolated from *A. koreanum*. Dendritic cells (DCs) produce IL-12, which plays a major role in resistance to tumors, bacterial, and viral infections (Trinchieri, 1998). Depending on the conditions, DCs can stimulate the outgrowth and activation of a variety of T cells, which affect the immune response differently. In the presence of mature DCs and of the IL-12 they produce (Cella *et al*., 1996; Koch *et al*., 1996; Reis e Sousa *et a*l., 1997), these T cells turn into IFN-γ producing Th1 cells. In the present study, we found that lignans from *A. koreanum* markedly enhanced IL-12 secretion in lymphoid and myeloid type dendritic cells after 48 hr of treatment. Three lignans, EE, TA (one carbohydrate removed from EE), or SY (two carbohydrates removed from EE) were used to treat dendrtic cells. The reason for this is to determine whether the hydrophobic/hydrophilic characteristics have different effects on cytokine secretion in the cells. For example, carbohydrates are highly soluble in water so when it is removed from the lignan, the compound becomes more hydrophobic. Small hydrophobic molecules will pass the cell membrane easily, but larger, hydrophilic molecules will require a transport protein to allow permeability. Since lignans are small molecules that have a low molecular weight, becoming more hydrophobic may allow the compound to penetrate the cell membrane easier. Since SY is the most hydrophobic lignan, we assumed that SY would be more effective than EE or TA. However, there were no significant differences in the cytokine stimulatory effects between EE, TA, or SY. This is probably due, in part, to the fact that although TA and SY are more hydrophobic than EE, this characteristic doesn't lead to enhanced cytokine secretion.

Most likely, cells are seldom exposed to only a single cytokine. Rather, combinations of cytokines and other

messenger molecules generate biologically relevant informational cues (Gabay and Kushner, 1999). Mature dendritic cells produce IL-12, essential not just for attraction of NK cells, cytolytic T cells, and for shifting helper T cell responses in a Th1 direction (Morse *et al*., 1998). Therefore, we used human NK cells to further evaluate the immunostimulating effects of *A. koreanum* lignans. After encounter with a pathogen or a danger, immature DCs mature and induce resting NK cell activation. NK cells are innate cytotoxic effectors but also regulatory cells releasing cytokines involved in innate resistance and adaptive immunity. In particular, IFN-γ is considered the prototypic NK cell cytokine, and its production by NK cells is known to shape the Th1 immune response (Mocikat *et al*., 2003). They are required in resistance to tumors expressing ligands for activating receptors, and in the regulation of B cell responses and autoimmunity (Shi *et al*., 2001; Zitvogel, 2002). As shown in the results above, treatment of lignans (EE, TA, or SY) of *A. koreanum* markedly induced IFN-γ secretion confirmed by ELISA assay. Smiliar to the IL-12 secretion results by dendritic cells, there were no significant differences in the IFN-γ stimulatory effects between EE, TA, or SY. IFN-γ activates the antitumor activities of macrophages and, together with IL-12, it promotes the differentiation of T cells into killer cells. So the capacity of DCs to produce IL-12 and Th1 cells will lead to tumor, viral, and microbial resistance (Banchereau and Steinman, 1998). Further research may find that components of *A. koreanum* affecting multiple cytokines can each generate a unique signature of immune perturbation dependent on the concerted effect on arrays of cytokines.

The reported therapeutic success of *A. koreanum* by traditional cultures may be partially due to their effects on cytokines. This study suggests that lignans from *A. koreanum* modulate cytokines, and that such modulation may provide the mechanism of action for many of their therapeutic effects.

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