**Natural Product Sciences**
13(4) : 283-288 (2007)

# Immunostimulatory effect of Korean traditional medicine Acanthopanacis Cortex

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**Abstract –** Acanthopanacis Cortex (AC) has been popularly used as an herbal medicine for medical treatment of rheumatoid arthritis, insomnia, impotence and diabetes. Here, we investigated immunostimulating effects of the aqueous extract of AC on macrophage. We studied nitric oxide (NO) and tumor necrosis factor (TNF)-α release in response to AC treatment, as they are important secretory products of macrophage. AC alone induce the NO and TNF-α production. AC increase c-Jun NH2-terminal kinase 1/2 (JNK) and extracellular signal-regulated kinase (ERK) phosphorylation but does not p38 activation in RAW 264.7 cells. Also AC resulted in the enhanced cell-surface expression of CD80 and CD14. In addition, AC resulted in enhanced T cell-stimulatory capacity and increased T cell secretion of interferon (IFN)-gamma. After feeding with AC to mouse for 10 days, the change of $CD28^+$ and $CD40^+$ population was analyzed. AC increased $CD28^+$ population in splenocytes *in vivo*. These studies indicate that AC induces macrophage activation and suggest the possible use of AC in macrophage-based immunotherapies
**Keywords –** Acanthopanacis Cortex, nitric oxide, TNF-α, CD80, CD86

## Introduction

The innate immunity is activated immediately after infection for fighting against the invading pathogens and therefore is the first line of host defense. Macrophages play a major role in host defense against infection and cancer (Yoon *et al*., 2003). Activated macrophages are the main source of inflammatory molecules like nitric oxide (NO), Tumor necrosis factor (TNF)-α, IFN-γ, and chemokines. Macrophages constitute the major group of phagocytic leukocytes and are crucial to immunosurveilance against invading pathogens and malignancies. Upon activation, macrophages express one or more cytotoxic effector molecule such as peroxidase, cytolytic protease, NO, and proinflammatory cytokines (Adams *et al*., 1983; Key *et al*., 1982).

NO is a short-lived biomolecule that mediates many biological functions, including host defense, vasoregulation, platelet aggregation and neurotransmission (Moncada *et al*., 1991; Nathan *et al*., 1992). The NO production by activated macrophages has been shown to mediate immune functions including antimicrobial and antitumor activities (MacMicking *et al*., 1997). TNF-α induces various biological responses including tissue injury, shock and apoptosis. IFN-γ is the major cytokine related to host defense by itself or through NO mediated pathway (Gessani *et al*., 1998), its production by the macrophages were investigated. It has been reported that macrophage can produce IFN-γ by itself (Munder *et al*., 1998). IFN-γ is as highly important cytokine in inflammation as well as in wound healing (Ishida *et al*., 2004). It also possesses potent immunostimulatory effects on a variety of immune cells in vitro as well as in vivo.

Mitogen-activated protein kinase (MAPK) are critical in immune and inflammatory process regulating the expression of a wide variety of cytokines including IL-2, IFN-γ, IL-12, TNF-α, IL-1β (Dong *et al*., 2002; Swantek *et al*., 1997; Rao *et al*., 2002). Its ubiquitous distribution, rapid induction and its apparent involvement in several diseases have made this pathway a potential target for the treatment of inflammatory disorders. MAPK signals transduction pathways in mammalian cells include the extracellular signal related kinase (ERK), c-Jun NH2-terminal kinase 1/2 (JNK) and p38 MAP kinase (Kyriakis *et al*., 2001; Rao *et al*., 2001). The presence of all the three kinases has been shown to play a critical role in the events leading to lymphocyte activation and production of inflammatory cytokines (Dumont *et al*., 1998).

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Plant materials long been used as tranditional medicines for the treatment of a wide variety of ailments and diseases. *Acabthopanacis cortex* (AC), this herb is increasingly being used for symptoms of deficiency such as general weakness and malaise, loss of petite, headache, insomnia, and impotence.

The aim of this study was to investigate the effect of aqueous extracts from commercially available powder of AC on the cytokines of murine macrophages and RAW264.7 cells. In summary, since regulation of theses cytokines, co-stimulatory molecules may induce stronger immune response than normal state, we investigated the effects of AC as immuno-stimulator on the phenotype of antigen presenting cell like macrophages, cytokine secretion capacity of splenocyte, expression levels of co-stimulatory molecules.

## Experimental

**Plant material** – Extract of *Acanthopanacis Cortex* (AC) was obtained by Plant Extract Bank (Korea, Daejeon). According to Plant Extract Bank, extract of AC was prepared by decocting the dried herb with boiling distilled water. The extract was filtered through filter paper. The dried extract was dissolved in PBS for *in vitro* experiment.

**Animal** – Female C57BL/6N mice were purchased from Orient Bio. Inc. (Jeonbuk, Iksan) and used 7 weeks of age. All animals were maintained under constant conditions (temperature: 22 ºC, 12 h dark/light cycle)

**Preparation of Peritoneal macrophages** – One milliliter of 3% thioglycollate was injected intraperitoneally into mice as a stimulant to elicit peritoneal macrophages. Four days later, the peritoneal exudate was collected by peritoneal lavage with 8 ml of RPMI 1640 medium. The exudate was centrifuged at 300 × g, 25 ºC for 20 min. The erythrocytes in the cell pellets were lysed by D.W. The cell suspension was centrifuged and the cells were washed twice and re-suspended in complete RPMI 1640 medium. The cell number was adjusted to $1 \times 10^6$ cell/mL.

**Preparation of mouse splenocytes** – Mice were sacrificed and their spleens were collected aseptically. Cell suspension was prepared by means of loose potter and flushing. After centrifugation at 300 × g, 37 ºC for 10 min, erythrocytes were lysed by D.W and the cell pellets were washed twice with RPMI 1640 medium. The cells were resuspended in complete RPMI medium and the cell number was adjusted to $1 \times 10^6$ cell/mL.

**MTT Assay and for cell viability** – Splenocytes viability after 24 hr of continuous exposure to the tested compounds were measured with a colorimetric assay based on the ability of mitocondria in viable cells to reduce MTT as described previously.

**Nitric oxide assay** – Nitrite concentration was determined using the Griess reagent. Briefly, 100 uL supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-1-napthylethylenediamine dihydrochloride in water) at room temperature for 10 min. The absorbance was measured at 540 nm by a microplate reader. Nitrite concentration in the supernatants was determined from a standard curve that was generated using known concentrations of sodium nitrite.

**Cytokine assay** – TNF-α and IFN-γ in the culture supernatant from macrophages or splenocytes were assayed with an enzyme-linked immunosorbent assay (ELISA kit., R&D Systems), as per the manufacturer's instructions.

**Western blot** – Cells were lysed in ice-cold buffer containing 25 mM monosodium phosphate (pH 7.4), 75 mM NaCl, 5 mM EDTA, 1% Triton X-100, 100 μg/mL phenylmethylsulphonylfluoride, 10 μg/mL antipain, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 20 μg/mL aprotinin, and 10 μg/mL trypsin inhibitor and centrifuged at 50,000 × g for 20 min at 4 ºC. The cytosolic proteins (20 μg per lane) were separated by 12% sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose filters, and then immunoblotted either with a rabbit anti-JNK, ERK, p38 polyclonal antibody. Anti-rabbit HRP-conjugated antibody was used as a secondary antibody at a dilution of 1 : 7500. Finally, the bands were visualized by ECL detection reagent (Amersham Biosciences) using X-ray film.

***In vivo* experiment** – Mice were treated perorally with the AC and PBS at daily doses of 0.1 g/kg, respectively, for 10 days. All extracts were dissolved in PBS, and control animals received PBS only. And then Spleen collected from the mice. Cell numbers were counted with a haemocytometer by trypan blue dye exclusion technique. Cell viability exceeded 95%. Mice were killed in accordance with National Institutes of Health animal care and use guidelines.

**Flow cytometric analysis** – The cell surface phenotypes were characterized by a two-color cytometric analysis. Harvested cells were incubated for 30 min at 4 ºC with Fc-blocker to prevent any non-specific binding and washed in ice colded PBS. Subsequent stainings with monoclonal antibodies (mAb) or isotype-matched controls were performed for 30 min on ice. Stained mAbs were described as follows: Fluorescein isothiocyanate (FITC) anti-mouse MHC class II (I-A/I-E), FITC anti-mouse CD80 (B7-1), CD86 (B7-2), and PE-anti-mouse CD14.

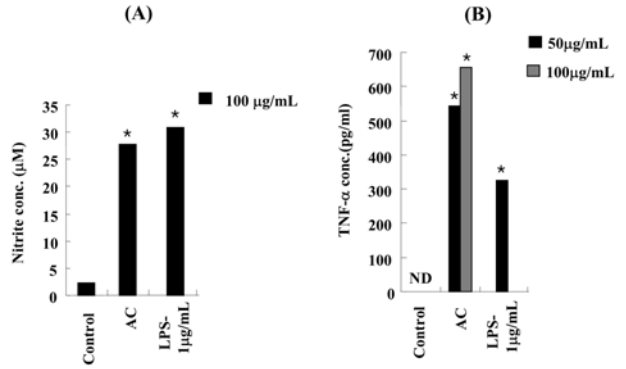


**Fig. 1.** Effect of AC induced NO and TNF-α production by splenocytes. NO production was determined by the Griess as nitrite accumulation in the medium after 24 hr in splenocytes (A). TNF-α secretion was subsequently analyzed by ELISA in the medium after 24 hr in splenocytes (B). Each data represents the mean of three independent experiments. *$p < 0.05$; significantly different from the control value.

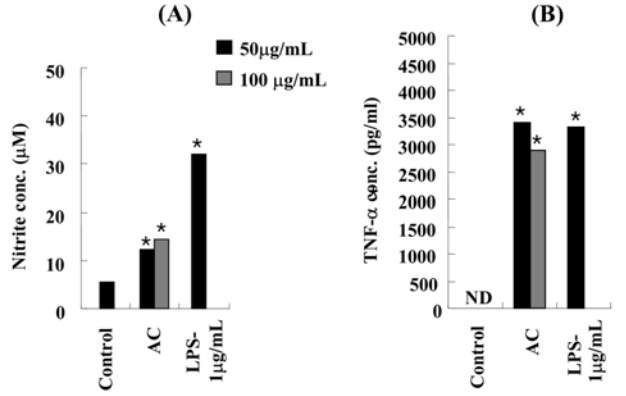


**Fig. 2.** Effect of AC induced NO and TNF-α production by peritoneal macrophages. NO production was determined by the Griess as nitrite accumulation in the medium after 24 hr in macrophages (A). TNF-α secretion was subsequently analyzed by ELISA in the medium after 24hr in macrophages (B). Each data represents the mean of three independent experiments. *$p < 0.05$; significantly different from the control value.

Immunofluorescent staining was analysed using a FACSCalibur (Becton-Dickinson, California) and FCS Express software (De Novo Software, Ont., Canada).

**Statistical analysis** – Each data represents mean of eachexperiment under the same conditions. A Students's *t*-test was used to make a statistical comparison between the groups. Results with $P < 0.05$ were considered statistically significant.

## Results

**Effect of AC induced NO and TNF-α production by splenocytes** – The effects of AC extract on NO, TNF-α production by splenocytes. Splenocytes were incubated

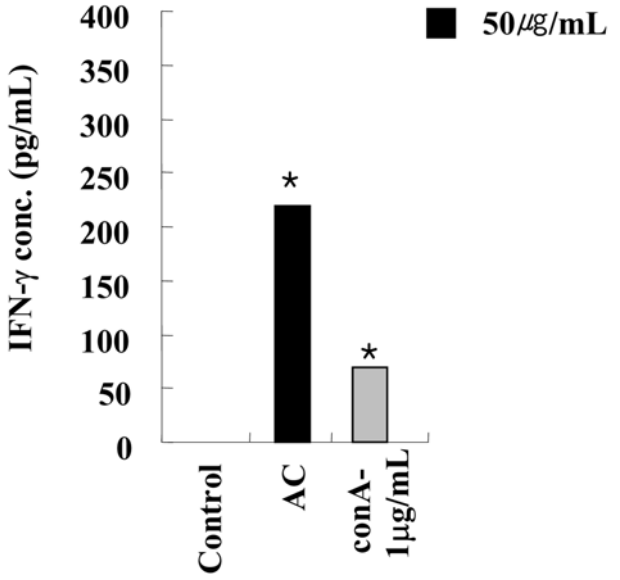


**Fig. 3.** Effect of the aqueous of AC on IFN-γ production in murine splenocytes. Splenocyte ($1 \times 10^6$/mL) of C57BL/6N mice were cultured with AC at five concentration at 37 °C in a humidified 5% $CO_2$ atmosphere for 24 hr. IFN-γ measurements in the culture supernatant were performed by a Mouse OptEIA™ ELISA IFN-γ set. Each data represents the mean of three independent experiments. *$p < 0.05$; significantly different from the control value.

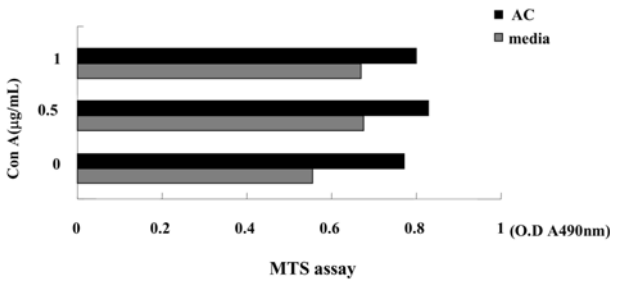


**Fig. 4.** Effect of the aqueous of AC on proliferation in murine splenocytes. Splenocytes ($1 \times 10^6$/mL) of C57BL/6N mice were cultured with at AC five concentration at 37 °C in a humidified 5% $CO_2$ atmosphere for 24hr. Proliferation of splenocytes was performed by MTS assay. Each data represents the mean of three independent experiments.

with AC extract (50, 100 μg/mL) for 24hr. The culture media were collected, and the nitrite, TNF-α content within the media was determined. As shown in Fig. 1, AC extract increased NO and TNF-α production.

**Effect of AC induced NO and TNF-α production by mouse peritoneal macrophages** – To assess the effect of AC extract on NO and TNF-α production of mouse peritoneal macrophages. Peritoneal macrophages were incubated with AC extract (50, 100 μg/mL) for 24 hr. The culture media were collected, and the nitrite, TNF-α content within the media was determined. As shown in Fig. 2, AC extract increased NO and TNF-α production.

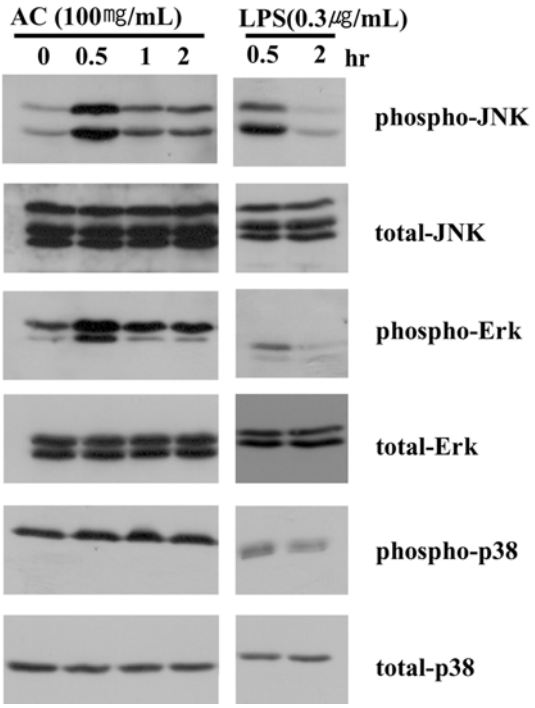


**Fig. 5.** AC induces the phosphorylation of p38 MAPK, p42/p44 ERK, and p46/52 JNK kinase. Mouse-derived splenocytes were treated with AC (100 μg/mL) for the indicated time periods and then collected the cell lysate, and the level of MAPK phosphorylations was assessed by western blotting with respective antityrosine-phosphorylated MAPK mb, and total mAb was for internal control.

**Effect of AC induced IFN-γ production by spelnocytes** – To investigate if AC influences on acquired immune response, the secretion of IFN-γ from splenocytes was assayed. Generally, IFN-γ was measured as a marker of acquired immune response. After 24 hr of incubation of the splenocytes with 50 μg/mL of AC extract, IFN-γ level in the culture supernatant was found to increase (Fig. 3).

**Effect of AC extract and pure molecule on cell cytotoxicity** – The unfaractionated spleen cells from C57BL/6N mice were cultured with AC for 24 hr, proliferative response was stimulated in normal spleen cells in a dose-dependent manner (Fig. 4).

**AC induces phosphorylation of members of the MAPK families in RAW264.7** – MAPK is a serine and threonine protein kinase whose activities are up-regulated through tyrosine and threonine residue phosphorylation by its upstream regulators. This experiment focused on p38 MAPK, p42/44 ERK, p46/54 JNK to further characterize the MAPK activation pathways involved in AC signaling. RAW264.7 cells were stimulated with AC or none at all, and the level of MAPK phosphorylations was assessed by Western blotting with respective antityrosine-phosphorylated MAPK mAb.

AC and LPS were found to induce the phosphorylation of ERK1/2 and JNK in RAW264.7 cells (Fig. 5).

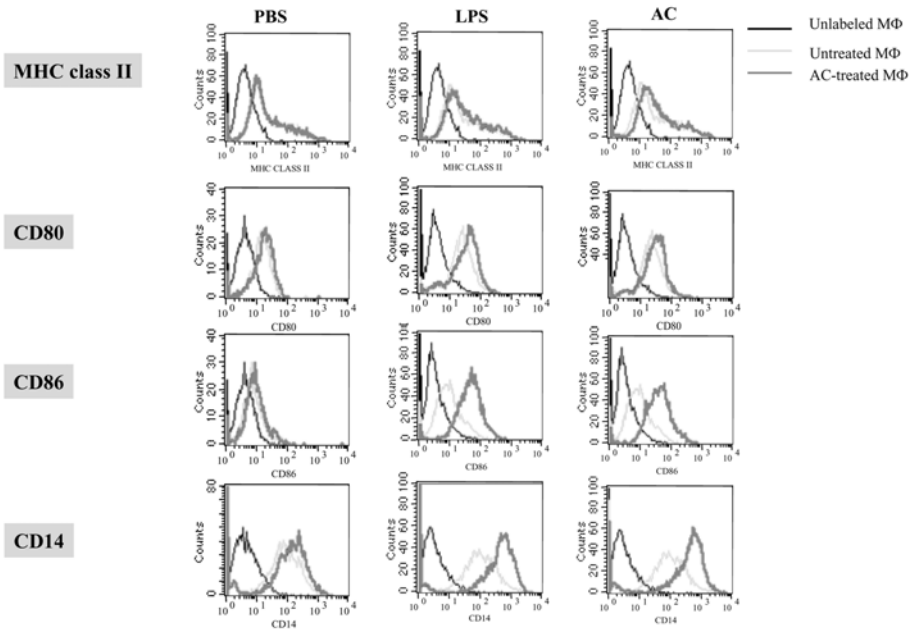


**Fig. 6.** Effect of the aqueous extract of AC on the expression of co-stimulatory molecules in vitro. Peritoneal macrophages were treated for 24 hr at 37 °C with PBS, AC extracts and LPS. After washing $1 \times 10^6$cells/tube were stained for 30 min at 37 °C with FITC-labeled monoclonal antibodies to MHC class II, CD80, CD86 and CD14. These cells were also stained with isotype-matched control antibody (black line). The expression levels of cells surface molecules were determined using FACS analysis. Data are representative of at least three independent experiments.

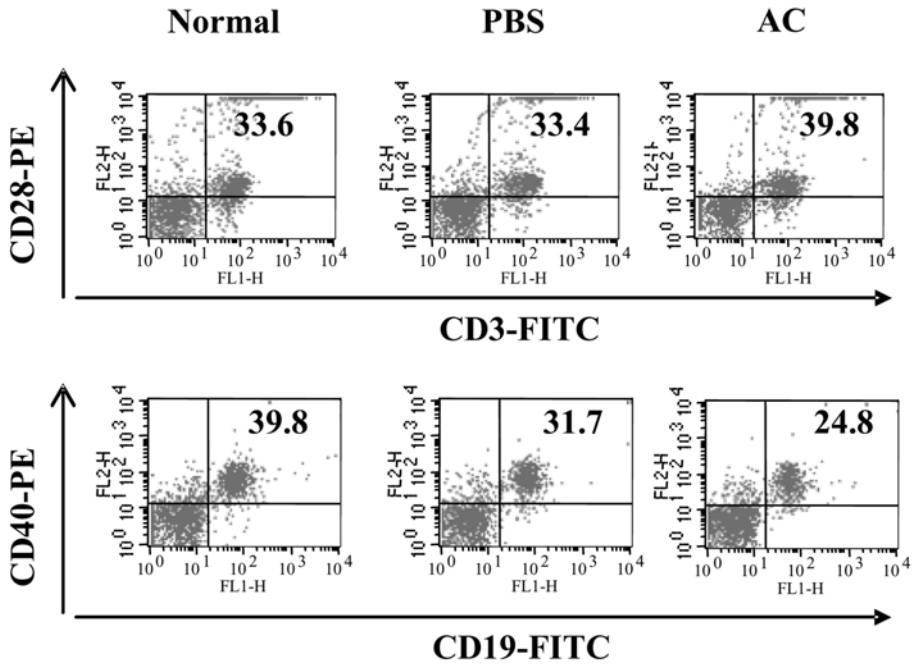


**Fig. 7.** Effect of AC on T & B cell activation *in vivo*. Three groups (3mice in each group) of 7-week-old C57BL/6N mice were fed orally with AC (0.1 g/kg) once daily for 10 days. A control group was given PBS. Splenocytes were isolated from orally fed C57BL/6N mice, and stained with CD3-FITC & CD28-PE for T cell subset and CD19-FITC & CD40-PE for B cell subset, respectively. The cells were analyzed using a flow cytometry.

**Regulation of the expression of cell surface molecules by AC** – Since changes in expression of a number of cell surface molecules are involved in Ag presentation and T-cell stimulation, the regulation of the expression of cell surface molecules by AC was evaluated by flow cytometry. $5 \times 10^5$ cells of peritoneal macrophages were treated with PBS as a negative control, 100 μg/mL of AC, and LPS (10 μg/mL) as a positive control *in vitro*. After washing $5 \times 10^5$ cells/tube, cells were stained with FITC-labeled monoclonal antibodies to mouse MHC-II, CD80, CD86, and CD14, 24 hr for 30 min at 4 °C. As shown in Fig. 6, the expression of co-stimulatory molecules (CD86) and CD14 were increased by AC (Fig. 6). These results suggest that up-regulation of these molecules by the AC can activate innate immune response against tumor or parasite through the up-regulation of co-stimulatory molecules.

***In vivo* immuno-stimulating activities of AC** – To investigate whether AC effects on T & B cell activation *in vivo*, three groups (3 mice in each group) of 7-week-old C57BL/6N mice were fed orally with AC (0.1 g/kg), once daily for 10 days. A control group was given PBS. Splenocytes were isolated from orally fed C57BL/6N mice, and stained with CD3-FITC & CD28-PE for T cell subset and CD19-FITC & CD40-PE for B cell subset, respectively. The cells were analyzed using a flow cytometry. The extent of T cell activation in AC was increased more than compared to control (Fig. 7).

## Discussion

In the recent, increased concern has been focused on the pharmacology and clinical utility of herbal extract and derivatives as a drug or adjunct to chemotherapy and immunotherapy. Since herbal extracts are often recognized as highly "foreign" by the host immune system, it can have immunostimulatory effect by virus of local accumulation and increase in immune cells (Morikawa *et al*., 1984). Macrophages play a major role in host defense against infection and cancer (Yoon *et al*., 2003). The other effect of macrophages on pathogens is the secretion of cytokines, including TNF-α, and inflammatory mediators, such as NO. The present report shows that AC extract induce NO and TNF-α production by mouse peritoneal macrophages. IFN-γ is the major cytokine related to host defense by itself or through NO mediated pathway (Gessani *et al*., 1998). IFN-γ is a highly important cytokine in inflammation as well as in wound healing (Ishida *et al*., 2004). It also possesses potent immuno-stimulatory effects on a variety of immune cells *in vitro* as well as *in vivo*. IFN-γ level increased after the splenocytes were incubated with the AC extract. Phosphorylation of

extracellular signal-regulated kinase (ERK)1/2, JNK were also confirmed by western blotting. Two classes of MAPK, ERK and JNK are stimulataneously activated by AC in RAW264.7 cells with maximal activation occurring 30 min poststimulation. Innate immunity against extracellular stimuli such as pathogen and herbal extract can be executed by immune cells, and the biological result by these cell types is expression of co-stimulatory molecules. Activation of macrophages was characterized by an increased co-stimulatory molecules CD86 and CD14. After feeding with AC to mouse for 10 days, the change of $CD28^+$ and $CD40^+$ population was analyzed. AC increased $CD28^+$ population in splenocytes *in vivo*.

The immunostimulating effets obtained in vivo and in vitro are in agreement with the stimulation of AC. It also activates macrophges, and T cells and stimulates cytokine production (*e.g.,* IFN-γ), heightens cell-mediated immune response, prevents the weakening of the immune response associated and presumably bolsters the ability to protect against infection. AC is thus an effective protective agent.

## Acknowlegments

This work was supported by grant No. RTI05-03-02 from the Regional Technology Innovation Program of the Ministry of Commerce, Industry and Energy (MOCIE).

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(Accepted September 3, 2007)