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# Effects of Proanthocyanidin-rich Extract from *Pinus radiata* Bark on Immune Responses of Broiler Chickens

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***ABSTRACT*** We evaluated the immunomodulatory effects of proanthocyanidin-rich extract (PAE) from *Pinus radiata* bark in broiler. Proliferation of peripheral blood mononuclear cells and thymocytes was significantly enhanced in 2.5, 5, 10 mg/kg PAE-treated broiler chickens. Proliferation of splenocytes was significantly enhanced in 1.25, 2.5, 5, 10 mg/kg PAE-treated broiler chickens. These effects were markedly enhanced by the presence of LPS, which acts on B cells responsible for humoral immunity, and Con A, which acts directly on T cells involved in cell mediated immunity. PAE significantly promoted the expression of interleukin-18 and interleukin-1$\beta$. Thus, PAE from *P. radiata* possesses immunomodulatory effects in broiler chickens.
(Key words : *Pinus radiata* bark, proanthocyanidin-rich extract (PAE), broiler chicken, immunomodulatory effect)

## INTRODUCTION

Oligomeric proanthocyanidins are some of the most abundant polyphenolic substances in the plant kingdom. Proanthocyanidins (PAs) are an integral part of the human diet, found in high concentration in fruits, vegetables and seeds as well as in most types of tea and red wine (Middleton et al., 2000). PAs are potent free radical scavengers (Prior and Gu, 2005; Ku and Mun, 2008a), anti-bacterial agents (Shan et al., 2007), and effective enzyme inhibitors (Stevens et al., 2002; Ku and Mun, 2008b). In addition, they also exhibit vasodilatory, anti-allergic, anti-inflammatory (Torras et al., 2005), cardioprotective (Kohama et al., 2004), immune-stimulating, anti-viral, and estrogenic activities (Bagchi et al., 2000). Numerous studies have reported that flavonoids have potent antioxidant effects through scavenging of superoxide and hydroxyl radicals (Jorge et al., 1991) and anti-proliferative actions via inhibition of metabolic pathways and inhibition of intra-cellular signal transduction (Jang et al., 1997). However, there have been few reports related to the immunomodulatory effects of PAs. Phenolic compounds such as PAs can stimulate or suppress the immune system due to the presence of hydroxyl groups in the structure. These groups can affect the enzyme or electron-transfer system, resulting in immunomodulation of specific responses, especially phagocytosis (Manosroi et al., 2003). Incorporation of grape seed proanthocyanidin extracts into diet is able to significantly reduce the mortality and improve the chicken performance after *E. tenella* infection in broiler chickens (Wang et al., 2008). *P. radiata* bark is one of the important sources for PAs (Ku et al, 2007).

In our previous studies, we found that a mild alkaline extraction was a useful method for preparing a high yield of PA-rich extract (PAE) from *P. radiata* bark (Mun and Kim, 2009). The objective of this study was to evaluate the immunomodulatory effects of PAE in broiler chickens.

## MATERIALS AND METHODS

### 1. Preparation of PAE

Five kilograms of oven-dried *P. radiata* bark powder was passed through a 1 mm screen, put into a 200 mesh polypropylene bag, and extracted with 25 L of 0.4% $NaHCO_3$ at boiling temperature for 5 min in a 55 L stainless steel extractor (KR-101, Koryeo Industry, Korea) equipped with a gas burner. After

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extraction, the slurry was pressed to obtain the extract and the residue mat was washed with 10 L of distilled water and pressed again. The washed mat was transferred to a hydroextractor and centrifuged for 10 min at 1,700 rpm. All filtrates were combined together and re-filtered using a bag filter. The filtrate was then lyophilized to dryness. In this experiment, the lyophilized pine bark extract was defined as PAE (Fig. 1).

## 2. Chickens and Treatment

1-day-old broiler chickens (Avian 48) were used to determine the immunomodulatory effects of PAE. The chickens were divided into five groups. Groups 1～4 ($n$=10 per group) were treated with 1.25, 2.5, 5, and 10 mg/kg body weight PAE, respectively, once daily by oral gavage. Group 5 ($n$=10) received an equal volume of normal saline on the same schedule and served as the treatment control. All chickens were treated for a total duration of 5 weeks. Proliferation of peripheral blood mononuclear cells (PBMCs) was checked at 2, 5 weeks. Each chicken was sacrificed at the age of 5 weeks.

The chickens were wing-banded individually and reared under uniform management care in isolator. They were brooded initially at 31 to 33℃ in the first 5 d and with following weekly reduction of 2 to 3℃ until the temperature reached 22 to 23℃.

The birds had free access to water and feed.

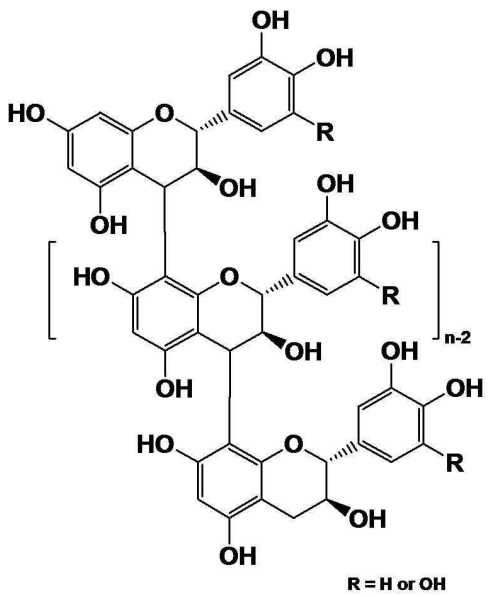



**Fig. 1.** Chemical structure of proanthocyanidin isolated from *P. radiata* bark.

## 3. PBMC, Splenocyte and Thymocyte Isolation

Whole blood was collected via wing-vein puncture into an ethylenediaminetetraacetic acid containing tube. The blood was layered on 1077 Histopaque (Sigma-Aldrich, St. Louis, MO) and spun at 2,500 rpm for 25 min. PBMCs were collected from the gradient interface, and the plasma suspension was combined and washed three times with Dulbecco's modified Eagle's medium (DMEM).

Spleens and thymuses were dissociated between the frosted ends of two microscope slides, and erythrocytes were lysed in RBC lysis buffer for 5min at room temperature and spun at 1,500 rpm for 5 min. Splenocytes and thymocytes washed two times with DMEM.

## 4. Proliferation Assay

To test whether PAE promoted or inhibited PBMCs, splenocytes and thymocytes proliferation, PBMCs, splenocytes and thymocytes were isolated and cultured alone or with added concanavalin A (Con A; 10 $\mu$g/mL) or lipopolysaccharide (LPS; 1 $\mu$g/mL) at a density of $10^6$ cells/mL. The cells were cultured at 37℃ and 5% $CO_2$ in DMEM containing 10% fetal bovine serum (FBS) and antibiotics. Cell proliferation was determined by the MTS assay (Buttke et al., 1993), using a CellTiter 96 aqueous non-radioactive cell proliferation assay kit (Promega, Madison, WI). Absorbance was measured using an enzyme-linked immunosorbent assay reader at 490 nm.

## 5. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNAs were isolated by using easy-BLUE$^{TM}$ Total RNA Extraction Kit (Intron Biotech, Sungnam, Korea), and reverse transcription was performed by using random hexamers and MultiScribe reverse transcriptase. The following transcripts were PCR-amplified: interleukin (IL)-6, -1$\beta$, and -18; interferon (IFN)-$\gamma$ and -$\alpha$; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). For amplification, the reaction mixtures (total volume, 20 $\mu$L) containing each primer at a concentration of 10 pM were prepared using 10 X *nTaq* reaction buffer, 2.5 U of *nTaq* polymerase (Enzinomics, Daejeon, Korea), 2 $\mu$L of 2.5 mM dNTP. Conditions for PCR on the MyCycler (Bio-Rad, Hercules, CA) were initial denaturation at 95℃ for 5 min; 30 amplification cycles with denaturation at 95℃ for 30 sec, annea-

ling at 62℃ for 30 sec, and extension at 72℃ for 3 min; and a final incubation at 72℃ for 10 min. The primer pairs used in these reactions are summarized in Table 1.

### 6. Statistical Analysis

All data were analyzed by the SPSS 12.0 statistical software. The data are expressed as the mean ± SD. Statistical differences were examined independently using the Student's *t*-test and the Pearson Correlation Test. A $p$ value < 0.05 was considered significant.

## RESULTS

### 1. Effect of PAE on PBMC Proliferation

PAE was orally administered to broiler chickens at doses of 1.25, 2.5, 5, and 10 mg/kg body weight per day for 5 weeks. After 2 weeks of treatment, the PBMC proliferation in chickens treated with 2.5, 5, 10 mg/kg PAE was significantly higher than those of the control chickens (cultured alone : 100% vs 111.54～137.89% , cultured with ConA : 100% vs 108.94～132.33% , cultured with LPS : 100% vs 121.34～154.99%) (Fig. 2(A)). But, after 5 weeks of treatment, the PBMC proliferation in the PAE-treated chickens was similar to that of the control chickens (Fig. 2(B)).

### 2. Effect of PAE on Splenocyte and Thymocyte Proliferation

The splenocyte proliferation in chickens treated with 2.5, 5, 10 mg/kg PAE was significantly higher than those of the control chickens (cultured alone : 100% vs 132.47～163.22% , cultured with ConA : 100% vs 127.72～153.86%, cultured with LPS : 100% vs 122.57～153.36%) (Fig. 3(A)).

The thymocyte proliferation in chickens treated with 2.5, 5, 10 mg/kg PAE was significantly higher than those of the control chickens (cultured alone : 100% vs 114.26～139.23%, cultured with ConA : 100% vs 119.52～140.38%, cultured with LPS : 100% vs 124.53～161.71%) (Fig. 3(B)).

### 3. Effect of PAE on Cytokine Expression

After 5 weeks of treatment, splenocytes were isolated from PAE-treated chickens. RT-PCR was performed to analyze the effect of PAE on the expression of various cytokines. At PAE doses of 1.25, 2.5, 5, and 10 mg/kg, production of IL-18 mRNA was elevated in splenocytes. Also, At PAE doses of 2.5 and 5 mg/kg, production of IL-1$\beta$ mRNA was elevated in splenocytes. However, the levels of IFN-$\alpha$ mRNA was similar to that of the control chickens (Fig. 4). IFN-$\gamma$ and IL-6 were not detected in splenocytes.

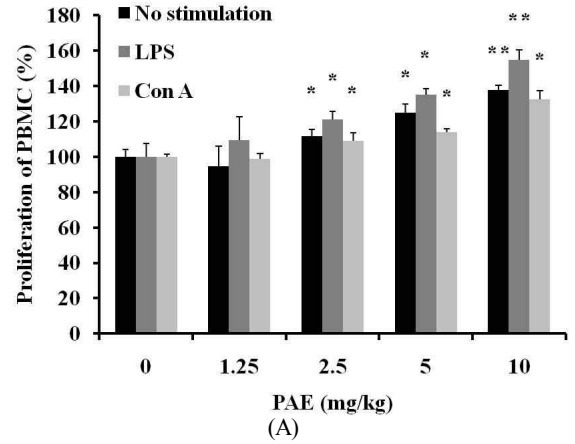
## DISCUSSION

Proanthocyanidins are natural polyphenolic compounds that are widely distributed in many plants, and which have long been utilized as food supplements in human diets for many years. The present results provide evidence PAE from *P. radiate* bark exhibits immunomodulatory activities in broiler chickens.

After 2 weeks of treatment with 2.5, 5, 10 mg/kg PAE, PBMC proliferation in broiler chickens was significantly higher than those of the control chickens. But, after 5 weeks of treatment, the PBMC proliferation in the PAE-treated chickens was similar to that of the control chickens. Also, after 5 weeks of treatment, the proliferation of splenocytes and thymocytes in

**Table 1.** Sequence of the primers used in RT-PCR

| Primers | Sequences | | Annealing temperature |
|---|---|---|---|
| | Forward | Reverse | |
| IL-6 | 5'-CAAGGTGACGGAGGAGGAC-3' | 5'-TGGCGAGGAGGGATTTCT-3' | 62℃ |
| IL-1$\beta$ | 5'-TGGGCATCAAGGGCTACA-3' | 5'-TCGGGTTGGTTGGTGATG-3' | 62℃ |
| IL-18 | 5'-GGAATGCGATGCCTTTTG-3' | 5'-ATTTTCCCATGCTCTTTCTCA-3' | 62℃ |
| IFN-$\gamma$ | 5'-AGCTGACGGTGGACCTATTATT-3' | 5'-GGCTTTGCGCTGGATTC-3' | 62℃ |
| IFN-$\alpha$ | 5'-GACATCCTTCAGCATCTCTTCA-3' | 5'-AGGCGCTGTAATCGTTGTCT-3' | 62℃ |
| GAPDH | 5'-GGTGGTGCTAAGCGTGTTAT-3' | 5'-ACCTCTGTCATCTCTCCACA-3' | 62℃ |



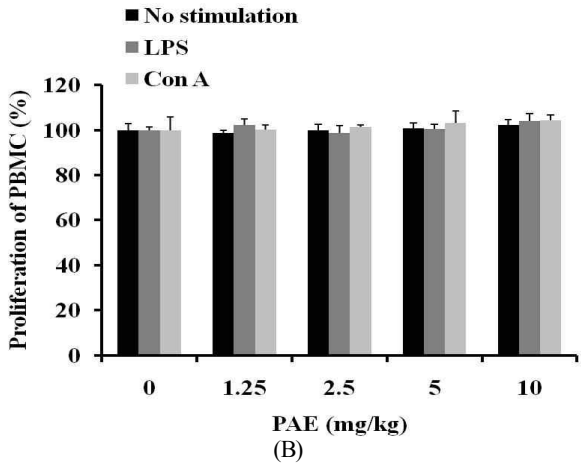
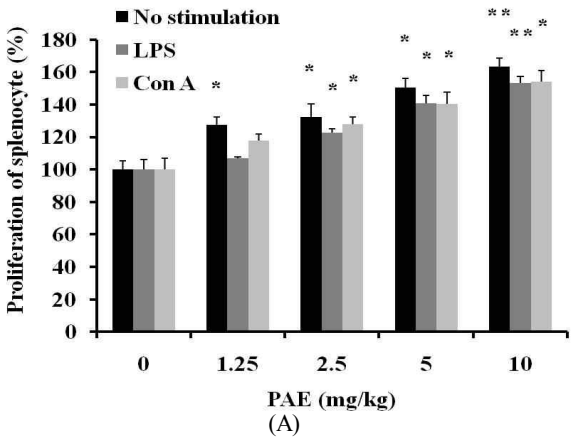




**Fig. 2.** PAE promotes PBMC proliferation. PBMCs were cultured in triplicate wells with the stimulants in a 5% $CO_2$ atmosphere at 37℃ for 48 h, and cell proliferation was determined by the MTS assay. (A) PBMC proliferation after 2 weeks of treatment. At 2.5, 5, 10 mg/kg, PAE enhanced the proliferation of chicken PBMCs. (B) PBMC proliferation after 5 weeks of treatment. Proliferation was similar to the control chickens. Con A = concanavalin A. LPS = lipopolysaccharide. $^{*}P < 0.05$.



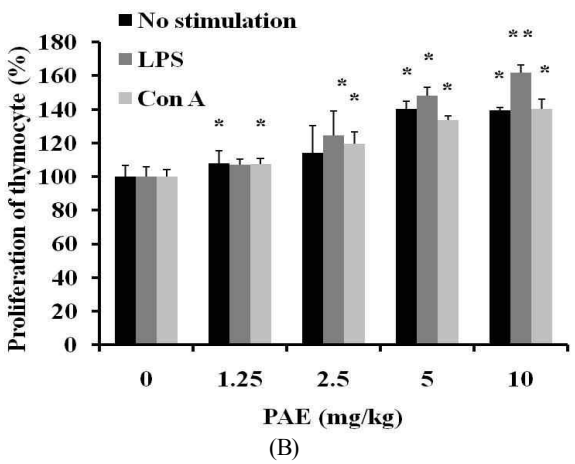




**Fig. 3.** PAE promotes splenocyte and thymocyte proliferation. Splenocytes (A) and thymocytes (B) were cultured in triplicate wells with the stimulants in a 5% $CO_2$ atmosphere at 37℃ for 48 h, and then the cell proliferation was determined by the MTS assay. At PAE doses 1.25, 2.5, 5 and 10 mg/kg, the proliferation of broiler chicken splenocytes and thymocytes were enhanced. Con A = concanavalin A. LPS = lipopolysaccharide. $^{*}P<0.05$, $^{**}P<0.005$.

the PAE-treated chickens were significantly higher than those of the control chickens. This results indicate that PAE enhances the early events in PBMC proliferation.

A number of studies reported that certain compounds can enhance the immunocyte proliferation that was induced by Con A or LPS and suggested an important role in immune function (Gupta et al., 2006). The evaluation of substances that either promote or inhibit immunocyte proliferation is crucial to the study of immunomodulation and drug discovery. PAE significantly increased the proliferation of PBMCs, splenocytes and thymocytes at concentrations of 2.5～10 mg/kg, and these effects were markedly enhanced by the presence of LPS and Con A. Con A act directly on T cells, while LPS acts on B cells. T cells are involved in cell mediated immunity, whereas B cells are primarily responsible for humoral immunity. Therefore, PAE could enhance both cellular and humoral immunity in broiler chickens.

Macrophages play an important role in the defense mecha-

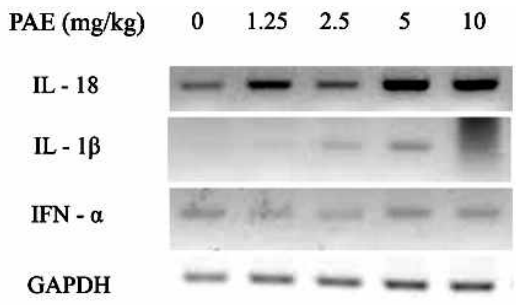



**Fig. 4.** Analysis of cytokine mRNA levels by RT-PCR in splenocytes of PAE treated chickens. At PAE doses of 1.25, 2.5, 5, and 10 mg/kg, production of IL-18 mRNA was elevated in splenocytes. At PAE doses of 2.5 and 5 mg/kg, production of IL-1$\beta$ mRNA was elevated in splenocytes. However, the levels of IFN-$\alpha$ mRNA was similar to that of the control chickens. IFN-$\gamma$ and IL-6 were not detected in splenocytes.

nism against host infections and in the killing of tumor cells (Kang et al., 2002). IL-18 and IL-1$\beta$ are a cytokine produced by macrophages. IL-18 was originally discovered as an IFN-$\gamma$-inducing factor (Hunter et al., 1997). IL-18 acts as a strong co-inducer of IFN-$\gamma$ and granulocyte macrophage colony-stimulating factor production in T cells, natural killer cells, B cells, and macrophages (Okamura et al., 1995, 1998; Dinarello et al., 1998; Munder et al., 1998). IL-1$\beta$ acts as a co-stimulates activator in Th cells. RT-PCR was performed to compare cytokines expression in splenocytes of PAE-treated chickens and control chickens. The present results show that the expression of IL-18 and IL-1$\beta$ are enhanced in splenocytes of PAE-treated chickens. Thus, PAE appears to induce acivation of macrophages in broiler chickens.

In summary, this study demonstrated that PAE elevated cell proliferation of PBMCs, splenocytes and thymocyte. The levels of IL-18 and IL-1$\beta$ are enhanced in splenocytes of PAE-treated chickens. These results indicate that PAE might have immunomodulatory properties in broiler chickens.

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