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Immune-enhancing effect of *Acanthopanax Koreanum* and its component, Eleutheroside E on the protein-energy malnourished C57bl/6 mice

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SUMMARY

Acanthopanax Koreanum stem (AK) has been used in Korea as a tonic and sedative as well as a drug with ginseng like activities. The purpose of our present study was to investigate the effects of AK extract (AKE) and Eleutheroside E, major component of AKE on an exacerbated immune function through utilization of protein-energy malnutrition (PEM) diet by using forced swimming test (FST). The immobility time were significantly decreased in the AKE or Eleutheroside E-administrated group compared with the control group on the FST ($P < 0.05$). The level of blood parameters were not changed significantly. PEM-induced weight loss of mice was reduced by oral administration of 500 mg/kg AKE. AKE oral administration improved the nutritional status such as the food efficiency ratio and the adrenal gland weight. AKE treatment significantly increased the production of interferon (IFN)-γ compared with unstimulated splenocytes but not interleukin (IL)-4. Eleutheroside E also significantly increased the IFN-γ production but not IL-2 and IL-4 in T cell line, MOLT-4 cells. These results suggest that AKE and Eleutheroside E may influence to immune-enhancing through increasing the physical endurance capacity and immune cell activation.

Key words: *Acanthopanax Koreanum*; Protein energy malnutrition; Forced swimming test; Immune-enhancing effect

INTRODUCTION

Acanthopanax species are widely distributed in Korea, Japan, China, and the far-eastern region of Russia. The stem barks of these plants have been used as a tonic and sedative, as well as in the treatment of rheumatism and diabetes (Perry and Metzger, 1981). The major active components of *Acanthopanax Koreanum* stems (AK) are eleutherosides, chiisanosides, isofraxidin, acanthosides, daucosterine, β-sitosterol, sesamine, and savinine (Davydov and Krikorian, 2000). AK extracts (AKE) have been used as popular health supplements to treat stress-induced physiological changes (Fujikawa *et al.*, 1996; Gaffney *et al.*, 2001). as well as various allergic conditions, cancer, and inflammation (Yi *et al.*, 2002; Lee *et al.*, 2004; Yamazaki *et al.*, 2007; Lin *et al.*, 2008).

Protein-energy malnutrition (PEM) is a result of unavailability of food, poverty and the lack of means to buy food, the negligence of food, and the presence of debilitating diseases that affect caloric intake, absorption of foodstuffs, or energy expenditure

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(Cunningham-Rundles *et al.*, 2005). PEM is responsible, directly or indirectly, for 54% of the 10.8 million deaths per year in children under 5 years and contributes to 53% of death associated with infectious diseases among children belonging to this age-group in developing countries (World Health Organization, 2005). PEM affects both the cellular and humoral immunity, it influences the phagocytic function, cytokine production, complement factors generation, and secretory immunoglobulin (Ig) A production (Chandra RK, 2002; Keusch, 2003). In a previous study, the body weights of young adult female PEM mice were 30% less at 4 weeks and 43% less at 6 weeks compared with their respective normal diet mice. Liver, kidney, heart, and spleen weights were significantly lower in PEM mice than normal mice. A disproportionately large reduction in spleen weight compared with other organ weights was determined. The lower spleen weight corresponded to a reduction in the numbers of splenocyte in PEM mice compared with normal mice (Taylor, 1997). The capacity of T cells to produce interferon-γ (IFN-γ) is consistently depressed in rodent models of acute protein and energy.

The forced swimming test (FST) is one of the most commonly used animal models of behavioral despair, and has been used widely as a pre-clinical diagnostic tool for the evaluation of novel anti-depressants and immune-enhancing agents (Porsolt, *et al.*, 1978; Connor *et al.*, 1998, An *et al.*, 2006). It has been reported that FST exposure induces alterations in both cellular and noncellular immunity (Delbende *et al.*, 1994).

Therefore, in the current study, we examined the nutritional status and anti-immobility effects of the AKE as well as the contents of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose (Glc), total protein (TP), and lactate dehydrogenase (LDH) in the serum of PEM groups. Furthermore, to investigate the effect of AKE and Eleutheroside E on the production of cytokines, we analyzed the production of IFN-γ, IL-2 and IL-4 in splenocytes and T cell line, MOLT-4 cells.

MATERIALS AND METHODS

Reagents

Avidin-peroxidase and 2-AZINO-bis (3-ethylbenzithiazoline-6-sulfonic acid) tablet substrate (ABTS) were purchased from Sigma (St. Louis, MO, U.S.A.). Anti-mouse IL-2, IL-4, and IFN-γ, biotinylated anti-mouse IL-2, IL-4 and IFN-γ and recombinant mouse IL-2, IL-4 and IFN-γ were purchased from BD Biosciences (San Jose, CA, U.S.A.).

Preparation of AKE

AKE was provided by the SKSOGAPY CO. Ltd. (Chungnam, Republic of Korea). The AKE was dissolved in distilled water at 150 and 500 mg/kg dosages.

Animals

Male C57BL/6J mice weighing 20 - 22 g (Daehan Biolink Co. Daejeon, Korea) were used in these experiments. They were housed under following laboratory conditions: temperature 23 ± 1°C, humidity 40 - 60 %, 12:12 h light/dark cycle, lights on at 07:00 h. Food and water were available *ad libitum*. Thirty

Table 1. Composition of experimental diets[a]

Ingredients	Standard (g/kg diet)	PEM (g/kg diet)
Casein (> 85 % protein)	200	40
Sucrose	100	100
Fiber	10	10
Corn oil	80	80
Mineral mixture[b]	40	40
Vitamin mixture[b]	10	10
L-Methionine	1.5	1.5
Choline bitartrate	2.5	2.5
Cornstarch	556.5	716.5

Composition of experimental diets.
[a]Isocaloric diets providing 1716.3 kJ/100g.
[b]Mineral and vitamin mixtures were prepared according to the 1993 recommendations of the American Institue of Nutrition for adult mice (Reeves PG, 1993)

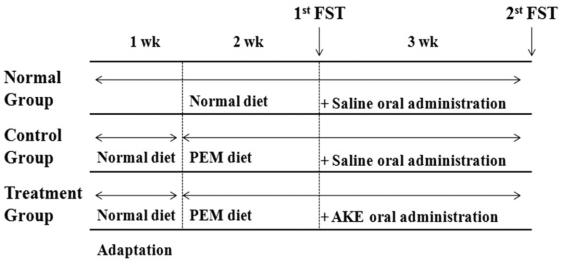

Fig. 1. PEM period diagram. First, all mice left 1 week for the adaptation. After 2 weeks on the starting PEM diet, the mice were performed the first FST. And the mice were administered each drugs, respectively. The other PEM diet group and the control diet group were administered the saline. The drugs were administered orally for 3 weeks.

male C57BL/6J mice were divided into six groups of 5 mice each. The control group was fed a control diet, and mice in other five groups were fed a PEM diet for 35 days (Table 1 and Fig. 1). Mice were treated in accordance with the current law and the NIH Guide for Care and Use of Laboratory Animals.

FST

After 2 weeks on the starting PEM diet, the mice was performed the first FST. And the mice were administered 300 mg/kg of CVE, 150 and 500 mg/kg of AKE and 10 mg/kg of Eleutheroside E, respectively. The other PEM diet group and the control diet group were administered the saline. The CVE, AKE, and saline were administered orally for 3 weeks. For 6 min of FST, the duration of immobility was measured, as previously described by Porsolt and co-workers (Porsolt *et al.*, 1978). The apparatus consisted of two Plexiglas cylinders (height: 25 cm, diameter: 10 cm) placed side by side in a Makrolon cage filled with water (10 cm height) at 23 - 25°C. Two mice were tested simultaneously for a 6 min period inside vertical Plexiglas cylinders; a nontransparent screen placed between the two cylinders prevented the mice from seeing each other. The total duration of immobility, after a delay of 2 min, was measured during a period of 4 min. Each mouse was considered to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water.

Preparation and ingredient analysis of blood serum

Mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg). After anesthetization, blood was withdrawn from the heart of FST mice into syringes. Then, serum was prepared by centrifugation at 12,000 rpm at 4 °C for 20 min. The contents of ALP, AST, ALT, Glc, TP, and LDH were determined by an autoanalyzer (Hitachi 747, Hitachi, Japan).

Enzyme-linked immunosorbent assay (ELISA)

Sandwich ELISA for IFN-γ, IL-2, and IL-4 was carried out in duplicate in 96-well ELISA plates (Nunc, Denmark) coated with each of 100 ml aliquots of anti-human IFN-γ, IL-2, and IL-4 monoclonal antibodies at 1.0 mg/ml in PBS at pH 7.4, followed by incubation overnight at 4 °C. The plates were washed in PBS containing 0.05% Tween-20 (Sigma, St. Louis, MO, U.S.A.) and blocked with PBS containing 1% BSA, 5% sucrose, and 0.05% NaN3 for 1 h. After additional washes, sample or IFN-γ, IL-2 and IL-4 standards were added and incubated at 37 °C for 2 h. After 2 h incubation at 37 °C, the wells were washed, then each of 0.2 mg/ml of biotinylated anti-mouse IFN-γ, IL-2, and IL-4 were added and again incubated at 37 °C for 2 h. After washing the wells, avidin-peroxidase was added, and the plates were incubated for 20 min at 37 °C. Wells were again washed and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant IFN-γ, IL-2, and IL-4 in serial dilutions.

Statistical analysis

The data obtained was analyzed by a Student's t-test and ANOVA with a Turkey's *post hoc* test. Statistical significance was compared among each treated group from at least three experiments. The

results are presented as the mean ± standard error of mean (S.E.M.). Results with $P < 0.05$ were considered statistically significant.

RESULTS

Effect of AKE on FST

After the first measurement of the immobility time, the mice were divided into a control diet group (saline) and PEM diet groups (saline, 300 mg/kg of CVE, 150 and 500 mg/kg of AKE, and 10 mg/kg of Eleutheroside E) to match the swimming time in each group. CVE, known as an immune-enhancing material, was used as a reference agent. The immobility times with the saline, CVE, and AKE treated groups were measured before the administration. Compared with the saline administrated group, the immobility time of mice was not changed significantly in the other administrated groups. However, CVE, AKE, or Eleutherosid E administration for 3 weeks significantly decreased the immobility time compare with the PEM diet group (Fig. 2).

Effect of AKE on the blood biochemical parameters

As shown in Table 2, we investigated the blood parameters on the PEM-induced mice. The ALP level of the Eleutheroside E (10 mg/kg) groups decreased significantly in comparison with that of the PEM diet group ($P < 0.05$). The other blood parameters levels were not shown the significant effect in CVE, AKE, or Eleutheroside E group compared with PEM diet group.

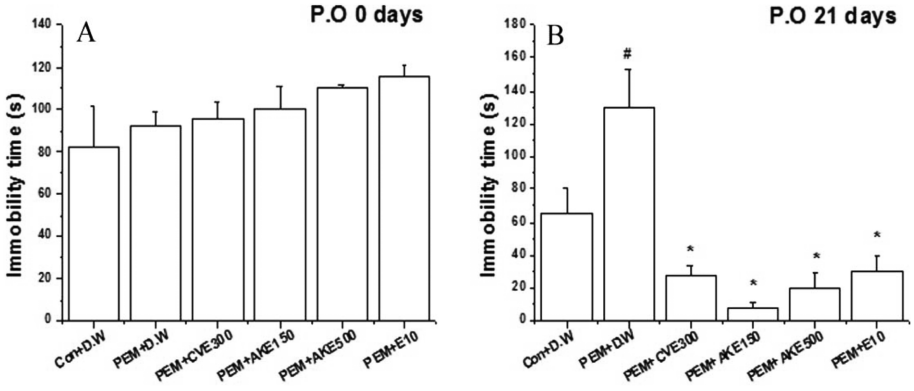

Fig. 2. Effect of AKE and Eleutheroside on the immobility time in the FST. The mice were administrated for 21 days at the same time. During the FST, the administration of saline, CVE, and AKE was executed 1 h before the test. Immobility time recorded during 4 min in FST in mouse given saline (control group), CVE (300 mg/kg), and AKE (150 and 500 mg/kg). $^{\#}P < 0.05$ versus a control diet group. $^{*}P < 0.05$ versus a PEM diet group.

Table 2. Concentration of ALP, AST, ALT, Glc, TP, and LDH after last forced swimming test

	ALP (U/l)	AST (U/l)	ALT (U/l)	Glc (mg/dl)	TP (g/dl)	LDH (IU/l)
Con+Saline	81.0 ± 10.2	88.0 ± 26.8	19.3 ± 1.9	427.3 ± 13.1	5.3 ± 0.1	446.3 ± 90.6
PEM+Saline	135.3 ± 3.5$^{\#}$	74.3 ± 5.8	21.0 ± 2.0	361.0 ± 31.0	4.5 ± 0.1	530.0 ± 102.6
PEM+CVE	120.7 ± 6.9	68.7 ± 3.2	20.7 ± 1.2	392.3 ± 17.3	4.5 ± 0.1	476.7 ± 25.0
PEM+AKE150	122.0 ± 4.5	62.0 ± 8.3	23.0 ± 3.2	287.0 ± 39.5	4.4 ± 0.1	421.3 ± 40.2
PEM+AKE500	116.3 ± 4.8	69.0 ± 10.1	18.3 ± 2.2	248.3 ± 10.4	4.3 ± 0.0	326.3 ± 58.5
PEM+E10	90.7 ± 7.1*	142.0 ± 45.0	28.7 ± 2.2	292.3 ± 14.1	4.3 ± 0.2	811.7 ± 43.5

Saline, CV (300 mg/kg), AKE (150 and 500 mg/kg), and Eleutheroside E (10 mg/kg) was administered orally to mice, we have measured the levels of ALP, AST, ALT, Glc, Tp, and LDH in the serum ($n = 5$). Values are means ± S.E.M. $^{\#}P < 0.05$ versus a saline-treated control diet group. $^{*}P < 0.05$ versus a saline-treated PEM diet group.

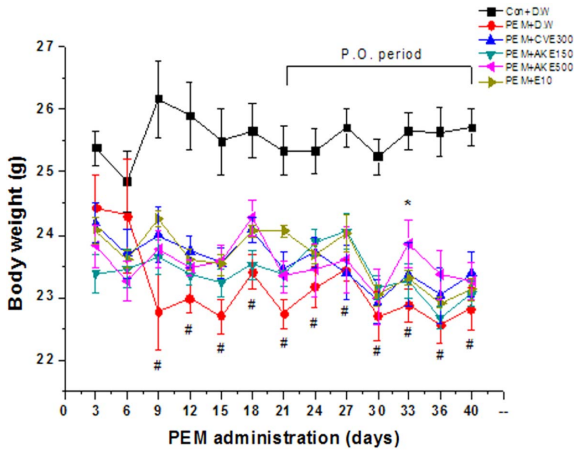

Fig. 3. Effect of AKE and Eleutheroside E on the evaluation of the body weights of PEM diet mice. Con (normal diet), PEM (PEM diet), CVE (300 mg/kg), AKE (150 and 500 mg/kg), and Eleutheroside E (10 mg/kg). Data are reported as means ± S.E.M. $^{\#}P < 0.05$ versus a control diet group. $^{*}P < 0.05$ versus a PEM diet group.

Evaluation of the body weights, food intake, water intake, and food efficiency ratio

In order to examine the nutritional status, body weight was evaluated weekly in mice that were fed with control or PEM diet. At 7 days, it was possible to observe a statistical difference in body weight among mice fed with control or PEM diet. But after 500 mg/kg of AKE oral administration significantly increased the body weights of mice (Fig. 3). Food intake and water intake was not changed significantly (Fig. 4A and B). Food efficiency ratio in all treatment-groups was increased significantly ($P < 0.05$) compared with PEM diet groups (Fig. 4C).

Effect AKE on the immune organ weight such as adrenal gland, spleen, and thymus

To examine the effect of AKE on the deteriorated immunity caused by the PEM diet, the crucial immune organ weight of adrenal gland, spleen, and thymus was measured. The weight of adrenal gland was increased in PEM diet group compared with control group. After CVE or AKE oral administration, the weight of adrenal gland were decreased significantly ($P < 0.05$). But oral administration of Eleutheroside E did not affect the weight of

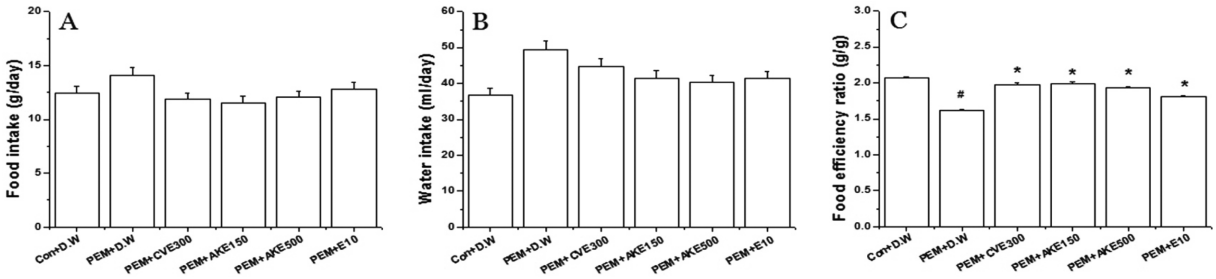

Fig. 4. Effect of AKE and Eleutheroside E on the nutritional status of PEM diet mice. Con (normal diet), PEM (PEM diet), CVE (300 mg/kg), AKE (150 and 500 mg/kg), and Eleutheroside E (10 mg/kg). Data are reported as means ± S.E.M. $^{\#}P < 0.05$ versus a control diet group. $^{*}P < 0.05$ versus a PEM diet group.

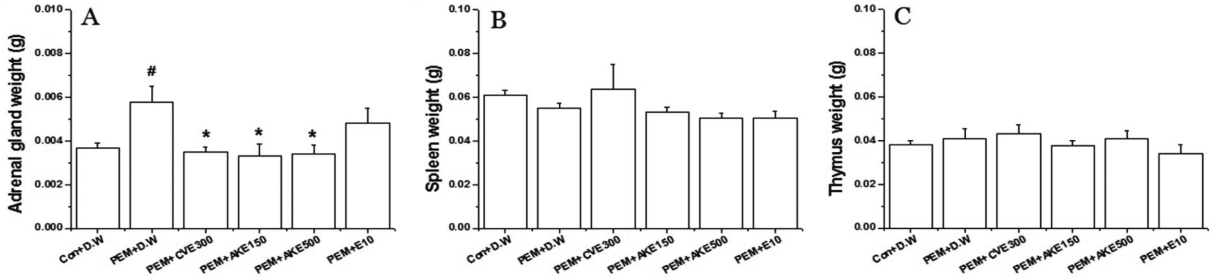

Fig. 5. Effect of AKE and Eleutheroside E on the immune organ of PEM diet mice. Data are reported as means ± S.E.M. $^{\#}P < 0.05$ versus a control diet group. $^{*}P < 0.05$ versus a PEM diet group.

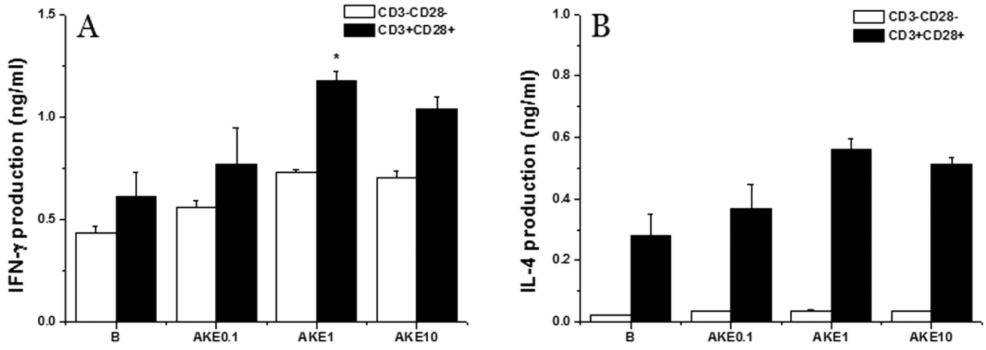

Fig. 6. Effect of AKE on the cytokine production from splenocytes. Cells (5×10^5) were treated with various concentrations (0.1 to 10 mg/ml) of AEK for 24 h. Cytokine levels in the culture supernatant were measured using ELISA. Values represent the mean ± S.E.M. $^*P < 0.05$ versus a control group. B, unstimulated cells.

adrenal gland. CVE, AKE, or Eleutheroside E oral administration did not affect the weight of spleen and thymus (Fig. 5).

Effect of AKE on the cytokine production from splenocytes

To assess the effects of AKE on the cytokines production, the levels of IFN-γ, IL-2, and IL-4 in splenocytes were analyzed by the ELISA method. As shown in Fig. 6A and B, 1 μg/ml of AKE significantly increased the IFN-γ level compared with the control group ($P < 0.05$). But AKE did not affect the IL-4 production. IL-2 production in CD3 +CD28+stimulated cell was not changed significantly compared with unstimulated cells. AKE also did not affect the IL-2 production (data not shown).

Effect of Eleutheroside E on the cell proliferation and cytokine production in T cell line, MOLT-4 cells

The effect of Eleutheroside E on the cell proliferation of T cells was investigated by using MTT assay. As shown in Fig. 7A, treatment with Eleutheroside E

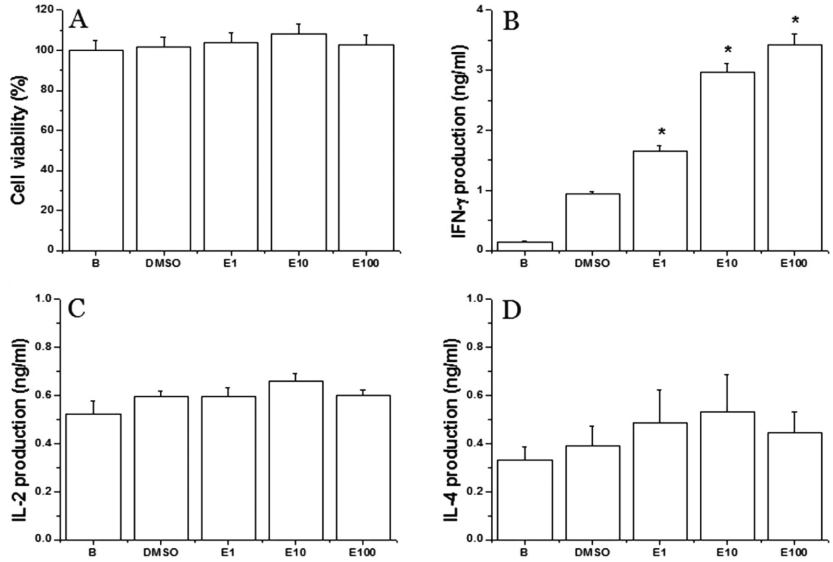

Fig. 7. Effect of Eleutheroside E on the cell proliferation and the cytokine production in MOLT-4 cells. Cells (5×10^5) were treated with various concentrations (1 to 100 μM) of Eleutheroside E for 24 h. Cytokine levels in the culture supernatant were measured using ELISA. Values represent the mean ± S.E.M. $^*P < 0.05$ versus a control group. B, unstimulated cells.

had no effect on cell proliferation under of the tested conditions. To investigate the effects of Eleutheroside E on the cytokine production, the levels of IFN-γ, IL-2, and IL-4 in MOTL-4 cells were analyzed by the ELISA method. Ttreatment with Eleutheroside E significantly increased the IFN-γ level compared with the control group ($P < 0.05$). But Eleutheroside E did not affect the IL-2 and IL-4 production.

DISCUSSION

AKE, a traditional Korean medicine was considered to play an important role on the treatment of a variety of diseases. However, the effect of AKE on immune-enhancing effect in the PEM diet model has not been clearly clarified.

PEM is the major cause of secondary immunodeficiency in the world (Cunningham-Rundles *et al.*, 2004). Many studies have observed that PEM can lead to clinically significant immune deficiency and infections in children (Scrimshaw *et al.*, 2003). Among the effects that PEM has on immunocompetence, the most striking are: (i) atrophy of the lymphoid tissue, particularly in the thymus; (ii) a reduction in delayed cutaneous hypersensitivity; (iii) a reduction in the number of T cells, especially T helper cells; (iv) a decrease in thymulin activity; (v) decreased secretory immunoglobulin A antibody response; and (vi) a reduced concentration and activity of complement components and phagocyte dysfunction (Malafaia *et al.*, 2009). So, we investigated the relationship between the immune system and the nutritional function on AKE treated PEM diet mice. Consequently, AKE and Eleutheroside E administration significantly increased the food efficiency ratio on PEM diet mice, thereby having a positive nutritional function. In addition, the adrenal gland weight was significantly decreased by AKE treatment on PEM diet mice. But the spleen and the thymus on PEM diet mice were not affected by AKE or Eleutheroside E administration. In this study, AKE or Eleutheroside E decreased the immobility time. From this, these results indicate that AKE has an immune-enhancing effect on immune deficiency model.

In most cases, the swimming exercise is known to induce the biochemical changes in blood (De-Mello, 1992). Thus, we assessed the blood biochemical parameters related to fatigue. ALP, AST, ALT, Glc, TP, and LDH contents in the blood of the mice were examined after the FST. In the present study, the ALP levels was significantly increased by Eleutheroside E treatment, but the other blood parameters level were not changed in blood serum of the AKE or Eleutheroside E treated PEM diet group compared with a saline-treated PEM diet group.

T cells play a crucial role in immune functions as they act both as effectors like cytotoxic T cells, and regulators like T_H cells and suppressor T cells. T_H cells mediate the link between the antigen-presenting and triggering of other cellular and humoral components of the immune response (Stephens *et al.*, 2002). T_H cells have two different subsets, T_H1 and T_H2. In particular, T_H1 cytokine, like the IFN-γ, IL-2 and tumor necrosis factor (TNF), plays an important role in the immune response in protecting against various intracellular microorganisms and tumors (Riddell *et al.*, 2002). Previous reports have demonstrated that the induction of T_H1-promoting cytokine, using specific adjuvants, can enhance anti-tumor immunity and can reduce or even prevent tumor growth (Dredge *et al.*, 2002). Many cancer vaccines in combination with immune adjuvants, elicit strong cellular immune responses leading to the production of T_H1 type cytokines such as IFN-γ, IL-2, and TNF (Dalgleish, 2000). IFN-γ is also an important cytokine in the host defense against infection by viral and microbial pathogens (Samuel, 2001). IFN-γ induces a variety of physiologically significant responses that contribute to immunity. IL-2 is a T cell growth factors and it has multiple immunoregulatory functions and biological properties (Kim *et al.*, 2006). IL-2 was conjunct with the antigens, mitogens, or anti-

immunoglobulin antibodies, and then it controls B cell proliferation and differentiation into antibody-producing plasma cells (Jelinek and Lipsky, 1987). In addition, IL-2 promotes immunoglobulin production by B cells and regulates the proliferation and apoptosis of the activated T cells (Dalgleish, 2000). IL-4, also known as the prototypic immunoregulatory cytokine, is a particularly important cytokine in the type 2 immune response. This cytokine can drive the development andexpansion of T_H2 cells and mediate downstream effector functions, such as B-cell activation. IL-4 also has various direct effects on non-lymphoid tissue including mucosal epithelial cells, goblet cells, and smooth muscle cells (Liu *et al*., 2004). In this study, we observed that T_H1 and T_H2 cytokines, such as IFN-γ, IL-2, and IL-4 in splenocytes and T cells. The level of IFN-γ, but not IL-2 and IL-4, was significantly increased to affect by AKE and Eleutheroside E treatment. Therefore, it was thought that AKE may regulate production of T_H1 and T_H2 cytokine via activation of splenocytes and T cells.

Conclusively, our study showed that the administration of AKE and Eleutheroside E improves the nutritional status and decreases the immobility times after FST in PEM diet mice. AKE administration enhanced the adrenal gland weight in PEM mice. In addition, the level of IFN-γ was significantly increased by AKE or Eleutheroside E in splenocytes and T cells. Therefore, our results provide for the therapeutic potential of AKE for immune deficiency diseases.

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