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Immunostimulating Effects of Extract of *Acanthopanax sessiliflorus*

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Abstract: As malfunction/absence of immune cells causes a variety of immunosuppressive disorders and chemical synthetic drugs for curing these diseases have many adverse effects, vigorous studies are being conducted. The *Acanthopanax* family has been used as traditional medicines for gastric ulcer, diabetes, etc. and culinary materials in East-South Asia. In this study, the immunostimulating properties of *A. sessiliflorus* were evaluated. *A. sessiliflorus* increased not only the splenocyte number but also immune-related cytokines such as TNF- α . However, it could not upregulate the expressions of IFN- γ and IL-2. *A. sessiliflorus* increased the swimming time, and comparison of organ weights relative to body weights for immune-related organs such as the spleen and thymus after a forced swim test showed that it could recover the spleen and thymus weights. It also increased the expression of TNF- α and slightly increased the concentration of IFN- γ but not IL-2. From the results, we concluded that as *A. sessiliflorus* has not only a host defense effect but also a stress-ameliorating property, further study it will be a promising material of immunostimulating material.

Key words: *Acanthopanax sessiliflorus*, IFN- γ , immunostimulating, rat, TNF- α

Introduction

The immune system is made up in the various organs in the body such as bone marrow, lymph nodes, the spleen, and the thymus, and when the immune response is feeble or absent, it is easy for many pathogens to invade living organisms. So in order to protect against pathogens it is important to strengthen the immune system.

TNF- α is produced by a lot of immune cells, such as T cells, B cells, NK cells, and macrophage, and modulates not only cell survival but also cell death (apoptosis),

and it is referred to as a “double-edged sword” [1]. In particular, it is important in regulation of the inflammation and host defense is response to bacterial infection [16]. IFN- γ is a type II interferon that is regulated with two types of receptor such as IFN- γ R1 and IFN- γ R2 [7], and activates JAK1, JAK2, and then STAT1 [24]; it was recently approved by the US FDA as a material for improving chronic granulomatous disease and malignant osteopetrosis [21]. IL-2 regulates the functions of lymphocytes such as differentiation, immune responses, and homeostasis and especially has a key role of controlling regulatory T (T_{Reg}) cells and differentiating CD4 T cells

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[5].

The *Acanthopanax* family has been used as traditional medicines for gastric ulcer, diabetes, etc. and culinary materials in East-South Asia [10, 11]. Recently, the biological efficacies of single molecules isolated from *Acanthopanax* species such as the platelet anti-aggregation effect of triterpenoids [13], immunostimulating properties of biopolymers [9, 12], and apoptosis-inducing effects of calenduloside E 6'-methyl ester [15] have been reported.

In this study, we investigated immunostimulating effects of *Acanthopanax sessiliflorus* (*A. sessiliflorus*) through *in vitro* and *in vivo* studies. Splenocyte culture was used for the *in vitro* study, and a modified Porsolt forced swim test was used for *in vivo* study. In order to evaluate *A. sessiliflorus*'s evaluate immunostimulating effects, the changes in immunity-related cytokines such as TNF- α , IFN- γ , and IL-2, splenocyte proliferation, the changes in immunity-related organ weights such those of the thymus and spleen, swimming time, etc. were analyzed.

Materials and Methods

A. sessiliflorus extract preparation

The roots of *A. sessiliflorus* were collected in the area of Jecheon, Chungbuk, Republic of Korea, in January and February 2012. A voucher specimen (HV-REP-7-B-1207-17-9-ASE) was deposited with the Jecheon Traditional Korean Medicine Farming Association, Republic of Korea. The roots (6.5 kg) were soaked with 100°C water (120 l) for 4 h. The extracts were combined and concentrated by freeze-drying to yield a deep brown residue (682 g).

Animals

Thirty-six male SD rats were purchased from Samtaco Korea (Osan, Korea) and acclimated for 7 days. All animals were housed in a temperature- and relative humidity-controlled environment ($22 \pm 3^\circ\text{C}$, 12-h light/dark cycle) during acclimation and the experiment and fed *ad libitum* with Purina diet (Purina Korea, Korea) and water.

One male rat was used for collecting splenocytes, and 7 male rats per group (the number for the animal study: 35 rats) were treated with saline or *A. sessiliflorus* oral administration (0, 30, 100, or 300 mg/kg/day). All experiments were approved by the Institutional Animal

Care and Use Committee at Wonkwang University (Approval No. WKU12-47).

Splenocyte proliferation assay and cytokines analysis

Collected splenocytes were seeded on a 96-well plate with 2×10^5 cells/well/90 μl with 100 U/ml penicillin-streptomycin and RPMI 1640 containing 10% fetal bovine serum (growth media), and *A. sessiliflorus*(0, 1, 10, 100, or 250 $\mu\text{g}/\text{ml}$), LPS (10 $\mu\text{g}/\text{ml}$) or Con A (2.5 $\mu\text{g}/\text{ml}$) was added to the wells. The final volume per well was 100 μl . Cells were incubated in a CO₂ incubator (5% at 37°C) under humidified conditions for 24 h. A WST-1 assay kit (ITSBio, Seoul, Korea) for splenocyte proliferation rate measurement and cytokine activation analysis kits for TNF- α (RTA00, R&D Systems, Minneapolis, MN, USA), IFN- γ (RIF00, R&D Systems), and IL-2 (R2000, R&D Systems) were used according to the manufacturer's instructions, and the results were determined using a Microplate-Reader (Molecular Device, Sunnyvale, CA, USA).

Modified Porsolt forced swim test

The Porsolt forced swim test [17] was modified. For the forced swim test, a Plexglass cylinder (150 cm (height) \times 80 cm (diameter)) was made with nontransparent materials to prevent the animals from seeing each other. The test was conducted at 1 h after *A. sessiliflorus* treatment on Day 28, which was the final treatment day. The animal experiment was performing using 5 groups, the normal group, which was not subjected the forced swim test and 4 treated with 0, 30, 100, or 300 mg/kg/day *A. sessiliflorus*. Before the test, each animal was weighed, and about 10% of the animal's weight was placed on its tail. The duration from putting the animal putting in the apparatus to 10 seconds after the animal stopped moving was checked and recorded.

CBC, organ weight measurement, and cytokine analysis

Seven male rats per group were treated with saline or *A. sessiliflorus* oral administration (0, 30, 100, or 300 mg/kg/day) once a day for 28 days respectively. Their body weights were checked every 7 days. After the final administration, the rats were weighed, and their appearances were judged. They were then anesthetized with diethyl ether, whole blood was collected through the abdominal vena cava, and they were finally sacrificed using diethyl ether. Using the collected whole blood, each element of the blood cells, including neutrophil,

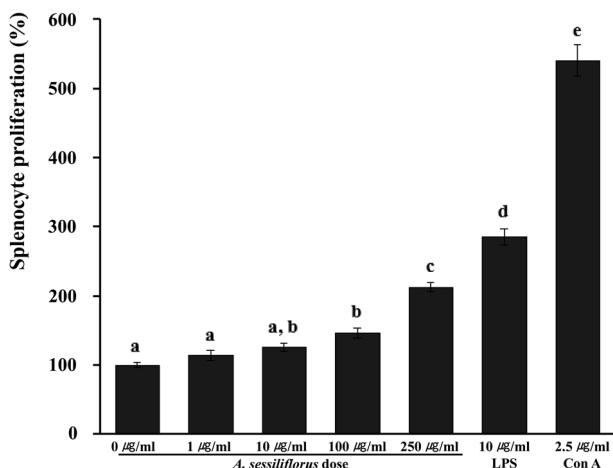


Fig. 1. Effect of *Acanthopanax sessiliflorus* on splenocyte proliferation. *A. sessiliflorus* induced splenocytes proliferation (up to 213%) in the 300 mg/kg/day *A. sessiliflorus* treatment. After collection, splenocytes were seeded on a 96-well plate, and *A. sessiliflorus* (0, 1, 10, 100, or 250 µg/ml), LPS (10 µg/ml), or Con A (2.5 µg/ml) was added. Cells were incubated in a CO₂ incubator (5% at 37°C) under humidified conditions for 24 h and then the proliferation rate was assessed. ^{a, b, c, d, e}Values in the same row with different superscripts are significantly different, *P*<0.05. Data are shown as the mean ± SE (*n*=3).

eosinophil, basophil, and monocyte/macrophage, was measured with a Hemavet950 (Drew Scientific Group, Dallas, TX, USA). TNF-α (RTA00, R&D Systems), IFN-γ (RIF00, R&D Systems), and IL-2 (R2000, R&D Systems) were analyzed with serum using a Microplate-Reader (Molecular Devices).

Statistical analysis

Results are shown as the average ± standard error (SE). A one-way analysis of variance (ANOVA) test was employed followed by Tukey's multiple range tests to compare each group. A *P*-value less than 0.05 was considered statistically significant.

Results

A. sessiliflorus stimulates splenocytes proliferation and induces TNF-α expression in a dose-dependent manner

To evaluate the splenocyte proliferation for 24 h, splenocytes were incubated with 2.5 µg/ml Con A, 10 µg/ml LPS, or *A. sessiliflorus* (0, 1, 10, 100, or 250 µg/ml) after they were collected (Fig. 1). The number of splenocytes increased in a dose-dependent manner, and the percentage of proliferation with the 250 µg/ml *A. ses-*

siliflorus treatment was up to 213%. This was higher than that obtained with the LPS treatment although it was lower than that obtained with the Con A treatment.

As shown in Fig. 2, the concentration of TNF-α 24 h after splenocytes were incubated with 250 µg/ml *A. sessiliflorus* (953.30 ± 44.07 pg/ml; in the 0 µg/ml *A. sessiliflorus*-treated group, 90.57 ± 2.87 pg/ml) was significantly increased (Fig. 2A), but the concentrations of IFN-γ (Fig. 2B) and IL-2 (Fig. 2C) were not changed compared with the other groups.

A. sessiliflorus increases swimming time and lymphocytes

In order to evaluate the physiological change in rats, modified Porsolt swim test was conducted. *A. sessiliflorus* increased the swimming time (Fig. 3). Although the swimming time in the 30 mg/kg/day *A. sessiliflorus* treatment group (7.61 ± 0.98 min) was guessed to be similar to that non-treated group (6.22 ± 0.47 min), but the result might be like those of the 100 mg/kg/day (8.62 ± 1.13 min) and 300 mg/kg/day treatment groups (8.64 ± 0.77 min). The result in the low-dose group might be similar to those in the middle- and high-dose groups but it is important that the swimming time increased in the *A. sessiliflorus*-treated groups.

As shown in Table 1, *A. sessiliflorus* improved the number of white blood cells and especially lymphocytes.

A. sessiliflorus recovers the weights of the spleen and thymus after forced swim test and increases the concentrations of TNF-α and IFN-γ but not IL-2

The spleen weight relative to the body weight in the group not subjected to the swimming test was 0.19 ± 0.00% and this decreased to 0.17 ± 0.1% after the swimming test (Fig. 4). When *A. sessiliflorus* was administered, the spleen weight relative to the body weight recovered in a dose-dependent manner (in the 300 mg/kg/day *A. sessiliflorus* treatment group, 0.19 ± 0.00%). The thymus weight relative to the body weight was similar to the spleen weight; in group not subjected to the swimming test, it was 0.11 ± 0.001%; in the 0 mg/kg/day *A. sessiliflorus* treatment group after the swimming test, it was 0.09 ± 0.00%; in the 300 mg/kg/day *A. sessiliflorus* treatment group after the swimming test, it was 0.11 ± 0.01%. Both the spleen weight and thymus weight dose-dependently recovered to the weights found in the group not subjected to the swimming test.

The expression of TNF-α increased in a dose-dependent manner (Fig. 5), and in the 300 mg/kg/day *A. ses-*

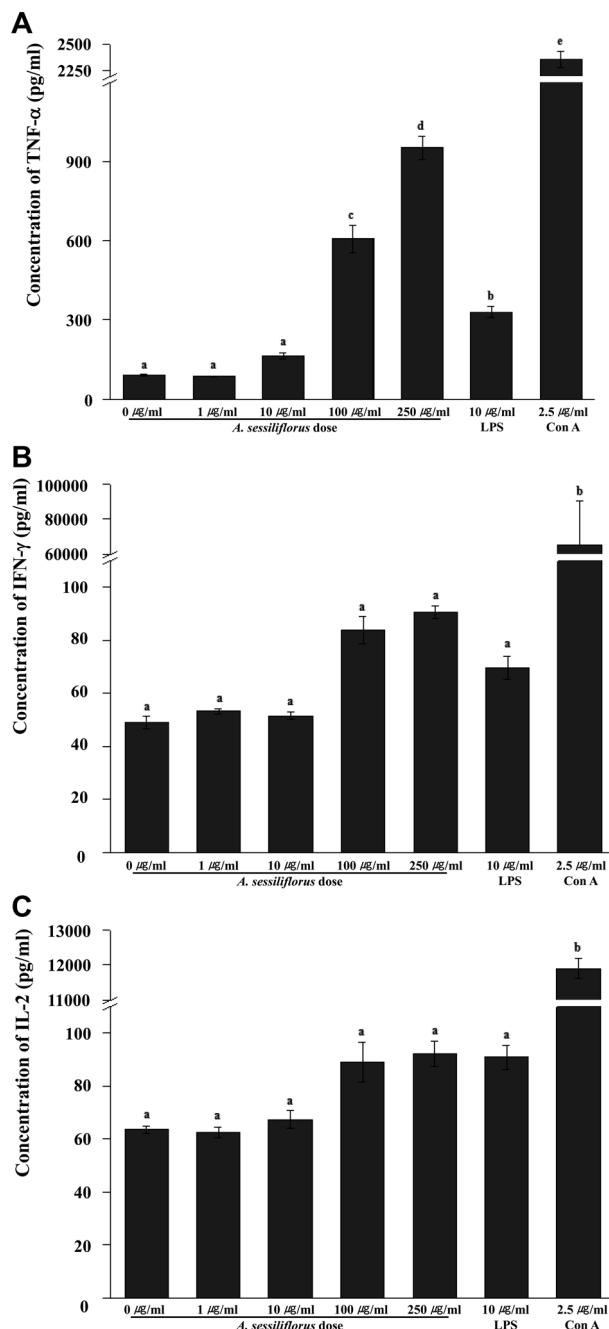


Fig. 2. Effect of *Acanthopanax sessiliflorus* on the concentration of TNF- α , IFN- γ , and IL-2 in the splenocyte. The concentration of TNF- α in the 250 $\mu\text{g}/\text{ml}$ *A. sessiliflorus* treatment was upregulated to 953.30 ± 44.07 pg/ml and it increased in a dose-dependent manner (A); the concentrations of IFN- γ (B) and IL-2 (C) are unchanged compared with the other groups. After collection, splenocytes were seeded on a 96-well plate, and *A. sessiliflorus* (0, 1, 10, 100, or 250 $\mu\text{g}/\text{ml}$), LPS (10 $\mu\text{g}/\text{ml}$), or Con A (2.5 $\mu\text{g}/\text{ml}$) was added. Cells were incubated in a CO₂ incubator (5% at 37°C) under humidified conditions for 24 h and the activation of cytokines such as TNF- α , IFN- γ , and IL-2 was analyzed. ^{a, b, c, d, e}Values in the same row with different superscripts are significantly different, $P<0.05$. Data are shown as the mean \pm SE ($n=3$).

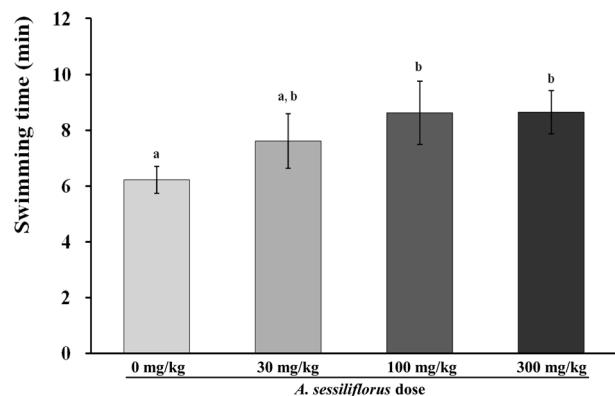


Fig. 3. Effect of *Acanthopanax sessiliflorus* on the forced swim test. *A. sessiliflorus* increased the swimming time. The test was conducted at 1 h after *A. sessiliflorus* treatment on day 28, which was the final day of treatment. The animal experiment was performed using 5 groups, the normal group, which was not subjected to the forced swim test, and 4 groups, which were treated with 0, 30, 100, or 300 mg/kg/day *A. sessiliflorus*. Before the test, the animal was weighed, and about 10% its body weight was placed on its tail. The duration from putting the animal in the apparatus to 10 seconds after the animal stopped moving was checked and recorded. ^{a, b}Values in the same row with different superscripts are significantly different, $P<0.05$. Data are shown as the mean \pm SE ($n=7$).

Table 1. Effect of *Acanthopanax sessiliflorus* on the absolute number of WBCs, lymphocytes, and monocytes in the whole blood of rats

Group	WBC ($\times 10^3$ cells)		
	WBC	Monocyte	Lymphocyte
Normal	$7.06 \pm 1.13^{\text{a}, \text{b}}$	$0.15 \pm 0.02^{\text{a}}$	$5.36 \pm 1.32^{\text{a}}$
0 mg/kg/day	$5.59 \pm 1.10^{\text{a}}$	$0.15 \pm 0.03^{\text{a}}$	$5.25 \pm 1.03^{\text{a}}$
30 mg/kg/day	$9.50 \pm 0.67^{\text{b}, \text{c}}$	$0.22 \pm 0.02^{\text{a}, \text{b}}$	$8.93 \pm 0.64^{\text{b}}$
100 mg/kg/day	$8.54 \pm 0.60^{\text{b}, \text{c}}$	$0.20 \pm 0.02^{\text{a}}$	$8.04 \pm 0.57^{\text{a}, \text{b}}$
300 mg/kg/day	$10.97 \pm 0.89^{\text{c}}$	$0.28 \pm 0.03^{\text{b}}$	$10.18 \pm 0.84^{\text{b}}$

^{a, b, c}Values in the same row with different superscripts are significantly different, $P<0.05$. Data are shown as the mean \pm SE ($n=7$).

sessiliflorus treatment group (11.12 ± 0.968 pg/ml), it was significantly upregulated compared with the 0 mg/kg/day *A. sessiliflorus* treatment group (8.06 ± 0.881 pg/ml). *A. sessiliflorus* might increase the concentration of IFN- γ (in the 0 mg/kg/day *A. sessiliflorus* treatment group, 12.53 ± 2.201 pg/ml; in the 300 mg/kg/day *A. sessiliflorus* treatment group, 23.54 ± 4.072 pg/ml). However, although the variation in each animal in the 30 mg/kg/day *A. sessiliflorus* treatment group was large, it is hard to conclude exactly that *A. sessiliflorus* is able to increase the expression of INF- γ or that *A. sessiliflorus*

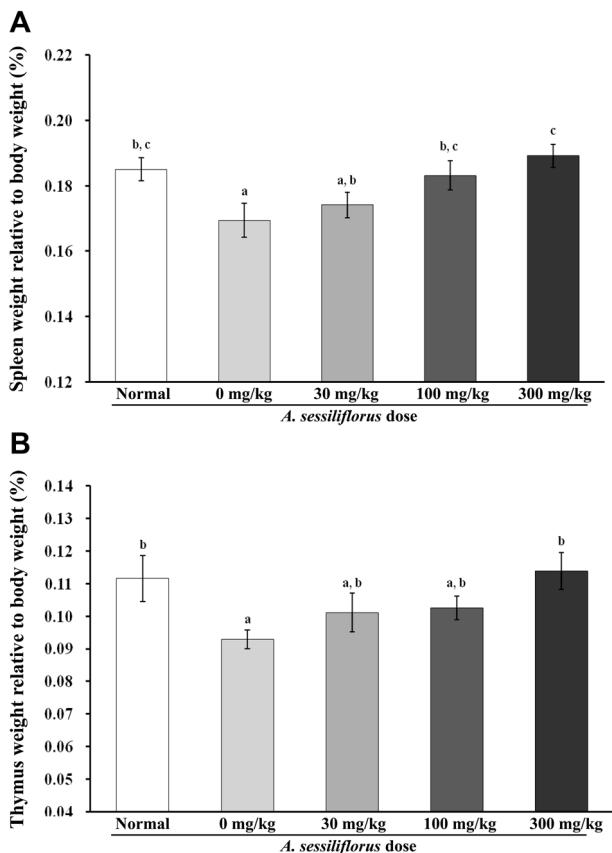


Fig. 4. Effect of *Acanthopanax sessiliflorus* on organ weights relative to body weight. When *A. sessiliflorus* was administered, the spleen weight relative to the body weight recovered in a dose-dependent manner (up to $0.19 \pm 0.00\%$) in 300 mg/kg/day *A. sessiliflorus* treatment group compared with $0.19 \pm 0.00\%$ in the group not subjected to the swimming test (A). The thymus weight relative to the body weight showed changes similar to those of the spleen. In the group not subjected to the swimming test, the change was $0.11 \pm 0.001\%$; in the 300 mg/kg/day *A. sessiliflorus* treatment group, the change was $0.11 \pm 0.01\%$. After the final *A. sessiliflorus* administration, all animals were weighed, their appearances were judge, they were anesthetized with diethyl ether, whole blood was collected through abdominal vena cava, and the animals were then sacrificed using diethyl ether. The weights of the spleen and thymus were also measured. a, b, c Values in the same row with different superscripts are significantly different, $P < 0.05$. Data are shown as the mean \pm SE ($n=7$).

rus tends to up-regulate it. The concentration of IL-2 was not changed by *A. sessiliflorus* treatment.

Discussion

In this study, we investigated the relation of *A. sessiliflorus* and immune modulation. *A. sessiliflorus* stimulated dose-dependently splenocyte proliferation, and in

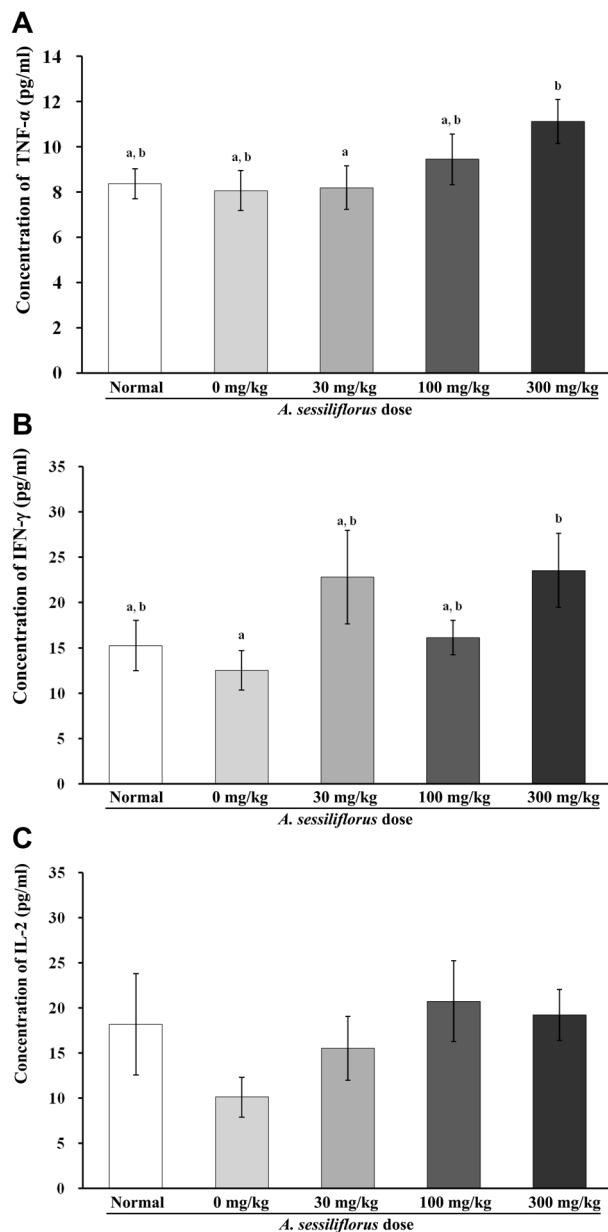


Fig. 5. Effect of *Acanthopanax sessiliflorus* on TNF- α , IFN- γ , and IL-2 in the serum of rats. The expression of TNF- α increased in a dose-dependent manner (A). *A. sessiliflorus* increased relatively the concentration of IFN- γ (B). The concentration of IL-2 was unchanged by *A. sessiliflorus* treatment. After the final *A. sessiliflorus* administration, all animals were weighed, their appearances were judged, they were anesthetized with diethyl ether, whole blood was collected through the vena cava, and the animals were then sacrificed with diethyl ether. TNF- α , IFN- γ , and IL-2 were analyzed with serum using a Microplate Reader. a, b Values in the same row with different superscripts are significantly different, $P < 0.05$. Data are shown as the mean \pm SE ($n=7$).

the 250 µg/ml *A. sessiliflorus* treatment group the proliferation rate of splenocytes reached to 213%. *A. sessiliflorus* increased not only the splenocyte number but also immune-related cytokines such as TNF-α. However, it could not upregulate the expressions of IFN-γ and IL-2. In order to evaluate the physiological change in rats, a modified Porsolt swim test was conducted. The results showed that *A. sessiliflorus* increased the swimming time. This means that *A. sessiliflorus* might enhance resistance against stress. Comparison of the organ weights relative to body weights with regard to immune-related organs such as the spleen and thymus showed that forced swimming, which is stressful situation decreased the body weights. *A. sessiliflorus* could recover the relative immune-related organ weights in a dose-dependent manner, and the 300 mg/kg/day *A. sessiliflorus* treatment in particular recovered the weights of the spleen and thymus to levels similar to those in the group not subjected to the swimming test. The results for the immunity-related cytokines in the animal study were similar to those in the cell study. *A. sessiliflorus* increased the expression of TNF-α and slightly increased the concentration of IFN-γ but not IL-2.

Immunosuppression is a very critical symptom in bio-organisms as it means they cannot defend themselves from various infections. The older they get, and the weaker their immune system become (immune cells shrink and white blood cells decrease) [3, 18]. It causes a variety of immunosuppressed situations such as Ataxia-telangiectasia [4], hypogammaglobulinemia [6], combined immunodeficiency disease [9], and DiGeorge syndrome [2]. Cytokines are produced by a variety of immune cells such as B cells, T cells, macrophages, and monocytes and have a key role in immune modulation in bio-organisms such as in regulating cell survival, cell death, inflammation, host defense against bacterial infection, lymphocytes' differentiation, and immune responses [1, 5, 16].

TNF-α is produced by many immune cells such as T cells, B cells, NK cells, and macrophage and modulates not only cell survivals but also cell death (apoptosis), and it is referred to as a “double-edged sword” [1]. TNF-α regulates the inflammation and host defense in response to bacterial infection [16], and acute stress (swim stress etc.) suppresses TNF-α [8]. IFN-γ improves chronic granulomatous disease and malignant osteopetrosis [23]; IL-2 regulates lymphocyte differentiation, immune responses, and homeostasis and especially controls regula-

tory T (T_{Reg}) cells and differentiating CD4 T cells [10]; and IFN-γ and IL-2 are decreased by acute swim stress [14].

Recently, research to find immune modulating materials in natural products is being actively carried out [19–23]. This research has produced many immune modulating candidates including those with immunostimulating properties such as *Stephania delavayi* Diels., *Zanthoxylum schinifolium*, *Alpinia galanga*, and *Panax ginseng*, although the method of activation differs between the materials. As chemical synthetic drugs have many adverse effects, vigorous studies are being conducted. However, as natural products consist of thousands of elements at least, it is hard to illuminate the specific mechanism of immune modulation. For this reason, the immune modulation pathways of each element in natural products need to be determined.

From the results in this study, we concluded that as *A. sessiliflorus* has not only a host defense effect but also a stress-ameliorating property, further study is needed to determine if it will be a promising immunostimulating candidate material.

Acknowledgement

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