RESEARCH ARTICLE

Stimulatory Effects of *Acanthus ebracteatus* Vahl. Aqueous Root Extract on Innate Immune Response in Mice

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Abstract

Acanthus ebracteatus Vahl. (family Acanthaceae), a mangrove plant, is used in Thai traditional medicine for treatment of various ailments such as skin diseases and cancers, and for health promotion and longevity. Previous reports have shown that aqueous extract of *A. ebracteatus* (AE) roots exhibits an immunostimulatory effect on macrophage cells *in vitro*. However, *in vivo* effects of its extract on immune response are still largely unknown. In this study, the stimulatory effect of AE extract on macrophage function was further assessed in cyclophosphamide (CTX)-induced immunosuppressive BALB/c mice. The mechanism involving alterations of immune responses *in vitro* and *in vivo* by AE extract was also evaluated. The results demonstrated that 14-day pretreatment with AE extract (0.06, 0.6 and 3.0 g/kg body weight/day) markedly enhanced phagocytic activity of peritoneal macrophages and affected hematological profiles especially white blood cells and platelet counts as well as the serum levels of some cytokines in CTX-induced immunosuppressive mice, compared to healthy mice. Correspondingly, *in vitro* treatment of J774A.1 murine macrophage cells by non-toxic doses of AE extract (32.5-500 µg/mL) significantly increased phagocytic activity as well as mRNA cytokine expressions of interleukin (IL)-1β and tumor necrosis factor (TNF)-α. In conclusion, the aqueous extract of AE roots exhibited a stimulatory effect on innate immunity through macrophage activations *in vitro* and *in vivo*. These also imply its therapeutic uses in regulating immune dysfunction diseases.

Keywords: *Acanthus ebracteatus* extract, innate immunity, murine macrophages, cyclophosphamide-induced immunosuppression

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ผลของสารสกัดน้ำจากรากเหงือกปลาหมอต่อการกระตุ้นภูมิคุ้มกันแบบไม่จำเพาะเจาะจงในหนูไมซ์

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บทคัดย่อ

เหงือกปลาหมอ (*Acanthus ebracteatus* Vahl.) วงศ์ Acanthaceae เป็นพืชสมุนไพรป่าชายเลน ในการแพทย์แผนไทยใช้เป็นยารักษาโรคต่าง ๆ เช่น โรคผิวหนัง โรคมะเร็ง บำรุงร่างกายและใช้เป็นยาอายุวัฒนะ ผลงานวิจัยที่ผ่านมาพบว่า สารสกัดน้ำจากรากเหงือกปลาหมอมีฤทธิ์กระตุ้นการทำงานของเซลล์แมคโครเฟจในหลอดทดลอง อย่างไรก็ตามยังมีรายงานผลของสารสกัดดังกล่าวต่อการตอบสนองทางภูมิคุ้มกันในสัตว์ทดลองน้อยมาก งานวิจัยนี้จึงมุ่งศึกษาผลของสารสกัดน้ำจากรากเหงือกปลาหมอต่อการกระตุ้นการทำงานของเซลล์แมคโครเฟจในหนูไมซ์สายพันธุ์ BALB/c ที่ถูกกดภูมิคุ้มกันด้วยยาไซโคลฟอสฟาไมด์ รวมถึงกลไกของสารสกัดดังกล่าวต่อการเปลี่ยนแปลงการตอบสนองทางภูมิคุ้มกันทั้งในหลอดทดลองและสัตว์ทดลอง ผลการศึกษาพบว่า การให้สารสกัดน้ำจากรากเหงือกปลาหมอ (ความเข้มข้น 0.06, 0.6 และ 3.0 กรัม/กิโลกรัมน้ำหนักตัว/วัน) เป็นเวลา 14 วัน ก่อนกดภูมิคุ้มกันด้วยยาไซโคลฟอสฟาไมด์ เพิ่มประสิทธิภาพการกลืนกินของเซลล์แมคโครเฟจ และมีผลเปลี่ยนแปลงค่าทางโลหิตวิทยาโดยเฉพาะเซลล์เม็ดเลือดขาวและเกล็ดเลือด รวมทั้งระดับไซโตไคน์บางชนิดในซีรั่มของหนูไมซ์ที่ถูกกดภูมิคุ้มกันด้วยยาไซโคลฟอสฟาไมด์ได้อย่างชัดเจน เมื่อเปรียบเทียบกับหนูกลุ่มปกติ ซึ่งสอดคล้องกับผลการศึกษาในหลอดทดลองที่พบว่า สารสกัดดังกล่าวในระดับความเข้มข้นที่ไม่เป็นพิษ (32.5-500 ไมโครกรัมต่อมิลลิลิตร) ต่อเซลล์แมคโครเฟจ J774A.1 สามารถเพิ่มประสิทธิภาพการกลืนกินของเซลล์ที่ใช้ทดสอบ และเพิ่มการแสดงออกของ mRNA ของไซโตไคน์ชนิด IL-1β และ TNF-α ได้อย่างมีนัยสำคัญ โดยสรุป สารสกัดน้ำจากรากเหงือกปลาหมอมีผลกระตุ้นระบบภูมิคุ้มกันแบบไม่จำเพาะเจาะจง โดยกระตุ้นการทำงานของเซลล์แมคโครเฟจทั้งในหลอดทดลองและสัตว์ทดลอง ซึ่งอาจจะนำไปสู่การพัฒนาต่อยอดเพื่อใช้รักษาโรคที่เกี่ยวข้องกับความผิดปกติของระบบภูมิคุ้มกันได้

คำสำคัญ: สารสกัดเหงือกปลาหมอ, ภูมิคุ้มกันแบบไม่จำเพาะเจาะจง, เซลล์แมคโครเฟจของหนู, การกดภูมิคุ้มกันโดยใช้ยาไซโคลฟอสฟาไมด์

Introduction

Suppression of immune responses and bone marrow activity are the major drawback of conventional chemotherapy drugs which limits their use in cancer patients. Therefore, immunostimulatory agents are needed in addition to chemotherapy drugs in overcoming cancers.[1] Immunostimulatory agent is an agent that enhances the immune system. Medicinal plants are a rich source of substances known to possess immunostimulatory properties, which generally act by stimulating both specific and non-specific immunities. Some of these can induce both humoral and cell-mediated immunities. Therefore, herbal-based immunostimulatory agents may be used as an add-on therapy to enhance the immunity of cancer patients undergoing chemotherapy.[2]

Acanthus ebracteatus Vahl. (*A. ebracteatus*), a selected mangrove plant belonging to family Acanthaceae, is widely distributed throughout Southeast Asia. In Thai traditional medicines, both *A. ebracteatus* Vahl. and *A. ilicifolius* Linn. are used in a similar way. They have been used for the treatment of asthma, diabetes, hepatitis, inflammation, skin diseases and various types of cancers as well as promoting health and longevity.[3-11] The acanthus plants contain many bioactive compounds such as alkaloids, flavonoids, phenolic compounds, lignans, steroids and terpenoids.[12-18] Potential pharmacological compounds are accumulated in various parts of these plants. The leaf, root, stem and bark of acanthus plants have been used for preventing tumor growth and cancer progression.[3-9] Moreover, Masathien and co-workers[10] reported that the extract of *A. ebracteatus* roots exhibited immunopotentiation *in vitro*. Yahuafai et al.[11] reported that the aqueous extract from *A. ebracteatus* roots at concentrations ranging from 32.35 to 500 µg/mL significantly stimulated nitric oxide (NO) production, as well as enhanced the mRNA expression of inducible nitric oxide synthase (iNOS) in a dose-dependent manner in activated J774A.1 macrophage-like cells. Thus, the extract may activate macrophage function. Although there have been many reports about biological activities of Acanthus family members, the *in vivo* immuno-modulatory activity of the aqueous extract of *A. ebracteatus* roots (AE) has not yet been explored. Cyclophosphamide (CTX) is an anticancer drug and also used to suppress the immune system in patients with autoimmune and immune-related diseases.[19] CTX-induced immunosuppressive mouse model has been characterized and used to study the immunomodulatory effects of test compounds *in vivo*.[20-21]

In the present study, immunomodulatory potential of AE extract was investigated by evaluating its effects on peritoneal macrophage phagocytic activity, hematological parameters and spleen index, and alterations of pro-inflammatory cytokines in sera of CTX-induced immunosuppressive mice. Moreover, *in vitro* phagocytic activity and changes in mRNA expression of cytokines induced by AE extract in J774A.1 murine macrophage cells were also explored.

Materials and Methods

Chemicals and reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent, nitroblue tetrazolium (NBT), trypan blue, dimethylsulfoxide (DMSO), CTX and

lipopolysaccharide (LPS) from *E. coli* 055 were purchased from Sigma-Aldrich (St. Loius, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute Medium (RPMI-1640), fetal bovine serum (FBS) and penicillin-streptomycin solution were obtained from Gibco-BRL (Grand Island, NY, USA). Improme II^{TM} reverse transcription system and Oligo $(dT)_{15}$ primer were purchased from Life Technologies (Grand Island, NY, USA) and TRIzol reagent was from Invitrogen (Paisley, UK). The multiplex bead mouse cytokine assay kit was obtained from Merck (Darmstadt, Germany). Other chemicals and reagents used in the study were analytical grade.

Plant material and aqueous root extract preparation

Fresh roots of *A. ebracteatus* Vahl. were collected from Samut Songkhram province, Thailand. This plant was identified by comparison with authentic specimens at the Forest Herbarium (BKF), Department of National Parks, Wildlife and Plant Conservation, Bangkok, Thailand. The voucher specimen has been preserved in the Herbarium of Natural Products and Integrative Medicine Research Section, Research Division, National Cancer Institute, Bangkok, Thailand as standard reference. AE extract was prepared as previously described.[11] In brief, the dried coarsely powder of the roots (600 g) was made into a decoction in distilled water (8 L) for 2-3 h and being extracted twice. The extract was filtered and concentrated under reduced pressure using a rotary evaporator, then lyophilized to dryness. Dark brownish powder of AE extract (140 g) was obtained and kept at -20°C for further use. The solutions of AE extract were freshly prepared in distilled water at the tested concentrations and sonicated prior to use for *in vitro* and *in vivo* experiments.

Cell cultures

Murine macrophage cells (J774A.1) were gifted from Associate Professor Dr. Wacharee Limpanasithikul, Department of Pharmacology, Chulalongkorn University. The cells were grown in DMEM supplemented with 10% heated-inactivated FBS and 1% penicillin-streptomycin at 37°C with 5% CO_2 in a humidified atmosphere for 3-4 days to reach the logarithmic phase for using in all experiments.

In vitro phagocytic activity assay

The *in vitro* non-specific phagocytic activity was measured using NBT dye reduction assay according to the slightly modified method by Rainard.[22] Briefly, J774A.1 murine macrophage cells (4×10^5 cells/mL) cultured in 96-well plates were treated with AE extract solutions (31.25, 62.5, 125, 250, 500 µg/mL) or LPS (100 ng/mL). After incubation at 37°C for 24 h, the cells were washed twice with DMEM and then incubated with zymosan (800 µg/mL) and NBT (600 µg/mL) in phosphate buffer saline (PBS) for 1 h. The supernatant was carefully removed and the adherent macrophages were rinsed with methanol, air dried and then 2M potassium hydroxide (KOH, 120 µL) and DMSO (140 µL) were added to each well. The phagocytic ability of macrophages was measured at 570 nm using a microplate reader (model 550, BIO-RAD, Hercules, CA, USA). The percentage of NBT reduction (reflecting phagocytic activity) was calculated from the following equation:

$$\text{Phagocytic activity (\%)} = \frac{OD_{570}\,(\text{sample}) - OD_{570}\,(\text{control})}{OD_{570}\,(\text{control})} \times 100$$

Determination of IL-1β and TNF-α mRNA expressions in J774A.1 cells

The *in vitro* effect of AE extract on cytokine expressions of J774A.1 macrophage cells was performed using real-time polymerase chain reaction (RT-PCR) technique as previously described.[11] Briefly, J774A.1 cells (4×10^5 cells/mL) were treated with AE extracts (31.25, 62.5, 125, 250 and 500 µg/mL) and incubated at 37˚C for 6 h. The LPS (100 ng/mL) was used as a positive control. After incubation, cells were harvested and total RNA were isolated using TRIzol reagent. The RNA samples were then reverse transcribed to cDNA using Improme II^{TM} reverse transcription system with oligo $(dT)_{15}$ primer, following the manufacturer's instructions. PCR was performed using the cDNA sample (1 µL) and PCR reaction mixture (24 µL) containing target gene-specific primers (IL-1β and TNF-α) as shown in Table 1. β-Actin was used as an internal standard. Amplification was done for 30 cycles with initial denaturation at 94˚C, 55˚C, 72˚C and final extension at 72˚C for 7 min. The PCR products were run on 1.5% (w/v) agarose electrophoresis, stained with ethidium bromide and visualized under UV light. The density of the PCR bands was measured by gel documentation system (Bio-Rad, Hercules, CA, USA) and expressed as a ratio of the band density divided by that of the house-keeping gene (β-actin).

Table 1. Oligonucleotide sequences of primers used in this study.

Genes	Primer Sequences
IL-1β	F 5'- TGCTTGAGAGGTGCTGATGT-3'
	R 5'-TGCTTGAGAGGTGCTGATGT-3'
TNF-α	F 5'-CTCCCAGGTTCTCTTCAA GG-3'
	R 5'TGG AAGACTCCTCCCAGG TA-3'
β-actin	F 5'-GTGGGCCGCCCTAGGCACCAG-3'
	R 5'-GGAGGAAGAGGATGCGGCAGT-3'

Animals

Sixty-three male BALB/c mice (5 weeks old, weighing 20-25 g) were purchased from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom province, Thailand. All mice were acclimatized for 1 week before experimentation and were housed in polypropylene cages under standard laboratory conditions of the Animal Laboratory of National Cancer Institute, Bangkok, Thailand (22±2˚C, 40-60% humidity and a 12 h light/12 h dark cycle). They were fed with the standard pellet diet and filter water *ad libitum*. All animal experiments were performed in accordance to the Guidelines in the Care and Use of Animals and Experiment Protocol and were approved by the Institutional Animal Ethics Committee of the National Cancer Institute, Thailand.

Preparation of sheep red blood cells

Fresh sheep red blood cells (SRBC) purchased from the Department of Animal Husbandry, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand were mixed with sterile Alsever's solution (1:1) and stored at 4°C. The erythrocytes were centrifuged at 3,000 rpm for 5 min and the supernatant was discarded before use in the experiment. The SRBC pellets were washed three times with 10 mL of PBS and the viability of SRBC was determined by the trypan blue dye exclusion technique. Subsequently, the SRBC pellets were suspended in PBS (2.5×10^9 cells/mL) for *in vivo* treatment.[23]

In vivo treatment protocol

The effects of 14-day pretreatment with AE extract on innate immune response in mice with immunosuppression induced by CTX, a standard immunosuppressant drug, were evaluated in comparison with normal mice. Before the experiment, sixty-three BALB/c male mice were immunized by intraperitoneally injecting 0.2 mL of SRBC suspension (approximately 5×10^8 cells/animal) three times (days 0, 9 and 14). After immunization on day 0, all mice were randomly divided into two groups; normal mice group (n=28) and CTX-induced immunosuppressed mice group (n=35).

Normal mice group Twenty-eight immunized mice were randomly divided into 4 groups (n=7). Each group received different concentrations of AE extracts (0.06, 0.6 and 3.0 g/kg body weight) or deionized (DI) water (control) orally for 14 consecutive days.

CTX-induced immunosuppressed mice group Thirty-five immunized mice were randomly divided into 5 groups (n=7). Group I and II served as the normal control group (DI water) and CTX-induced immunosuppressed mice control group (DI water+CTX), respectively. The other three groups were received AE extracts (0.06, 0.6 and 3.0 g/kg body weight) orally for 14 consecutive days, followed by a daily intraperitoneal injection of CTX (40 mg/kg body weight) for 3 days (days 15, 16 and 17) in order to induce immunosuppression.

Body weight of animals were recorded daily for 2 weeks. All mice were then sacrificed and the peritoneal macrophage fluid was collected. The blood samples were collected by cardiac puncture for hematological and immunological assays. The spleen was also removed from each mouse and weighed.

Preparation of peritoneal macrophage cells

Peritoneal macrophages were collected as previously described.[24-25] Briefly, 10 mL of ice-cold sterile PBS was infused into the peritoneal cavity. After gentle massaging, the peritoneal exudates were collected by peritoneal lavage with RPMI-1640 medium. This step was repeated three times for each mouse. The exudates were pooled, centrifuged at 3,000 rpm for 10 min at 4°C and the erythrocytes were lysed by hypotonic lysis. The cell pellets were washed three times with PBS and resuspended in RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin. The viability of peritoneal macrophage cells was determined using a hemocytometer and trypan blue exclusion technique.

Ex vivo phagocytic activity assay

Phagocytic activity was assessed using NBT dye reduction assay previously described with a slight modification.[25-27] Briefly, peritoneal macrophages from each mice group were seeded in 96-well culture plates (1×10^5 cells/well) at 37°C with 5% CO_2 in a humidified atmosphere. After 24 h incubation, the cell culture media were removed. A mixture solution of zymosan A and NBT (8:6) was added to each well and then incubated at 37°C for 4 h. Subsequently, the solution was removed and the macrophages were washed three times with PBS and rinsed with methanol. A mixture solution of 2M KOH and DMSO (6:7) were added to each well. The absorbance of the solutions was determined at 570 nm by a microplate reader (Infinite M200 PRO, TECAN, Grödig, Austria). The phagocytic index (PI) was calculated by the following equation: PI = (OD_{570} (sample)/OD_{570} (control)) $\times100$

Hematological analysis

After treatment with AE extract and CTX, blood samples were drawn by cardiac puncture from the treated mice and transferred into the tubes containing ethylenediaminetetraacetic acid (EDTA) for hematological analysis. Hematological profiles including total white blood cell count (WBC), total red blood cell count (RBC), platelet count (PLT), hemoglobin concentration (Hb) and hematocrit level (Hct) were determined, in comparison between the normal and CTX-induced immunosuppressed mice groups, using an automated hematology analyzer (LH 500, Beckman Coulter, Fullerton, CA, USA).

Determination of pro-inflammatory cytokines production

The serum cytokine levels (granulocyte colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), TNF-α, interferon (IFN)-γ, IL-1α, IL-1β, IL-6 and IL-12 (p40)) of both normal and CTX-induced immunosuppressed mice groups were quantitatively determined using multiplex immunoassay kit (Merck, Darmstadt, Germany). Briefly, antibody-coated magnetic beads were captured by adding 25 µL of beads to each well of 96-well plates. A total of 25 µL of serum samples were mixed with the captured antibody-coated magnetic beads. Reconstituted Quality Control 1 and Quality Control 2 (25 µL) were also added into the wells. Subsequently, 25 µL of assay buffer was added to each well and incubated on a magnetic shaker with 500 rpm speed at 4°C overnight in the dark. Afterwards, the beads were washed thrice with washing buffer. Then, 25 µL of detection antibody was mixed into each well, and the samples were further incubated for 60 min in the dark at room temperature. The washing steps were repeated after incubation as mentioned previously. Later, 25 µL of streptavidin-phycoerythrin solution was added into each well and incubated on a plate shaker at room temperature for 30 min. After incubation, the suspension was removed and the plate was washed twice. Lastly, 150 µL of sheath fluid was added to each well. The cytokine levels were measured using Magpix System (Luminex, Austin, USA) and the data were analyzed by xPONENT 3.1 software (Luminex, Austin, USA). Cytokine concentrations in the sera were determined from the appropriate calibration curve.

Determination of spleen index

All mice were sacrificed at the end of experiments on day 15 (for normal mice) and day 18 (for CTX-induced immunosuppressed mice). The spleens were removed and weighed. The spleen index of each animal was calculated as the ratio between spleen weight (mg) and body weight (g).

Statistical analysis

The experiments were performed in triplicate (*in vitro*) or in duplicate (*in vivo*). Data were expressed as the mean±standard deviation (SD) or standard error of mean (SEM). Statistical significance was analyzed using analysis of variance (ANOVA) for multiple comparisons. *P*-value less than 0.05 were considered as significant.

Results

In vitro effect of AE extract on the phagocytic activity in J774A.1 cells

To investigate the *in vitro* effect of AE extract on innate immune response in J774A.1 murine macrophage cells, the phagocytic activity of AE extract-treated cells was assessed using the NBT dye reduction assay. The LPS (100 ng/mL) was used as a positive control. As shown in Figure 1, AE extract at the non-toxic concentrations (32.25, 62.5, 125, 250 and 500 µg/mL) significantly enhanced phagocytic activity of J774A.1 cells compared with the vehicle control. The effect was in a concentration-dependent manner with maximum effect of 250 µg/mL.

In vitro effect of AE extract on IL-1β and TNF-α mRNA expressions in J774A.1 cells

A significant increase in mRNA expression of IL-1β was observed in J774A.1 murine macrophage cells treated with AE extract at all tested concentrations (31.25-500 µg/mL). TNF-α mRNA expression was also markedly increased, especially at the concentrations of 250 and 500 µg/mL, which was quite similar to the response induced by LPS (100 ng/mL) (Figure 2).

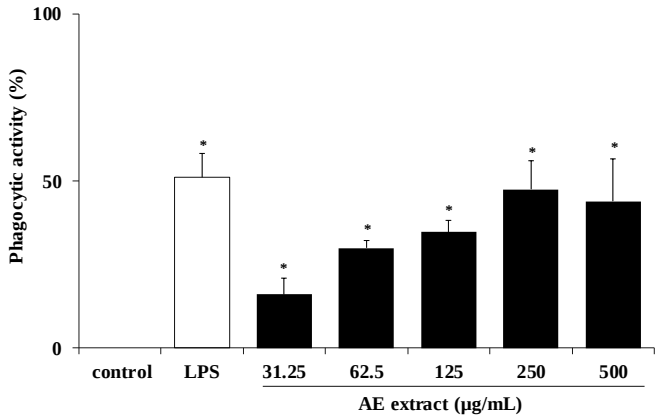
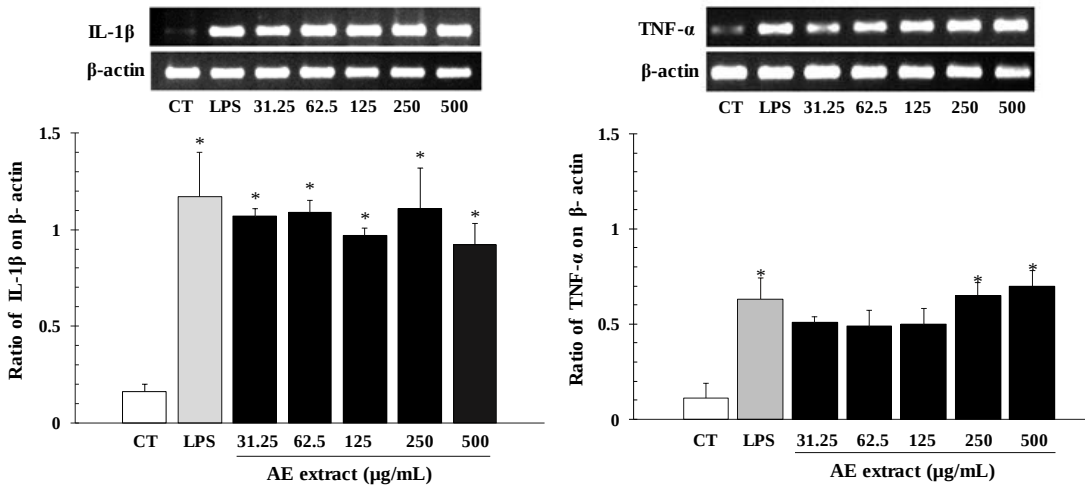


Figure 1. Effect of AE extract (31.25-500 µg/mL) on the phagocytic activity in J774A.1 cells**.** Data are presented as mean±SEM of three independent experiments (n=3). *Significant difference compared with the vehicle control (p<0.05).



(A) (B)

Figure 2. Effect of AE extract (31.25-500 µg/mL) on the mRNA expressions of (A) IL-1β and (B) TNF-α in murine macrophage J774A.1 cells. Data are presented as mean±SEM of two independent experiments (n=2). *Significant difference compared with the vehicle control (p<0.05).

In vivo effects of AE extract in normal and CTX-induced immunosuppressed BALB/c mice

Body weight In normal mice, the increases in body weight were observed in all dose groups (0.06, 0.6 and 3.0 g/kg body weight) receiving oral 14-day treatment of AE extract after SRBC challenge. However, there was no significant difference in body weight gain between the AE extract treated group and the vehicle control group (Figure 3A). The CTX-induced immunosuppressed mice also gained body weight in all dose groups but the reduction of body weight was observed during CTX treatment (days 15-17). The pattern of body weight change in animals receiving AE extract was not significantly different from that of CTX-induced immunosuppressed control mice (Figure 3B).

Phagocytic activity of mouse peritoneal macrophage cells Phagocytosis by macrophages is one of the most important non-specific immune responses in the human body.[28] The effects of AE extract on phagocytic activity of peritoneal macrophage cells from normal and CTX-induced immunosuppressed mice were determined using NBT dye reduction assay. The results showed that AE extract slightly increased phagocytic activity of peritoneal macrophage cells at all dose concentrations in non-immunosuppressed mice but not significant when compared to the vehicle control group (Figure 4A). Increases in phagocytic activity of macrophages was also observed in CTX-induced immunosuppressed mice treated with 0.06 and 0.6 mg/kg AE extract, although a reduction in phagocytic rate was detected in the highest dose group (3.0 g/kg) (Figure 4B). Nonetheless, no significant difference in phagocytic activity of macrophages was observed in any dose groups of normal and immunosuppressed mice treated with AE extract.

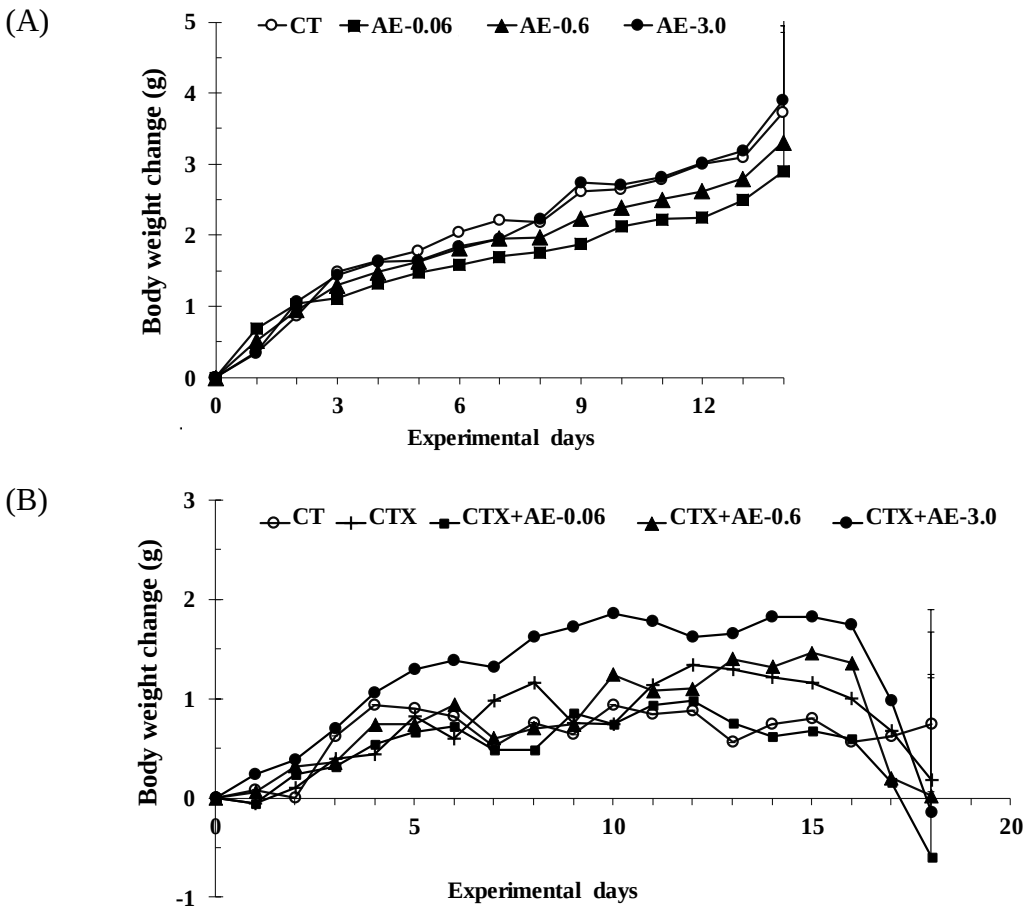


Figure 3. Effect of AE extract (0.06, 0.6 and 3.0 g/kg) on body weight in (A) normal mice and (B) CTX-induced immunosuppressed mice. Each value is presented as mean±SEM (n=2).

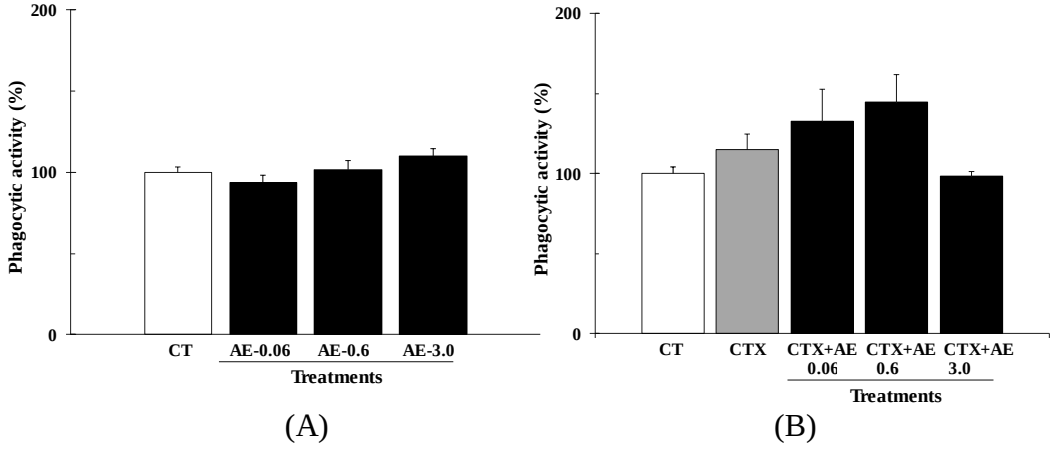


Figure 4. Effect of AE extract (0.06, 0.6 and 3.0 g/kg) on phagocytic activity of peritoneal macrophages in (A) normal mice and (B) CTX-induced immunosuppressed mice. Each value is presented as mean±SEM (n=2).

Hematological profiles As shown in Table 2, with oral 14-day administration of AE extract (0.06, 0.6 and 3.0 g/kg body weight) in the SRBC-immunized normal mice, no significant effect on hematological parameters (total WBC, RBC, PLT and Hb) in response to SRBC challenge was observed when compared to the vehicle control normal mice.

On the contrary, intraperitoneal injection with CTX (40 mg/kg) for 3 consecutive days in the SRBC-immunized normal mice significantly reduced the total WBC and PLT counts, compared with those of the normal control mice (WBC (10^3/µL): 0.35±0.30 *vs.* 6.35±1.11; $p<0.001$ and PLT (10^3/µL): 235.0±80.23 *vs.* 440.0±70.71; $p<0.01$, respectively). Pretreatment of AE extract at doses of 0.06, 0.6 and 3.0 g/kg body weight for 14 days before CTX administration, under the same conditions, slightly increased the total WBC and PLT counts in a dose-dependent manner when compared to those of CTX-treated control mice (WBC (10^3/µL): 0.43±0.35, 0.53±0.27, 0.63±0.25 *vs.* 0.35±0.30 and PLT (10^3/µL): 259.0± 90.86, 274.0±72.66, 319.0±82.42 *vs.* 235.0±80.23). The results indicate that the reduction of hematological indices was markedly restored with oral pretreatment with AE extract in CTX-induced immunosuppressive mice.

Table 2. Effect of AE extract on hematological profiles in normal and CTX-induced immunosuppressed mice.

Groups		Hematological Parameters			
		WBC (10^3/µL)	**RBC (10^3/µL)**	**PLT (10^3/µL)**	**Hb (g/dL)**
Normal Mice	Normal control	9.20±1.66	10.71±0.63	604.63±112.76	17.09±0.79
	AE-0.06 g/kg	9.78±0.80	11.02±1.16	599.14±82.95	17.51±1.54
	AE-0.6 g/kg	9.05±1.19	10.50±0.74	644.25±49.72	16.69±1.80
	AE-3.0 g/kg	9.38±2.48	10.04±1.58	628.67±52.55	16.22±2.28
CTX-induced immunosuppressed mice	Normal control	6.35±1.11	11.28±0.65	440.0±70.71	16.60±1.32
	CTX- control	0.35±0.30**	11.23±0.83	235.0±80.23*	16.70±1.35
	CTX-AE-0.06 g/kg	0.43±0.35	11.23±0.90	259.0±90.86	16.50±1.17
	CTX-AE-0.6 g/kg	0.53±0.27	12.00±1.32	274.0±72.66	17.70±2.25
	CTX-AE-3.0 g/kg	0.63±0.25	11.88±0.47	319.0±82.42	17.90±0.65

Each value is presented as mean±SD (n=7). *$p<0.01$ and **$p<0.001$ compared to normal control.

Mouse spleen index The results demonstrated that there was no change in spleen index of normal mice receiving AE extract at all dose concentrations, compared to the vehicle control normal mice (Figure 5A). However, a slight increase in the spleen index was observed in CTX-induced immunosuppressed mice receiving 0.06 g/kg of AE extract but it was not significantly different when compared with the CTX-treated control mice (Figure 5B).

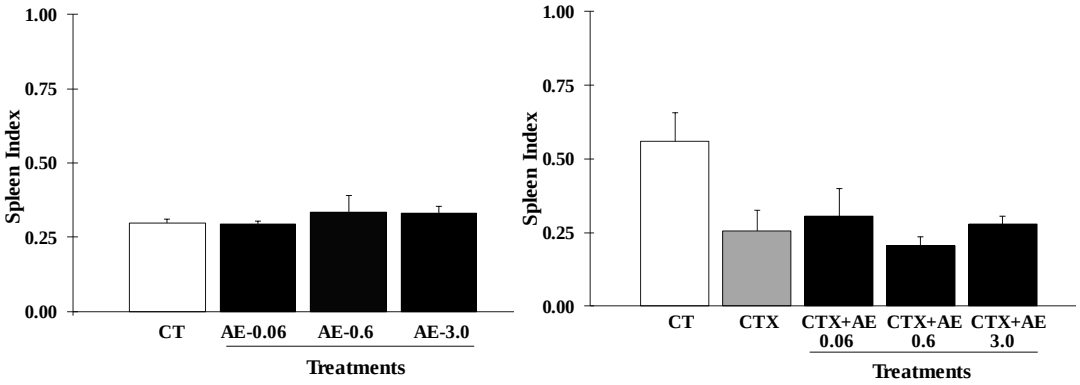


Figure 5. Effects of AE extract (0.06, 0.6 and 3.0 g/kg) on mouse spleen index in (A) normal mice and (B) CTX-induced immunosuppressed mice. Each value is presented as mean±SEM (n=2).

Pro-inflammatory cytokine productions Figure 6 showed that some serum levels of cytokines G-CSF, IL-6 and IL-12 (p40)) markedly increased in CTX-induced immunosuppressed mice receiving AE extract at doses of 0.06, 0.6 and 3.0 g/kg body weight for 14 consecutive days, compared with those of CTX-treated control mice. Only the alteration of G-CSF production in CTX-treated mice receiving 0.6 g/kg body weight of AE extract was statistically significant ($p<0.05$) However, no alteration in the serum level of other five pro-inflammatory cytokine (GM-CFS, TNF-α IFN-γ, IL-1α and IL-1β) was observed.

Discussion

Macrophages are important immune cells, which play a significant role in the host defense mechanism. Many stimuli are able to activate macrophages to generate both innate and adaptive immune responses. Once activated, they activate phagocytic activity, produce and release reactive oxygen species (ROS) and nitric oxide (NO) in response to stimulation with various agents and can inhibit the growth of various tumor cells and microorganisms.[29] Macrophages also secrete cytokines and chemokines, such as TNF-α, IL-1, IL-6β, IL-8, IL-12, IFN-γ and IFN-β2.[30] Therefore, stimulating macrophages is a major target for therapeutic application. In previous study, Yahuafai et al.[11] reported that treatment of the activated J774A.1 murine macrophage cells with an aqueous extract from *A. ebractetatus* roots (AE) at non-toxic doses of 32.25, 62.5, 125, 250 and 500 µg/mL enhanced NO production and mRNA expression of iNOS. Our study demonstrated that AE extract at similar test doses significantly increased phagocytic activity as well as the levels of mRNA cytokine expression of IL-1β and TNF-α in J774A.1 cells in a dose-dependent manner. This indicates for the first time that AE extract-mediated activation of macrophages may contribute to immunostimulatory activity and AE extract is a potential source of immunostimulatory agents.

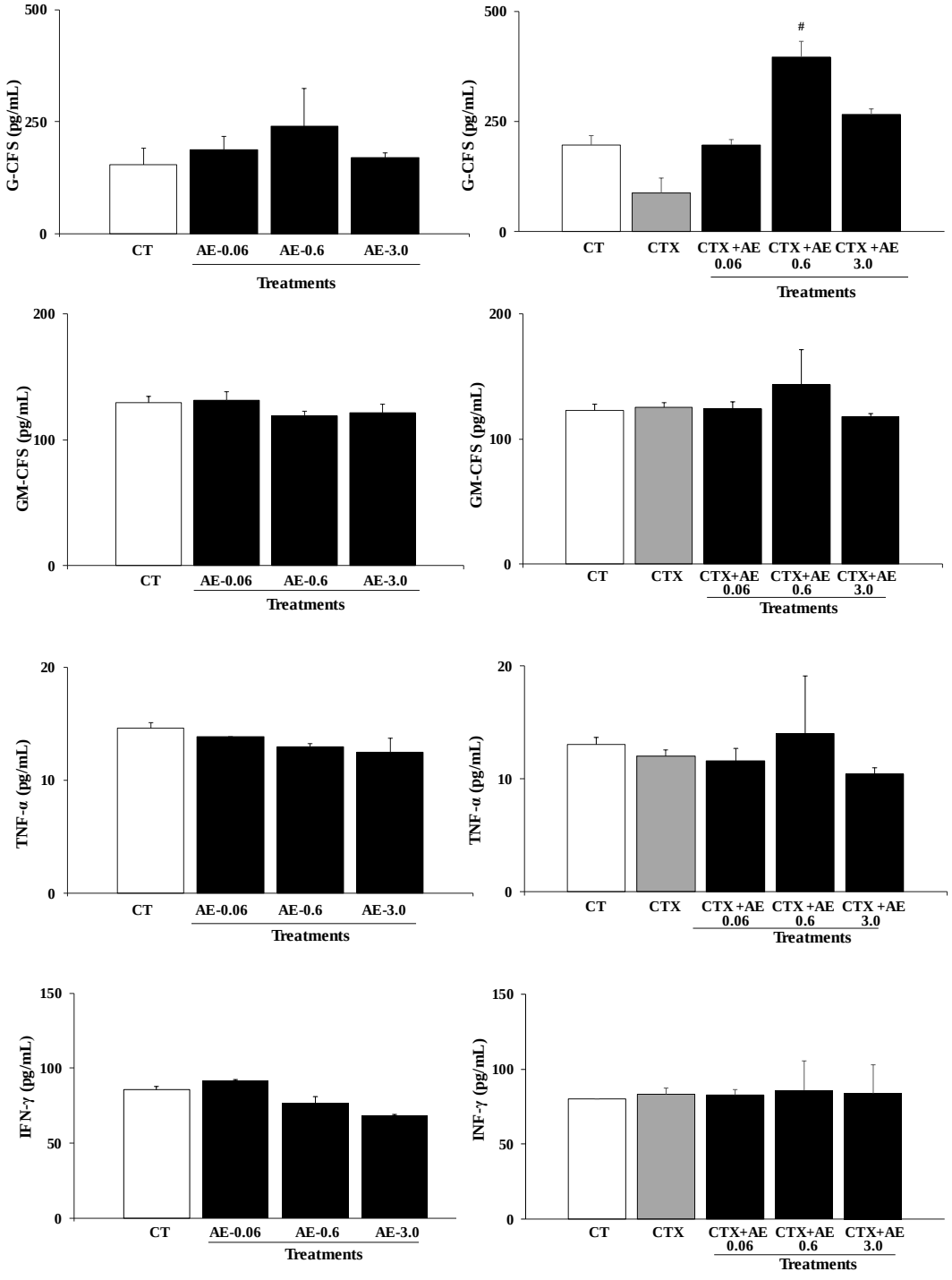


Figure 6. Effects of AE extract (0.06, 0.6 and 3.0 g/kg) on pro-inflammatory cytokine productions in (A) normal mice and (B) CTX-induced immunosuppressive mice. Each value is presented as mean±SEM (n=2). $^{\#}p<0.05$ compared to CTX control group.

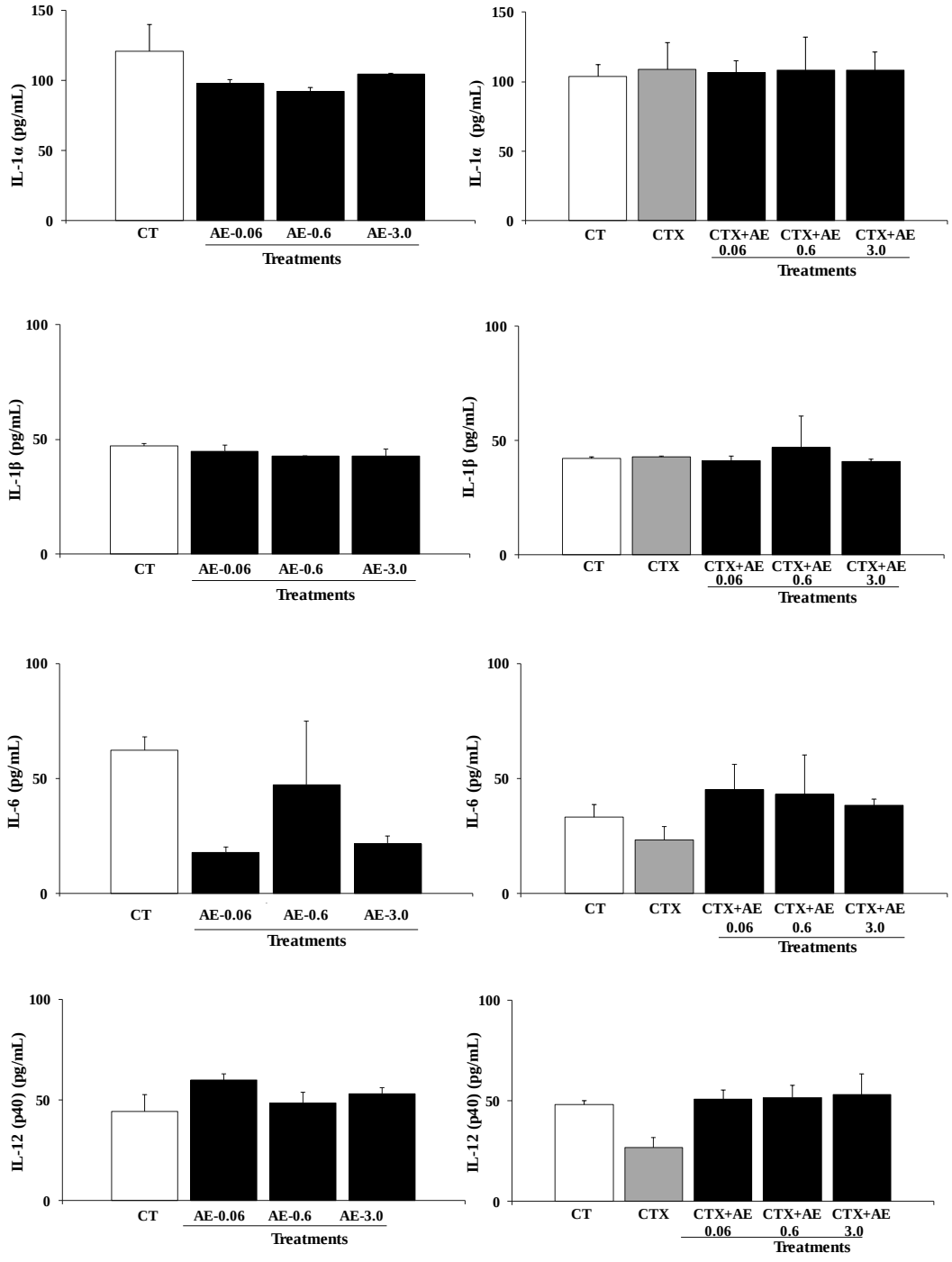


Figure 6. Continued.

Phagocytosis is one of the important functions of macrophage. We further confirmed the immunostimulatory effects of the AE extract *in vivo*. In correspondence with *in vitro* findings, administration of AE extract (0.06, 0.6 and 3.0 g/kg body weight) in CTX-induced immunosuppressed BALB/c mice for 14 consecutive days also enhanced phagocytic activity of mouse peritoneal macrophages.

Immune activation is capable of stimulating the secretion of various cytokines. Cytokines exert an effect on hematopoiesis and immune process which regulate both cellular and humoral immune response. In the present study, some serum levels of G-CSF, IL-6 and IL-12 increased in CTX-induced immunosuppressed mice, compared with those of healthy and CTX-treated control mice. However, only a serum level of G-CSF significantly increased ($p<0.05$). AE extract at the dose of 0.6 g/kg produced the maximum level of pro-inflammatory cytokines in sera of CTX-treated mice. This may occur through the activation of macrophages, resulting in secreting some cytokines such as G-CSF and IL-1.[31] In contrast, no alteration in serum levels of GM-GSF, TNF-α, IFN-γ, IL-1(α and β) was observed, which may be related to T-cell depletion induced by CTX. Moreover, a marked decrease in stimulating cytokines such as TNF-α, IL-1(α and β) and IFN-γ levels may be due to decline in circulating immune cells after CTX treatment.[32] Therefore, AE extract may improve cellular immunity by stimulating macrophage functions.

Cancer chemotherapy can cause a number of serious side effects, particularly myelosuppression and anaemia.[32-33] The anemia encountered in tumor bearing mice is mainly due to the reductions in RBC or Hb, which may result from iron deficiency and hemolytic or myelopathic conditions.[34] Our study showed that pretreatment with AE extract at doses of 0.06, 0.6 and 3.0 g/kg body weight can restore some hematological profiles such as WBC and PLT counts in CTX-induced immunosuppressive mice, compared with CTX-treated control mice. In contrast, AE extract did not produce any significant effect on hematopoietic challenge in healthy mice. This indicates that AE extract possesses a protective action on the hematopoietic system. Furthermore, no significant changes in body weights in healthy mice were seen, whereas CTX-induced immunosuppressive mice had a slight weight reduction after intraperitoneal administration with CTX (40 mg/kg) over three consecutive days. These observations are in agreement with the results from chronic toxicity study on similar doses of AE extract, which showed no effect on normal Wistar rats after 12 months exposure[35], and those of Manjunatha et al.[31] which exhibited the protective effect of *Terminalia bellirica* extract on CTX-induced neutropenia in mice, suggesting its therapeutic usefulness in immunocompromised conditions.

Phytochemical analysis studies of acanthus plants have revealed the presence of several chemical constituents in the extract including alkaloids, flavonoids, triterpenoids, sterols, aliphatic glycosides, lignan, phenolic glycosides, quanternary amino acid and polysaccahrides.[3-5] It is well known that crude water-soluble extract from *A. ebracteatus* Vahl. roots exhibited immunostimulatory,[10-11] antitumor[3-9] and anti-angiogenic[5] effects both *in vitro* and *in vivo* experimental models. However, the bioactive constituents of the extract have not yet been identified.[12-13] Chakraborty et al.[8] reported that the aqueous extract from *A. ilicifolius* leaves restored hematological and hepatic histological profiles and lengthened the survival of the animals against the proliferation of ascites tumor *in vivo*. *A. ilicifolius*

alkaloids A (4-hydroxyl-2(3H)-benzoxazolone, HBOA) and its derivatives showed potent hepatoprotective effect against carbon tetrachloride-induced liver damage in mice.[18] Okoli et al.[36] have found that the aqueous root extract of *A. montonus*, a medicinal plant used for treatment of furuncles, had effects on phagocytosis and specific cell-mediated immune response. Phytochemical constituents such as alkaloids and carbohydrate may be responsible for this activity. Hence, purification and structural evaluation of active compounds responsible for immunostimulatory activity in AE extract should be further investigated as well as the clarification of intracellular process related to macrophage activation.

Conclusion

Our findings can conclude that the aqueous extract of *A. ebracteatus* roots exhibits a stimulatory effect on innate immunity through macrophage activation *in vitro* and *in vivo*. These imply the therapeutic usefulness of *A. ebracteatus* in immune dysfunction diseases and as a Thai herbal remedy for promoting health and longevity.

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