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Suppressive Effects of Lotus Plumule (*Nelumbo nucifera* Geartn.) Supplementation on LPS-Induced Systemic Inflammation in a BALB/c Mouse Model

JIN-YUARN LIN*, ANN-RU WU, CHIEN-JUNG LIU AND YING-SHU LAI

Nutritional Immunology Laboratory, Department of Food Science and Biotechnology, College of Agriculture and Natural Resources National Chung Hsing University, 250 Kuokuang Road, Taichung 402, Taiwan, R.O.C.

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ABSTRACT

To determine the prophylactic effects of lotus plumule (*Nelumbo nucifera* Geartn.) supplementation *in vivo* on acute systemic inflammation, the mediators secreted in serum and by cultured peritoneal macrophages from the lipopolysaccharide (LPS)-challenged mice were measured. The female BALB/c mice were continuously supplemented with lotus plumule for 3 weeks and then administrated with an intra-peritoneal (i.p.) LPS injection at a concentration of 10 mg/kg body weight (BW) to induce acute systemic inflammation. After 24 hours of LPS injection, the mice were sacrificed to determine the inflammatory mediators. The results showed that high dose supplementation (20 mg/day/mouse) with lotus plumule significantly ($P < 0.05$) decreased the pro-inflammatory cytokine of tumor necrosis factor-α (TNF-α) level in the serum of LPS-challenged mice. Simultaneously, supplementation with lotus plumule significantly increased the levels of anti-inflammatory cytokine IL-10 produced by peritoneal macrophages from LPS-challenged mice. The results indicated that lotus plumule supplementation significantly inhibited the production of pro-inflammatory cytokine TNF-α and increased that of anti-inflammatory cytokine IL-10. It can be concluded that lotus plumule supplementation before systemic inflammation attenuates the acute inflammation status *in vivo*.

Key words: Lotus plumule, Lipopolysaccharide, Tumor necrosis factor-α (TNF-α), Interleukin-10 (IL-10), Peritoneal macrophages, Acute inflammation

INTRODUCTION

Lotus (*Nelumbo nucifera* Geartn.), a southeast Asian aquatic edible plant, is traditionally used as a Chinese medicine to treat different disorders, particularly inflammation[1]. Lotus seeds are commonly consumed by people in Taiwan. However, the plumule, a germ of lotus seed, is usually removed from lotus seeds due to its bitter taste. Lotus seed plumule is found to have antioxidant activities[2] and is recognized as a "cooling food"[3] with anti-inflammatory activities. However, the research of lotus plumule against inflammation *in vivo* is still limited. In this study, we attempted to determine the effects of lotus plumule supplementation *in vivo* on an acute systemic inflammation.

Sepsis is accompanied by severe systemic inflammation with a high mortality rate of 40~60 percent[4]. The severity of inflammation may serve as a predictor for survival and be detected by leukocyte (neutrophil and monocyte) activation in the circulation and the inflammatory mediator production during sepsis[5]. Thus, serum inflammatory mediators and leukocyte function, especially monocytes/macrophages play crucial indictors of systemic inflammation. TNF-α, IL-1, and IL-6 are recognized as pro-inflammatory cytokines[6-7]. Nitric oxide (NO) and prostaglandin E2 (PGE2) produced from lipopolysaccharide (LPS)-induced inflammatory cells, especially macrophages or chondrocytes, are served as pro-inflammatory indicators[8] and regarded as secondary inflammatory mediators[4] during inflammation. However, IL-10 plays an anti-inflammatory cytokine and exhibits its potent anti-inflammatory activities via inhibiting the synthesis of pro-inflammatory cytokines, the release of reactive oxygen and nitrogen intermediates, and the antigen-presenting capacity of mononuclear phagocytes or dendritic cells[9].

Ancient herbal medicines or medicinal foods might provide a modern hope for the treatment of sepsis or acute systemic inflammation. For example *Sedum kamtschaticum*, a folk medicine in Northeast Asia for treating inflammatory disorders, has been reported to inhibit cyclooxygenase-2 expression of LPS-treated RAW 264.7 and demonstrates a potent anti-inflammatory activity[10]. The aqueous extract of sword brake fern (*Pteris ensiformis* Burm), a component in most of the traditional herbal beverage formulas in Taiwan, attenuates inflammatory mediator (TNF-α, IL-1β, IL-6, NO, and PGE_2) synthesis of activated macrophages (RAW 264.7) partially through a NF-κB-dependent pathway[11]. Lotus plumule is also traditionally used as an anti-inflammatory agent in Chinese medicine formulas. In this study, we investigated the effects of lotus plumule on acute systemic inflammation *in vivo*. To evaluate the prophylactic effects of lotus plumule, female BALB/c mice were consecutively supplemented with lotus plumule powder through 3 weeks supplementation before systemic administration of LPS and

* Author for correspondence. Tel: + 886-4-22851857; Fax: +886-4-22851857; E-mail: jinlin@nchu.edu.tw.

following intra-peritoneal injection of endotoxin LPS. After LPS challenge for 24 hours, the cytokines and inflammatory mediators in serum and peritoneal macrophage cultures from the LPS-challenged mice were measured.

MATERIALS AND METHODS

I. *Plant Material and Sample Preparation*

Fresh lotus seeds from a variety of *Nelumbo nucifera* Geartn. were provided by a local farmer in Tainan, Taiwan. The lotus seeds were harvested in the late summer of 2003. The lotus plumule was carefully collected and air dried at 40°C oven for one night. Then the air-dried lotus plumule was milled into powder, sealed in a plastic bottle, and stored at -20°C until use.

II. *Experimental Animals and Dietary Groups*

Female BALB/cByJNarl mice (6 weeks old) were obtained from the National Laboratory Animal Center, National Applied Research Laboratories, National Science Council in Taipei, Taiwan and maintained in the Department of Food Science and Biotechnology at National Chung Hsing University, College of Agriculture and Natural Resources in Taichung, Taiwan. The mice were housed and kept on a chow diet (laboratory standard diet; BioLASCO Taiwan Co., Ltd.). The animal room was kept on a 12-h-light and 12-h-dark cycle. Constant temperature (25±2°C) and relative humidity (50-60%) were maintained. After the mice acclimatized for 2 weeks, the mice were divided into four groups (n = 6) including non-sensitized control (SC, 0 mg lotus plumule/day/mouse), dietary control (DC, 0 mg lotus plumule/day/mouse), low dose supplementation group (L, 5 mg lotus plumule/day/mouse), and high dose supplementation group (H, 20 mg lotus plumule/day/mouse). A series of preliminary experiments were conducted to determine the effective lotus plumule dosage. To test the prophylactic effects of lotus plumule on acute systemic inflammation, the lotus plumule was supplemented daily for 3 weeks with tube feeding. Aliquots of 0.5 mL double distilled water containing different concentrations of lotus plumule powder were respectively supplemented to each mouse for 3 consecutive weeks before LPS challenge. Mouse body weight was measured every 4 days during the study period. No significant difference in body weight was found among the four groups during the experimental period (data are not shown).

III. *Challenge with LPS by Intra-peritoneal (i.p.) Injection to Induce Acute Systemic Inflammation in a Female BALB/c Mouse Model*

To test the effects of lotus plumule supplementation on systemic inflammation, the mice (11 weeks old) which had been supplemented with lotus plumule powder for 3 consecutive weeks were challenged with *Escherichia coli* LPS (O127:B8, Sigma-Aldrich Co., L-3129) to establish a systemic inflammation murine model. Mice were given an intraperitoneal injection of LPS from *Escherichia coli* O127:B8 (Sigma-Aldrich Co., L-3129) at a concentration of 10 mg/kg body weight (BW). Each animal received this concentration of LPS in a volume of 100 μL of LPS dissolved in sterilized phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2 μm filtered) using aliquots from a single lot of LPS. The control animals (SC group) received the same volume of PBS. At 24 hours after injection of LPS, all experimental animals were sacrificed. The sera and peritoneal macrophages were collected for cytokine assay.

IV. *Animal Sample Collection*

(I) *Serum Collection*

At 24 hours after injection of LPS, the animals were weighed, anaesthetized with a diethyl ether and immediately exsanguinated by retro-orbital venous plexus puncture. The blood was respectively collected into a 1.5 mL vial, allowed to stand for 2 h at room temperature, and then centrifuged at 12,000 × g for 15 min at 4°C to separate the serum. The serum from each mouse was respectively collected and stored at -70°C for later analysis.

(II) *Collection of Peritoneal Macrophage Cell Cultures*

Anesthetized mice were exsanguinated before CO_2 euthanasia. Peritoneal cells were prepared by lavaging the peritoneal cavity with 2 aliquots of 5 mL sterile Hank,s balanced salts solution (HBSS) for a total of 10 mL through peritoneum. The peritoneal lavage fluid was centrifuged at 200 × g for 10 min at 4°C. The cell pellets were collected and re-suspended in TCM medium. The peritoneal adherent cells (mostly macrophages) from each animal were adjusted to 3×10^6 cells/mL in TCM medium with a hemocytometer using trypan blue dye exclusion method. Peritoneal adherent cells (0.50 mL/well) in the absence or presence of stimulus (0.50 mL/well, LPS (Sigma-Aldrich Co., L-2654) at a final concentration of 10 μg/mL) were plated in 24 well plates. The plates were incubated at 37°C in a humidified incubator with 5% CO_2 and 95% air for 48 hours. The plates were centrifuged at 200 × g for 10 min. The supernatants in cell cultures were collected and stored at -70°C for cytokine and inflammatory mediator assays.

V. *Analytical Methods*

(I) *Measurement of Cytokine Levels in Sera and Peritoneal Cell Cultures by an Enzyme-linked Immunosorbent Assay (ELISA)*

Cytokine (IL-2, IL-6, IL-10, or TNF-α) levels were determined by sandwich ELISA kits, respectively. The cytokine concentrations were assayed according to the

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Table 1. Effects of lotus plumule on serum mediator levels from female BALB/c mice after three weeks of consecutive tube feeding and treatment with lipopolysaccharide i.p. for 24 hours

	Serum mediators				
groups	IL-2 (pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)	NO (μM)	TNF-α (pg/mL)
non-sensitized control group (SC)	353 ± 108	—*	1863 ± 303	18.9 ± 4.8	—*
dietary control group (DC)	444 ± 112	563 ± 211	1321 ± 359	9.8 ± 1.3	14.5 ± 1.1
low dose group (5 mg /mouse/day)	376 ± 139	354 ± 67	743 ± 234	9.8 ± 1.6	9.1 ± 5.9
high dose group (20 mg/mouse/day)	193 ± 74	394 ± 97	640 ± 199	14.8 ± 2.7	—*

Each value represents a mean ± S.E. (n = 6). Asterisk (*) means $P < 0.05$ vs. dietary control (DC) group in the same column. -: undetectable.

manufacturer's cytokine ELISA protocol (mouse DuoSet ELISA Development system, R&D Systems). The sensitivity of these cytokine assays was about 15.6 pg/mL.

(II) *Inflammatory Mediator Levels of Nitric Oxide (NO) in Sera or Peritoneal Macrophage Cell Cultures*

Aliquots of 80 μLof samples and standards (0-100 μM sodium nitrite (Sigma-Aldrich Co., S-2252) dissolved in double distilled water) were pipeted into the 96 microplate wells (Nunc), respectively. Aliquots of 160 μL of Griess reagent were then added into each respective well to develop the color. The Griess reagent was freshly prepared from Reagent A and B at a ratio of 1:1 (Reagent A: 2% (w/v) sulfanilamide (Sigma-Aldrich Co., S-9251) dissolved in 2.5% (v/v) phosphoric acid; Reagent B: 0.2% (w/v) N-1-naphthylethylene diamide dihydrochloride (Sigma-Aldrich Co., N-9125) dissolved in 2.5% (v/v) phosphoric acid). After incubation for 10 min, the plate was read on a plate reader (ELISA reader, ASYS Hitech GmbH, Austria) at 540 nm. Using the standard curve, the NO concentration for each unknown sample was determined.

VI. *Statistical Analysis of Data*

Data are expressed as mean ± S.E. and analyzed statistically using Dunnett's t-test of parametric type. Differences between the dietary control and other groups were considered statistically significant if $P < 0.05$.

RESULTS

I. *Effects of Lotus Plumule Supplementation on Serum Mediators of LPS-challenged Mice*

The serum mediator levels of female BALB/c mice fed with lotus plumule powder and administrated with LPS i.p. are shown in Table 1. The results showed that neither LPS challenge nor lotus plumule supplementation significantly affect the IL-2, IL-10, and NO levels in the serum. However, lotus plumule supplementation decreased IL-2 (from 444 ± 112 to 193 ± 74 pg/mL) and IL-10 (from 1321 ± 359 to 640 ± 119 pg/mL) levels in the sera of LPS-challenged mice. The levels of serum IL-6 and TNF-α are too low to be detectable in the non-sensitized control group (SC group). Predictably, the serum levels of IL-6 (from 0 to 563 ± 211 pg/mL) and TNF-α (from 0 to 14.5 ± 1.1 pg/mL) from LPS-challenged mice increased. Supplementation with lotus plumule decreased serum IL-6 (from 563 ± 211 to 394 ± 97 pg/mL) levels of LPS-challenged mice, but it did not reach statistical significance at the dosage used in this study. Lotus plumule supplementation decreased the levels of serum TNF-α in a dose-dependent manner, while supplemented with high dose significantly inhibited the production of serum TNF-α (from 14.5 ± 1.1 to 0 pg/mL) in LPS-challenged mice. Closer inspection of the data indicated that TNF-α levels in serum were lower than that of the ELISA kit sensitivity (15.6 pg/mL). The low TNF-α level in serum suggests that LPS challenge resulted in sub-septic rather than septic inflammation in this study. Simultaneously, low level of cytokine might result in experimental deviation.

II. *Effects of Lotus Plumule Supplementation on the Levels of IL-10 in Peritoneal Macrophage Cultures from LPS-challenged mice*

To investigate the anti-inflammatory effects of lotus plumule supplementation *in vivo* on the peritoneal macrophages, the anti-inflammatory cytokine IL-10 levels in peritoneal macrophage cultures from LPS-challenged mice were determined (Figure 1). The peritoneal macrophages were cultured in the absence (spontaneous) and presence of stimulus (LPS-stimulated). Figure 1 shows that LPS challenge did not significantly affect spontaneous and LPS-stimulated IL-10 levels in peritoneal macrophage cultures. However, lotus plumule supplementation significantly increased the levels of spontaneous and LPS-stimulated IL-10 (from 0 to 387 ± 75 pg/10^6 cells; from 8.6 ± 4.3 to 558 ± 136 pg/10^6 cells, respectively) secreted by peritoneal exudate cells.

III. *Effects of Lotus Plumule Supplementation on the Level of IL-6 in Peritoneal Macrophage Cultures from LPS-challenged Mice*

To evaluate the effects of lotus plumule supplementation on the cytokine secretions of macrophages, the amounts

of IL-6 in peritoneal macrophage cultures were measured (Figure 2). The results showed that LPS challenge significantly inhibited spontaneous IL-6 production (from 7.43 ± 1.51 to 2.48 ± 1.56 ng/10^6 cells) of macrophages, suggesting that LPS challenge affected the immune responses of macrophages. Supplemented with lotus plumule decreased IL-6 production in a dose-dependent manner in both spontaneous (from 2.48 ± 1.56 to 1.66 ± 1.33 ng/10^6 cells) and LPS-stimulated (from 1.10 ±0.10 to 0.71 ± 0.31 ng/10^6 cells) macrophage cultures. However it did not reach statistical significance at the dosage used in this study.

IV. *Effects of Lotus Plumule Supplementation on the Levels of NO in Peritoneal Macrophage Cultures from LPS-challenged Mice*

To further investigate the effects of lotus plumule supplementation on the peritoneal macrophages, the levels of pro-inflammatory mediator NO in peritoneal macrophage cultures from LPS-challenged mice were determined (Figure 3). The results showed that LPS challenge increased spontaneous and LPS-stimulated NO production from 2.42 ± 1.89 to 2.74 ± 1.03 n mole/10^6 cells and from 0.98 ± 0.34 to 11.5 ± 5.4 n mole/10^6 cells, respectively, in peritoneal macrophage cultures. Lotus plumule supplementation decreased LPS-stimulated NO levels from 11.5 ± 5.4 to 3.51 ± 1.78 n mole/10^6 cells in a dose-dependent manner in the peritoneal macrophage cultures from LPS-challenged mice. However it did not significantly decrease the levels of NO.

DISCUSSION

Severe sepsis may not be reversible and generally result in high mortality. In this study, we established a mild systemic inflammation model, in which female BALB/c mice were challenged i.p. by a single dose of 10 mg LPS/kg BW. According to our preliminary data, the LPS-challenged mice were sick from LPS challenge, but none died during one week of observation period. A sub-lethal endotoxic shock murine model has been established. The sub-septic doses of endotoxin LPS can activate distinct immune responses, such as systemic inflammation, and have been used in previous studies for different purposes[12-14]. Using our established sub-septic (sub-lethal) animal model, the effects of lotus plumule supplementation *in vivo* on acute systemic inflammation were measured. The results showed that LPS challenge (DC group) significantly increased serum mediator levels of IL-6 or TNF-α, suggesting that systemic inflammation was induced. Supplementation with lotus plumule daily before systemic inflammation decreased serum IL-6 levels of LPS-challenged mice and decreased pro-inflammatory cytokine TNF-α levels in serum in a dose-dependent manner (Table 1). IL-6 plays a critical role in the manifestation of fever and is believed to be a pro-inflammatory cytokine[7]. TNF-α, produced by macrophages, natural killer (NK) cells, and T cells, has been suggested to play an important role in endotoxin-mediated shock and inflammation[15]. The effects on serum mediators suggest

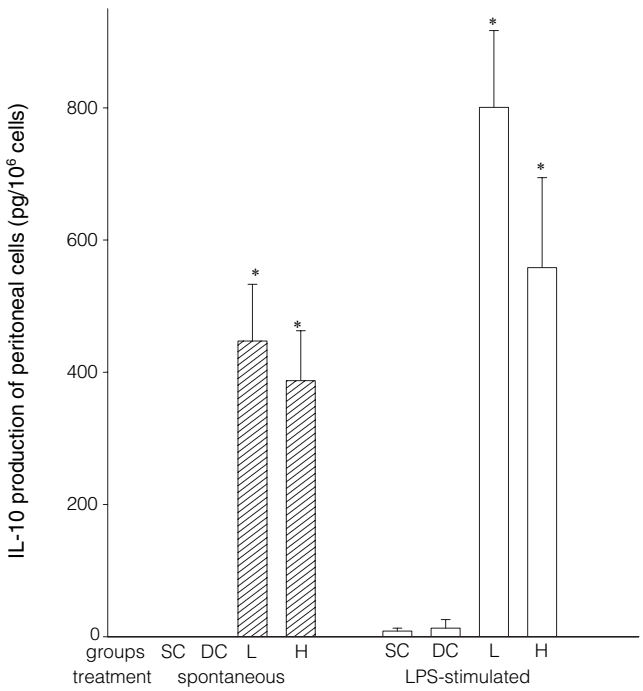

Figure 1. Effects of lotus plumule on IL-10 production of peritoneal macrophage cultures from female BALB/c mice after three weeks of tube feeding and treatment with lipopolysaccharide i.p. for 24 hours. Each value represents mean ± S.E. (n = 6). Asterisk (*) means $P < 0.05$ vs. dietary control (DC) group. SC: non-sensitized control group (0 mg of lotus plumule /mouse/day) ; L: low dose group (5 mg of lotus plumule/mouse/day); H: high dose group (20 mg of lotus plumule /mouse/day).

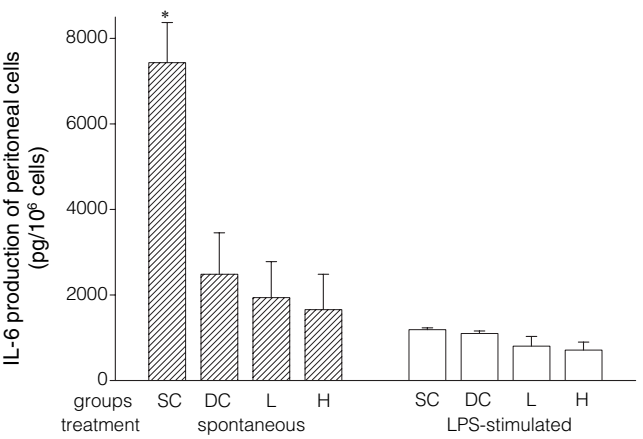

Figure 2. Effects of lotus plumule on IL-6 production of peritoneal macrophage cultures from female BALB/c mice after three weeks of tube feeding and treatment with lipopolysaccharide i.p. for 24 hours. Each value represents mean ± S.E. (n = 6). Asterisk (*) means $P < 0.05$ vs. dietary control (DC) group. SC: non-sensitized control group (0 mg of lotus plumule /mouse/day); L: low dose group (5 mg of lotus plumule/mouse/day); H: high dose group (20 mg lotus plumule /mouse/day).

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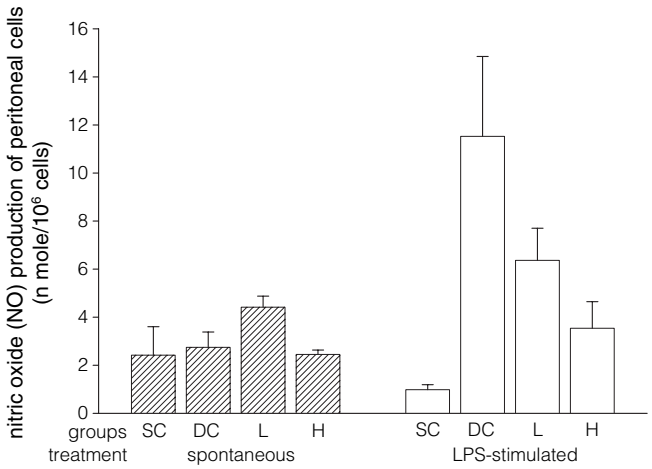

Figure 3. Effects of lotus plumule on nitric oxide production of peritoneal macrophage cultures from female BALB/c mice after three weeks of tube feeding and treatment with lipopolysaccharide i.p. for 24 hours. Each value represents mean ± S.E. (n = 6). No statistical difference between experimental groups and dietary control groups (DC). SC: non-sensitized control group (0 mg of lotus plumule / mouse/day); L: low dose group (5 mg of lotus plumule/mouse/day); H: high dose group (20 mg of lotus plumule /mouse/day).

that lotus plumule supplementation before acute systemic inflammation alleviates acute inflammation status via inhibiting serum IL-6 and TNF-α production.

Macrophages are large mononuclear phagocytic cells involving innate and adaptive immunity *in vivo*. They are migratory cells and are found in most tissues of the body. The releases of IL-1, IL-6, and TNF-α by macrophages may induce local protective effects, but they can have damaging effects when released systemically. However, IL-10 produced by T cells and macrophages is a cytokine synthesis inhibitory factor which might provide host defense against systemic inflammation. IL-10 reduces the severity of local and systemic inflammation in a murine model of intestinal ischemia/reperfusion when given before or after reperfusion injury. It is suggested that IL-10 may exert anti-inflammatory effects via blocking cytokine production and distant organ neutrophil accumulation[16]. Kupffer cell-derived IL-10 is also found to play a critical role in host defense in murine septic peritonitis[9]. In this study, lotus plumule supplementation decreased pro-inflammatory mediators IL-6 and NO production in both spontaneous and LPS-stimulated macrophage cultures in a dose-dependent manner (Figures 2 and 3). Simultaneously, supplementation with lotus plumule significantly increased the levels of spontaneous and LPS-stimulated IL-10 (Figure 1). These results suggest that lotus plumule might demonstrate an anti-inflammatory effect via enhancing the secretion of anti-inflammatory cytokine IL-10 by macrophages. We assumed that supplemented with low dose of lotus plumule for 3 weeks before LPS challenge administration could stimulate IL-10 production and inhibit TNF-α secretion during systemic inflammation. These results suggest that supplementation of lotus plumule might exhibit prophylactic effect on acute systemic inflammation *in vivo*.

Lotus plumule is traditionally used anti-inflammatory agent. Lotus seeds were reported to be effective in anti-oxidant properties and protecting DNA damage in human lymphocytes *in vitro*[17-18]. Hydro-alcoholic extract of lotus plumule is found to have heapto- and renal-protective activities against carbon tetracholoride (CCl4) damage and demonstrate an antioxidant activity *in vivo* and *in vitro*[19]. Liu *et al.*[20] reported that ethanolic extracts of *Nelumbo nucifera* seeds, NN-B-4, suppresses human peripheral blood mononuclear cells (PMBC) proliferation via inhibiting cyclin-dependent kinase (cdk) 4 mRNA expression. NN-B-4 also suppresses the production and mRNA expression of IL-2, IL-4, IL-10, and IFN-γ *in vitro* in activated PMBC[20]. In this study, lotus plumule was first introduced to treat acute systemic inflammation. Lotus plumule supplementation decreased serum IL-2, IL-6, IL-10, and TNF-α levels in LPS-challenged mice (Table 1). The *in vitro* and *in vivo* effects of lotus plumule on serum cytokine secretion are accordant. However, lotus plumule supplementation enhanced IL-10 production by peritoneal macrophages from LPS-challenged mice. The *in vivo* effects of lotus plumule on serum and macrophage IL-10 expression seemed not corresponding. We assume different effects of lotus plumule on different cells. The immunomodulatory mechanisms and pharmacological effects of lotus plumule against systemic inflammation need further investigation.

Lotus plumule contains alkaloids, saponins, phenolics and carbohydrates[19]. Recently, lotus seed extracts are found to contain phenolics including caffeic acid, chlorogenic acid, *p*-hydroxybenzoic acid, gallic acid and large amounts of phenolic compounds which are suggested to be responsible for the antioxidant activity[17-18]. Three novel flavonols, myricetin-3,-O-(6,,-*p*-coumaroyl) glucoside, myricetin-3-O-rhamnoside and pentagalloyl glucose have been isolated from the wild water lily *Nymphaea lotus* L.[21]. Dietary isoflavonones including daidzin, glycitin, and genistin, have been found to suppress endotoxin-induced inflammatory reactions in the liver and intestine[22]. We presume that phenolic compounds, especially flavonoids, in lotus plumule might contribute to the anti-inflammatory effects against systemic inflammation. However, the effective components of lotus plumule against systemic inflammation remain to be elucidated.

CONCLUSIONS

This study showed that lotus plumule supplementation before acute systemic inflammation significantly decreased serum TNF-α level and increased peritoneal macrophages derived IL-10 production. These results suggest that lotus plumule exhibits an anti-inflammatory effect against acute systemic inflammation. Lotus plumule can be used to develop anti-inflammatory agent in the future.

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