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Modulation of Activated Murine Peritoneal Macrophages Functions by Emodin, Aloe-emodin and Barbaloin Isolated from *Aloe barbadensis*

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ABSTRACT

Activated murine macrophages produce high level of nitric oxide by inducible nitric oxide synthase (iNOS), which is an important mechanism in macrophage-induced inflammation. In the present study, we isolated emodin, aloe-emodin and barbaloin compounds from *Aloe barbadensis* and investigated their inhibitory effects on the production of nitric oxide (NO), tumor necrosis factor-α (TNF-α) and interleukin (IL-12) from murine macrophages activated by lipopolysaccharide (LPS) and interferon-gamma (IFN-γ).

Our results showed that the 50% inhibition concentration (IC_{50}) of emodin, aloe-emodin and barbaloin on NO, TNF-α and IL-12 from activated peritoneal macrophages were 120, 30 and 200 μM respectively. Our experimental results showed that reduction of NO level by emodin might inhibit both at the pretreated LPS/IFN-γ activation and post-LPS/IFN-γ activation pathway. Activities of aloe-emodin and barbaloin could be caused by inhibition at the pretreated LPS/IFN-γ activation event. Chemical structures of all the isolated components were identified by comparison of NMR, mass spectral data and melting points with those reported in literature.

Key words: *Aloe barbadensis*, liliaceae, emodin, aloe-emodin, tumor necrosis factor, nitric oxide, anti-inflammatory activity, lipopolysaccharide

INTRODUCTION

Macrophage activation plays an important role in both the innate and acquired immune responses[1]. They influence a range of immune responses by antigen recognition, capture, clearance and transport. Macrophages are activated by lipopolysaccharide (LPS, the major component of gram-negative bacteria cell wall) and interferon-gamma (IFN-γ) and release several inflammatory mediators such as nitric oxide (NO), proinflammatory cytokines, tumor necrosis factor-α (TNF-α), and interleukins (IL-1, IL-6, and IL-12). The production of NO is regulated by intracellular NOS, and three types of nitric oxide synthase (NOS), endothelium NO synthase (eNOS), neutral NO synthase (nNOS) and inducible NO synthase (iNOS), have been identified. Exposure to stimulators such as bacterial toxin or viral infection, results in the production of iNOS in various cells such as macrophages, smooth muscle cells, and hepatocytes. The study of NO has created considerable interest in the identification of inhibitors of NOS with potential clinical use in humans. Increasing evidence shows that NO is involved in various pathophysiological processes including inflammation and sepsis[2]. Although NO production induced by iNOS may initiate several cellular responses, excessive amount of NO leads to diseases, such as inflammation, sepsis, and stroke[3-5].

Proinflammatory cytokines are low molecular weight proteins that play a crucial role in the normal regulation of the immune system as well as in pathological conditions. TNF-γ is one of the proinflammatory cytokines that is primarily secreted by activated macrophages and monocytes[6]. TNF-α enhances the production of other proinflammatory cytokines and activates other inflammatory cells. On the other hand, excessive amount of TNF-α has also been implicated in the pathogenesis of many chronic inflammatory diseases[7]. Significant effort has been focused on developing therapeutic drugs that interfere with TNF-α production or action due to its pivotal role in pathogenesis[8-9]. IL-12 has been shown to be a major Th1 promoting cytokine and several *in vivo* studies indicated that using anti-IL-12 antibody or IL-12 deficient animals show impaired Th1 responses and inflammation[10-11].

Natural plants are potential sources of novel anti-inflammatory drugs. Experiments have been carried out to study effects of aloe leaf components on various biochemical and microbiological systems relevant to many inflammatory responses[12-13]. It was suggested that aloe works as an anti-inflammatory when used tropically or taken orally. An animal study also examined the anti-inflammatory activity of extracts derived from aloe gel[14]. These results concluded that the activity was probably due to inhibition of the arachidonic pathway through cyclooxygenase[15]. Plants belonging to the genus aloe (Liliaceae), with nearly 420 species confined mainly to Africa, has over the years been proven to be one of the most important sources of biologically active compounds. More than 130 compounds

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belonging to different classes including anthrones, chromones, pyrones, coumarins, alkaloids, glycoproteins, naphthalenes and flavanoids have been reported from the genus aloe. *Aloe barbadensis* is a short-stemmed succulent herb that is widely distributed in Asia and other tropical regains. *Aloe barbadensis* has a very ancient history of healing wounds and also finds use in cosmetics[16-17]. Its leaves are well reputed in folk medicine for the treatment of asthma, gastrointestinal ulcers, burns and chronic wounds. Biological studies revealed that the leaves of aloe possess anti-tumor, anti-diabetic, anti-tyrosinase and anti-inflammatory activities[17-21]. In the present paper, we examined and reported the influence of emodin, aloe-emodin and barbaloin on NO production in activated murine peritoneal macrophages.

MATERIALS AND METHODS

I. *Instruments and Reagents*

All chemicals, solvents and reagents used in the experimental were of reagent grade. TLC was monitored over precoated Merk Silica gel 60F254 aluminum plates. Melting points were determined on a Kofler hot stage apparatus. NMR spectral data were recorded on a Varian Unity Inova-600 VXR-300/51 spectrometer operating at 600 MHz, in DMSO-d_6; and tetra methyl silane (TMS) is used as an internal standard. EI-MS was recorded on a Thermo-Finigan LCQ Adavntage system. Column chromatography was performed using Merck grade Silica gel 60. Morin hydrate, lipopolysaccharide (LPS, from *Escherichia coli* 055: B5), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Griess reagent were purchased from Sigma Chemical (St. Louis, MO, USA). RPMI-1640 medium, Hank's balanced salt solution (HBSS), penicillin, streptomycin, L-glutamine and fetal calf serum were purchased from Gibco BRL (Grand Island, NY, USA).

Female BALB/c mice were obtained from the Animal Center of the College of Medicine, National Taiwan University and maintained in the Animal Center of China Medical University. The animal room was on a 12-hr light and dark cycle with a constant temperature and humidity. All mice were 8 weeks old, and used to obtain cells from peritoneal exudates. All procedures conformed to the Guide for the Care and Use of Laboratory Animals (NRC, USA). All animals were sacrificed under anesthesia.

II. *Plant Material*

The fresh leaves of aloe were collected from Kalimantan, Indonesia in 2003. The plants were identified by Prof. C. C. Chen, China Medical University, Taichung, Taiwan. A voucher specimen of the plants has been deposited at China Medical University herbarium, Taichung, Taiwan.

III. *Extraction and Isolation*

Fresh aloe leaves (1 kg) were washed with water, cut at their base and yellow sap and allowed to exude. This sample was then collected and dissolved in ethanol and concentrated under reduced pressure to give thick mass (21 g). Afterwards the mass obtained was partitioned between ethyl acetate and water. Ethyl acetate residue (8 g) obtained after usual workup dissolved in hexane. The hexane insolubles were partitioned with 70% ethanol and hexane (1:1). The ethanol layer was extracted with ethyl acetate and concentrated under reduced pressure to get dark green mass (5.5 g). The ethyl acetate residue was subjected to column chromatography (Hexane, ethyl acetate and methanol in order of increasing polarity) which afforded four fractions (fractions I-IV). Fraction I showed major spot on TLC (hexane and ethyl acetate, 7:3), whereas fraction II exhibited yellow fluorescence over TLC (hexane and ethyl acetate, 1:1). On further purification by column chromatography using hexane and ethyl acetate as eluants with increasing polarity, both fraction resulted in isolation of emodin [mp 254~256°C (lit.[22] 255~256°C) TLC, hexane and ethyl acetate (7:3), *Rf* 0.60, 15 mg] and aloe-emodin [mp 222~224°C (lit.[23] 223~25°C) TLC, hexane and ethyl acetate (1:1), *Rf* 0.75, 18 mg] respectively. Fraction III on recrystalization from a mixture of chloroform and methanol (1:1) gave yellow powder [mp 146~148°C (lit.[24] 147~148°C); TLC, EtOAc/MeOH/H_2O, 10:2:1, *Rf* 0.52, 20 mg] which was identified as barbaloin. On the other hand, more polar fraction IV on further purification yielded betasitosterol glycoside [mp 289~290°C (lit.[24] 290~292°C), 150 mg]. The structures of all the isolated compounds were established using NMR, mass spectral data and comparison with those of authentic samples[22-24].

IV. *Anti-inflammatory Activity Evaluation*

(I) *Macrophage Cultures*

Mouse peritoneal excluded macrophages were obtained by lavage with 10 mL of cold Hank's balanced salt solution (HBSS; Life Technologies, GIBCO-BRL, Gaithersburg, MD) 3 days after intraperitoneal (*i.p.*) injection of 2 mL of 3% thioglycollate in saline (1.5 mL per mouse, Difco, Detroit, MI)[25]. Cells were maintained in RPMI-1640 supplemented with 10% (v/v) fetal calf serum and antibiotics (100 U/mL of penicillin, 100 µg/mL of streptomycin) and seeded in 96-well cluster plates at a density of 2×10^6 cells/mL and incubated at 37°C in humidified 5% CO_2 incubator to allow macrophages adherence. Two hours later, non-adherent cells were removed by washing with 37°C PBS and the remaining cells (90% macrophages, judged by non-specific esterase stain) were incubated with medium containing various concentrations of test compounds. Control cells were grown under identical conditions but were not exposed to the test compounds or LPS/IFN-γ.

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(II) *Cell Viability*

Test compounds emodin, aloe-emodin and barbaloin were dissolved in few drops of DMSO and diluted with water to make the final concentrations. Various concentrations of compounds were incubated in a 96-well plate for 24 hr. Mitochondrial respiration-dependent MTT assay was employed to determine their cytotoxicity[26]. MTT was a pale yellow substance that was reduced by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even fresh dead cells cannot reduce significant amount of MTT. MTT (0.1 mg) in PBS was added into each well and then incubated at 37°C for 4 hr. The MTT formazan (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) crystals formed from dye reduction by viable cells were dissolved using acidified isopropanol and mixed at room temperature. After 20 min, index of cell viability was calculated by measuring the optical density (OD) of the color produced by MTT dye reduction with a microplate reader (BIO-RAD, model 3550, USA) at 570 nm ($OD_{570-620}$). The mean OD value of the content of four wells was used to assess the cell viability expressed as % of control.

(III) *NO Determination*

The production of NO was estimated from the accumulation of nitrite (NO_2^-), the metabolic end product of NO metabolism, in the medium using the Griess reagent[27]. Cells were incubated with medium containing various concentrations of test compounds in the presence or absence of LPS (2 μg/mL) plus IFN-γ (10 U/mL) for 24 hr. Equal volumes of culture supernatant or serum and Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid, and 0.1% α-naphthylethylenediamine dihydrochloride in distilled water) were mixed and incubated for 15 min at room temperature. Absorbance was measured at 540 nm on a spectrophotometer. The absorbance was referred to a nitrite standard curve to determine the nitrate concentration in supernatants.

(IV) *Cytokine Determination*

TNF-α and IL-12 concentrations in supernatants from macrophage cultures were determined by enzyme-linked immunosorbent assay (ELISA) using antibody from PharMingen, according to manufacturer's instruction[28]. Cells were incubated with LPS/IFN-γ in the presence of different concentrations of compounds for 24 hr. The supernatants were collected and stored in -80°C before analysis. Standards were prepared from recombinant mouse TNF-α and IL-12 separately (PharMingen, San Diego, CA, USA). The sensitivity of TNF-α and IL-12 were 15.6 pg/mL. Cell viability was assessed by the trypan blue dye exclusion method and was always found to be greater than 95%.

(V) *Statistical Analysis*

All experimental data are shown as means ± S.D. Statistical analysis was performed using one-way ANOVA followed by Dunnetts italicize test, and the significant difference was set at ${}^{a}p < 0.05$, ${}^{b}p < 0.01$ and ${}^{c}p < 0.001$.

RESULTS AND DISCUSSION

We examined ethyl acetate extract of *A. barbadensis* for its potential to inhibit nitric oxide production in activated murine macrophages. In the preliminary studies yellow sap of aloe leaf was exuded into ethanol and concentrated under reduced pressure. The resulting thick mass obtained was fractionated with ethyl acetate and water. Ethyl acetate extract obtained after usual workup was treated with hexane and 70% ethanol (1:1). Hexane insolubles were fractionated with ethyl acetate and yielded to crude extract that were examined for its activity. It showed appreciable dose dependent activity on activated murine macrophages (data not shown). To isolate the compounds responsible for the activity, the ethyl acetate extract was further fractionated using column and thin-layer chromatography. Further purification resulted in isolation of emodin, aloe-emodin and barbaloin.

To analyze potential anti-inflammatory properties of emodin, aloe-emodin and barbaloin and to elucidate the underlying mechanisms, this is the first paper to use primary cells, rather than tumor cell, to study the possible anti-inflammatory effect of *A. barbadensis*. Murine peritoneal excluded macrophages, which can produce NO, TNF-α, and IL-12 upon stimulation by LPS/IFN-γ, thus provided a suitable model for studying inflammatory response in culture cells. The potential toxicity of emodin, aloe-emodin and barbaloin was assessed by MTT assay after 24-hr incubation in the macrophages. Cell viability was not affected up to the concentration of 400 μM (data not shown). At these concentrations, emodin, aloe-emodin and barbaloin caused dose-dependent inhibition of NO, TNF-α, and IL-12 (Figure 1). The 50% inhibition concentration (IC_{50}) values of emodin, aloe-emodin and barbaloin were found to be 120, 30, 200 μM, respectively. Aloe-emodin was significantly more active than others. However, no detectable level of NO and cytokines were measured when macrophages were cultured with compounds only (data not shown). Previous study indicated that the IC_{50} values of inhibition of NO production from LPS/IFN-γ activated RAW 264.7 cell line by emodin is 60.7 μM[29]. It was implied that the mitogenic responsive activity of macrophage cell line is more sensitive to emodin treatment than that of primary macrophages.

In order to examine whether the reduced NO level was a result of inhibition at the same moment of the LPS/IFN-γ activation, we tested whether emodin, aloe-emodin and barbaloin suppressed the NO production from macrophages in which inducible NOS expression had already been

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induced. Therefore, we compared the addition of each aloe isolated compounds individually and LPS/IFN-γ at the same time with peritoneal macrophages. These macrophages had been pretreated with LPS/IFN-γ for 24 hr before aloe isolated compounds were added. The experimental results showed that at the IC_{50} values of emodin reduce only 30~40% secretion of NO from macrophages that had been pretreated with LPS/IFN-γ for 24 hr as shown in Figure 2(A). In contrast, aloe-emodin and barbaloin failed to reduce the secretion of NO from pre-activated macrophages (Figure 2 B and C). Experiments showed that reduction of NO level by emodin might involve inhibition both the pretreated LPS/IFN-γ activation and post-LPS/IFN-γ activation pathway. Activities of aloe-emodin and barbaloin might be a result of inhibiting at the pretreated LPS/IFN-γ activation event.

Therapeutic agents that inhibit the biosynthesis of NO as well as proinflammatory cytokines (TNF-α and IL-12) production are known to be useful for the relief of inflammatory conditions. Based on the results of this study, we came to the conclusion that the components of *A. barbadensis* have significant inflammatory activity, and thus provide a scientific basis for the treatment of inflammatory processes in folk medicine.

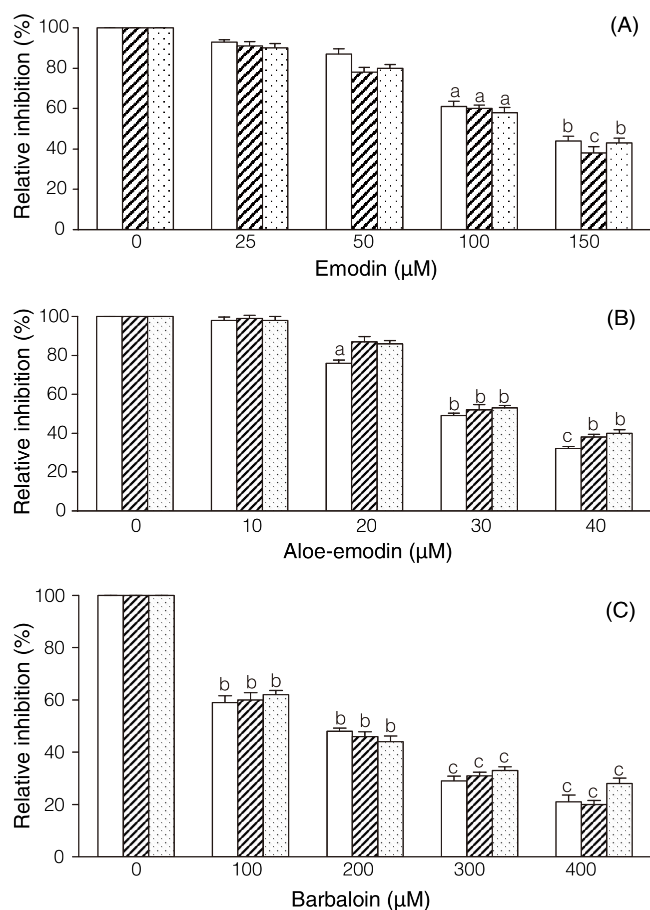

Figure 1. Effect of different concentrations of emodin (A), aloe-emodin (B) and barbaloin (C) on the production of nitric oxide (NO), tumor necrosis factor (TNF-α) and interleukin (IL-12). NO, TNF-α and IL-12 are white, slant and dot bar respectively. The statistical significance when compared with the values of NO: 45 μM; TNF-α: 53 pg/mL; IL-12: 102 pg/mL in the presence of LPS/IFN-γ were only, $^{a}p < 0.05$, $^{b}p < 0.01$ and $^{c}p < 0.001$.

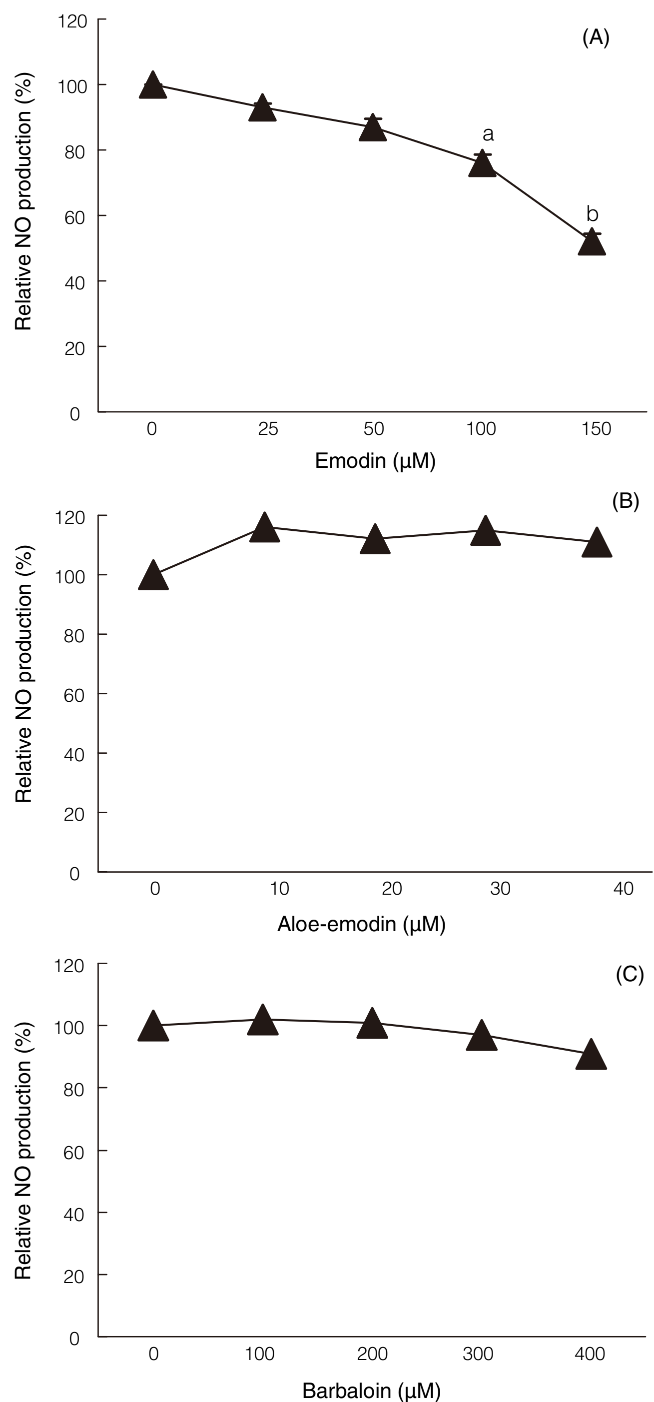

Figure 2. Effect of emodin (A), aloe-emodin (B) and barbaloin (C) on NO synthesis by LPS/IFN-γ pre-activated murine peritoneal macrophages. LPS/IFN-γ stimulated macrophages for 24 hr, after washing, isolated compounds were added for another 24 hr individually. The statistical significance when compared with the value of NO: 40 μM in the presence of LPS/IFN-γ were only, $^{a}p <$ 0.05 and $^{b}p < 0.01$.

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