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Anti-inflammatory Effects of the Roots of *Alpinia pricei* Hayata and Its Phenolic Compounds

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Alpinia pricei Hayata is cultivated throughout Asia and is an endemic plant in Taiwan. The leaf and root of this plant are used for traditional wrapping of food and as a cooking substitute for fresh ginger. The aim of this work was to study the *in vitro* anti-inflammatory effects of ethanol extracts from *A. pricei* Hayata (EEAP) and its phenolic compounds. High-performance liquid chromatography (HPLC) profiling indicated that EEAP contained caffeic acid, chlorogenic acid, ferulic acid, *p*-hydroxybenzoic acid, rutin, apigenin, curcumin and pinocembrin. EEAP and its phenolic compounds, apigenin, curcumin, and pinocembrin, inhibited lipopolysaccharide (LPS)-stimulated nitric oxide (NO) and prostaglandin E_2 (PGE_2) production in RAW 264.7 cells. Furthermore, EEAP, apigenin, curcumin, and pinocembrin decreased LPS-mediated induction of protein and mRNA expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in RAW 264.7 cells. In addition, EEAP and its major active compound pinocembrin inhibited LPS-induced nuclear translocation of nuclear factor-κB (NF-κB) and NF-κB-mediated reporter gene expression. EEAP and pinocembrin also significantly inhibited LPS-induced intracellular reactive oxygen species (ROS) production in RAW 264.7 cells. When these results are taken together, they indicate that EEAP and pinocembrin suppressed LPS-induced NO and PGE_2 production by inhibition of NF-κB nuclear translocation and ROS generation.

KEYWORDS: ***Alpinia pricei*** **Hayata; anti-inflammation; RAW 264.7 cells; lipopolysaccharide; pinocembrin**

INTRODUCTION

Excess nitric oxide (NO) functions as a toxic radical and causes many diseases, such as cancer and atherosclerosis, because of its mutagenic effects, in addition to its effects on cell apoptosis and necrosis (*1*, *2*). Macrophages play an important role in mediating the response to inflammation induced by NO, free radicals, and cytokines (*3*). The large amount of NO produced in response to lipopolysaccharide (LPS) contributes to inflammatory conditions (*4*). Salerno et al. (*5*) demonstrated that enhanced expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) genes is associated with inflammatory responses. iNOS is expressed in vascular smooth muscle cells, macrophages, and hepatocytes and its expression is induced in response to pro-inflammatory cytokines and bacterial LPS (*6*). COX-2 is thought to be the predominant cyclooxygenase involved in mediating the inflammatory response (*7*). Mann et al. (*8*) reported that COX-2-derived prostaglandin E_2 (PGE_2) promoted tumor growth and metastasis. Many studies have indicated that phenolic compounds exhibit pharmacological properties, such as antioxidant, antithrombosis, anti-inflammatory, and anticancer. However, little information is available on the benefits of the phenolic compounds that are found in *Alpinia pricei* Hayata.

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A. pricei Hayata is cultivated throughout Asia and is a traditional plant in Taiwan. The leaf of this plant is used for traditional wrapping of food, and its root is used as a traditional cooking substitute for fresh ginger. *Alpinia* plants have been reported to exhibit antioxidant, anti-inflammatory, anticancer, immunostimulating, and hepatoprotective activities. Chou et al. (*9*) reported that 70% ethanol extracts of roots from *A. pricei* Hayata reduced the metabolic syndrome induced by sucrose-containing drinking water in C57BL/6J mice. Shi et al. (*10*) demonstrated that protocatechuic acid isolated from *Alpinia* species inhibited H_2O_2-induced oxidative damage in PC12 cells and reduced oxidative stress in male Sprague−Dawley rats. Israf et al. (*11*) indicated that cardamonin from *Alpinia rafflesiana* inhibited NO and PGE_2 production in LPS-stimulated RAW 264.7 cells. Yadav et al. (*12*) and Lee et al. (*13*) indicated that diarylheptanoids from *Alpinia officinarum* significantly inhibit LPS-induced NO production and protein and mRNA expressions of iNOS and COX-2 by inhibiting NF-κB activation and phosphorylation of p44/42 MAPK in RAW 264.7 cells. *Alpinia* species exerted antiproliferative and growth-inhibition effects on human HT-1080 fibrosarcoma, murine colon 26-L5 carcinoma cells, CORL23 lung cancer cells, MCF7 breast cancer cells, and human carcinoma KB cells (*14*−*16*). Moreover, *Alpinia* species have been investigated phytochemically and biologically for immunostimulating (*17*) and hepatoprotective (*18*)

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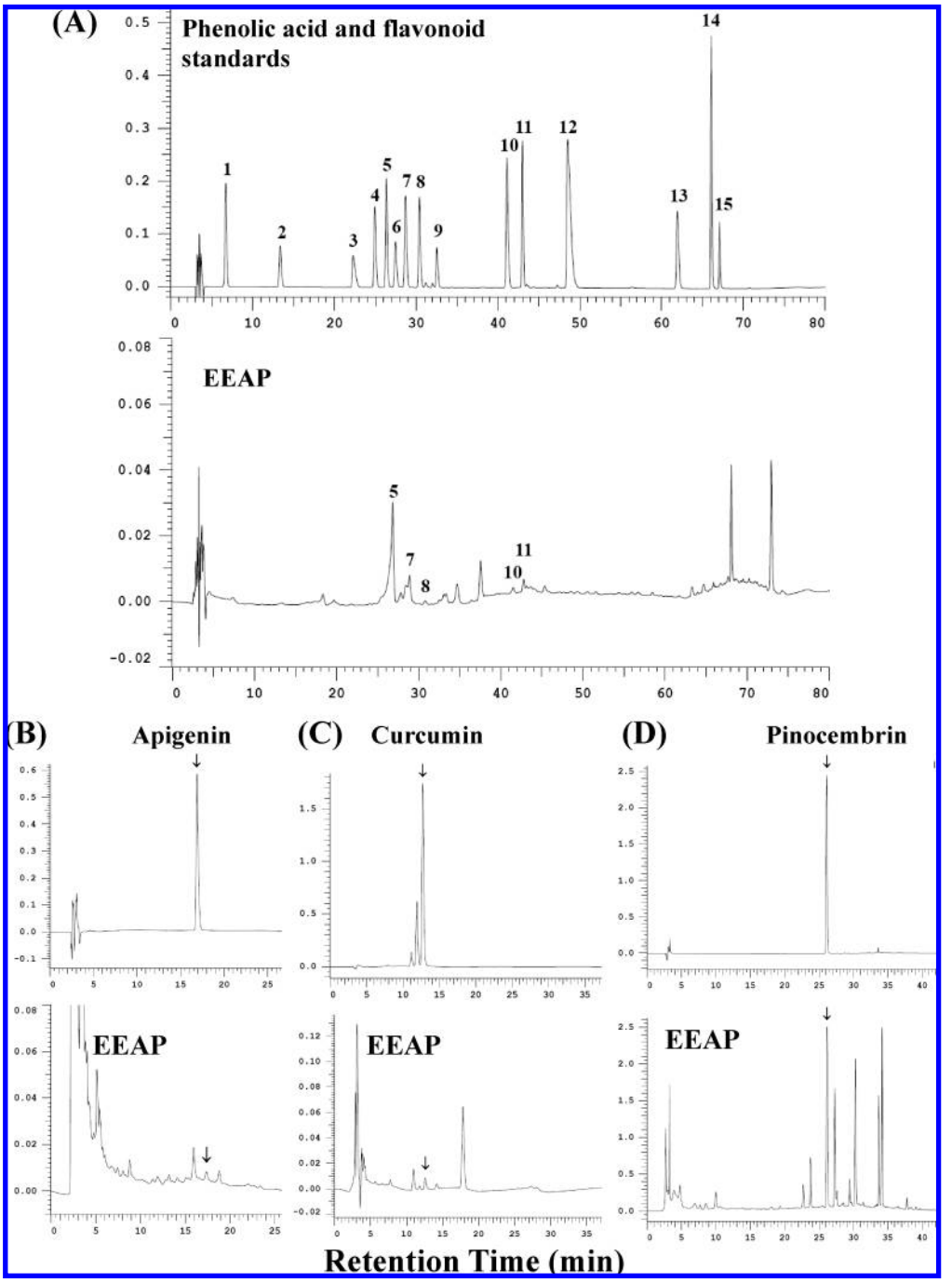

Figure 1. HPLC chromatograms for (A) some phenolic acid and flavonoid standards, (B) apigenin, (C) curcumin, and (D) pinocembrin and their profiles in EEAP. Identification of peaks: 1, gallic acid; 2, protocatechuic acid; 3, gentisic acid; 4, catechin; 5, chlorogenic acid; 6, vanillic acid; 7, *p*-hydroxybenzoic acid; 8, caffeic acid; 9, epicatechin; 10, ferulic acid; 11, rutin; 12, salicylic acid; 13, quercetin; 14, naringenin; and 15, kaempferol. EEAP, 70% ethanol extracts of *A. pricei* Hayata.

activities. *Alpinia* species have been reported to contain phenolic compounds, such as chlorogenic acid, rutin, quercetin, epicatechin, catechin, cardamonin, protocatechuic acid, zingerone, curcumin, apigenin, and pinocembrin (*10*, *19*−*24*). However, whether the ethanol extracts from *A. pricei* Hayata and its phenolic compounds exert anti-inflammatory activities is unclear.

The objective of this study was to investigate the anti-inflammatory effects of the ethanol extracts of roots from *A. pricei* Hayata (EEAP) and its phenolic compounds in LPS-stimulated RAW 264.7 murine macrophage cells. In this study, the protective effect of EEAP, mediated by its anti-inflammatory properties as a result of modulation of iNOS and COX-2 expression, NF-κB activation, and MAPK phosphorylation, was investigated.

MATERIALS AND METHODS

Materials. *A. pricei* Hayata was collected at Ping-tung, Taiwan, in March of 2006. A total of 70% ethanol extracts of roots from *A. pricei* Hayata (EEAP) were provided by Dr. Sheng-Yang Wang (Department of Forestry, National Chung Hsing University, Taichung, Taiwan). Lipopolysaccharide (LPS), 2′-7′-dichlorofluorescin diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye, pinocembrin, sulfanilamide, and anti-β-actin antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethylsulfoxide

Table 1. Contents of Phenolic Compounds in EEAP

compounds	contents[a]
caffeic acid (mg/g extract)	3.1 ± 0.6
chlorogenic acid (mg/g extract)	48.4 ± 0.2
ferulic acid (mg/g extract)	1.2 ± 0.1
p-hydroxybenzoic acid (mg/g extract)	0.56 ± 0.02
rutin (mg/g extract)	0.9 ± 0.2
apigenin (μg/g extract)	4.6 ± 0.6
curcumin (μg/g extract)	4.5 ± 0.03
pinocembrin (mg/g extract)	12.3 ± 0.07

[a] Reported values are the means ± SD ($n = 3$). EEAP, 70% ethanol extracts of *A. pricei* Hayata.

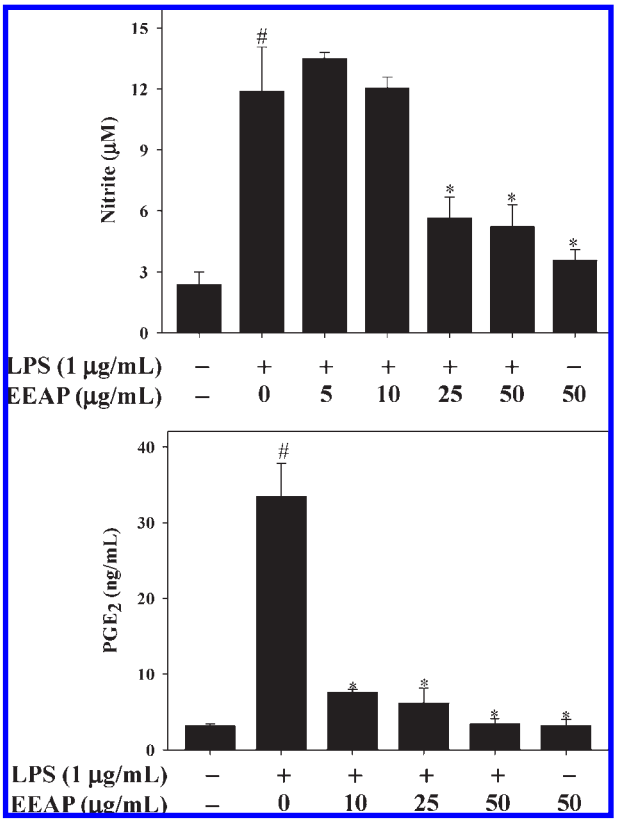

Figure 2. Effect of EEAP on LPS-induced nitrite and PGE_2 production in RAW 264.7 cells. Cells were treated with 0−50 μg/mL EEAP in the absence or presence of LPS (1 μg/mL) for 24 h. Reported values are the means ± SD ($n = 3$). (#) $p < 0.05$ indicates significant differences from the untreated group. (∗) $p < 0.05$ indicates significant differences from the LPS treatment alone.

(DMSO) was purchased from Merck Co. (Darmstadt, Germany). Dulbecco's modified Eagle's medium, fetal bovine serum, L-glutamine, and the antibiotic mixture (penicillin−streptomycin) were purchased from Invitrogen Co. (Carlsbad, CA). Anti-COX-2 and anti-iNOS antibodies were purchased from ABcam (Cambridge, MA). Antilamin B1 antibody was purchased from BioVision (Mountain View, CA). Antiphospho-NF-κB p65 antibody was purchased from Cell Signaling Technology (Beverly, MA). Anti-rabbit and anti-mouse secondary horseradish peroxidase antibodies were purchased from Bethyl Laboratories (Montgomery, TX). Protein molecular mass markers were obtained from Pharmacia Biotech (Saclay, France). Polyvinyldifluoride (PVDF) membranes for Western blotting were obtained from Millipore (Bedford, MA). All other chemicals were of reagent grade.

High-Performance Liquid Chromatography (HPLC) Analysis. The HPLC analysis was performed using a Hitachi L-6200 intelligent pump equipped with a photodiode array detector Hitachi L-7455 (Hitachi, Ltd., Tokyo, Japan) and a Mightysil RP-18 column (250 × 4.6 mm, 5 μm)

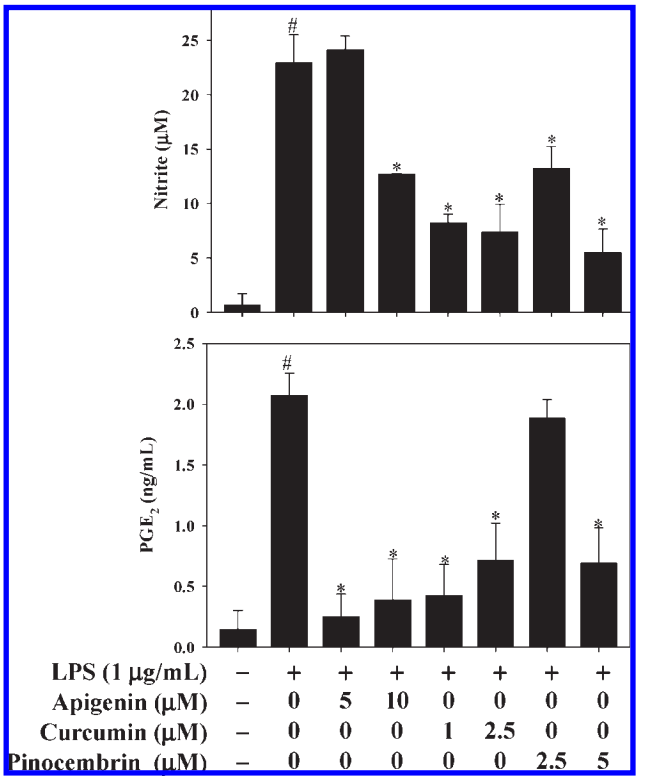

Figure 3. Effects of apigenin, curcumin, and pinocembrin on LPS-induced nitrite and PGE_2 production in RAW 264.7 cells. Cells were treated with different concentrations of apigenin, curcumin, and pinocembrin in the absence or presence of LPS (1 μg/mL) for 24 h. Reported values are the means ± SD ($n = 3$). (#) $p < 0.05$ indicates significant differences from the untreated group. (∗) $p < 0.05$ indicates significant differences from the LPS treatment alone.

(Kanto Chemical Co., Inc., Tokyo, Japan). The HPLC analyses for the quantitative determination of some phenolic acid and flavonoid contents in EEAP were carried out as described by Schieber et al. (*25*). The HPLC analyses for the quantitative determination of apigenin, curcumin, and pinocembrin content in EEAP were carried out as described by Janeska et al. (*26*), Jang et al. (*27*), and Quiroga et al. (*28*), respectively. Phenolic acids, flavonoids, apigenin, curcumin, and pinocembrin were identified by a comparison of their retention time (Rt) values and UV−vis spectra to those of known standards and were quantified by peak areas from the chromatograms.

Cell Culture. The RAW 264.7 cell line (BCRC 60001) was obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan). Cells were cultured at 37 °C in a humidified 5% CO_2 incubator in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 2 mM L-glutamine, and 100 units/mL penicillin−streptomycin.

Cell Viability Assay. The MTT assay was performed according to the method of Mosmann (*29*). RAW 264.7 cells were plated into 96-well microtiter plates at a density of 1×10^4 cells/well. After 24 h, the culture medium was replaced with 200 μL serial dilutions of EEAP (0−100 μg/mL) or phenolic compounds (0−100 μM), and the cells were incubated for 24 h. The final concentration of solvent was less than 0.1% in cell culture medium. The culture medium was removed and replaced with 90 μL of fresh culture medium. A total of 10 μL of sterile filtered MTT solution (5 mg/mL) in phosphate-buffered saline (PBS, pH 7.4) was added to each well to obtain a final concentration of 0.5 mg of MTT/mL. After 5 h, the unreacted dye was removed and the insoluble formazan crystals were dissolved in 200 μL/well of DMSO and measured spectrophotometrically in a FLUOstar galaxy spectrophotometer (BMG Labtechnologies Ltd., Offenburg, Germany) at 570 nm. The cell viability (%) relative to control wells containing cell culture medium without samples was calculated by $A_{570\ nm}[\text{sample}]/A_{570\ nm}[\text{control}] \times 100$.

Measurement of Nitric Oxide/Nitrite. Nitrite levels in the cultured media, which reflect intracellular nitric oxide synthase activity, were determined by the Griess reaction. The cells were incubated with EEAP (0−50 μg/mL) or phenolic compounds (0−10 μM) in the absence or presence of LPS (1 μg/mL) for 24 h. Briefly, cells were dispensed into 96-well plates, and 100 μL of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 5% phosphoric acid) and incubated at room temperature for 10 min. Using sodium nitrite to generate a standard curve (*30*), the concentration of nitrite was determined by measuring the OD reading at 550 nm.

Measurement of Prostaglandin E_2 (PGE_2). The cells were incubated with EEAP (0−50 μg/mL) or phenolic compounds (0−10 μM) in the absence or presence of LPS (1 μg/mL) for 24 h. PGE_2 levels were determined using a prostaglandin E_2 expression EIA kit (Cayman Chemical Company, Ann Arbor, MI). The concentration of PGE_2 was photometrically determined using a microplate reader (Awareness Technology, Palm City, FL) at 405 nm.

Western Blot Analysis. The cells were incubated with EEAP (0−50 μg/mL) or phenolic compounds (0−10 μM) in the absence or presence of LPS (1 μg/mL) for 0−6 h. After stimulation, cells were collected and lysed in ice-cold lysis buffer [20 mM Tris-HCl (pH 7.4), 2 mM ethylenediaminetetraacetic acid (EDTA), 500 μM sodium orthovanadate, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 10 μg/mL leupeptin, and 1 mM phenylmethylsulphonyl fluoride (PMSF)]. The nuclear fraction of cells was isolated using the nuclear/cytosol fractionation kit (BioVision, Mountain View, CA) according to the protocol of the manufacturer. The protein concentration of cell lysates was estimated using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin as the standard. Total proteins (50−60 μg) were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) using a 12% polyacrylamide gel. The proteins in the gel were transferred to a PVDF membrane. The membrane was blocked with 5% skimmed milk in PBST [0.05% (v/v) Tween-20 in PBS at pH 7.2] for 1 h. Membranes were incubated with primary antibody (1:5000) at 4 °C overnight and then with secondary antibody (1:5000) for 1 h. Membranes were washed 3 times in PBST for 10 min between each step. The signal was detected using the Amersham ECL system (Amersham-Pharmacia Biotech, Arlington Heights, IL). The relative protein expression was densitometrically quantified using the LabWorks 4.5 software and calculated according to the β-actin reference bands.

RNA Extraction and Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR). Real-time RT-PCR was performed to determine the level of gene expression in RAW 264.7 macrophages. The total RNA from RAW 264.7 cells was isolated using the TRIzol method (Life Technologies, Rockville, MD) according to the protocol of the manufacturer. The cDNA was synthesized from the total RNA (200 ng) by reverse transcription PCR using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) according to the instructions of the manufacturer. The following primer pairs were used: iNOS (accession number NM010927), 5′-TCCTACACCACACCAAAC-3′ (forward) and 5′-CTCCAATCTCTGCCTATCC-3′ (reverse); COX-2 (accession number NM011198), 5′-CCTCTGCGATGCTCTTCC-3′ (forward) and 5′-TCACACTTATACTGGTCAAATCC-3′ (reverse); interleukin-6 (IL-6) (accession number NM031168), 5′-TTCTCTGGGAAATCGTGGAAA-3′ (forward) and 5′-TCAGAATTGCCATTGCACAAC-3′ (reverse); and GAPDH (accession number NM008084), 5′-TCAACGGCACAGTCAAGG-3′ (forward) and 5′-ACTCCACGACATACTCAGC-3′ (reverse). Relative real-time RT-PCR for detection of gene expression levels was carried out using an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA). The reaction mixture (total volume of 25 μL) contained 1× power SYBR green PCR master mix, 300 nM forward primer, 300 nM reverse primer, cDNA, and DEPC-H_2O, as well as commercial reagents (Applied Biosystems, Foster City, CA). The thermal profile was established according to the protocol of the manufacturer: 95 °C for 10 min for enzyme activation, followed by denaturing at 95 °C for 15 s, and annealing and elongation at 60 °C for 1 min, with a total of 40 cycles. Relative levels of gene expression were quantified using the ΔΔCt method, which results in a ratio of target gene expression relative to equally expressed housekeeping genes.

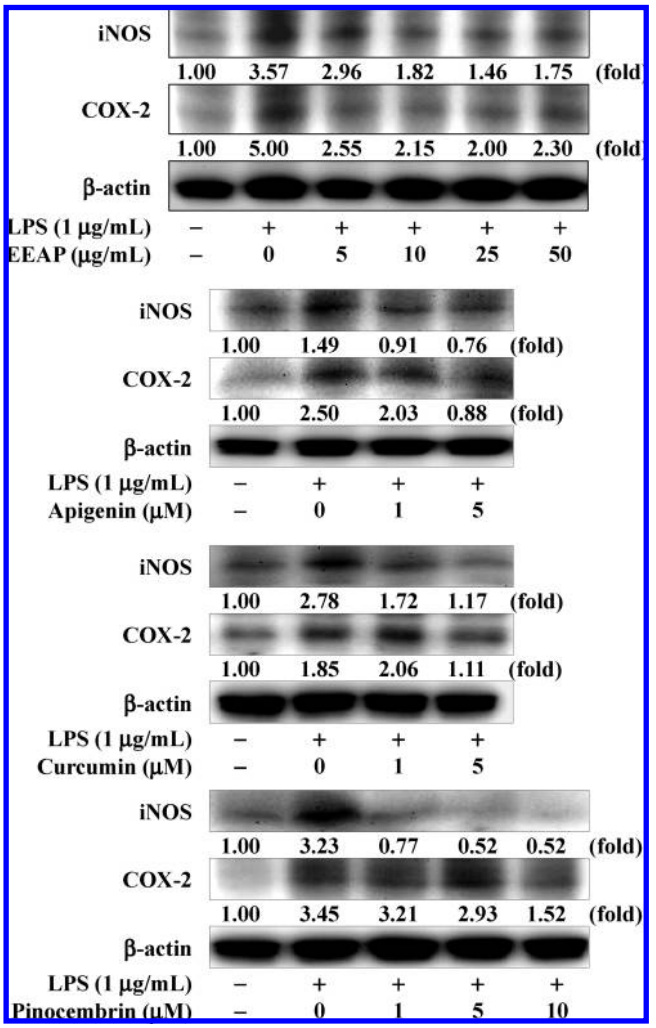

Figure 4. Effects of EEAP, apigenin, curcumin, and pinocembrin on LPS-induced iNOS and COX-2 protein levels in RAW 264.7 cells. Cells were treated with different concentrations of EEAP, apigenin, curcumin, or pinocembrin in the absence or presence of LPS (1 μg/mL) for 6 h. The relative expression of proteins was densitometrically quantified using LabWorks 4.5 software and calculated according to the β-actin reference bands.

Transfection and NF-κB-Luciferase Assays. RAW 264.7 cells (1×10^5 cells/well) were cultured in 6-well plates and transfected the following day using TransFast transfection reagent (Promega, Madison, WI). After 24 h, the medium was aspirated and replaced with culture medium. To assay the effect of the pNF-κB-Luc, cells were co-transfected with pNF-κB-Luc plasmid reporter gene (Stratagene, Jalla, CA) for 1 h and then treated with EEAP (0−25 μg/mL) or phenolic compounds (0−5 μM) in the absence or presence of LPS (1 μg/mL) for 1 h. Luciferase activity was determined using a Bright-Glo luciferase system (Promega, Madison, WI). The level of induction of luciferase activity was compared as a ratio of cells with and without treatment.

Determination of Intracellular Reactive Oxygen Species (ROS) Production. Intracellular ROS production was measured using the oxidant-sensitive fluorescent probe DCFH-DA. DCFH, which is converted from DCFH-DA by deacetylase within the cells, is oxidized by a variety of intracellular ROS to DCF, a highly fluorescent compound. The cells were incubated with EEAP or its phenolic compounds in the absence or presence of LPS (1 μg/mL) for 1 h. Briefly, cells were harvested by trypsin−EDTA (TE) solution (0.05% trypsin and 0.02% EDTA in PBS) and washed twice with PBS. The cells were stained with 20 μM of DCFH-DA for 15 min at room temperature and subjected to determination of intracellular ROS production using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Approximately,

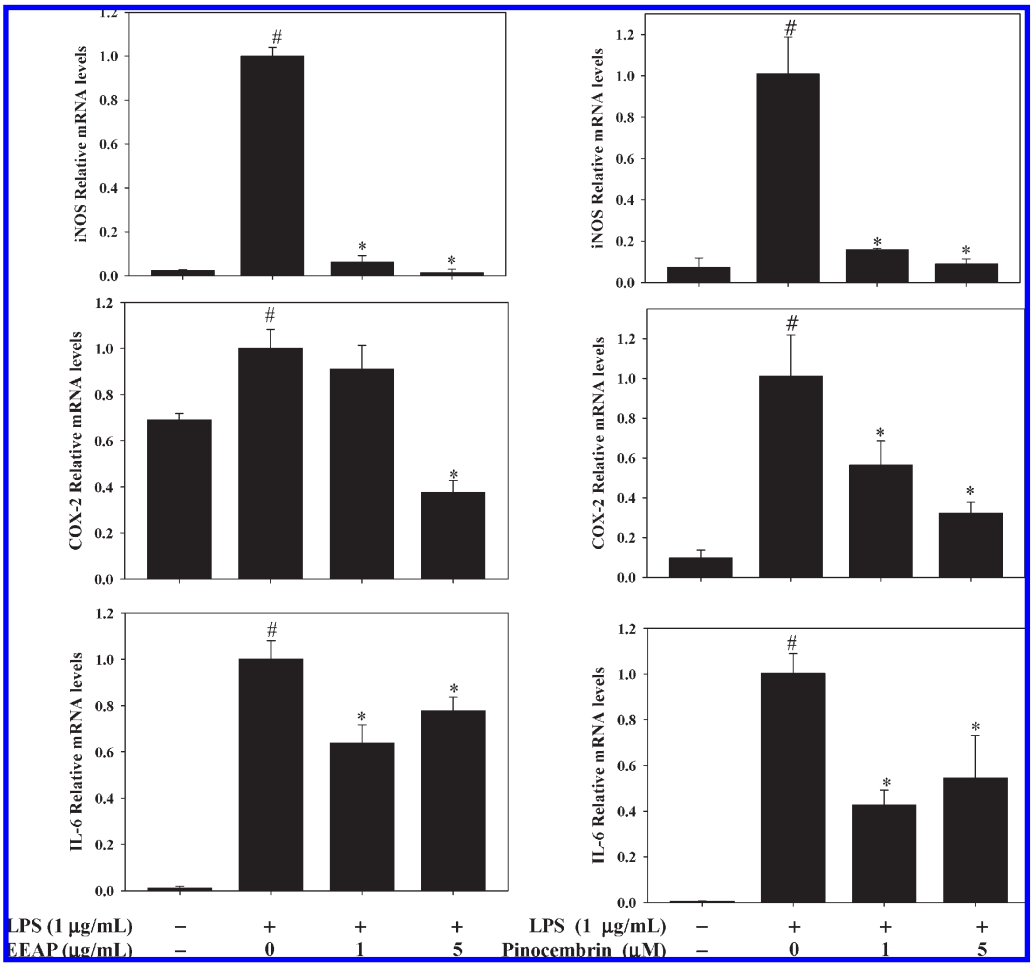

Figure 5. Effects of EEAP and pinocembrin on LPS-induced iNOS, COX-2, and IL-6 mRNA expression in RAW 264.7 cells. Cells were treated with EEAP (0−5 μg/mL) or pinocembrin (0−5 μM) in the absence or presence of LPS (1 μg/mL) for 0.5 h. Reported values are the means ± SD (n = 3). (#) $p < 0.05$ indicates significant differences from the untreated group. (∗) $p < 0.05$ indicates significant differences from the LPS treatment alone.

1×10^4 counts were taken for each sample. The ROS production (%) was calculated by CELL Quest software.

Statistical Analysis. Each experiment was performed in triplicate. The results were expressed as means ± standard deviation (SD). Statistical analysis was performed using SAS software. Analysis of variance (ANOVA) was performed using ANOVA procedures. Significant differences ($p < 0.05$) between the means were determined by Duncan's multiple range test.

RESULTS

Determination of Phenolic Compound Content in EEAP. The analytical plots of phenolic compounds in EEAP are listed in **Figure 1** and **Table 1**. The results from the chromatogram indicated that EEAP contained caffeic acid, chlorogenic acid, ferulic acid, *p*-hydroxybenzoic acid, rutin, apigenin, curcumin, and pinocembrin, as identified by comparison of their retention time values and UV spectra to those of known standards (data not shown). Among the eight identified phenolic compounds in EEAP, chlorogenic acid (48.4 mg/g extract) and pinocembrin (12.3 mg/g extract) were the most abundant.

Effects of EEAP, Apigenin, Curcumin, and Pinocembrin on NO and PGE_2 Production. Figure 2 shows the effects of EEAP on NO and PGE_2 production in LPS-stimulated RAW 264.7 cells. The NO production, measured as nitrite, was remarkably increased to 11.9 ± 2.1 μM when 1 μg/mL LPS was added to RAW 264.7 cells, as compared to 2.4 ± 0.6 μM for unstimulated control cells. EEAP (50 μg/mL) inhibited LPS-stimulated NO production (5.21 ± 1.1 μM) and had no cytotoxic effects (data not shown) on RAW 264.7 cells. EEAP also significantly reduced PGE_2 production in a dose-dependent manner. According to the data presented in **Figure 3**, apigenin, curcumin, and pinocembrin clearly inhibited NO and PGE_2 production in LPS-stimulated RAW 264.7 cells. The concentrations of apigenin (0−7.5 μM), curcumin (0−2.5 μM), and pinocembrin (0−10 μM) showed no cytotoxic effect on RAW 264.7 cells (data not shown). In addition, caffeic acid, chlorogenic acid, ferulic acid, *p*-hydroxybenzoic acid, and rutin did not affect NO production in LPS-stimulated RAW 264.7 cells (data not shown). Therefore, EEAP, apigenin, curcumin, and pinocembrin were selected for follow-up analysis.

Effects of EEAP, Apigenin, Curcumin, and Pinocembrin on Expression of iNOS and COX-2 Protein. The effects of EEAP, apigenin, curcumin, and pinocembrin on iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 cells was examined by Western blot analysis. **Figure 4** shows the effects of EEAP, apigenin, curcumin, and pinocembrin on iNOS and COX-2 protein expression in the LPS-stimulated RAW 264.7 cells. LPS (1 μg/mL) significantly increased iNOS and COX-2 protein expression as compared to the unstimulated control cells. When EEAP (0−50 μg/mL), apigenin (0−5 μM), curcumin (0−5 μM), or pinocembrin (0−10 μM) was simultaneously added to the medium with LPS (1 μg/mL), there was a dose-dependent inhibition of COX-2 and iNOS protein expression. The pinocembrin content (12.3 mg/g extract) was higher than apigenin

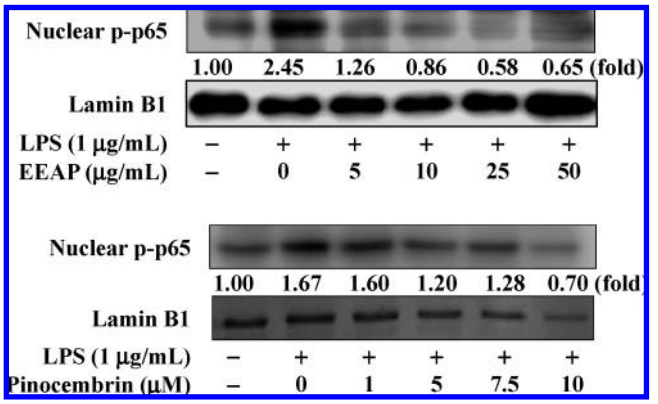

Figure 6. Effects of EEAP and pinocembrin on LPS-induced nuclear p-p65 protein expression in RAW 264.7 cells. Cells were treated with EEAP (0−50 μg/mL) or pinocembrin (0−10 μM) in the absence or presence of LPS (1 μg/mL) for 1 h. The relative expression of proteins was densitometrically quantified using the LabWorks 4.5 software and calculated according to the lamin B1 reference bands.

(4.6 μg/g extract) and curcumin (4.5 μg/g extract) in the EEAP extract; therefore, pinocembrin was selected for follow-up analysis.

Effects of EEAP and Pinocembrin on mRNA Expression of iNOS, COX-2, and IL-6. Figure 5 shows the effects of EEAP and pinocembrin on mRNA expression of iNOS, COX-2, and IL-6 in LPS-stimulated RAW 264.7 cells. LPS (1 μg/mL) significantly increased iNOS, COX-2, and IL-6 mRNA expression as compared to unstimulated control cells. When EEAP (0−5 μg/mL) or pinocembrin (0−5 μM) was simultaneously added to the medium with LPS, there were inhibitory effects of EEAP and pinocembrin on iNOS, COX-2, and IL-6 mRNA expression.

Effects of EEAP and Pinocembrin on Nuclear p-p65 Protein Levels and Transcriptional Activity of NF-κB. Figure 6 shows the effects of EEAP and pinocembrin on nuclear p-p65 protein expression in LPS-stimulated RAW 264.7 cells. We treated RAW 264.7 cells with EEAP or pinocembrin followed by LPS stimulation for 1 h. The EEAP and pinocembrin suppressed the LPS-induced nuclear expression of p-p65 in a dose-dependent manner. However, the transcriptional activity of NF-κB was evaluated to confirm whether EEAP and pinocembrin inhibited NF-κB binding activity in the LPS-stimulated RAW 264.7 cells. As shown in **Figure 7**, EEAP (10 and 25 μg/mL) and pinocembrin (5 μM) significantly ($p < 0.05$) inhibited LPS-induced NF-κB transcriptional activity in RAW 264.7 cells.

Effects of EEAP and Pinocembrin on Intracellular ROS Production. Figure 8 shows the effects of EEAP and pinocembrin on intracellular ROS production in LPS-stimulated RAW 264.7 cells. Intracellular ROS levels were measured using the fluorescent probe DCFH-DA. Treatment of RAW 264.7 cells with EEAP (0−50 μg/mL) or pinocembrin (0−10 μM) significantly inhibited LPS to induce intracellular ROS generation. Our data indicated that the NO and PGE_2 production in LPS-stimulated RAW 264.7 cells was inhibited by the removal of ROS.

DISCUSSION

Alpinia species are a rich source of phenolic compounds, such as chlorogenic acid, rutin, quercetin, epicatechin, catechin, cardamonin, protocatechuic acid, zingerone, curcumin, apigenin, and pinocembrin (*1*, *10*, *19*, *20*, *22*−*24*). In this study, quantitative determination of phenolic compounds in EEAP by HPLC indicated that EEAP contained caffeic acid, chlorogenic acid, ferulic acid, *p*-hydroxybenzoic acid, rutin, apigenin, curcumin,

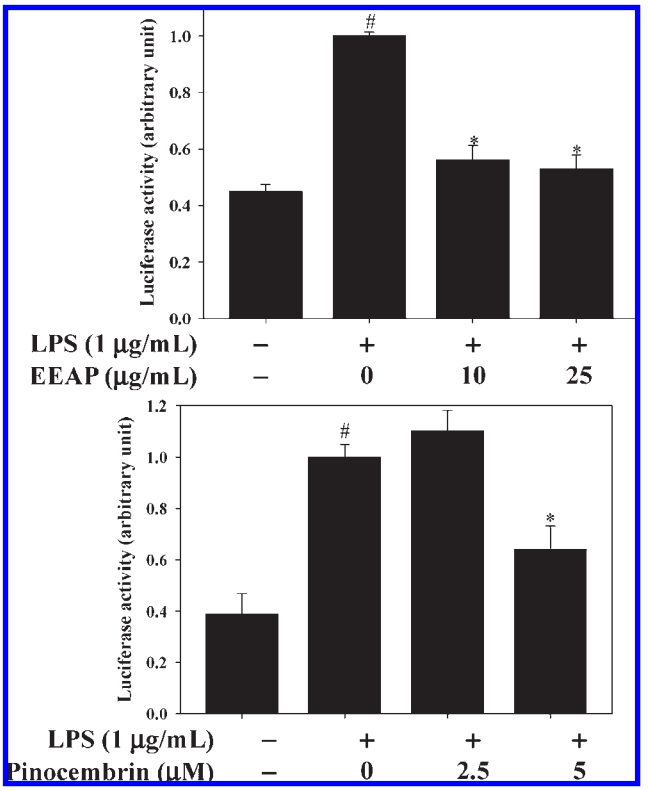

Figure 7. Effects of EEAP and pinocembrin on LPS-induced transcriptional activity of NF-κB in RAW 264.7 cells. Cells were transfected with 1 μg of pNF-κB-Luc reporter gene and then treated with EEAP (0−25 μg/mL) or pinocembrin (0−5 μM) in the absence or presence of LPS (1 μg/mL) for 1 h. Reported values are the means ± SD ($n = 3$). (#) $p < 0.05$ indicates significant differences from the untreated group. (*) $p < 0.05$ indicates significant differences from the LPS treatment alone.

and pinocembrin (**Figure 1** and **Table 1**). There is little information available on the anti-inflammatory effects of phenolic compounds from *A. pricei* Hayata. Therefore, we investigated the anti-inflammatory effects of EEAP and its phenolic compounds in LPS-stimulated RAW 264.7 cells. Our data indicated that EEAP, apigenin, curcumin, and pinocembrin dramatically inhibited NO and PGE_2 production in LPS-stimulated RAW 264.7 cells (**Figures 2** and **3**). However, caffeic acid, chlorogenic acid, ferulic acid, *p*-hydroxybenzoic acid, and rutin did not affect NO production in LPS-stimulated RAW 264.7 cells (data not shown). Many studies indicated that the active compounds (cardamonin and diarylheptanoid) from the *Alpinia* plants inhibit NO production in LPS-stimulated RAW 264.7 cells (*11*−*13*). An examination of the cell viability of EEAP and its phenolic compounds using the MTT assay indicated that these compounds did not affect the viability of the RAW 264.7 cells at all concentrations tested (data not shown). Thus, the inhibitory effects were not attributable to cytotoxicity. EEAP, apigenin, curcumin, and pinocembrin were selected for further analysis. We found that EEAP, apigenin, curcumin, and pinocembrin inhibited protein and mRNA expression of iNOS and COX-2 in the LPS-stimulated RAW 264.7 cells (**Figures 4** and **5**). Israf et al. (*11*) reported that cardamonin from *Alpinia* species inhibited NO production in LPS-stimulated RAW 264.7 cells through inhibition of p65NF-κB nuclear translocation and Iκ-B phosphorylation. Liang et al. (*31*) and Pan et al. (*32*) indicated that apigenin and curcumin have anti-inflammatory effects in LPS-stimulated RAW 264.7 cells. Among the potential phenolic compounds in EEAP, the content of pinocembrin (12.3 mg/g extract) was the

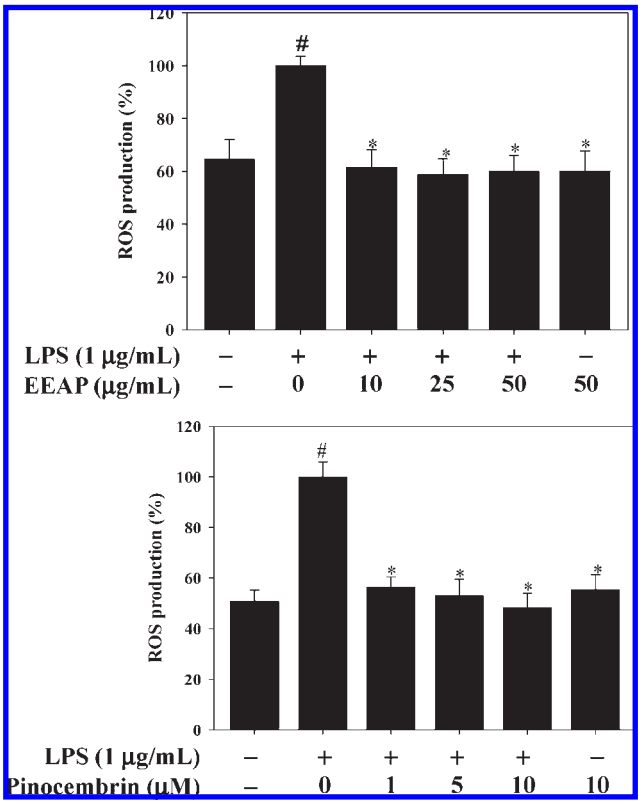

Figure 8. Effects of EEAP and pinocembrin on LPS-induced ROS production in RAW 264.7 cells. Cells were treated with EEAP (0−50 μg/mL) or pinocembrin (0−10 μM) in the absence or presence of LPS (1 μg/mL) for 1 h. Reported values are the means ± SD ($n = 3$). (#) $p < 0.05$ indicates significant differences from the untreated group. (∗) $p < 0.05$ indicates significant differences from the LPS treatment alone.

highest. The test concentrations of apigenin (0−10 μM) and curcumin (0−2.5 μM) do not exist in the composition of EEAP. Therefore, only pinocembrin was selected for a follow-up study.

LPS stimulation of macrophages resulted in the activation of several intracellular signaling pathways that include the MAPK and NF-κB pathway (*33*). Jung et al. (*34*) indicated that the phenolic-rich fraction from *Rhus verniciflua* Stokes suppressed the inflammatory response via modulation of NF-κB and JNK signaling. In the present study, EEAP or pinocembrin decreased p-p65 protein nuclear expression in the LPS-stimulated RAW 264.7 cells (**Figure 6**). EEAP and pinocembrin also significantly inhibited NF-κB transcriptional activity in the LPS-stimulated RAW 264.7 cells (**Figure 7**). The formation of intracellular ROS contributes to atherosclerosis, hypertension, diabetes, and cancer and causes lipid peroxidation, protein modification, and DNA damage (*35*). When macrophages were exposed to LPS, the fluorescence intensity was dramatically enhanced as compared to the unstimulated control cells, indicating that there was generation of intracellular ROS. Shi et al. (*36*) indicated that ROS serves as a messenger in NF-κB activation. NF-κB activates cytokines by acting as a transcriptional factor and binding to NF-κB promoter sequences. Ginn-Pease and Whisler (*37*) indicated that *N*-acetyl-L-cysteine inhibited inflammatory gene expression and NO production through the removal of ROS. In this study, treatment of EEAP or pinocembrin significantly inhibited intracellular ROS generation in LPS-stimulated RAW 264.7 cells (**Figure 8**).

In conclusion, 70% ethanol extracts of roots from *A. pricei* Hayata and its active phenolic compounds inhibited LPS-induced NO, PGE_2, and intracellular ROS production in RAW 264.7 cells. We found that EEAP and pinocembrin inhibited protein and mRNA expression of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells. The anti-inflammatory effects of EEAP and pinocembrin were associated with its strong inhibition of NF-κB nuclear translocation in LPS-stimulated RAW 264.7 cells. The roots of *A. pricei* Hayata may provide therapeutic benefits for inflammatory-based diseases.

ABBREVIATIONS USED

COX-2, cyclooxygenase-2; DCFH-DA, 2′-7′-dichlorofluorescin diacetate; DMSO, dimethylsulfoxide; EEAP, ethanol extracts from *A. pricei* Hayata; HPLC, high-performance liquid chromatography; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; PGE_2, prostaglandin E_2; PVDF, polyvinyldifluoride; ROS, reactive oxygen species; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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