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ARTICLE

Antioxidant and Anti-Inflammatory Effects of *Orthosiphon aristatus* and Its Bioactive Compounds

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Orthosiphon aristatus (Blume) Miq., which can be used as a food ingredient, is grown throughout Southeast Asia and Australia. *O. aristatus* is frequently used for the treatment of renal inflammation, kidney stones and dysuria. The focus of the current work was to study the antioxidant and anti-inflammatory effects of methanol, ethanol and water extracts from *O. aristatus* (abbreviated as MEOA, EEOA and WEOA, respectively). The evaluation of antioxidant activity was determined by total phenolics, Trolox equivalent antioxidant capacity (TEAC), oxygen-radical absorbance capacity (ORAC) and cellular antioxidant activity (CAA) assays. These assays demonstrated a relatively high antioxidant activity for MEOA and EEOA. These results revealed that EEOA had the most prominent inhibitory effect on lipopolysaccharide (LPS)-stimulated nitric oxide (NO), prostaglandin E_2 (PGE_2) and intracellular reactive oxygen species (ROS) production in RAW 264.7 cells. A high performance liquid chromatography profile indicated that MEOA and EEOA contained both ursolic acid and oleanolic acid. Moreover, ursolic acid significantly reduced NO production in LPS-stimulated RAW 264.7 cells. Both EEOA and ursolic acid inhibited LPS-stimulated protein and mRNA expression of both inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in these cells. These results demonstrate that EEOA and its bioactive compound, ursolic acid, suppress LPS-induced NO and PGE_2 production by inhibiting ROS generation, along with reducing expression of iNOS and COX-2 in RAW 264.7 cells.

KEYWORDS: *Orthosiphon aristatus*; antioxidant activity; anti-inflammation; RAW 264.7 cells; ursolic acid

INTRODUCTION

Nitric oxide (NO) is synthesized from the amino acid L-arginine by nitric oxide synthase (NOS). Activated macrophages release NO, a toxic radical that causes cellular alterations, including mutations in DNA, cell apoptosis, and necrosis, that lead to diseases such as cancer and atherosclerosis (*1*). A large amount of NO is produced in response to lipopolysaccharide (LPS), which plays an important role in inflammatory conditions (*2*). Levy et al. (*3*) indicated that inducible nitric oxide synthase (iNOS) is highly expressed in LPS-stimulated macrophages and plays a role in the development of inflammation. Cyclooxygenase-2 (COX-2) is thought to be the predominant cyclooxygenase involved in inflammatory responses (*4*). Cyclooxygenase converts arachidonic acid into prostaglandin E_2 (PGE_2). PGE_2 is overexpressed during inflammation (*5*). The expressions of iNOS and COX-2 are mainly regulated at the transcription level through the activation of several transcription factors, including nuclear factor-κB (NF-κB) (*6*). The DNA binding activity of NF-κB is regulated by a reduction/oxidation mechanism. NF-κB is a critical activator of both iNOS and COX-2 expression, (*7*).

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Java tea (*Orthosiphon aristatus*) (locally known as kidney tea, Misai Kuching, Kumis Kucing, Remujung or Yaa Nuat Maeo) is grown throughout Southeast Asia and Australia. Chau and Wu (*8*) describe the use of *O. aristatus* as a food ingredient in Taiwan. *O. aristatus* is used for the treatment of renal inflammation, kidney stones and dysuria. *O. aristatus* is one of the most popular medicinal plants used in Thai traditional medicine to treat dysuria. Ngamrojanavanich et al. (*9*) demonstrated that a hexane extract of *O. aristatus* can be used in the treatment of dysuria while the hexane extract has an inhibitory effect on the crude enzyme Na^+,K^+-ATPase from the rat brain.

Many studies indicate that the leaves of *O. aristatus* contain several compounds, including neoorthosiphols A, neoorthosiphols B, ursolic acid, oleanolic acid, acetovanillochromene, orthochromene A, orthosiphol A, orthosiphol B, orthosiphonone A, orthosiphonone B, lipophilic flavones, flavonol glycosides and caffeic acid derivatives (*10*−*14*). Methylripariochromene A isolated from the leaves of *O. aristatus* has been shown to treat several human ailments. Matsubara et al. (*15*) show that methylripariochromene A from *O. aristatus* can be used to treat hypertension, while Ohashi et al. (*10*) demonstrated that methylripariochromene A is able to continuously decrease systolic blood pressure in conscious stroke-prone spontaneously hypertensive rats (SHRSP) after subcutaneous administration. However, the

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literature regarding the antioxidant and anti-inflammatory effects of different solvent extracts from *O. aristatus* and its major compounds remains unclear.

The objective of this study was to investigate the antioxidant and anti-inflammatory effects of methanol, ethanol and water extracts from *O. aristatus* (abbreviated as MEOA, EEOA and WEOA, respectively). In addition, an aim was to determine the bioactive compounds in the LPS-stimulated RAW 264.7 murine macrophage cells. In the present work, MEOA, EEOA and WEOA were prepared and evaluated for their antioxidant activity by total phenolics, oxygen radical absorbance capacity (ORAC), Trolox equivalent antioxidant capacity (TEAC) and cellular antioxidant activity (CAA) assays. Moreover, we also examined the effects of MEOA, EEOA and WEOA, as well as the bioactive compounds on the generation of NO, PGE_2 and ROS. Furthermore, we examined the effect of EEOA on the protein and mRNA expression of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells.

MATERIALS AND METHODS

Materials. Lipopolysaccharide (LPS), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], sulfanilamide and anti-β-actin antibody were purchased from the Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from the Merck Co. (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine and the antibiotic mixture (penicillin−streptomycin) were purchased from the Invitrogen Co. (Carlsbad, CA). Anti-COX-2 and anti-iNOS antibodies were purchased from ABcam (Cambridge, MA). Anti-rabbit and anti-mouse secondary horseradish peroxidase conjugated antibodies were purchased from Bethyl Laboratories (Montgomery, TX). Protein molecular mass markers were obtained from Pharmacia Biotech (Saclay, France). Polyvinylidene fluoride (PVDF) membranes for Western blotting were obtained from Millipore (Bedford, MA). All other chemicals were reagent grade.

Sample Preparation. A 20 g dry powder of *O. aristatus* was extracted with methanol, ethanol or water (200 mL) on a rotary shaker at room temperature for 24 h. The methanol, ethanol and water extracts from *O. aristatus* were filtered through Whatman No. 1 filter paper, dried by a vacuum-evaporator and stored at −20 °C until use. The methanol, ethanol and water extracts from *O. aristatus* were named MEOA (methanol extract of *O. aristatus*), EEOA (ethanol extract of *O. aristatus*) and WEOA (water extract of *O. aristatus*).

Determination of Total Phenolic Content. The concentration of total phenolic was measured according to the method described by Taga et al. (*16*) and calculated using gallic acid as a standard. A sample (0.1 mL) was added to 2.0 mL of 0.02 g/mL Na_2CO_3. After 2 min, 50% Folin−Ciocalteu reagent (100 μL) was added to the mixture and then left for 30 min. Absorbance was measured at 750 nm using a spectrophotometer (BMG Labtechnologies, Offenburg, Germany). The total phenolics were calculated as a gallic acid equivalent using the regression equation between gallic acid standard and absorbance.

Trolox Equivalent Antioxidant Capacity (TEAC) Assay. Determination of TEAC was carried out using the method of Arnao et al. (*17*). $ABTS^{\bullet+}$ was generated by the interaction of ABTS (100 μmol/L), H_2O_2 (50 μmol/L), and peroxidase (4.4 U/mL). To measure antioxidant activity, 0.25 mL of serum was mixed well with an equal volume of ABTS, H_2O_2, peroxidase, and 1.5 mL of deionized water. The absorbance was measured at 734 nm after interacting with sample solution for 10 min. The decrease in absorption at 734 nm after the addition of the reactant was used to calculate the TEAC value. A dose−response curve was plotted for Trolox, and antioxidant ability was expressed as the TEAC. The higher the TEAC value of a sample, the stronger the antioxidant activity.

Oxygen Radical Absorbance Capacity (ORAC) Assay. The automated ORAC assay was carried out on a Fluostar Galaxy plate reader (BMG Labtechnologies, Offenburg, Germany) with a fluorescent filter (excitation wavelength of 540 nm and emission wavelength of 565 nm). The procedure was based on a previous report by Cao et al. (*18*) with slight modification. Briefly, in the final assay mixture, 16.7 nM β-phycoerythrin (β-PE) was used as a target of free radical (or oxidant) attack with AAPH (40 mM) as a peroxyl radical generator. Trolox (1 μM) was used as a standard and prepared fresh daily. The analyzer was programmed to record the fluorescence of β-PE every 5 min after AAPH was added. All fluorescence measurements were expressed relative to the initial reading. A final $ORAC_{ROO\cdot}$ value was calculated using the differences of area under the β-PE decay curves between the blank and the sample and expressed as μmol of Trolox equivalents per μmol of sample.

Cellular Antioxidant Activity (CAA) Assay. The quantification of cellular antioxidant activity was determined according to the method of Wolfe and Liu (*19*) with a slight modification. HepG2 cells were plated in a 96-well microtiter plate at a density of 5×10^4 cells/well. After 24 h, the cells were treated with 100 μL of quercetin or extract plus 25 μM DCFH-DA dissolved in the treatment medium for 1 h. The treatment medium was removed, and the cells were incubated with 100 μL of 1 mM AAPH. Fluorescence was measured (excitation wavelength of 485 nm and emission wavelength of 520 nm) with a FLUOstar galaxy fluorescence plate reader (BMG Labtechnologies, Offenburg, Germany) at 37 °C every 5 min for 1 h. Final CAA values were calculated using the CAA unit at each concentration of quercetin and extract and expressed as μmol of quercetin equivalents per g of extract.

High Performance Liquid Chromatography (HPLC) Analysis. The HPLC analysis (Hitachi L-6200 intelligent pump equipped with a photodiode array detector Hitachi L-7455; Hitachi, Tokyo, Japan) used a Mightysil RP-18 column (250 × 4.6 mm, 5 μm) (Kanto Chemical Co., Tokyo, Japan). The HPLC assay for the quantitative determination of ursolic acid and oleanolic acid in MEOA, EEOA and WEOA was carried out as described by Chen et al. (*20*). Elution was performed at room temperature and utilized acetonitrile as solvent A and 1.25% H_3PO_4 in water as solvent B. The mobile phase, consisting of solvent A and B in the proportions 86:14 v/v, was used for elution. The flow rate was 0.5 mL/min. The sample and the standards were injected at a volume of 20 μL each. Ursolic acid and oleanolic acid were identified by comparison of their retention time (t_R) values and UV−visible spectra with those of known standards and were quantified by peak areas from the chromatograms.

Cell Culture. 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 in DMEM with 10% FBS, 2 mM L-glutamine and 100 U/mL penicillin−streptomycin. The cells were cultured at 37 °C in a humidified 5% CO_2 incubator.

Cell Viability Assay. An MTT assay was performed according to the method of Mosmann (*21*). 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 extracts or its active compounds followed by a 24 h incubation. The final concentration of solvent was less than 0.1% in the cell culture medium. Culture medium was removed and replaced by 90 μL of fresh culture medium. Then, 10 μL of sterile filtered MTT solution (5 mg/mL) in phosphate buffered saline (PBS, pH = 7.4) was added to each well, reaching 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 by a FLUOstar galaxy spectrophotometer (BMG Labtechnologies, Offenburg, Germany) at 570 nm. The relative cell viability (presented as a percent) relative to control wells containing cell culture medium without samples was calculated using $A_{570nm}(\text{sample})/A_{570nm}(\text{control}) \times 100$.

Measurement of Nitric Oxide/Nitrite. Nitrite levels in the cultured media, which reflect NOS activity, were determined by Griess reaction. The cells were incubated with either the extracts or its active compounds in the presence or absence 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. Sodium nitrite was used to generate a standard curve (*22*), and the concentration of nitrite was measured by optical density reading at 550 nm.

Measurement of Prostaglandin E_2 (PGE_2). Cells were incubated with EEOA in the presence or absence of LPS (1 μg/mL) for 24 h. PGE_2 level was determined using the prostaglandin E_2 express 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.

Determination of Intracellular Reactive Oxygen Species (ROS) Production. The intracellular ROS production was measured using the oxidant-sensitive fluorescent probe, DCFH-DA. DCFH 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 EEOA in the presence or absence of LPS (1 μg/mL) for 4 h. The cells were harvested by trypsin−EDTA 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 1×10^4 counts were made for each sample. The ROS production (expressed as a percent) was calculated by CELL Quest software.

Western Blot Analysis. The cells were incubated with EEOA and ursolic acid in the presence or absence of LPS (1 μg/mL) for 12 h. After stimulation, cells were collected and lysed in ice-cold lysis buffer [20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 500 μM sodium orthovanadate, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 10 μg/mL leupeptin and 1 mM PMSF]. The protein concentration of the cell lysate was estimated by 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 and transferred to a PVDF membrane. The membrane was blocked with 5% skim milk in PBST (0.05% v/v Tween-20 in PBS, 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 three times in PBST for 10 min each. The signal was detected using the Amersham ECL system (Amersham-Pharmacia Biotech, Arlington Heights, IL). Relative protein expression was quantified by densitometry using the LabWorks 4.5 software and calculated relative to the β-actin reference band.

RNA Extraction and Real-Time RT-PCR. Real-time RT-PCR was performed to determine the level of RAW 264.7 macrophage gene expression. Total RNA from RAW 264.7 cells was isolated using the TRIzol RNA isolation kit (Life Technologies, Rockville, MD) following the manufacturer's protocol. cDNA was synthesized from total RNA (200 ng) by reverse transcription PCR using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The following primer pairs were used: iNOS (Accession No. NM010927), 5′-TCCTACACCACACCAAAC-3′ (forward) and 5′-CTCCAATCTCTGCCTATCC-3′ (reverse); COX-2 (Accession No. NM011198), 5′-CCTCTGCGATGCTCTTCC-3′ (forward) and 5′-TCACACTTATACTGGTCAAATCC-3′ (reverse); GAPDH (Accession No. NM008084), 5′-TCAACGGCACAGT-CAAGG-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 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 manufacturer's protocol. Briefly, this profile was 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, for a total of 40 cycles. Relative levels of gene expression were quantified using the $\Delta\Delta C_t$ method which results in a ratio of target gene expression to equally expressed housekeeping genes.

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

RESULTS

Antioxidant Activities of MEOA, EEOA and WEOA. The dry powders of *O. aristatus* were extracted using different solvents, including methanol, ethanol and water. The extract solutions were filtered, dried and diluted. The yields for MEOA, EEOA and WEOA were 9.18, 11.3 and 7.72%, respectively (**Table 1**). **Table 1** also shows the total phenolics and antioxidant activity of MEOA, EEOA and WEOA. The amounts of total phenolics in MEOA, EEOA and WEOA were 386, 227, and 69.0 mg/g extract, respectively. Antioxidant activities of MEOA, EEOA and WEOA were evaluated by the ORAC, TEAC and CAA assay. The data indicated that the TEAC values (Trolox equivalent, mM/g extract) of MEOA, EEOA and WEOA were 1.18, 0.85 and 0.72, respectively. The ORAC values (Trolox equivalent, mM/g extract) of MEOA, EEOA and WEOA were 17.4, 17.0 and 2.66, respectively. The CAA values (quercetin equivalent, mM/g extract) of MEOA, EEOA and WEOA were 322, 244 and 244, respectively. These data indicated that MEOA had the higher ORAC, TEAC and CAA values than both EEOA and WEOA.

Table 1. The Yields, Total Phenolics and Antioxidant Activities of Different Solvent Extracts from *O. aristatus*

extracts[a]	MEOA	EEOA	WEOA
yield (%)	9.18 ± 0.49	11.3 ± 0.15	7.72 ± 0.30
total phenolics (mg/g extract)	386 ± 19	227 ± 7	69.0 ± 4
TEAC (mmol of TE/g of extract)	1.18 ± 0.03	0.85 ± 0.09	0.72 ± 0.02
ORAC (mmol of TE/g of extract)	17.4 ± 0.87	17.0 ± 0.12	2.66 ± 0.44
CAA (mmol of QE/g of extract)	322 ± 6	244 ± 1	244 ± 6

[a] Reported values are the means ± SD (n = 3). MEOA, methanol extract of *O. aristatus*; EEOA, ethanol extract of *O. aristatus*; WEOA, water extract of *O. aristatus*; TE, Trolox equivalent; QE, quercetin equivalent.

Effects of MEOA, EEOA and WEOA on NO and PGE_2 Production. Figure 1 shows the effects of MEOA, EEOA and WEOA on NO production in LPS-stimulated RAW 264.7 cells. The NO production, measured as nitrite, increased to 38.5 ± 0.8 nM when 1 μg/mL of LPS was added compared to 4.82 ± 0.4 nM in the control cells without LPS. MEOA (50 μg/mL) and EEOA (25 μg/mL) inhibited LPS-stimulated NO production (32.5 ± 0.3 and 28.7 ± 2.0 nM, respective) with no cytotoxicity to the RAW 264.7 cells (**Figure 1A**,**B** and data not shown). However, WEOA did not affect the NO production in the LPS-stimulated RAW 264.7 cells (**Figure 1C**). EEOA had higher inhibition on LPS-stimulated NO production than MEOA. Therefore, we decided to follow EEOA activity. **Figure 2** shows the effect of EEOA on PGE_2 production in the LPS-stimulated RAW 264.7 cells. EEOA (25 μg/mL) significantly reduced LPS-stimulated PGE_2 production (6.35 ± 0.76 ng/mL) in these cells.

Effect of EEOA on Intracellular ROS Production. Intracellular ROS determination was measured using the fluorescent probe DCFH-DA. **Figure 3** shows the effect of EEOA on intracellular ROS production in the LPS-stimulated RAW 264.7 cells. Treatment of these cells with EEOA significantly inhibited the induction of intracellular ROS generation by LPS.

Determination of Major Compounds in MEOA, EEOA and WEOA. In the present study, the quantitative determination of ursolic acid and oleanolic acid in different solvent extracts from *O. aristatus* was performed by HPLC. The analytical plots of ursolic acid and oleanolic acid in MEOA, EEOA and WEOA are shown in **Figure 4**. The peaks corresponded to ursolic acid and oleanolic acid with the retention time of ursolic acid and oleanolic acid at 25.3 and 24.2 min, respectively (**Figure 4A**,**B**). The results from the chromatograms indicated that MEOA and EEOA contained ursolic acid and oleanolic acid as identified by comparison of their retention time values and UV spectra with those of known standards (**Figure 4C**,**D**). Using a standard curve of ursolic acid and oleanolic acid, the amounts of ursolic acid and oleanolic acid in the MEOA were calculated to be 9.38 ± 0.03 and 4.58 ± 0.01 mg/g extract, respectively (**Figure 5**). The levels

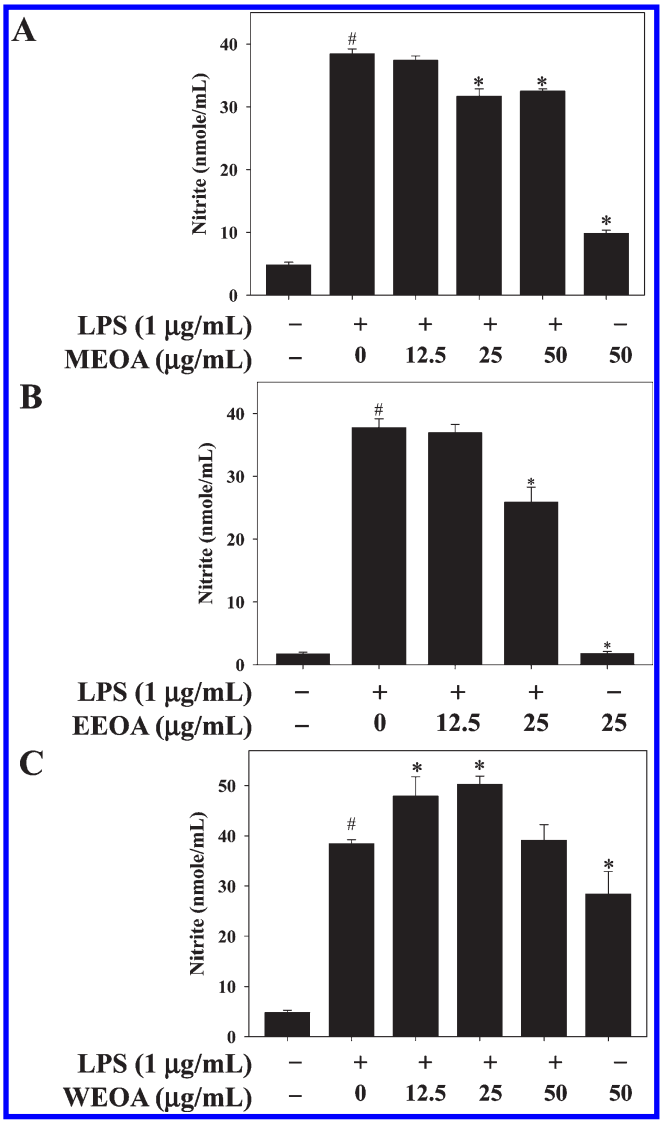

Figure 1. Effects of MEOA (**A**), EEOA (**B**) and WEOA (**C**) on LPS-induced nitrite production in RAW 264.7 cells. The cells were incubated with 0−50 μg/mL of extract in the presence or absence of LPS (1 μg/mL) for 24 h. Reported values are the means ± SD ($n = 3$). $^{\#}p < 0.05$ indicates significant differences from the control group. $^{*}p < 0.05$ indicates significant differences from the LPS treated group.

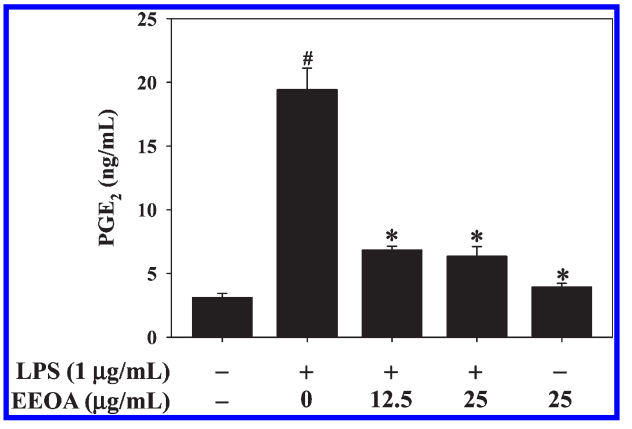

Figure 2. Effect of EEOA on LPS-induced PGE_2 production in RAW 264.7 cells. The cells were incubated with 0−25 μg/mL of EEOA in the presence or absence of LPS (1 μg/mL) for 24 h. Reported values are the means ± SD ($n = 3$). $^{\#}p < 0.05$ indicates significant differences from the control group. $^{*}p < 0.05$ indicates significant differences from the LPS treated group.

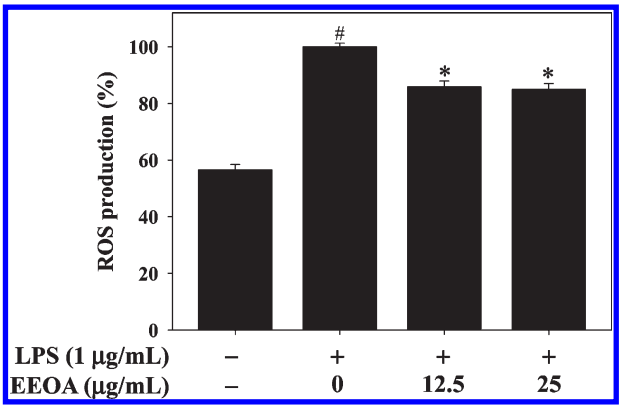

Figure 3. Effect of EEOA on LPS-induced ROS production in RAW 264.7 cells. The cells were incubated with 0−25 μg/mL of EEOA in the presence or absence of LPS (1 μg/mL) for 4 h. Reported values are the means ± SD ($n = 3$). $^{\#}p < 0.05$ indicates significant differences from the control group. $^{*}p < 0.05$ indicates significant differences from the LPS treated group.

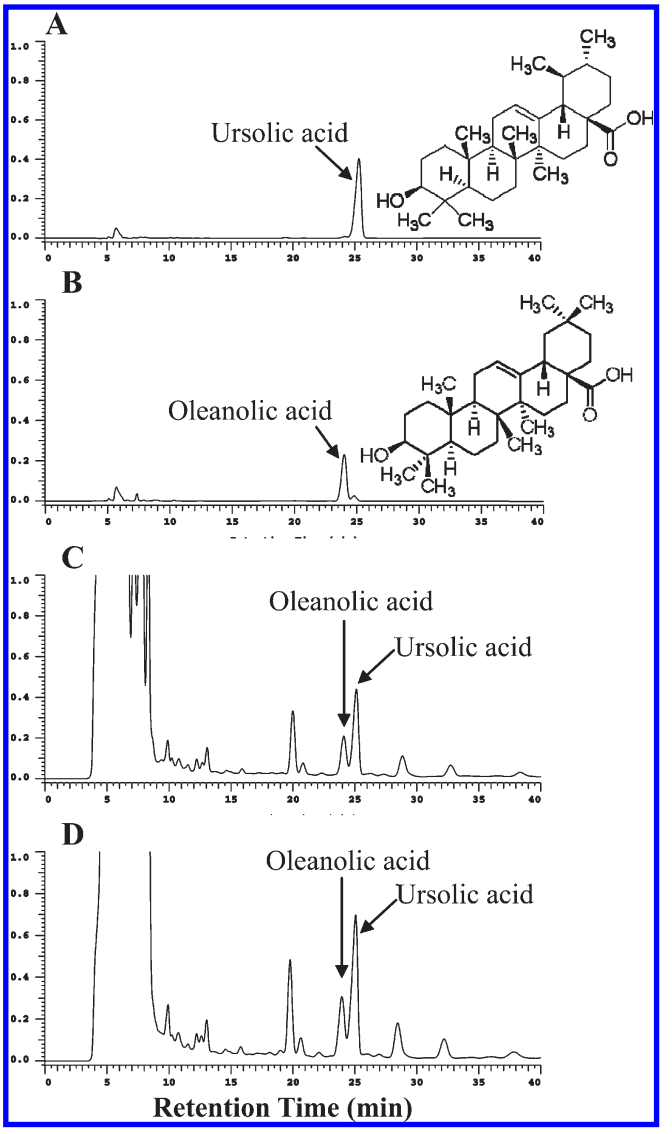

Figure 4. HPLC chromatograms of ursolic acid (**A**), oleanolic acid (**B**), MEOA (**C**) and EEOA (**D**).

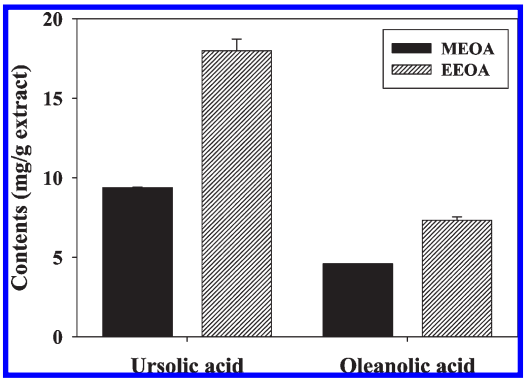

Figure 5. The amounts of ursolic acid and oleanolic acid in MEOA and EEOA. Reported values are the means ± SD (n = 3).

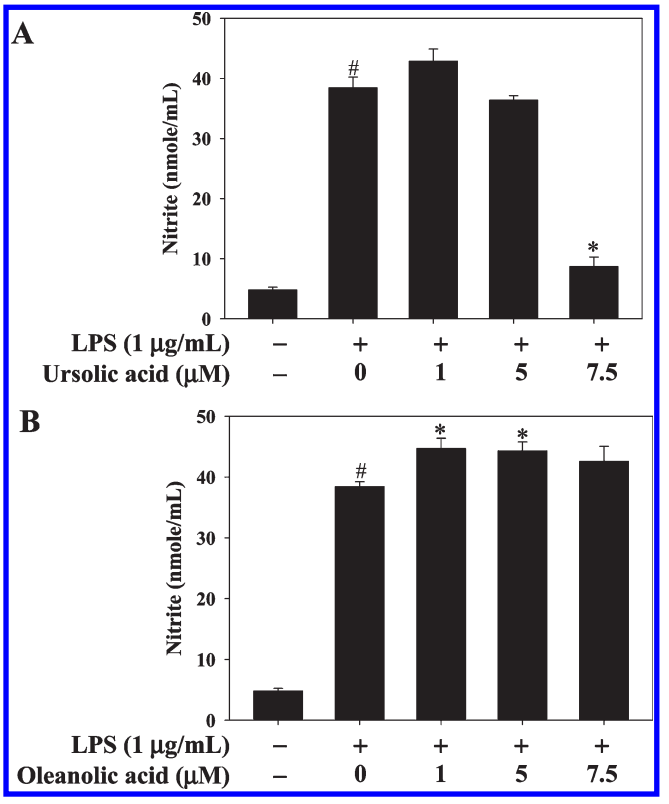

Figure 6. Effects of ursolic acid (**A**) and oleanolic acid (**B**) on LPS-induced nitrite production in RAW 264.7 cells. The cells were incubated with 0−7.5 μM of the compounds in the presence or absence of LPS (1 μg/mL) for 24 h. Reported values are the means ± SD (n = 3). $^{\#}p < 0.05$ indicates significant differences from the control group. $^{*}p < 0.05$ indicates significant differences from the LPS treated group.

of ursolic acid and oleanolic acid in the EEOA were 18.0 ± 0.72 and 7.32 ± 0.22 mg/g extract, respectively (**Figure 5**). Furthermore, it was determined that WEOA does not contain ursolic acid or oleanolic acid (data not shown).

Effects of Ursolic Acid and Oleanolic Acid on NO Production. **Figure 6** shows the effects of ursolic acid and oleanolic acid on NO production in the LPS-stimulated RAW 264.7 cells. These results indicated that ursolic acid (7.5 μM) inhibited LPS-stimulated NO production (decrease from 38.45 ± 1.77 to 8.72 ± 1.56 nM) and showed no cytotoxicity (data not shown) in RAW 264.7 cells (**Figure 6A**). The concentrations used in the present study were consistent with those used in other studies examining the anti-inflammatory effect of ursolic and oleanolic acids in RAW 264.7 cells (*23*). However, oleanolic acid did not inhibit LPS-stimulated

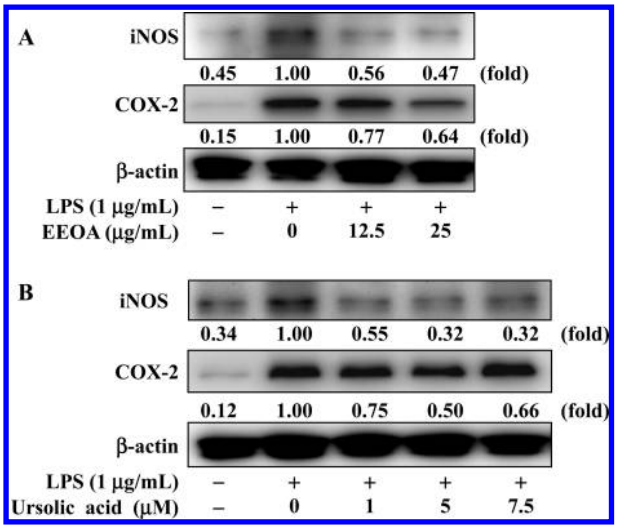

Figure 7. Effects of EEOA (**A**) and ursolic acid (**B**) on LPS-induced iNOS and COX-2 protein expression in RAW 264.7 cells. The cells were incubated with EEOA (0−25 μg/mL) or ursolic acid (0−7.5 μM) in the presence or absence of LPS (1 μg/mL) for 12 h. The relative protein expression was quantified using densitometry and LabWorks 4.5 software, and calculated in reference to the β-actin reference bands.

NO production in these cells (**Figure 6B**). Therefore, EEOA and ursolic acid were further examined.

Effects of EEOA and Ursolic Acid on Protein and mRNA Expression of iNOS and COX-2. The effects of EEOA and ursolic acid on iNOS and COX-2 protein expression in the LPS-stimulated RAW 264.7 cells were examined by Western blot analysis. **Figure 7** shows the effects of EEOA and ursolic acid on iNOS and COX-2 protein expression in the LPS-stimulated cells. LPS at 1 μg/mL induced a significant increase in iNOS and COX-2 protein expression compared to control cells without LPS. The addition of EEOA (0−25 μg/mL) or ursolic acid (0−7.5 μM) simultaneously with LPS (1 μg/mL) for 12 h resulted in an inhibitory effects of EEOA and ursolic acid on iNOS and COX-2 protein expression in a dose-dependent manner.

Figure 8 shows the effects of EEOA and ursolic acid on iNOS and COX-2 mRNA expression in the LPS-stimulated RAW 264.7 cells. When EEOA (0−25 μg/mL) or ursolic acid (0−7.5 μM) was added to the medium simultaneously with LPS (1 μg/mL) for 4 h, both EEOA and ursolic acid inhibited iNOS and COX-2 mRNA expression in the LPS-stimulated RAW 264.7 cells.

DISCUSSION

Java tea (*O. aristatus*) is used medicinally to treat renal inflammation, kidney stones and dysuria. Several studies have indicated that this plant contains many different compounds and derivatives (*10*−*15*). The objective of the current study was to investigate the antioxidant and anti-inflammatory effects of different solvent extracts from *O. aristatus* and identify its bioactive compounds in LPS-stimulated RAW 264.7 cells. Prior et al. (*24*) provides a basis and rationale for developing standardized antioxidant capacity methods. Three assays have been proposed for standardization, including total phenolics, ORAC and TEAC assay. In the present study, total phenolics, ORAC, TEAC and CAA methods were used for the evaluation of antioxidant activity of MEOA, EEOA and WEOA. The MEOA had the highest total phenolics, ORAC, TEAC and CAA values compared to EEOA and WEOA (**Table 1**).

NO is synthesized from L-arginine by NOS and plays an important role in the regulation of many diseases (*1*). Under pathological

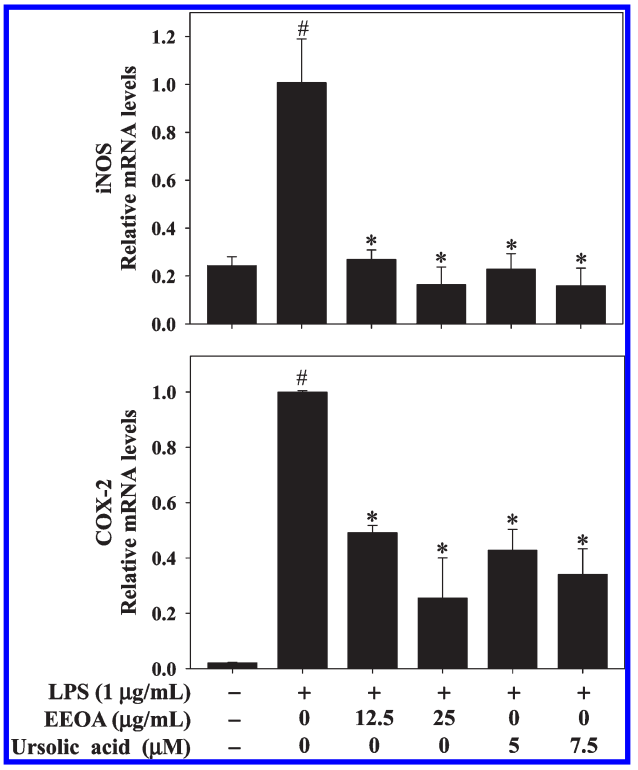

Figure 8. Effects of EEOA and ursolic acid on LPS-induced iNOS and COX-2 mRNA expression in RAW 264.7 cells. The cells were incubated with EEOA (0−25 μg/mL) or ursolic acid (0−7.5 μM) in the presence or absence of LPS (1 μg/mL) for 4 h. Reported values are the means ± SD ($n = 3$). $^{\#}p < 0.05$ indicates significant differences from the untreated group. $^{*}p < 0.05$ indicates significant differences from the LPS treatment alone.

conditions, NO production is increased by inducible NOS (iNOS), which subsequently brings about cytotoxicity and tissue damage (*25*). Our data shows that MEOA and EEOA have a marked inhibitory action toward NO production in LPS-stimulated RAW 264.7 cells (**Figure 1**). The inhibition of LPS-stimulated NO production was higher in EEOA treated cells compared to MEOA treated cells.

An examination of the cell viability in the presence of MEOA, EEOA and WEOA in RAW 264.7 cells indicated that the concentrations of these compounds used in this study did not affect the viability of the RAW 264.7 cells (data not shown). Thus, the inhibitory effects on NO production are not attributable to cytotoxic effects. Therefore, EEOA was further studied.

Murata et al. (*5*) indicated that PGE_2 is a principal mediator of inflammation in inflammatory diseases. Pong et al. (*26*) indicated that ROS induces oxidative damage in biomolecules and causes atherosclerosis, hypertension, diabetes and cancer. In the present study, EEOA significantly reduced LPS-stimulated PGE_2 and ROS production in RAW 264.7 cells (**Figures 2** and **3**).

Yoshimura et al. (*14*) suggested that *O. aristatus* contain triterpenoid compounds, such as ursolic acid and oleanolic acid. Ursolic acid is found in many plants and is known to have anti-inflammatory activity (*27*). Akowah et al. (*28*) reported that ursolic acid and oleanolic acid isolated from *Orthosiphon stamineus*, a related species, has free radical scavenging activity. In the present study, the quantitative identification of ursolic acid and oleanolic acid in MEOA, EEOA and WEOA was determined by HPLC. Among the triterpenoid compounds in MEOA, EEOA and WEOA, the amounts of ursolic acid and oleanolic acid in EEOA were higher than those of the other extracts (**Figures 4** and **5**). Moreover, ursolic acid significantly reduced NO production in LPS-stimulated RAW 264.7 cells (**Figure 6A**). However, oleanolic acid did not inhibit NO production in LPS-stimulated RAW 264.7 cells (**Figure 6B**). Wang et al. (*29*) also showed that oleanolic acid did not inhibit LPS-stimulated NO production in these cells.

NO, a toxic radical known to cause many diseases such as cancer and atherosclerosis, is released during inflammatory responses. Salerno et al. (*30*) indicated that enhanced gene expression of iNOS and COX-2 is also associated with inflammatory responses. iNOS is expressed in vascular smooth muscle cells, macrophages and hepatocytes. iNOS is induced in response to pro-inflammatory cytokines and bacterial LPS (*31*). COX appears to have an important role in the conversion of arachidonic acid to PGE_2 and is a rate-limiting enzyme in the biosynthesis of prostaglandins (*32*). Posadas et al. (*33*) showed that pro-inflammatory mediators, such as NO and PGE_2, are generated by iNOS and COX-2. As shown in **Figure 7**, EEOA and ursolic acid inhibited iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 cells. Our data also showed the inhibitory effects of EEOA and ursolic acid on iNOS and COX-2 mRNA expression in LPS-stimulated RAW 264.7 cells (**Figure 8**). These results suggest that EEOA and ursolic acid inhibit NO and PGE_2 production through the suppression of iNOS and COX-2 expression at both the protein and the mRNA level in LPS-stimulated RAW 264.7 cells.

In conclusion, the ethanol extract of *O. aristatus* and its bioactive compound (ursolic acid) are able to inhibit LPS-stimulated NO, PGE_2 and intracellular ROS production in RAW 264.7 cells. We observed that EEOA and ursolic acid are also able to inhibit protein and mRNA expression of iNOS and COX-2 in the LPS-stimulated RAW 264.7 cells. Taken together, the ethanol extract of *O. aristatus* may provide a beneficial effect for inflammatory-mediated diseases.

ABBREVIATIONS USED

COX-2, cyclooxygenase-2; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DMSO, dimethyl sulfoxide; EEOA, ethanol extract of *Orthosiphon aristatus*; HPLC, high performance liquid chromatography; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MEOA, methanol extract of *O. aristatus*; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate buffered saline; PGE_2, prostaglandin E_2; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TNF-α, tumor necrosis factor-alpha; WEOA, water extract of *O. aristatus*.

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