JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Bioassay Guided Isolation and Identification of Anti-inflammatory Active Compounds from the Root of *Ficus formosana*

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Supporting Information

ABSTRACT: Activity-directed fractionation and purification processes were employed to identify the anti-inflammatory active compounds using lipopolysaccharide (LPS)-stimulated mouse macrophage (RAW264.7) in vitro. Air-dried roots of *Ficus formosana* were extracted with methanol and separated into *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and water layers. Among them, the chloroform layer showed strong activity and was subjected to separation and purification by using various chromatographic techniques. Five compounds showing potent activity were identified by comparing spectral data to be β-sitosterol, stigmasterol, psoralen, kaempferol, carpachromene, and syringic aldehyde. When macrophages were treated with psoralen and kaempferol together with LPS, a concentration-dependent inhibition of nitric oxide (NO) and tumor necrosis factor (TNF-α) productions were detected. Western blotting revealed that kaempferol, psoralen, and carpachromene blocked protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in LPS-stimulated macrophages. The results confirmed that the traditional use of *F. formosana* could be a potential anti-inflammatory agent.

KEYWORDS: *Chinese herb, Ficus formosana, anti-inflammation, NO, TNF-α*

INTRODUCTION

Inflammation involves a complex series of reactions regulated by a cascade of cytokines, growth factors, and nitric oxide (NO) produced by active macrophages. Many scientific studies on anti-inflammatory compounds from natural products have investigated the potential inhibitory effects in vitro system, lipopolysaccharide (LPS)-stimulated macrophage.[1] Using this system, bacterial LPS has become one of the best characterized stimuli used to release of several inflammatory mediators including NO, TNF-α, and induce the up-regulation of proinflammatory proteins such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS).[2] When over-produced in response to LPS stimulation, excess NO can react with superoxide anion radicals to form peroxynitrite, which can cause a high oxidative stress.[3] High oxidative stress has been shown to be associated with chronic diseases, including asthma, rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, and Alzheimer's disease and various human cancers.[4]

Ficus formosana Maxim is a small evergreen tree with glabrous branchlets and membranaceous leaves, which is distributed in mainland China, Hong Kong, and Taiwan. The dried roots of *F. formosana* have been used in traditional oriental medicine food as expel wind, drain dampness, active blood, and ease pain in the Taiwan. It has curative effects for neuralgia, rheumatoid arthritis, and acute mastitis. Scientific studies reported that *F. formosana* has antitumor activities. Carpachromene, apigenin, and norartocarpetin from *F. formosana* exhibited cytotoxicity against HepG2, PLC/PRF/5, and Raji cancer cell lines in vitro.[5] However, little information is available on the anti-inflammatory effects of *F. formosana*. Therefore, we used this strategy, combined with chromatography to isolate the active components and used LPS-stimulated mouse macrophage RAW264.7 cells to confirm the in vitro anti-inflammatory effects of *F. formosana*.

MATERIALS AND METHODS

Chemicals. LPS (endotoxin from *Escherichia coli*, serotype 0127:B8), MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tumor necrosis factor (TNF-α) was purchased from Biosource International Inc. (Camarillo, CA, USA). Anti-iNOS, anti-COX-2, and anti-β-actin antibody (Santa Cruz, USA) and a protein assay kit (Bio-Rad Laboratories Ltd., Watford, Herts, U.K.) were obtained as indicated. Poly-(vinylidene fluoride) membrane (Immobilon-P) was obtained from Millipore Corp. (Bedford, MA, USA). The root of *F. formosana* was collected in Tai

Received: August 1, 2013
Revised: October 28, 2013
Accepted: October 30, 2013
Published: November 8, 2013

dx.doi.org/10.1021/jf4033766 | J. Agric. Food Chem. 2013, 61, 11008−11015

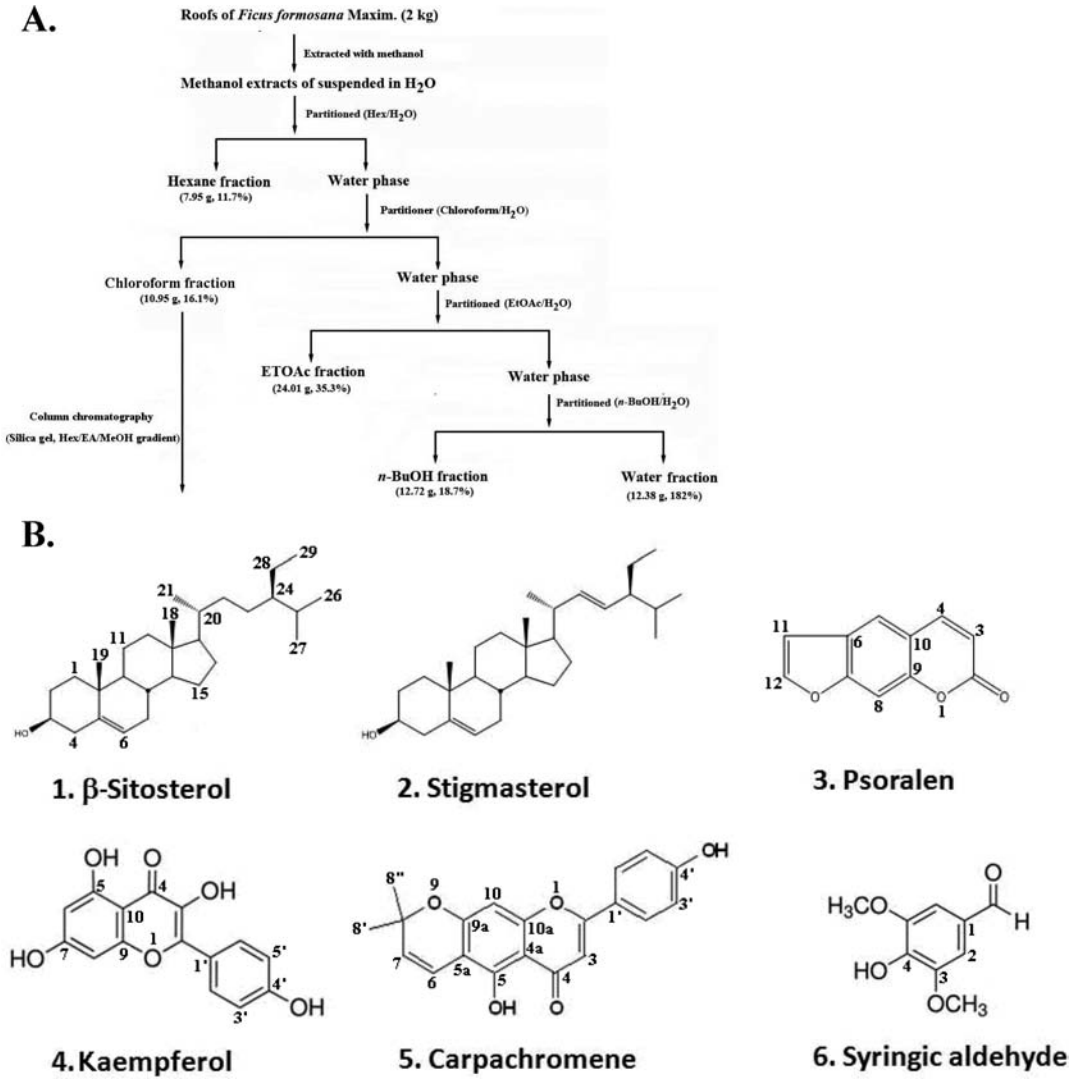

Figure 1. Flowchart of the separation methods used to isolate the anti-inflammatory agents of *F. formosana* Maxim. (FF) (A). The FF-chloroform fraction exhibited the most potent anti-inflammatory activities. The structures of *β*-sitosterol (**1**), stigmasterol (**2**), psoralen (**3**), kaempferol (**4**), carpachromene (**5**), and syringic aldehyde (**6**) (B).

Chung, Taiwan. Plant materials were collected from Taichung country of Taiwan. They were identified and authenticated by Dr. Shyh-Shyun Huang, Assistant Professor, School of Pharmacy, China Medical University, Taichung, Taiwan. Extracts were chromatographed on silica gel (Merk 230−400 mesh) and purified with crystallization. The purity was confirmed by using a semiperative normal phase HPLC column [250 mm × 10 mm, Phenomenex Luna 5*μ* Silica(2) 100A]. Voucher specimens (CMU 2010−01105) were deposited at Institute of Chinese Pharmaceutical Science, China Medical University, Taichung, Taiwan.

Isolation and Determination of the Active Compound. Air-dried pieces of *F. formosana* root (2 kg) were extracted three times with methanol (5 L) at room temperature (7 days each time). The extract was evaporated under reduced pressure using a rotavapor and then stored under light protection. A yield equivalent to 3.4% of the original weight was obtained. Next, methanol extract of *F. formosana* (68 g) was dissolved and suspended in 100 mL of water in a separatory funnel prior to be partitioned in sequence with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol (800 mL each for three times). Under reduced pressure, fractions were yielded and collected: *n*-hexane fraction (7.95 g, 11.7%), chloroform fraction (10.94 g, 11.1%), ethyl acetate fraction (24.01 g, 35.3%), *n*-butanol fraction (12.72 g, 18.7%), and aqueous fraction (12.38 g, 18.2%). All extracts were stored in the refrigerator before the use (Figure 1A). The chloroform fraction (10 g) was chromatographed on the open column of silica gel using *n*-hexane and EtOAc of increasing polarity as eluent. A mixture of *β*-sitosterol and stigrasterol and psoralen were eluted with *n*-hexane:EtOAc (90:10), and syringic aldehyde was isolated from the eluting with 20% EtOAc in *n*-hexane. Carpachromene and kaempferol were obtained from the eluent with 30% and 50% EtOAc in *n*-hexane, respectively. The normal phase HPLC was used to confirm the purity.

Cell Culture. A murine macrophage cell line RAW264.7 (BCRC no. 60001) was purchased from the Bioresources Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in plastic dishes containing Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA) in a CO_2 incubator (5% CO_2 in air) at 37 °C and subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin−0.02% EDTA in Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (DPBS).

Cell Viability. Cells (2×10^5) were cultured in 96-well plate containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were cultured with samples in the presence of 100 ng/mL LPS for 24 h. After that, the cells were washed twice with DPBS and incubated with 100 *μ*L of 0.5 mg/mL MTT for 2 h at 37 °C testing for cell viability. The medium was then discarded, and 100 *μ*L of dimethyl sulfoxide (DMSO) was added. After 30 min

incubation, absorbance at 570 nm was read by using a microplate reader (Molecular Devices, Orleans Drive, Sunnyvale, CA).

Measurement of Nitric Oxide/Nitrite. NO production was indirectly assessed by measuring the nitrite levels in the cultured media and serum determined by a colorimetric method based on the Griess reaction.[6] The cells were incubated with a test sample in the presence of LPS (100 ng/mL) at 37 °C for 24 h. Then, 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% naphthyl ethylenediamine dihydrochloride, and 5% phosphoric acid) and incubated at room temperature for 10 min, the absorbance was measured at 540 nm with a Micro-Reader (Molecular Devices, Orleans Drive, Sunnyvale, CA). By using sodium nitrite to generate a standard curve, the concentration of nitrite was measured form absorbance at 540 nm.

Measurement of TNF-α by an Enzyme-Linked Immunosorbent Assay (ELISA). The level of TNF-α was determined using a commercially available ELISA kit (Biosource International Inc., Camarillo, CA) according to the manufacturer's instruction. TNF-α was determined from a standard curve.

Protein Lysate Preparation and Western Blot Analysis of iNOS and COX-2. The stimulated murine macrophage cell line RAW264.7 cells were washed with PBS and lysed in an ice-cold lysis buffer [10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM EGTA, 10 mM NaF, 1 mM $Na_4P_2O_7$, 20 mM Tris buffer (pH 7.9), 100 mM β-glycerophosphate, 137 mM NaCl, 5 mM EDTA, and one protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA)] on ice for 1 h, followed by centrifugation at 12000*g* for 30 min at 4 °C, and 30 μg of protein from the supernatants was then separated on 10% sodium dodecylsulphate−polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. After transfer, the membrane was blocked for 2 h at room temperature with 5% skim milk in Tris-buffered saline−Tween (TBST; 20 mM Tris, 500 mM NaCl, pH 7.5, 0.1% Tween 20). The membranes were then incubated with mouse monoclonal anti-iNOS or anti-COX-2 (1:1000 dilution) antibody in 5% skim milk in TBST for 2 h at room temperature. The membranes were washed three times with TBST at room temperature and then incubated with a 1:2000 dilution of antimouse IgG secondary antibody conjugated to horseradish peroxidase (Sigma, St. Louis, MO, USA) in 2.5% skim milk in TBST for 1 h at room temperature. The membranes were washed three times, and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL) using hyperfilm and ECL reagent (Amersham International PLC, Buckinghamshire, UK). The results of Western blot analysis were quantified by measuring the relative intensity compared to the control using Kodak Molecular Imaging Software (version 4.0.5, Eastman Kodak Company, Rochester, NY) and represented in the relative intensities.

Statistical Analysis. Experimental results were presented as the mean ± standard deviation (SD) of three parallel measurements. IC_{50} values were estimated using a nonlinear regression algorithm (SigmaPlot 8.0; SPSS Inc. Chicago, IL). Statistical evaluation was carried out by one-way analysis of variance (ANOVA followed by Scheffe's multiple range tests). Statistical significance is expressed as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

RESULTS AND DISCUSSION

Effects of *F. formosana* and Partitioned Fractions on LPS-Induced NO and TNF-α Productions in RAW 264.7 Cells. Excessive NO produced by iNOS plays critical roles in the chronic inflammatory diseases such as organ dysfunction (severe sepsis), inflammatory hepatic dysfunctions, and inflammatory lung disease.[7] Many of disease conditions exhibit rapid development, often resulting in the failure of anti-inflammatory therapies; a suppression of NO production pathways may satisfy the unmet need for control of the inflammatory process.[6]

Macrophage cells or other cell lines are useful materials with a steady high-level production of NO in vitro models. The purpose of this paper was to identify the compounds in *F. formosana* (FF) that mediate the observed pro-inflammatory and anti-inflammatory effects. The effects of FF and its fractions (10−100 μg/mL) on the formation of NO in RAW 264.7 macrophages stimulated with LPS (100 ng/mL). Cell viability was determined by using an MTT assay in the effect of FF extract and fractions. Cells cultured with FF extract and fractions at the concentrations (0, 10, 20, and 40 μg/mL) used in the presence of 100 ng/mL LPS for 24 h did not change cell viability, significantly (Figure 2A). NO production decreased slightly in the presence of the FF (M), FF-hexane fraction (HF), FF-ethyl-acetate fraction (EAF), FF-butanol fraction (BF), and FF-water fraction (WF), whereas NO production was significantly inhibited by the FF-chloroform fraction (CF) in a dose-dependent manner (Figure 2B). The IC_{50} value for inhibition of NO production of FF-chloroform was about 39.2 ± 0.6 μg/mL.

TNF-α, a pro-inflammatory cytokine, mediates the actions of multiple cytokines during inflammation such as the production of interleukin-1 beta (IL-1β) and interleukin-6 (IL-6).[8] We examined the effect of FF-chloroform on LPS induced up-regulation of TNF-α. Very low levels for TNF-α protein were detected in controls (Figure 2C). When RAW264.7 macrophages were treated with FF-chloroform (10, 20, and 40 μg/mL) together with LPS for 24 h, a concentration-dependent inhibition of TNF-α production was detected. There was either a decrease in the TNF-α production of group treated with 10 μg/mL FF-chloroform ($p < 0.05$) or highly significant decrease of groups treated with 20 and 40 μg/mL of FF-chloroform when compared with the LPS-alone group ($p < 0.01$ or $p < 0.001$), respectively. The IC_{50} value of FF-chloroform for inhibition of TNF-α production was about 26.3 ± 0.2 μg/mL.

TNF-α is therefore thought to be a principal mediator in the LPS-inducible tissue injury. TNF-α elicits a number of physiological effects such as inflammation, cachexia, and cytotoxicity.[9,10] In this study, we evidenced that FF-chloroform fraction decreased the TNF-α level in LPS-induced RAW 264.7 Cells. Because FF-chloroform fraction was the more effective inhibition in the NO production, we used silica gel chromatography to separate this FF-chloroform fraction that was tested for their anti-inflammatory activity. β-Sitosterol (**1**), stigmasterol (**2**), psoralen (**3**), kaempferol (**4**), carpachromene (**5**), and syringic aldehyde (**6**) were identified in the FF-chloroform fraction. This is the first time that psoralen (**3**), kaempferol (**4**), and syringic aldehyde (**6**) have been found in FF.

Inhibition of LPS-Induced iNOS and COX-2 Protein by FF-Chloroform Fraction. INOS and COX-2 are important enzymes that mediate inflammatory pathways that produce NO, TNF-α, and IL-6.[11] In this study, Western blotting was used to determine whether the test samples inhibited the expression of iNOS and COX-2 in LPS-induced RAW 264.7 macrophages. The RAW 264.7 cells that were treated with LPS (100 ng/mL) dramatically increased their expression of COX-2 and iNOS. The results showed that incubation with FF-chloroform fraction in the presence of LPS for 24 h inhibited iNOS and COX-2 protein expressions in mouse macrophage RAW264.7 cells in a dose-dependent manner (Figure 3A). The intensity of protein bands were analyzed and showed an average of 63.8% and 60.7% down-regulation of iNOS and COX-2 proteins, respectively, after treatment with FF-chloroform fraction at 40 μg/mL compared with the LPS-alone (Figure 3B).

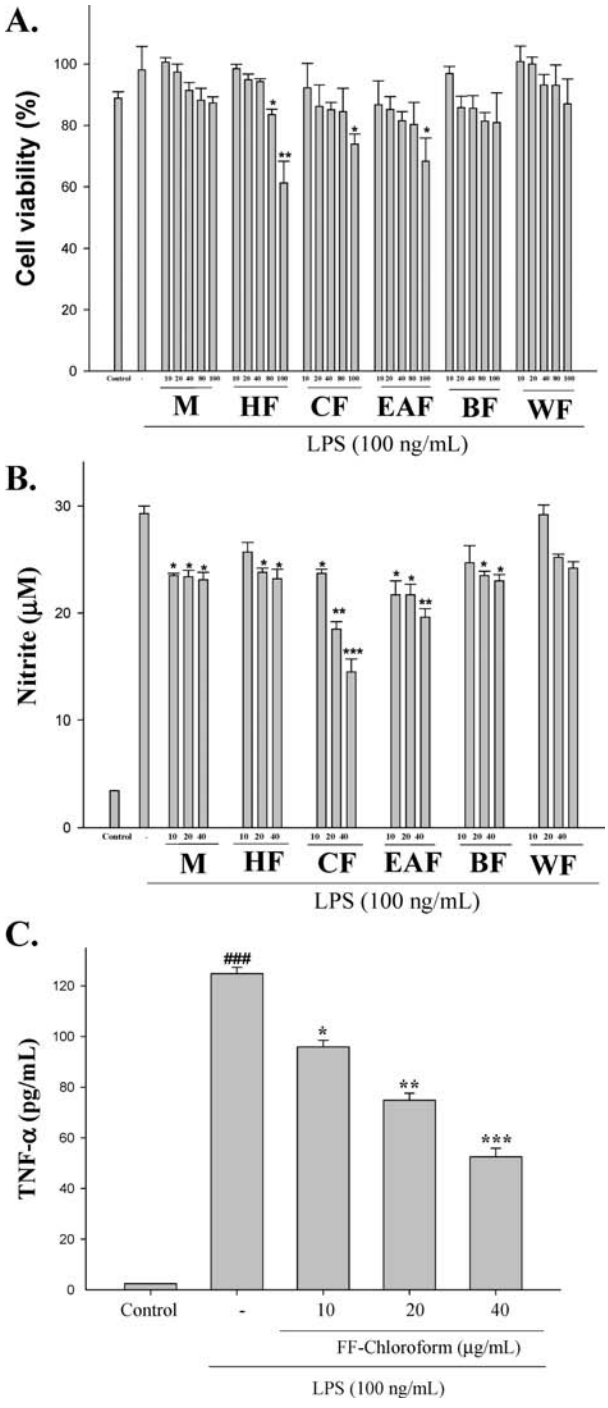

Figure 2. Cytotoxic effects of the methanol extract and fractions from the roots of *F. formosana* Maxim in RAW264.7 cell (A), and the effects of the methanol extract and fractions on LPS-induced NO (B) and TNF-α (C) productions of RAW264.7 macrophages. Cells were incubated for 24 h with 100 ng/mL of LPS in the absence or presence of samples (0, 10, 20, 40, 80, and 100 μg/mL). Samples were added 1 h before incubation with LPS. Cell viability assay was performed using MTT assay. Nitrite concentration in the medium was determined using Griess reagent. TNF-α level in the medium were determined using ELISA kit. M, methanol extract; HF, *n*-hexane fraction; CF, chloroform fraction; EAF, ethyl acetate fraction; BF, *n*-butanol fraction; WF, water fraction. The data were presented as mean $\pm$ SD for three different experiments performed in triplicate. ### compared with sample of control group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were compared with LPS-alone group.

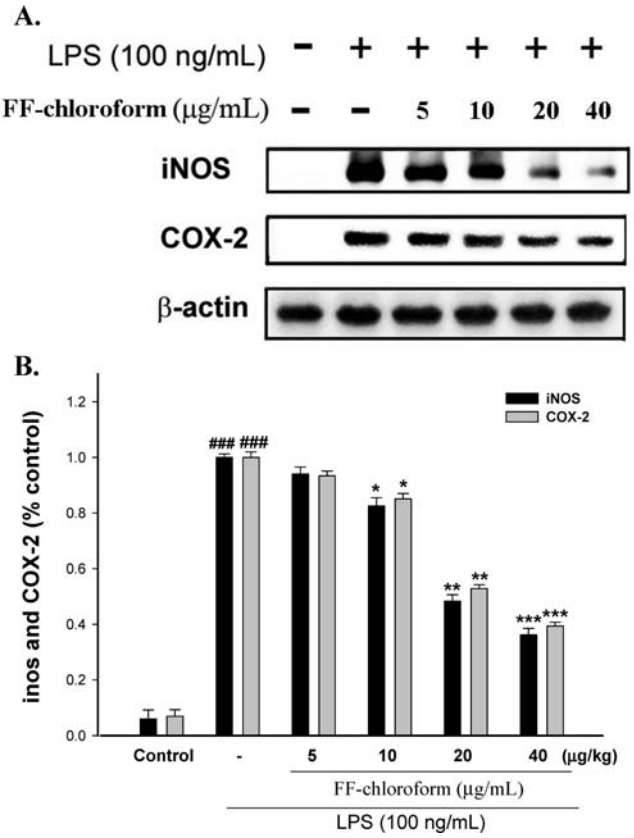

Figure 3. Inhibition of iNOS and COX-2 protein expressed by FF-chloroform in LPS-stimulated RAW264.7 cells. Cells were incubated for 24 h with 100 ng/mL of LPS in the absence or the presence of FF-chloroform (0, 10, 20, and 40 μg/mL). FF-chloroform was added 1 h before incubation with LPS. Lysed cells were then prepared and subjected to Western blotting using an antibody specific for iNOS and COX-2. β-actin was used as a quantity control. (A) A representative Western blot from two separate experiments is shown. (B) Relative iNOS and COX-2 protein levels were calculated with reference to a LPS-stimulated culture. The data were presented as mean $\pm$ SD for three different experiments performed in triplicate. ### compared with sample of control group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were compared with LPS-alone group.

NO is synthesized from L-arginine by the enzyme iNOS, thereby causing detrimental effects. NO is described as highly toxic and reactive, it has beneficial antiviral, microbicidal, antiparasital, and antitumor effects, and the excessive of NO causes cell injury by generating reactive radicals such as peroxynitrite.[12] The molecular mechanisms by which FF-chloroform fraction inhibits inflammatory activity have not been elucidated. Results in vitro showed that FF-chloroform fraction suppressed NO production in LPS-induced macrophage, the expression of inflammatory protein products such as iNOS and COX-2. Examination of the cytotoxicity of FF-chloroform fraction in RAW264.7 macrophages using MTT assay has indicated that FF-chloroform fraction even at 40 μg/mL did not affect the viability of RAW264.7 cells. Therefore, the inhibition of LPS-induced nitrite production by FF-chloroform fraction was not cytotoxic effect on RAW264.7 cells.

Effects of β-Sitosterol (1), Stigmasterol (2), Psoralen (3), Kaempferol (4), Carpachromene (5), and Syringic Aldehyde (6) on LPS-Induced NO and TNF-α Formation in RAW 264.7 Cells. Isolation of compounds (1.25−10 μg/

dx.doi.org/10.1021/jf4033766 | J. Agric. Food Chem. 2013, 61, 11008−11015

Table 1. Effects of Compounds (Mix of β-Sitosterol and Stigmasterol, Psoralen, Kaempferol, Carpachromene, and Syringic Aldehyde) Isolated from FF-Chloroform on Lipopolysaccharide (LPS)-Induced Cell Viability, NO, and TNF-α Productions of RAW 264.7 Macrophages[a]

	dose (μg/mL)	cell viability (% of control)	NO level (μM)	TNF-α (pg/mL)
control	(−)	98.5 ± 1.3	0.4 ± 0.1	2.3 ± 0.3
LPS	(+)	99.6 ± 2.8	32.7 ± 0.8$^{\#\#\#b}$	121.7 ± 0.3$^{\#\#\#b}$
β-sitosterol and stigmasterol	1.25	98.3 ± 3.5	30.4 ± 0.9	67.7 ± 1.9
	2.5	93.7 ± 5.2	29.4 ± 0.3	46.5 ± 1.5^{**d}
(mixed)	5	83.3 ± 4.1	28.7 ± 1.1	34.3 ± 1.3^{***e}
	10	80.5 ± 0.3	28.2 ± 0.1	21.9 ± 0.4^{***e}
psoralen	1.25	89.6 ± 1.1	17.0 ± 0.2^{**d}	110.2 ± 0.5
	2.5	84.7 ± 2.1	12.1 ± 0.4^{***e}	84.3 ± 1.1^{*c}
	5	80.6 ± 1.9	9.7 ± 0.3^{***e}	70.3 ± 0.6^{**d}
	10	74.8 ± 2.4	(−)	(−)
kaempferol	1.25	100.6 ± 2.9	25.9 ± 0.5	117.3 ± 0.4
	2.5	96.7 ± 1.4	23.3 ± 0.7^{*c}	115.8 ± 1.5
	5	95.7 ± 1.2	17.6 ± 0.7^{**d}	112.0 ± 0.4
	10	86.8 ± 3.9	5.5 ± 0.2^{***e}	93.7 ± 0.3^{*c}
carpachromene	1.25	85.1 ± 1.8	27.8 ± 0.7	119.6 ± 0.7
	2.5	80.1 ± 0.3	23.5 ± 0.4^{*c}	114.8 ± 1.3
	5	72.1 ± 3.1	(−)	(−)
	10	69.7 ± 1.6	(−)	(−)
syringic aldehyde	1.25	98.0 ± 1.5	33.3 ± 2.7	120.5 ± 0.8
	2.5	85.6 ± 0.4	28.4 ± 0.4	119.1 ± 1.2
	5	87.5 ± 2.6	26.5 ± 0.7	97.1 ± 0.7
	10	84.2 ± 1.7	24.4 ± 0.2^{*c}	89.3 ± 1.9^{*c}

[a]The data were presented as mean ± SD for three different experiments performed in triplicate. [b]compared with sample of control group. [c]$p < 0.05$. [d]$p < 0.01$, and. [e]$p < 0.001$ were compared with LPS-alone group.

mL) on the formation of NO from FF-chloroform fraction stimulated with LPS (100 ng/mL) in RAW 264.7 macrophages. The effect of β-sitosterol (**1**), stigmasterol (**2**), psoralen (**3**), kaempferol (**4**), carpachromene (**5**), and syringic aldehyde (**6**) on RAW264.7 cell viability was determined by a MTT assay. Cells cultured with β-sitosterol and stigmasterol (mixed) (**1**, **2**), kaempferol (**4**), and syringic aldehyde (**6**) at the concentrations (0, 1.25, 2.5, 5, and 10 μg/mL) used in the presence of 100 ng/mL LPS for 24 h did not change cell viability significantly (Table 1). Cells cultured with psoralen (**3**) at the concentrations (0, 1.25, 2.5, and 5 μg/mL) and carpachromene (**5**) at the concentrations (0, 1.25, and 2.5 μg/mL) and used in the presence of 100 ng/mL LPS for 24 h did not change cell viability significantly (Table 1). Thus, the concentrations of β-sitosterol and stigmasterol (mixed) (**1**, **2**), psoralen (**3**), kaempferol (**4**), carpachromene (**5**), and syringic aldehyde (**6**) did not affect the cell viability for further experimentation.

NO production slightly decreased in the presence of the β-sitosterol and stigmasterol (mixed) (**1**, **2**), carpachromene (**5**), and syringic aldehyde (**6**), whereas NO production was inhibited by psoralen (**3**) and kaempferol (**4**) in a dose-dependent manner significantly (Table 1). The IC_{50} value for inhibition of NO production of psoralen (**3**) and kaempferol (**4**) was about 1.5 ± 0.2 and 5.8 ± 0.5 μg/mL.

We also examined the effect of β-sitosterol and stigmasterol (mixed) (**1**, **2**), psoralen (**3**), kaempferol (**4**), carpachromene (**5**), and syringic aldehyde (**6**) on LPS induced up-regulation of TNF-α. A low amount of TNF-α protein was detected for TNF-α in controls (Table 1). When RAW264.7 macrophages were treated with different concentrations of β-sitosterol and stigmasterol (mixed) (**1**, **2**), psoralen (**3**), kaempferol (**4**), carpachromene (**5**), and syringic aldehyde (**6**) together with LPS for 24 h, a concentration-dependent inhibition of TNF-α production was detected significantly. There was either a decrease in the TNF-α production of group treated with 2.5, 5, or 10 μg/mL β-sitosterol and stigmasterol (mixed) (**1**, **2**) ($p < 0.01$ or $p < 0.001$), treated with 2.5 and 5 μg/mL psoralen (**3**) ($p < 0.05$ or $p < 0.01$) or highly decrease of groups treated respectively with 10 μg/mL kaempferol (**4**) and syringic aldehyde (**6**) when compared with the LPS-alone group ($p < 0.05$). The IC_{50} value for inhibition of TNF-α production of β-sitosterol and stigmasterol (mixed) (**1**, **2**) were about 1.8 ± 0.4 μg/mL.

Effects of β-Sitosterol and Stigmasterol (Mixed), Psoralen, Kaempferol, Carpachromene, and Syringic Aldehyde on LPS-Induced iNOS and COX-2 Expression in RAW 264.7 Macrophages. The effects of β-sitosterol and stigmasterol (mixed) (**1**, **2**), psoralen (**3**), kaempferol (**4**), carpachromene (**5**), and syringic aldehyde (**6**) on LPS-stimulated iNOS and COX-2 expression in RAW 264.7 cells were examined. As shown in Figure 4A, RAW264.7 cell treated with LPS had increased iNOS and COX-2 expressions as compared with the control group in the absence of LPS. β-Sitosterol and stigmasterol (mixed) (**1**, **2**), kaempferol (**4**), and syringic aldehyde (**6**), at concentrations of about 5 and 10 μg/mL, were added to the medium with LPS. Psoralen (**3**) (2.5

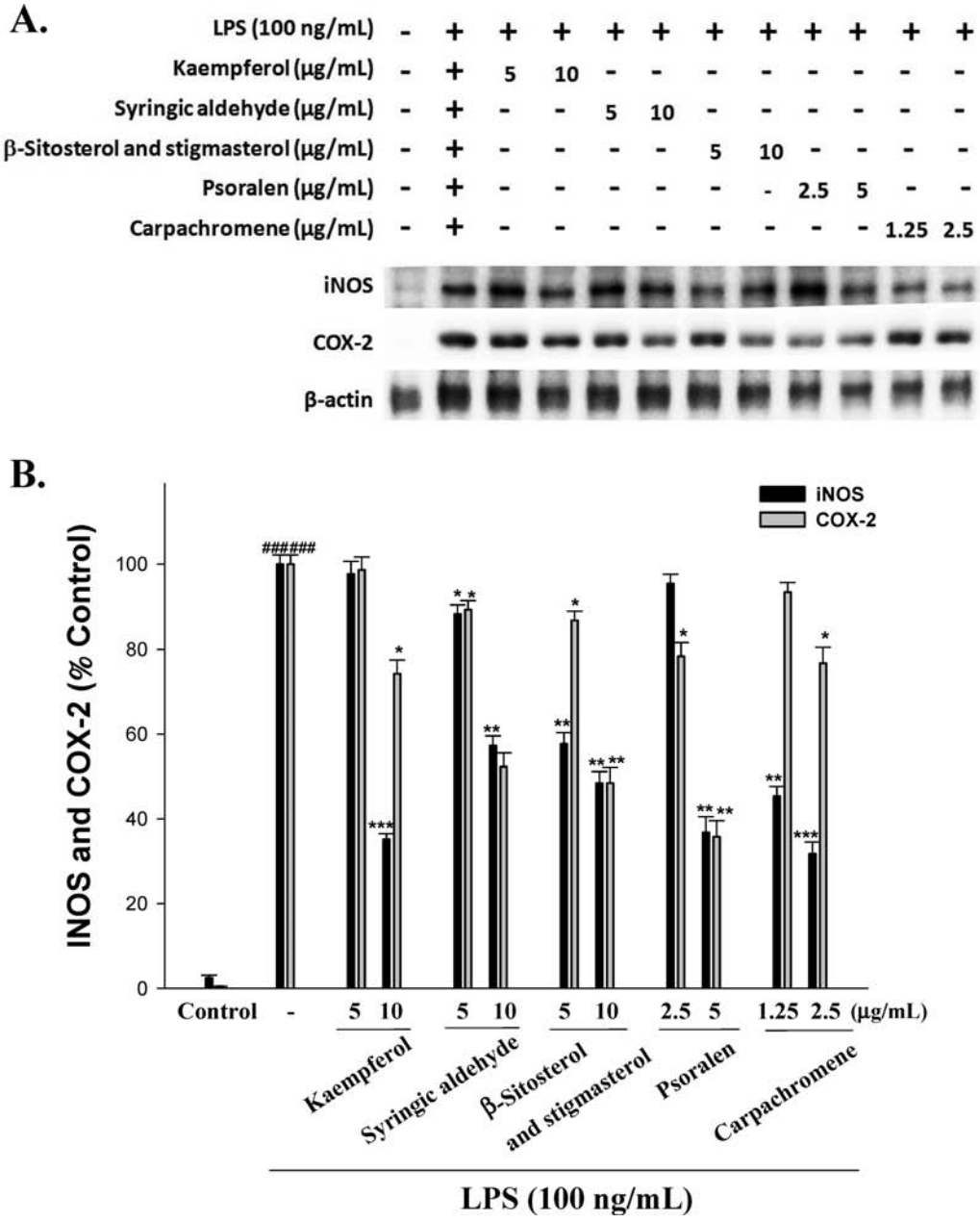

Figure 4. Inhibition of iNOS and COX-2 protein expressed by the isolated compounds of FF-chloroform [β-sitosterol and stigmasterol (mixed), psoralen, kaempferol, carpachromene, and syringic aldehyde] in LPS-stimulated RAW264.7 cells. Cells were incubated for 24 h with 100 ng/mL of LPS in the absence or the presence of samples. Samples were added 1 h before incubation with LPS. Lysed cells were then prepared and subjected to Western blotting using an antibody specific for iNOS and COX-2. β-actin was used as a quantity control. (A) A representative Western blot from two separate experiments is shown. (B) Relative iNOS and COX-2 protein levels were calculated with reference to a LPS-stimulated culture. The data were presented as mean ± SD for three different experiments performed in triplicate. ### compared with sample of control group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were compared with LPS-alone group.

and 5 μg/mL) and carpachromene (**5**) (1.25 and 2.5 μg/mL) were added to the medium with LPS. Those compounds also inhibited iNOS and COX-2 expressions. However, iNOS and COX-2 expression was slightly down-regulated in the presence of 5 and 10 μg/mL β-sitosterol and stigmasterol (mixed) (**1**, **2**) and syringic aldehyde (**6**). Kaempferol (**4**) (5 and 10 μg/mL), psoralen (**3**) (2.5 and 5 μg/mL), and carpachromene (**5**) (1.25 and 2.5 μg/mL) inhibited iNOS and COX-2 expression in a dose-dependent manner in LPS-induced RAW264.7 cells.

Plant sterols are cholesterol like molecules present in all plants. Little is known about the role of sterols in inflammatory effects. Three sterols (campesterol, stigmasterol, and β-sitosterol) were administrated in the adjuvant-induced arthritis mouse mode that paw edema was decreased in inflamed tissues as well as the level of superoxide ions.[13] Regarding β-sitosterol, it has important anti-inflammatory properties; it inhibits vascular adhesion molecule-1, intracellular adhesion molecule-1, and the phosphorylation of NF-κB p65 expression in TNF-α stimulated human aortic endothelial cell.[14] In addition, β-sitosterol also reduces IL-6 and TNF-α secretion in LPS-stimulated RAW264.7 cell and reduces the inflammatory effect by the carrageenan using a murine air pouch model.[15] Stigmasterol is a plant sterol able to bind to chondrocyte membrane and possesses potential anti-inflammatory and

anticatabolic properties.[13] Stigmasterol counteracts the expression of the matrix metalloproteinases involved in cartilage degradation along with an inhibitory effect on the pro-inflammatory mediator PGE_2, at least in part via the inhibition of the NF-κB pathway. Thus, stigmasterol, by preventing the expression of deleterious mediators, should be considered as a target if future in vivo experiments confirm these in vitro data.[13]

Psoralen is one of the furanocoumarins. Furanocoumarins, based on the position of the furan ring, are divided into the angelicin type (furan-ring at position 6 and 7) and the psoralen type (furan-ring at position 7 and 8). For the angelicin type, the C5-methoxy group is important for activity. For the psoralen type, the C8-methoxy or the double bond of the dimethylallyoxy group saturated by two hydroxyl groups at the C5 position enhances the activity.[16] Furanocoumarins have biological functions including antidiabetic, anticonvulsant, increased cell differentiation in osteoblasts, and reduction in liver steatosis.[17] The IC_{50} of inhibitory activity on NF-κB/DNA interactions of psoralen is about 3.0 ± 0.2 μM.

The biological effects of flavonoids, such as kaempferol and carpachromene, have been identified, including antioxidant, anticancer, and anti-inflammatory activities.[18] Although flavonoids with multiple beneficial biological functions, application of flavonoids is not popular due to their poor absorptive efficacy. Kaempferol might be an effective strategy for the suppression of LPS-induced NO production and induced HO-1 expression.[19] Kaempferol dose-dependently repressed iNOS expression and prostaglandin E2 production, through an attenuating NF-κB signaling pathway.[16] Carpachromene exhibited cytotoxicity against HepG2, PLC/PRF/5, and Raji cancer cell lines in vitro.[5] We also found carpachromene the suppression of LPS-induced NO and TNF-α productions for the first time.

Phenolic compounds also have a influence on human health such as a decrease of coronary heart diseases, prevention of several kinds of cancer, anti-inflammatory, and antimutagenic activities.[20−23] Syringic aldehyde moderately inhibited COX-2 activity with an IC_{50} of 3.5 μg/mL.[21] The IC_{50} value of the FF-chloroform fraction for inhibition of LPS-induced NO formation in RAW 264.7 macrophages was 21.5 ± 0.6 μg/mL, higher than that found for the psoralen (1.5 ± 0.2 μg/mL) and kaempferol (5.8 ± 0.5 μg/mL). Therefore, phytochemicals of the FF-chloroform fraction may act synergistically to inhibit the LPS-induced inflammatory response.

In conclusion, it appears that psoralen and kaempferol isolated from FF are at least partially responsible for the observed anti-inflammatory effects of the FF-chloroform fraction. They do so by inhibiting TNF-α and NO formation and down-regulating iNOS and COX-2 expressions. Thus, the FF-chloroform fraction and/or components may be efficacious for the treatment of inflammatory-mediated disease.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR data for compounds **1**−**6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Funding

We thank the financial support from the National Science Council (NSC101-2313-B-039-002-MY3, NSC102-2320-B-039-002, and NSC102-2320-B-468-004), China Medical University (CMU) (CMU100-ASIA-15 and CMU101-AWARD-08), Asia University (100-ASIA-15), and Taiwan Department of Heath Clinical Trial and Research Center of Excellence (DOH102-TD-B-111-004).

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Wang, A. Y.; Zhou, M. Y.; Lin, W. C. Antioxidative and anti-inflammatory properties of *Citrus sulcata* extracts. *Food Chem.* **2011**, *124*, 958−963.

(2) Chang, C. T.; Huang, S. S.; Lin, S. S.; Amagaya, S.; Ho, H. Y.; Hou, W. C.; Shie, P. H.; Wu, J. B.; Huang, G. J. Anti-inflammatory activities of tormentic acid from suspension cells of Eriobotrya Japonica ex vivo and in vivo. *Food Chem.* **2011**, *127*, 1131−1137.

(3) Huang, G. J.; Huang, S. S.; Lin, S. S.; Hsieh, I. C.; Hou, W. C.; Shie, P. H.; Kuo, Y. H. Anti-inflammatory activities of 6beta-acetoxy-7alpha-hydroxyroyleanone from *Taiwania cryptomerioides* Hayata ex vivo and in vivo. *J. Agric. Food Chem.* **2011**, *59*, 11211−11218.

(4) Huang, S. S.; Chiu, C. S.; Lin, T. H.; Lee, M. M.; Lee, C. Y.; Chang, S. J.; Hou, W. C.; Huang, G. J.; Deng, J. S. Antioxidant and anti-inflammatory activities of aqueous extract of *Centipeda minima*. *J. Ethnopharmacol.* **2013**, *147*, 395−405.

(5) Sheu, Y. W.; Chiang, L. C.; Chen, I. S.; Chen, Y. C.; Tsai, I. L. Cytotoxic flavonoids and new chromenes from *Ficus formosana* f. *formosana*. *Planta Med.* **2005**, *71*, 1165−1167.

(6) Chang, H. Y.; Sheu, M. J.; Yang, C. H.; Leu, Z. C.; Chang, Y. S.; Peng, W. H.; Huang, S. S.; Huang, G. J. Analgesic effects and the mechanisms of anti-inflammation of hispolon in mice. *Evidence-Based Complementary Altern. Med.* **2011**, *2011*, 478246.

(7) Huang, G. J.; Huang, S. S.; Lin, S. S.; Shao, Y. Y.; Chen, C. C.; Hou, W. C.; Kuo, Y. H. Analgesic effects and the mechanisms of anti-inflammation of ergostatrien-3β-ol from *Antrodia camphorata* submerged whole broth in mice. *J. Agric. Food Chem.* **2010**, *58*, 7445−7452.

(8) Wen, C. L.; Chang, C. C.; Huang, S. S.; Kuo, C. L.; Hsu, S. L.; Deng, J. S.; Huang, G. J. Anti-inflammatory effects of methanol extract of *Antrodia cinnamomea* mycelia both in vitro and in vivo. *J. Ethnopharmacol.* **2011**, *137*, 575−584.

(9) Sheu, M. J.; Deng, J. S.; Huang, M. H.; Wu, C. H.; Huang, S. S.; Huang, G. J. Antioxidant and anti-inflammatory properties of *Dichondra repens* Forst. and its reference compounds. *Food Chem.* **2012**, *132*, 1010−1018.

(10) Wang, C. Y.; Kao, T. C.; Lo, W. H.; Yen, G. C. Glycyrrhizic acid and 18β-glycyrrhetinic acid modulate lipopolysaccharide-induced inflammatory response by suppression of NF-κB through PI3K p110δ and p110γ inhibitions. *J. Agric. Food Chem.* **2011**, *59*, 7726−7733.

(11) Chiu, C. S.; Deng, J. S.; Chang, H. Y.; Chen, Y. C.; Lee, M. M.; Hou, W. C.; Lee, C. Y.; Huang, S. S.; Huang, G. J. Antioxidant and anti-inflammatory properties of taiwanese yam (*Dioscorea japonica* Thunb. var. pseudojaponica (Hayata) Yamam.) and its reference compounds. *Food Chem.* **2013**, *141*, 1087−1096.

(12) Wang, L.; Tu, Y. C.; Lian, T. W.; Hung, J. T.; Yen, J. H.; Wu, M. J. Distinctive antioxidant and antiinflammatory effects of flavonols. *J. Agric. Food Chem.* **2000**, *54*, 9798−9804.

(13) Gabay, O.; Sanchez, C.; Salvat, C.; Chevy, F.; Breton, M.; Nourissat, G.; Wolf, C.; Jacques, C.; Berenbaum, F. Stigmasterol: a phytosterol with potential anti-osteoarthritic properties. *Osteoarthritis Cartilage* **2010**, *18*, 106−116.

(14) Loizou, S.; Lekakis, I.; Chrousos, G. P.; Moutsatsou, P. beta-Sitosterol exhibits anti-inflammatory activity in human aortic endothelial cells. *Mol. Nutr. Food Res.* **2010**, *54*, 551−558.
(15) Kurano, M.; Iso-O, N.; Hara, M.; Noiri, E.; Koike, K.; Kadowaki, T.; Tsukamoto, K. Plant sterols increased IL-6 and TNF-α secretion from macrophages, but to a lesser extent than cholesterol. *J. Atheroscler. Thromb.* **2011**, *18*, 373−383.
(16) Wang, C. C.; Lai, J. E.; Chen, L. G.; Yen, K. Y.; Yang, L. L. Inducible nitric oxide synthase inhibitors of Chinese herbs. Part 2: naturally occurring furanocoumarins. *Bioorg. Med. Chem.* **2000**, *8*, 2701−2707.
(17) Borgatti, M.; Chilin, A.; Piccagli, L.; Lampronti, I.; Bianchi, N.; Mancini, I.; Mancini, I.; Marzaro, G.; dall'Acqua, F.; Guiotto, A.; Gambari, R. Development of a novel furocoumarin derivative inhibiting NF-κB dependent biological functions: design, synthesis and biological effects. *Eur. J. Med. Chem.* **2011**, *46*, 4870−4877.
(18) Yao, L. H.; Jiang, Y. M.; Shi, J.; Tomás-Barberán, F. A.; Datta, N.; Singanusong, R.; Chen, S. S. Flavonoids in food and their health benefits. *Plant Foods Hum. Nutr.* **2004**, *59*, 113−122.
(19) Lin, H. Y.; Juan, S. H.; Shen, S. C.; Hsu, F. L.; Chen, Y. C. Inhibition of lipopolysaccharide-induced nitric oxide production by flavonoids in RAW264.7 macrophages involves heme oxygenase-1. *Biochem. Pharmacol.* **2003**, *66*, 1821−1832.
(20) Anderson, K. J.; Teuber, S. S.; Gobeille, A.; Cremin, P.; Waterhouse, A. L.; Steinberg, F. M. Walnut polyphenolics inhibit in vitro human plasma and LDL oxidation. *J. Nutr.* **2001**, *131*, 2837−2842.
(21) Stanikunaite, R.; Khan, S. I.; Trappe, J. M.; Ross, S. A. Cyclooxygenase-2 inhibitory and antioxidant compounds from the truffle *Elaphomyces granulatus*. *Phytother. Res.* **2009**, *23*, 575−578.
(22) Lai, P. K.; Chan, J. Y.; Wu, S. B.; Cheng, L.; Ho, G. K.; Lau, C. P.; Kennelly, E. J.; Leung, P. C.; Fung, K. P.; Lau, C. B. Anti-inflammatory activities of an active fraction isolated from the root of *Astragalus membranaceus* in RAW 264.7 macrophages. *Phytother. Res.* **2013**, DOI: 10.1002/ptr.5002.
(23) Umar, M. I.; Asmawi, M. Z.; Sadikun, A.; Atangwho, I. J.; Yam, M. F.; Altaf, R.; Ahmed, A. Bioactivity-guided isolation of ethyl-*p*-methoxycinnamate, an anti-inflammatory constituent, from *Kaempferia galanga* L. extracts. *Molecules* **2012**, *17*, 8720−8734.