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. 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). 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. 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.

*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 norarctocarpin from *F. formosana* exhibited cytotoxicity against HepG2, PLC/PRF/5, and Raji cancer cell lines in vitro. 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 O127: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...
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 being 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 stigmasterol 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 elution 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 CO2 incubator (5% CO2 in air) at 37 °C and subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin−0.02% EDTA in Ca2+-, Mg2+-free phosphate-buffered saline (DPBS).

Cell Viability. Cells (2 × 105) 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 μL of 0.5 mg/mL MTT for 2 h at 37 °C testing for cell viability. The medium was then discarded, and 100 μL of dimethyl sulfoxide (DMSO) was added. After 30 min
incubation, absorbance at 570 nm was read by using a microplate reader (Molecular Devices, Orlando 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.\(^7\) 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 dispersed into 96-well plates, and 100 μL of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylene diamine 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, Orlando 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 by 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 Na3VO4, 1 mM EGTA, 10 μM NaF, 1 mM Na2P2O7, 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 12000g 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 μM 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. IC50 values were estimated using a nonlinear regression algorithm (SigmaPlot 8.0; SSPS 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. formsana 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.\(^6\)

Macrophase 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. formsana* (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) were 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) 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 IC50 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 < 0.05 or p < 0.001), respectively. The IC50 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).
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.

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/mL)
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

<table>
<thead>
<tr>
<th>dose (µg/mL)</th>
<th>cell viability (% of control)</th>
<th>NO level (µM)</th>
<th>TNF-α (pg/mL)</th>
</tr>
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<tr>
<td>control</td>
<td>(-)</td>
<td>98.5 ± 1.3</td>
<td>0.4 ± 0.1</td>
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<td>LPS (+)</td>
<td></td>
<td>99.6 ± 2.8</td>
<td>32.7 ± 0.8**</td>
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<td>β-sitosterol and stigmasterol</td>
<td>1.25</td>
<td>98.3 ± 3.5</td>
<td>30.4 ± 0.9</td>
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<td></td>
<td>2.5</td>
<td>93.7 ± 5.2</td>
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<td>(mixed)</td>
<td>5</td>
<td>83.3 ± 4.1</td>
<td>28.7 ± 1.1</td>
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<tr>
<td></td>
<td>10</td>
<td>80.2 ± 0.3</td>
<td>28.2 ± 0.1</td>
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<td>psoralen</td>
<td>1.25</td>
<td>89.6 ± 1.1</td>
<td>17.0 ± 0.2**</td>
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<td></td>
<td>2.5</td>
<td>84.7 ± 2.1</td>
<td>12.1 ± 0.4***</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>80.6 ± 1.9</td>
<td>9.7 ± 0.3***</td>
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<td></td>
<td>10</td>
<td>74.8 ± 2.4</td>
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<td>kaempferol</td>
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<td>100.6 ± 2.9</td>
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<td>2.5</td>
<td>96.7 ± 1.4</td>
<td>23.3 ± 0.7**</td>
</tr>
<tr>
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<td>5</td>
<td>95.7 ± 1.2</td>
<td>17.6 ± 0.7**</td>
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<td></td>
<td>10</td>
<td>86.8 ± 3.9</td>
<td>5.5 ± 0.6***</td>
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<tr>
<td>carpachromene</td>
<td>1.25</td>
<td>85.1 ± 1.8</td>
<td>27.8 ± 0.7</td>
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<tr>
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<td>2.5</td>
<td>80.1 ± 0.3</td>
<td>23.3 ± 0.4**</td>
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<td></td>
<td>5</td>
<td>72.1 ± 3.1</td>
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<tr>
<td></td>
<td>10</td>
<td>66.7 ± 1.6</td>
<td>(-)</td>
</tr>
<tr>
<td>syringic aldehyde</td>
<td>1.25</td>
<td>98.0 ± 1.5</td>
<td>33.3 ± 2.7</td>
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<td>2.5</td>
<td>85.6 ± 0.4</td>
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<td>87.5 ± 2.6</td>
<td>26.5 ± 0.7</td>
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<td>10</td>
<td>84.2 ± 1.7</td>
<td>24.4 ± 0.2**</td>
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The data were presented as mean ± SD for three different experiments performed in triplicate. *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. 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. 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. Stigmasterol is a plant sterol able to bind to chondrocyte membrane and possesses potential anti-inflammatory and

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.
anticatabolic properties. Stigmasterol counteracts the expression of the matrix metalloproteinases involved in cartilage degradation along with an inhibitory effect on the pro-inflammatory mediator PGE₂, 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.

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 dimethyloxy group saturated by two hydroxyl groups at the C₅ position enhances the activity. Furanocoumarins have biological functions including anti-diabetic, anti-convulsant, immunomodulatory functions including antidiabetic, anti-convulsant, in-ducement of liver steatosis. The IC₅₀ of inhibitory activity on NF-κB/DNA interaction of psoralen is about 3.0 ± 0.2 μM.

The biological effects of flavonoids, such as kaempferol and carpachromene, have been identified, including antioxidant, anti-cancer, and anti-inflammatory activities. 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. Kaempferol dose-dependently repressed iNOS expression and prostaglandin E₂ production, through an attenuating NF-κB signaling pathway. Carpachromene exhibited cytotoxicity against HepG2, PLC/PRF/5, and Raji cancer cell lines in vitro. 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. Syringic aldehyde moderately inhibited COX-2 activity with an IC₅₀ of 3.5 μg/mL. The IC₅₀ value of the FF-chloroform fraction for inhibition of LPS-induced NO formation in RAW 264.7 macrophages was 21.5 ± 0.9 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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Notes
The authors declare no competing financial interest.

REFERENCES


