# Anti-Inflammatory Principles from *Balanophora laxiflora*

### WEN-FEI CHIOU[1], CHIEN-CHANG SHEN[1] AND LIE-CHWEN LIN[1,2*]

[1.] *National Research Institute of Chinese Medicine, Taipei, Taiwan, R.O.C.*

[2.] *Graduate Institute of Integrated Medicine, China Medical University, Taichung, Taiwan, R.O.C.*

## ABSTRACT

Eighteen known compounds, including lignans, phenylpropanoids, triterpenoids, and phytosterols, were isolated from *Balanophora laxiflora* (Balanophoraceae). The inhibitory activities of these compounds on the production of nitric oxide (NO), a pro-inflammatory mediator, were evaluated. Compounds isolariciresinol (1), ethyl caffeate (13) and ferulic aldehyde (16) showed potent to mild inhibitory activities on LPS-stimulated NO production in RAW 264.7 macrophages with $IC_{50}$ values of 0.81, 7.29, and 98.67 μM, respectively. The results showed that compound 1 had a potent effect on TNF-α production with an $IC_{50}$ value of 0.87 μM, but practically no effect on IL-6 synthesis. In addition, an inhibitory effect of compound 1 on nuclear factor-κB (NF-κB) activation was shown by the repression of LPS-stimulated β-lactamase reporter activity. Taken together, these results demonstrated that compound 1 modulates the production of inflammatory mediators through the attenuation of NF-κB transcription signaling.

Key words: *Balanophora laxiflora*, Isolariciresinol, Nitric oxide (NO), TNF-α, NF-κB

## INTRODUCTION

*Balanophora laxiflora* Hemsl. *ex* Forbes & Hemsl. (synonym: *Balanophora spicata* Hayata, Balanophoraceae) is a parasitic herb without chlorophyll growing in roots or rhizomes of various hosts. With reduced leaves, scale-like objects hugging the thick stem, the outward appearance of *B. laxiflora* looks more like a fungus than a flowering plant. *B. laxiflora* can be found in low to medium altitude throughout the island of Taiwan[1] and has been used as antipyretic, antidote, and tonic agent in Formosan folk medicines[2]. Some lignans and hydrolysable tannis isolated from *B. laxiflora* have been proven to possess DPPH radical scavenging activity[3,4]. However, it remained unclear whether this plant displayed anti-inflammatory activity. Our preliminary screening showed the ethanol (EtOH) extract of *B. laxiflora* exhibited an inhibitory activity on NO production in cultured murine macrophage RAW 264.7 cells activated with bacterial LPS. The high amount of NO is potentially cytotoxic, which is capable of injuring the surrounding cells and tissues indiscriminately. Indeed, it has been reported that excess production of NO by macrophages and other cells exposed to endotoxin may contribute to local, e.g. polyarthritis and osteoarthritis, or systemic inflammatory

diseases[5,6]. Thus, it was proposed that inhibition of NO over-production may have the therapeutic benefit in patients with inflammatory diseases. Based on the finding that the EtOH extract of *B. laxiflora* significantly repressed LPS-induced NO production, it is suggested that this herb may have anti-inflammatory benefit. In order to identify the active components, a phytochemical investigation of *B. laxiflora* was carried out. Through solvents partition and repeatedly chromatographic purification, 18 known compounds from the ethanolic extract of *B. laxiflora* were isolated. The structures of these compounds were established by extensive spectroscopic analysis and by comparison with the spectral data in literatures. The abilities of isolated compounds to inhibit the production of the pro-inflammatory mediator, nitric oxide (NO), were evaluated in LPS-stimulated RAW 264.7 macrophages.

## MATERIALS AND METHODS

### I. *Equipment*

^{1}H-, ^{13}C- and 2D-NMR spectra were acquired on a Varian Unity Inova-500 spectrometer. APCI-MS and ESI-MS were recorded on a Thermo Finnigan LCQ spectrometer. Column chromatography was performed on Sephadex LH-20

* Author for correspondence. Tel: +886-2-28201999 ext. 7101;
　Fax: +886-2-28264276; E-mail: lclin@nricm.edu.tw

(Pharmacia). Silica gel 60 (Merck, Darmstadt, Germany) was used for TLC (0.25 mm) and column chromatography (230 - 400 mesh). The preparative HPLC system consisted of a chromatographic pump (LC-8A, Shimadzu, Kyoto, Japan) and a UV-Visible detector (SPD-10A vp, Shimadzu, Kyoto, Japan). A Cosmosil 5C18-AR-II column (20 × 250 mm; particle size 5 μm; Nacalai tesque, Kyoto, Japan) was used for separation.

## II. *Plant Material*

The herbs of *B. laxiflora* Hemsl. *ex* Forbes & Hemsl. were collected in November 2008, in San-Xia, Taipei, Taiwan. Identification of plant materials were confirmed by authors by comparison with a voucher specimen (NHP-00577), which was deposited in the Herbarium of National Research Institute of Chinese Medicine, Taipei, Taiwan.

## III. *Extraction and Isolation*

The dried tuberous rhizome of *B. laxiflora* (2.6 Kg) was extracted with 95% EtOH (6 L × 4). The ethanolic extract was evaporated to dryness (500 g) and partitioned successively between $H_2O$ and *n*-hexane, EtOAc and *n*-BuOH (each 1 L × 3). The *n*-hexane fraction (89 g) was subjected to column chromatography on a silica gel (10 × 75 cm), with a gradient elution of EtOAc in *n*-hexane from 5 to 100% to give 11 fractions (Fr.H1-H11). Fraction H2 (1.9 g) was further fractionated by silica gel eluting with 10% EtOAc/*n*-hexane to yield lupeol (7, 206.9 mg) and lupa-12,20(29)-dien-3$\beta$-ol (8, 95.3 mg). The solid precipitates were separated from Fr.H3 and H6, and recrystalized from $CHCl_3$/*n*-hexane individually to give $\beta$-sitosterol (10, 234.8 mg) and monoglyceryl stearate (9, 58.7 mg), respectively. Fr. H9 (1.3 g) was chromatographed on a Sephadex-LH-20 with acetone elution and delivered eight subfractions, Fr. H9-1 - Fr. H9-8. Fr. H9-5 (70.9 mg) was chromatographed on a semipreparative HPLC (30% ACN-$H_2O$, 15 mL/min) to afford secoisolariciresinol (2, 19.1 mg). Further chromatography on a Sephadex-LH-20 column with MeOH elution gave lariciresinol (3, 43.1 mg) and ethyl caffeate (13, 6.9 mg) from Fr. H9-6 (128.3 mg) and isolariciresinol (1, 58.4 mg) from Fr. H9-8 (188.7 mg). Fr. H8 (594 mg) was chromatographed on a Sephadex-LH-20 (MeOH) to afford pinoresinol (4, 119.4 mg).

The EtOAc fraction (145 g) was fractioned on a silica gel column (10 × 120 cm) with a gradient of EtOAc in *n*-hexane and 9 fractions (Fr.E1-E9) were collected. Fr. E4 (460 mg) was chromatographed on a semipreparative HPLC (35% ACN-$H_2O$, 15 mL/min) to afford vanillin (6, 18.3 mg) and ferulic aldehyde (16, 28.1 mg). Fr. E6 (1.4 g) yielded lariciresinol (3, 72.1 mg), cinnamic acid (15, 12.6 mg) and ferulic aldehyde (16, 33.8 mg) after separation on a Sephadex LH-20 column (MeOH) and a semipreparative HPLC (35% ACN-$H_2O$, 15 mL/min). Fr. E8 (6.9 g) yielded pinoresinol (4, 307.5 mg), caffeic acid (12, 226.7 mg), and $\beta$-sitosterol glucoside (11, 72.7 mg) after separation on a Sephadex LH-20 column (MeOH) and a silica gel column

(10% MeOH-$CHCl_3$). Part of Fr. E9 (2 g) was chromatographed on a silica gel column (10% MeOH-$CHCl_3$) to give isolariciresinol (1, 84.0 mg).

*n*-BuOH fraction (100 g) was subjected to Sephadex LH-20 column (10 × 100 cm), with MeOH elution to give 7 fractions (Fr.B1-B7). Fr. B2 (3.1 g) yielded coniferin (17, 72.7 mg), methylconiferin (18, 7 mg) and pinoresinol-4-$\beta$-$O$-glucoside (5, 63.4 mg) after separation on a Sephadex LH-20 column (MeOH) and a silica gel column (10% MeOH-$CHCl_3$). Fr. B3 (7.2 g) yielded 1-$O$-$E$-caffeoyl-$\beta$-glucose (14, 4.8 g) after separation on a Sephadex LH-20 column (MeOH).

### *Isolariciresinol (1):*

$^1$H NMR ($CD_3OD$) δ 1.75 (1H, m, H-8′), 2.00 (1H, m, H-8), 2.76 (2H, d, *J* = 7.5 Hz, $H_2$-7), 3.39 (1H, dd, *J* = 12.0, 5.5 Hz, Ha-9′), 3.66 (3H, m, $H_2$-9, Hb-9′), 3.76 (3H, s, 3′-$OCH_3$), 3.78 (3H, s, 5-$OCH_3$), 6.18 (1H, s, H-3), 6.60 (1H, dd, *J* = 8.0, 2.0 Hz, H-6′), 6.64 (1H, s, H-6), 6.67 (1H, d, *J* = 2.0 Hz, H-2′), 6.73 (1H, d, *J* = 8.0 Hz, H-5′); $^{13}$C NMR ($CD_3OD$) δ 33.6 (C-7), 40.0 (C-8), 48.0 (C-7′/-8′), 56.3/56.4 (5, 3′-$OCH_3$), 62.2 (C-9′), 65.9 (C-9), 112.3 (C-6), 113.8 (C-2′), 116.0 (C-5′), 117.3 (C-3), 123.2 (C-6′), 129.0 (C-1), 134.1 (C-2), 138.6 (C-1′), 145.2 (C-4), 145.9 (C-4′), 147.2 (C-5), 149.0 (C-3′); $[\alpha]^{23}_D$ +16° (MeOH, c 0.4); EIMS *m/z* 360 [M]$^+$.

### *Secoisolariciresinol (2):*

$^1$H NMR ($CD_3OD$) δ 1.90 (2H, br. s, H-8, 8′), 2.55 (2H, dd, *J* = 13.8, 7.8 Hz, Ha-7, 7′), 2.65 (2H, dd, *J* = 13.8, 7.2 Hz, Hb-7, 7′), 3.58 (4H, m, $H_2$-9, 9′), 3.73 (6H, s, 3, 3′-$OCH_3$), 6.54 (2H, dd, *J* = 7.8, 1.2 Hz, H-6, 6′), 6.58 (2H, d, *J* = 1.2 Hz, H-2, 2′), 6.65 (2H, d, *J* = 7.8 Hz, H-5, 5′); $^{13}$C NMR ($CD_3OD$) δ 36.0 (C-7, 7′), 44.1 (C-8, 8′), 56.2 (3, 3′-$OCH_3$), 62.1 (C-9, 9′), 113.3 (C-2, 2′), 115.8 (C-5, 5′), 122.7 (C-6, 6′), 133.9 (C-1, 1′), 145.5 (C-4, 4′), 148.8 (C-3, 3′); ESIMS *m/z* 361 [M-H]$^-$.

### *Lariciresinol (3):*

$^1$H NMR ($CD_3OD$) δ 2.35 (1H, m, H-8), 2.48 (1H, dd, *J* = 12.6, 12.0 Hz, Ha-7′), 2.72 (1H, m, H-8′), 2.92 (1H, dd, *J* = 12.6, 4.8 Hz, Hb-7′), 3.63 (1H, dd, *J* = 10.8, 6.6 Hz, Ha-9), 3.71 (1H, dd, dd, *J* = 7.8, 6.0 Hz, Ha-9′), 3.83 (1H, m, Hb-9), 3.82/3.83 (each 3H, s, 3, 3′-$OCH_3$), 3.97 (1H, dd, *J* = 7.8, 6.6 Hz, Hb-9′), 4.74 (1H, d, *J* = 6.6 Hz, H-7), 6.63 (1H, dd, *J* = 8.4, 2.0 Hz, H-6′), 6.71 (1H, d, *J* = 7.8 Hz, H-5′), 6.76 (2H, s, H-5, 6), 6.79 (1H, d, *J* = 2.0 Hz, H-2′), 6.90 (1H, s, H-2); $^{13}$C NMR ($CD_3OD$) δ 33.7 (C-7′), 43.9 (C-8′), 54.1 (C-8), 56.3/56.4 (3, 3′-$OCH_3$), 60.5 (C-9), 73.5 (C-9′), 84.0 (C-7), 110.6 (C-2), 113.4 (C-2′), 116.0 (C-5), 116.2 (C-5′), 119.8 (C-6), 122.2 (C-6′), 133.5 (C-1′), 135.7 (C-1), 145.8 (C-4′), 147.0 (C-4), 149.0 (C-3, 3′); ESIMS *m/z* 383 [M+Na]$^+$.

### *Pinoresinol (4):*

$^1$H NMR ($CD_3OD$) δ 3.12 (2H, br. s, H-8, 8′), 3.82 (2H,

504

m, Ha-9, 9′), 3.84 (6H, s, 3, 3′-OCH$_3$), 4.22 (2H, dd, $J$ = 9.0, 7.0 Hz, Hb-9, 9′), 4.69 (2H, d, $J$ = 4.0 Hz, H-7, 7′), 6.76 (2H, d, $J$ = 8.0 Hz, H-5, 5′), 6.80 (2H, dd, $J$ = 8.0, 1.5 Hz, H-6, 6′), 6.93 (2H, d, $J$ = 1.5 Hz, H-2, 2′); $^{13}$C NMR (CD$_3$OD) δ 55.4 (C-8, 8′), 56.4 (3, 3′-OCH$_3$), 72.6 (C-9, 9′), 87.5 (C-7, 7′), 111.0 (C-2, 2′), 116.1 (C-5, 5′), 120.1 (C-6, 6′), 133.8 (C-1, 1′), 147.3 (C-4, 4′), 149.1 (C-3, 3′); EIMS $m/z$ 358 [M]$^+$.

*Pinoresinol-4-O-β-Glucoside (5):*

$^1$H NMR (CD$_3$OD) δ 3.11 (2H, br. s, H-8, 8′), 3.40 (2H, m, H-4″,5″), 3.46 (2H, m, H-2″, 3″), 3.68 (1H, d, $J$ = 9.0 Hz, Ha-6″), 3.84 (1H, m, Hb-6″), 3.84 (3H, s, 3′-OCH$_3$), 3.85 (3H, s, 3-OCH$_3$), 3.84/4.22 (each 2H, H$_2$-9, 9′), 4.68 (1H, d, $J$ = 4.0 Hz, H-7′), 4.74 (1H, d, $J$ = 3.5 Hz, H-7), 4.88 (1H, d, $J$ = 7.5 Hz, H-1″), 6.76 (1H, d, $J$ = 8.0Hz, H-5′), 6.79 (1H, d, $J$ = 8.0 Hz, H-6′), 6.89 (1H, d, $J$ = 8.5 Hz, H-6), 6.93 (1H, s, H-2′), 7.01 (1H, s, H-2), 7.13 (1H, d, $J$ = 8.5 Hz, H-5); $^{13}$C NMR (CD$_3$OD) δ 55.3/55.5 (C-8, 8′), 56.4 (3′-OCH$_3$), 56.8 (3-OCH$_3$), 62.4 (C-6″), 71.3 (C-4″), 72.7 (C-9, 9′), 74.9 (C-2″), 77.8 (C-3″), 78.1 (C-5″), 87.0 (C-7), 87.4 (C-7′), 102.8 (C-1″), 111.0 (C-2′), 111.6 (C-2), 116.1 (C-5′), 118.0 (C-5), 119.8 (C-6), 120.0 (C-6′), 133.7 (C-1′), 137.5 (C-1), 147.2/147.4 (C-4, 4′), 149.1 (C-3′), 150.9 (C-3); ESIMS $m/z$ 519 [M-H]$^-$.

*Ethyl Caffeate (13):*

$^1$H NMR (CD$_3$OD) δ 1.30 (3H, t, $J$ = 7.0 Hz, H-2′), 4.20 (2H, q, $J$ = 7.0 Hz, H-1′), 6.24(1H, d, $J$ = 16.0 Hz, H-8), 6.77 (1H, d, $J$ = 8.0 Hz, H-5), 6.93 (1H, dd, $J$ = 8.0, 1.5 Hz, H-6), 7.03 (1H, d, $J$ = 1.5 Hz, H-2), 7.52 (1H, d, $J$ = 16.0 Hz, H-7); $^{13}$C NMR (CD$_3$OD) δ 14.6 (C-2′), 61.5 (C-1′), 115.1 (C-8), 115.2 (C-2), 116.5 (C-5), 122.8 (C-6), 127.7 (C-1), 146.8 (C-3, 7), 149.5 (C-4), 169.4 (C-9); EIMS $m/z$ 208 [M]$^+$.

*1-O-E-caffeoyl-β-Glucose (14):*

$^1$H NMR (CD$_3$OD) δ 3.39-3.44 (4H, m, sugar-H), 3.68 (1H, dd, $J$ = 12.5, 4.5 Hz, Ha-6′), 3.84 (1H, dd, $J$ = 12.5, 2.0 Hz, Hb-6′), 5.56 (1H, d, $J$ = 8.0 Hz, H-1′), 6.29 (1H, d, $J$ = 15.5 Hz, H-8), 6.77 (1H, d, $J$ = 8.0 Hz, H-5), 6.96 (1H, dd, $J$ = 2.0, 8.0 Hz, H-6), 7.05 (1H, d, $J$ = 2.0 Hz, H-2), 7.65 (1H, d, $J$ = 15.5 Hz, H-7); $^{13}$C NMR (CD$_3$OD) δ 62.3 (C-6′), 71.1 (C-4′), 74.0 (C-2′), 78.0 (C-3′), 78.8 (C-5′), 95.8 (C-1′), 114.4 (C-8), 115.2 (C-2), 116.5 (C-5), 123.2 (C-6), 127.6 (C-1), 146.9 (C-3), 148.3 (C-7), 149.9 (C-4), 167.7 (C-9); ESIMS $m/z$ 341 [M-H]$^-$.

*Coniferin (17):*

$^1$H NMR (CD$_3$OD) δ 3.40 (2H, m, H-4′, 5′), 3.46 (1H, m, H-3′), 3.49 (1H, t, $J$ = 9.0 Hz, H-2′), 3.68 (1H, dd, $J$ = 12.0, 5.4 Hz, Ha-6′), 3.86 (3H, s, 3-OCH$_3$), 3.87 (1H, m, Hb-6′), 4.20 (2H, d, $J$ = 5.4 Hz, H-9), 4.88 (1H, d, $J$ = 7.2 Hz, H-1′), 6.26 (1H, dt, $J$ = 15.6, 5.4 Hz, H-8), 6.53 (1H, d, $J$ = 15.6 Hz, H-7), 6.94 (1H, dd, $J$ = 1.8, 8.4 Hz, H-6), 7.06 (1H, d, $J$ = 1.8 Hz, H-2), 7.09 (1H, d, $J$ = 8.4 Hz, H-5); $^{13}$C NMR (CD$_3$OD) δ 56.7 (3-OCH$_3$), 62.5 (C-6′), 63.7 (C-9), 71.3 (C-4′), 74.9 (C-2′), 77.8 (C-3′), 78.2 (C-5′), 102.7 (C-1′), 111.3 (C-2), 117.9 (C-5), 120.7 (C-6), 128.9 (C-8), 131.3 (C-7), 133.6 (C-1), 147.6 (C-4), 150.9 (C-3); ESIMS $m/z$ 365 [M+Na]$^+$.

*Methylconiferin (18):*

$^1$H NMR (CD$_3$OD) δ 3.36 (3H, s, 9-OCH$_3$), 3.87 (3H, s, 3-OCH$_3$), 3.40 (2H, m, H-4′, 5′), 3.45 (1H, t, $J$ = 7.8 Hz, H-3′), 3.49 (1H, t, $J$ = 9.0 Hz, H-2′), 3.68 (1H, dd, $J$ = 12.0, 4.8 Hz, Ha-6′), 3.86 (1H, m, Hb-6′), 4.07 (2H, dd, $J$ = 6.0, 1.2 Hz, H-9), 4.89 (1H, d, $J$ = 7.8 Hz, H-1′), 6.22 (1H, dt, $J$ = 15.6, 6.0 Hz, H-8), 6.56 (1H, d, $J$ = 15.6 Hz, H-7), 6.95 (1H, dd, $J$ = 1.8, 8.4 Hz, H-6), 7.03 (1H, d, $J$ = 1.8 Hz, H-2), 7.10 (1H, d, $J$ = 8.4 Hz, H-5); $^{13}$C NMR (CD$_3$OD) δ 56.7 (3-OCH$_3$), 58.1 (9-OCH$_3$), 62.5 (C-6′), 71.3 (C-4′), 74.2 (C-9), 74.9 (C-2′), 77.8 (C-3′), 78.2 (C-5′), 102.7 (C-1′), 111.4 (C-2), 117.8 (C-5), 120.9 (C-6), 125.6 (C-8), 133.3 (C-1), 133.6 (C-7), 147.8 (C-4), 150.9 (C-3); ESIMS $m/z$ 379 [M+Na]$^+$.

### IV. *Cell Culture and Nitrite Assay*

The macrophage cell line RAW 264.7 was obtained from ATCC (Rockville, MD), cultured in DMEM containing 10% heat-inactivated fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin and grown at 37°C with 5% CO$_2$ in fully humidified air. Cells were plated at a density of $2 \times 10^5$ cells/well in a 96-well culture plate and stimulated with LPS (1 μg/mL) for 18 h in the presence or absence of various concentrations of tested compounds simultaneously. All compounds were dissolved in dimethyl sulfoxide (DMSO) and further diluted with sterile phosphate buffered saline (PBS) and sterilized via a 0.2 μm filter. Nitrite (NO$_2^-$) accumulation in the medium, as an indicator of NO production, was measured by adding Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid). Nitrite production was determined by measuring the optical density at 550 nm and NaNO$_2$ was used as a standard. All experiments were performed in triplicate. NO production by LPS stimulation was designated as 100% for each experiment.

### V. *Enzyme-Linked Immunosorbent Assay (ELISA)*

TNF-α and IL-6 contents in the culture medium were measured by ELISA using anti-mouse TNF-α or IL-6 antibodies and biotinylated secondary antibody according to the manufacturer's instruction (R&D Systems, Inc., USA).

### VI. *Cell Viability*

Cell respiration, an indicator of cell viability, was determined by the mitochondrial-dependent reduction of 3-(3,4-dimehylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. The extent of the reduction of MTT to formazan within cells was quantified by measuring the optical density at 550 nm.

### VII. *NF-κB Activity Assay*

The CellSensor NF-κB-*bla* RAW 264.6 cells from Invitrogen (Carlsbad, CA), which stably express a plasmid containing *β*-lactamase under the control of a NF-κB promoter, and the LiveBLAzer FRET B/G Loading Kit (Invitrogen) were used. All protocol was performed according to the manufacturing guideline. Imiquimod (a Toll-like receptor 7 activator) was introduced to the assay system to stimulate NF-κB transcription while pyrrolidine dithiocarbamate (PDTC, an NF-κB inhibitor) was used to repress. Fluorescence was read on a fluorescent plate reader in bottom read mode (409 nm excitation, 460 nm emission followed by 409 nm excitation, 530 nm emission). The background, defined as the fluorescence of the substrate with no cells present, was subtracted from each reading, and then the ratio of the emission at 460 nm to the emission at 530 nm was calculated and represented as NF-κB activity.

### VIII. *Statistical Analysis*

Results were expressed as mean ± SE Nitrite production was indicated as absolute concentrations in μM. Computation of 50% effective concentration ($EC_{50}$) to inhibit LPS-induced NO production was computer-assisted (PHARM/PCS v.4.2).

## RESULTS AND DISCUSSION

The ethanolic extract from the herb of *B. laxiflora* was partitioned using *n*-hexane, EtOAc, *n*-BuOH and $H_2O$. These four fractions exhibited middle to high inhibitory effects on NO production in LPS-activated macrophages, and all of them did not show harmful effects on cell viability. The $IC_{50}$ values of *n*-hexane, EtOAc and *n*-BuOH fractions were 28.85, 2.44, and 1.56 μg/mL, respectively. Upon repeated column chromatographic purification, compounds 1 - 18 were isolated from these fractions.

Compounds 1 - 11 were identified, on the basis of their spectral data of $^1H$, $^{13}C$ NMR and mass spectra, as the known isolariciresinol (1)[7], secoisolariciresinol (2)[7], lariciresinol (3)[7], pinoresinol (4)[8], pinoresinol-4-*O*-*β*-glucoside (5)[9], lupeol (7)[10], lupa-12,20(29)-dien-3*β*-ol (8)[11], *β*-sitosterol (10), and *β*-sitosterol glycoside (11), vanillin (6) and mono-glyceryl stearate (9).

Compounds 12 - 16 were considered to be aromatic compounds with *trans* styrene groups according to the NMR spectral data which were identified as caffeic acid (12), ethyl caffeate (13), 1-*O*-E-caffeoyl-*β*-glucose (14)[12], cinnamic acid (15), and ferulic aldehyde (16). Examining the $^1H$ and $^{13}C$ NMR spectra of 17 showed a coniferyl alcohol and a glucosyl moiety in the molecule. HMBC correlations of 17 from anomeric-H (δ 4.87) to C-4 (δ 147.8) indicated the linkage of C-4 and glucosyl moiety. Therefore, 17 was deduced as coniferin[13]. Comparison of $^1H$ and $^{13}C$ spectra of 18 with those of 17 suggested that the main difference

between them was an additional methoxy group in 18. A low-field shifted of C-9 from δc 63.7 (17) to δc 74.2 (18) indicated that the additional methoxy group lays in C-9 of 18. Thus, 18 was deduced as methylconiferin[14]. Compound 18 could be existential in plant originally or be an artificial product of 17 derived from the purification process, which could not be differentiated here.

The abilities of isolated compounds to inhibit the production of NO in LPS-stimulated RAW 264.7 macrophage cells were evaluated. Aminoguanidine (AMG), a well known inducible NO synthase (iNOS) inhibitor, was used as a reference control. The results (Table 1) showed that compounds 1 and 13 displayed significant NO inhibitory activities with $IC_{50}$ value of 0.81 μM and 7.29 μM, respectively, and 16 showed mild inhibitory activity with $IC_{50}$ of 98.67 μM. The effects of compounds were significantly distinguished from the vehicle (data not shown). Cell viability under this condition was still greater than 98% as measured by MTT assay, indicating that the inhibition of nitrite production by compounds 1 and 13 was not due to cell death. As shown in Table 1, the 50% toxic concentrations ($TC_{50}$) of the tested compounds for killing the cells were greater than 100 μM and even greater than 1000 μM in some cases.

Since compound 1 showed the most potent effect to repress NO production, we further analyzed whether compound 1 inhibited the synthesis of other proinflammatory cytokines such as TNF-α and IL-6. TNF-α and IL-6 production in resting cells was very low (0.1 vs. 25.4 pg/mL), but their release was significantly enhanced in response to LPS (1 μg/mL) for 18 h stimulation (6446.3 vs. 2102.9 pg/mL)

**Table 1.** $TC_{50}$ and $IC_{50}$ of isolated compounds from *B. laxiflora* on nitrite production induced by LPS in RAW 264.7 macrophages

| Compound | $TC_{50}$ (μM) | $IC_{50}$ (μM) |
|---|---|---|
| (1) | >100 | 0.81 ± 0.12 |
| (2) | >100 | >100 |
| (3) | >100 | >100 |
| (4) | >100 | >100 |
| (5) | >100 | >100 |
| (6) | >1000 | >100 |
| (9) | >100 | >100 |
| (12) | >1000 | >100 |
| (13) | >100 | 7.29 ± 1.45 |
| (14) | >1000 | >100 |
| (15) | >1000 | >100 |
| (16) | >1000 | 98.67 ± 4.06 |
| (17) | >100 | >100 |
| AMG | >1000 | 20.94 ± 2.13 |

$TC_{50}$ indicated the 50% toxic concentration to induce cells death. $IC_{50}$ represented the 50% effective concentration to inhibit LPS-induced NO production. Data are expressed as mean ± standard deviation (n = 6). AMG: positive control.

(Figure 2). Compound 1 treatment seriously and concentration-dependently inhibited TNF-α production (IC$_{50}$ value of 0.87 μM) as equal potent to repress NO synthesis, but failed to affect IL-6 level. The proinflammatory cytokines such as TNF-α and IL-6 are small secreted proteins, which mediate and regulate immunity and inflammation. Bacterial LPS acts on macrophages to release NO and TNF-α, and the secreted TNF or LPS then induces the cells to release secondary IL-6[15-16]. We found that the suppressive effects of compound 1 on NO or TNF-α production were greater than that on IL-6, which supports that compound 1 has a greater inhibitory effect on the production of primary cytokines. These results also suggested that the effects of compound 1 on inflammatory mediators are focused, regulatory and specific in nature.

NF-κB has been implicated in the transcriptional activation of various inflammatory mediators including NO and TNF-α. Lines of evidence further pointed out that the promotor region of TNF-α contained a strong NF-κB binding motif and such binding site is required for the maximal induction by LPS[17]. Therefore, the effect of compound 1 on this signaling pathway was further investigated by β-lactamase reporter assay as described in methods. As shown in Figure 3, NF-κB activity was dramatically enhanced by LPS stimulation for 18 h. Compound 1 treatment significantly and concentration-dependently suppressed the upregulated NF-κB activity as the reference control PDTC did (data not shown). This result indicated that compound 1-mediated suppression of NF-κB activation might be one of the possible mechanisms underlying its inhibitory actions on NO and TNF-α productions.
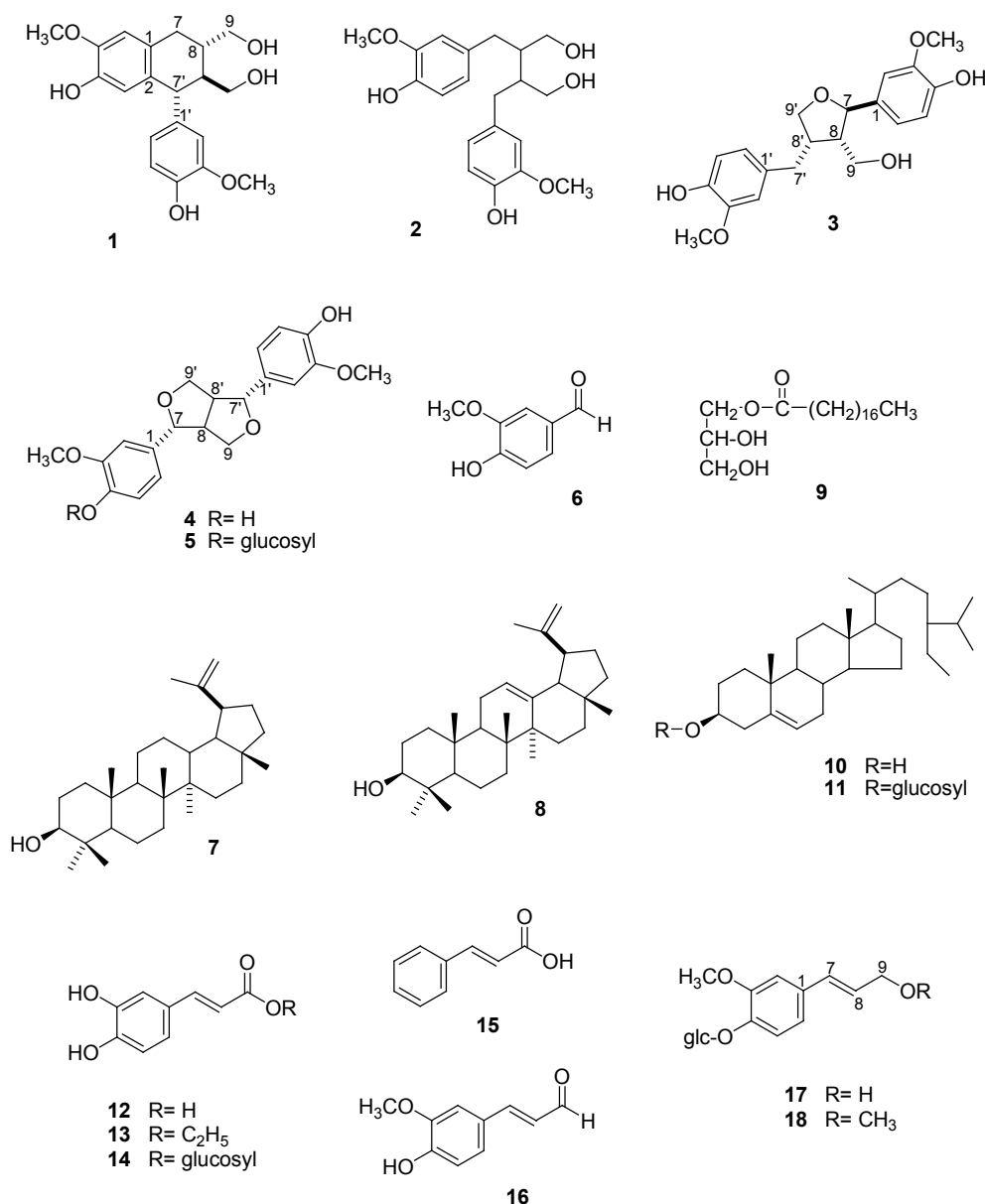


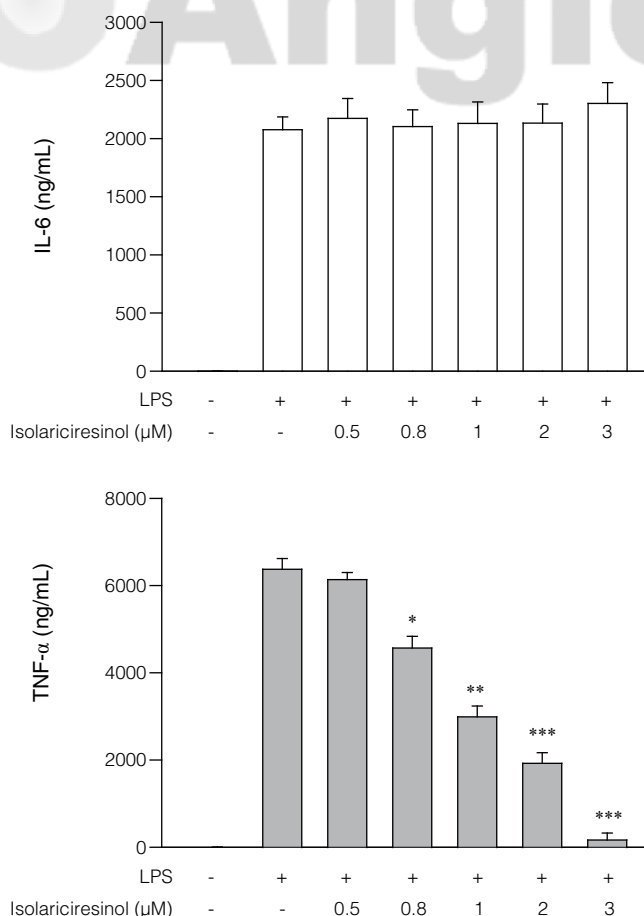**Figure 1.** Chemical structures of isolated compounds from *B. laxiflora*.

**Figure 2.** Effect of isolariciresinol (compound 1) on LPS-induced productions of IL-6 and TNF-α in RAW 264.7 macrophages. Results are expressed as the mean ± SEM of five independent experiments each performed in triplicate. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, compared with LPS alone.



**Figure 3.** Effect of isolariciresinol (compound 1) on imiquimod-induced NF-κB activity in NF-κB-*bla* RAW 264.6 cells. Results are expressed as the mean ± SEM of three independent experiments each performed in duplicate. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, compared with imiquimod alone.

Previously, Cho *et al.*[18] also found that isolariciresinol (1) inhibited NO (IC$_{50}$: 17.8 μM) and TNF-α (IC$_{50}$: 123.8 μM) productions in LPS/IFN-γ co-stimulated RAW 264.7 cells but at much higher effective concentration as compared to our system. The possible explanation is that combined stimulation with LPS plus IFN-γ in their assay system might trigger more complex signaling pathways or other transcription factors different from NF-κB participating in regulating NO and/or TNF-α synthesis as compared to LPS alone. In fact, evidence has demonstrated that IFN-γ alone was able to stimulate the expression of inducible iNOS and the subsequent production of NO through the activation of Janus kinase-2 (JAK-2)-mediated phosphorylation and activation of the signal transducers, and activation of transcription-1 (STAT-1), a pivotal transcription factor in IFN-γ-induced expression of inflammatory genes[19]. Held *et al.*[20] also reported that IFN-γ augmented macrophage activation by LPS at the signal transduction level. That is, a synergy between IFN-γ and LPS by which they cross-regulate the signal-transducing molecules was observed. These phenomenon may account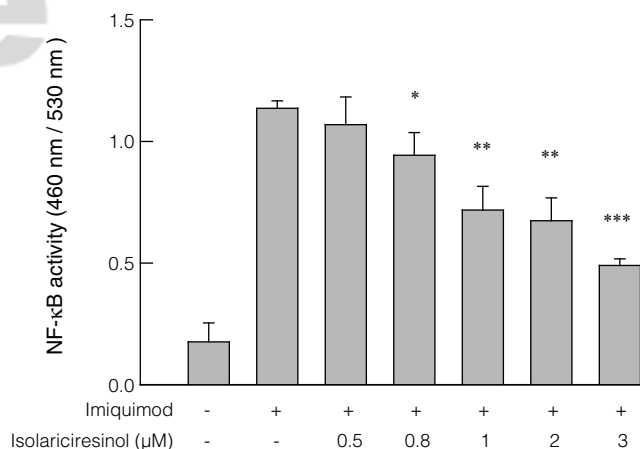 for the requirement of higher concentration of compound 1 to suppress NO production in response to combined stimulation with LPS and IFN-γ in Cho's study.

## CONCLUSIONS

The results demonstrated that phenylpropanoids and lignans are two important components in the title plant. Compounds 1 and 13 from *B. laxiflora* exerted inhibition activity on NO production, especially compound 1, which significantly repressed not only NO, but also TNF-α production at an impressively low effective concentration by blocking NF-κB transcription. This suggested that compound 1 may be useful for the treatment of inflammatory-related diseases. Nevertheless, further studies would be carried to determine the biological efficacy of compound 1 in *ex vivo* or *in vivo* studies.

## ACKNOWLEDGMENTS

## REFERENCES

1. Huang, S. F. and Huang, T. C. 1996. Flora of Taiwan. In "Balanophoraceae" 2nd ed. Vol. II, pp 287-293. Editorial Committee of the Flora of Taiwan ed. & pub. Taipei, Taiwan.
2. Chiu, N. Y. and Chang, K. H. 1986. The Illustrated Medicinal Plants of Taiwan. Vol. 2. p. 35. SMC

508

Publishers, Taipei, Taiwan.

3. Dai, Z., Wang, F., Wang, G. L. and Lin, R. C. 2006. Studies on chemical constituents of *Balanophora spicata*, Zhongguo Zhong Yao Za Zhi. 31: 1798-1800.

4. She, G. M., Zhang, Y. J. and Yang, C. R. 2009. Phenolic constituents from *Balanophora laxiflora* with DPPH radical-scavenging activity. Chem. Biodivers. 6: 875-880.

5. Stefanovic-Racic, M., Stadler, J. and Evans, C. H. 1993. Nitric oxide and arthritis. Arthritis and Rheumatism 36: 1036-1044.

6. Connor, J. R., Manning, P. T., Settle, S. L., Moore, W. M., Jeroma, G. M, Webber, R. K., Tjoeng, F. S. and Currie, M. G. 1995. Suppression of adjuvant-induced arthritis by selective inhibition of inducible nitric oxide synthase. Eur. J. Pharmacol. 273: 15-24.

7. Achenbach, H., Waibel, R. and Addae-Mensah, I. 1983. Lignans and other constituents from *Carissa edulis*. Phytochemistry 22: 749-753.

8. Harrison, L. J., Sia, G. L., Sim, K. Y., Tan, H. T. W., Connolly, J. D., *et al.* 1995. A ferulic acid ester of sucrose and other constituents of *Bhesa paniculata*. Phytochemistry 38: 1497-1500.

9. Vermes, B., Seligmanin, O. and Wagner, H. 1991. Synthesis of biological active tetrahydrofurofuran-lignan-(syringin, pinoresinol)-mono- and bis-glucosides. Phytochemistry 30: 3087-3089.

10. Reynolds, W. F., McLean, S., Poplawski, J., Enriquez, R. G., Escobar, L. I. and Leon, I. 1986. Total assignment of $^{13}$C and $^{1}$H spectra of three isomeric triterpenol derivatives by 2D NMR: An investigation of the potential utility of $^{1}$H chemical shifts in structural investigation of complex natural products. Tetrahedron 42: 3419-3428.

11. Jin, Y. P., Wei, X. N. and Shi, Y. P. 2007. Chemical constituents from *Cynogiossum gansuense*. Helv. Chim. Acta. 90: 776-782.

12. Villegas, R. J. A. and Kojima, M. 1985. Sweet potato root enzyme which catalyzes the formation of chlorogenic acid from 1-O-caffeoyl-D-glucose and D-quinic Acid. Agric. Biol. Chem. 49: 263 - 266.

13. Sugiyama, M., Nagayama, E. and Kikuchi, M. 1993. Lignan and phenylpropanoid glycosides from *Osmanthus asiaticus*. Phytochemistry 33: 1215-1220.

14. Luo, B., Zou, K., Guo, Z., Dan, F., Wang, J. and Wang, H. 2009. Balanoinvolin, a new steroid derivative from *Balanophora involucrate*. Chem. Nat. Comp. 45: 371-373.

15. West, M. A, Seatter, S. C., Bellingham, J. and Clair, L. 1995. Mechanisms of reprogrammed macrophage endotoxin signal transduction after lipopolysaccharide pretreatment. Surgery 118: 220-228.

16. Jean-Baptiste, E. 2007. Cellular mechanisms in sepsis. J. Intensive Care Med. 22: 63-72.

17. Trede, N. S., Tsytsykova, A. V., Chatila, T., Goldfeld, A. E. and Geha, R. S. 1995. Transcriptional activation of the human TNF-a promoter by superantigen in human monocytic cells: role of NF-kB. J. Immunol. 155: 902-908.

18. Cho, J. Y., Kim, A. R. and Park, M. H. 2001. Lignans from the rhizomes of *Coptis japonica* differentially act as anti-inflammatory principles. Planta Med. 67: 312-316.

19. Lowenstein, C. J., Alley, E. W., Raval, P., Snowman, A. M., Snyder, S. H., Russell, S. W., *et al.* 1993. Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon γ and lipopolysaccharide. Proc. Natl. Acad. Sci. USA 90: 9730-9734.

20. Held, T. K., Weihua, X., Yuan, L., Kalvakolanu, D. V. and Cross, A. S. 1999. Gamma interferon augments macrophage activation by lipopolysaccharide by two distinct mechanisms, at the signal transduction level and via an autocrine mechanism involving tumor necrosis factor alpha and interleukin-1. Infect. Immun. 67: 206-212.