The Effect of the Aerial Part of *Lindera akoensis* on Lipopolysaccharides (LPS)-Induced Nitric Oxide Production in RAW264.7 Cells

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**Abstract:** Four new secondary metabolites, 3α-((E)-Dodec-1-enyl)-4β-hydroxy-5β-methylidihydrofuran-2-one (1), linderinol (6), 4′-O-methylkaempferol 3-O-α-L-(4′″-E-p-coumaroyl)rhamnoside (11) and kaempferol 3-O-α-L-(4′″-Z-p-coumaroyl)rhamnoside (12) with eleven known compounds—3-epilistenolide D1 (2),...
3-epilistenolide D₂ (3), (3Z,4α,5β)-3-(dodec-11-ynylidene)-4-hydroxy-5-methylbutanolide (4), (3E,4β,5β)-3-(dodec-11-ynylidene)-4-hydroxy-5-methylbutanolide (5), matairesinol (7), syringaresinol (8), (+)-pinoresinol (9), salicifoliol (10), 4''-p-coumaroylafzelin (13), catechin (14) and epicatechin (15)—were first isolated from the aerial part of Lindera akoensis. Their structures were determined by detailed analysis of 1D- and 2D-NMR spectroscopic data. All of the compounds isolated from Lindera akoensis showed that in vitro anti-inflammatory activity decreases the LPS-stimulated production of nitric oxide (NO) in RAW 264.7 cell, with IC₅₀ values of 4.1–413.8 µM.

**Keywords:** Chinese herb; Lindera akoensis; butanolide; lignans; flavonoids; anti-inflammatory

### 1. Introduction

*Lindera akoensis* (Lauraceae) is an endemic evergreen tree that grows in broad-leaved forests in lowlands throughout Taiwan; it is often used as a fence. Aporphines [1], alkaloids [2], sesquiterpenoids [3–5], flavonoids [6], butanolides [6], furanoids [7], chalconoids [8] and phenolic compounds [9,10] are widely distributed in the plants of the genus of Lindera. Some isolates exhibit biological activities, including suppressed the contraction of thoracic aorta [1], anti-mycobacterial [6], anti-inflammatory [11], against human lung cancer cell (SBC-3) [12], inhibitory osteoclast differentiation [10], slowing down of the progression of diabetic nephropathy in mice [12], anti-nociceptivity [13], inhibition on human acyl-coenzyme A cholesterol acyltransferase activity and antioxidation of low density lipoprotein [9]. Only one piece of literature had reported the chemical constituents and anti-mycobacterial activity from the root of *L. akoensis* [6].

The folk usage of *L. akoensis* is in the treatment of trauma and inflammation [14]. Butanolides showed anti-inflammation in previous studies [15,16]. In a random screening for inhibitory activity of various Chinese traditional medicines toward nitric oxide (NO) production in vitro by RAW264.7 cells, the EtOH extract of the aerial parts of *L. akoensis* showed a significant activity. Thus, the constituents of *L. akoensis* were investigated. This paper deals with the structure elucidation of the new compounds, and the inhibitory activity of the isolates toward nitric oxide (NO) production towards RAW264.7 cells is also discussed.

### 2. Results and Discussion

**Isolation and Structural Elucidation**

The aerial part of *L. akoensis* was air-dried and then extracted by EtOH and purified. Extensive normal phase Si gel column chromatographic purification of the EtOAc-soluble fraction afforded four new compounds, 3α-((E)-Dodec-1-enyl)-4β-hydroxy-5β-methylidihydrofuran-2-one (1), linderinol (6), 4''-O-methylkaempferol 3-O-α-L-(4''-E-p-coumaroyl)rhamnoside (11), kaempferol 3-O-α-L-(4''-Z-p-coumaroyl)rhamnoside (12), as well as eleven known compounds, 3-epilistenolide D₁ (2) [17], 3-epilistenolide D₂ (3) [17], (3Z,4α,5β)-3-(dodec-11-ynylidene)-4-hydroxy-5-methylbutanolide
(4) [18], (3E,4β,5β)-3-(dodec-11-ynylidene)-4-hydroxy-5-methylbutanolide (5) [19], matairesinol (7) [20], syringaresinol (8) [21], (+)-piroresinol (9) [22], salicifoliol (10) [23], 4''-p-coumaroylafzelin (13) [24], catechin (14) [25] and epicatechin (15) [25] (Figure 1).

**Figure 1.** Structures of 1–16.
Compound 1 was isolated as an optically inactive colorless oil (\([\alpha]_D^2 + 0 (c 0.4, \text{CHCl}_3)\)) and showed the presence of hydroxy (3401 cm\(^{-1}\)), olefin (1682 cm\(^{-1}\)) and \(\gamma\)-lactone (1759 cm\(^{-1}\)) functionalities groups in its infrared (IR) spectrum. The high resolution electron impact mass spectrometry (HREIMS) data determined the molecular formula to be C\(_{17}\)H\(_{30}\)O\(_3\) (\(m/z\) 282.2198 ([M]+; calcd 282.2195)). The 1H-NMR spectrum showed signals similar to those of (3\(\beta\),4\(\beta\),5\(\beta\))-3-dodecyl-4-hydroxy-5-methyldihydrofuran-2-one (16) (not purified in this research) [18] at \(\delta_H\) 3.19 (1H, dd, \(J = 6.5, 4.7\) Hz), \(\delta_H\) 4.23 (dd, \(J = 4.7, 4.5\)), \(\delta_H\) 4.64 (qd, \(J = 6.5, 4.5\)) were assigned to H-3, H-4 and H-5, respectively (Table 1). The chemical shift and coupling patterns of H-4 and H-5 suggested that the relative configuration of 1 was identified similar to that of 16. This conclusion was supported by comparison of the \(^1\)H and \(^13\)C NMR data of 1 with those of reported compounds having a cis-relationship between H-4 and H-5. \(^1\)H-NMR spectrum of 1 was similar to that of compounds 3 with 4\(\beta\)-hydroxy-5\(\beta\)-methyl groups. Two olefinic H-atoms were assigned the signals at \(\delta_H\) 5.37 (1H, dd, \(J = 15.4, 6.5\) Hz, H-7), \(\delta_H\) 5.72(1H, dt, \(J = 15.4, 7.4\) Hz, H-8), and nine CH\(_2\)-group signals were observed (\(\delta_H\) 2.04 (2H, q, \(J = 7.4\) Hz, H-9), \(\delta_H\) 1.24 (16H, m, H-10-17)). The H-7 was coupled with H-3 and H-8, with coupling constant 6.5 Hz and 15.4 Hz, respectively, establishing the trans-geometry of \(\Delta^7\). Compared with 3\(\beta\)-((E)-dodec-1-enyl)-4\(\beta\)-hydroxy-5\(\beta\)-methyldihydrofuran-2-one in our previous study [26], the only difference was the configuration of the H-3((\(\delta_H\) 3.19, dd, \(J = 6.5, 4.7\) Hz), \(\delta_C\) 52.7 (C-3)). The key correlation of NOESY spectrum, H-3, has correlation with H-6 and no correlation with H-5, moreover H-4 and H-5 having NOESY correlation, confirmed that H-4 and H-5 in the same phase and H-3 was in opposite side of H-3 (Figure 2). The zero optical rotation value indicated that there may exist in compound 1 a racemic mixture. All protons and carbons were confirmed by 1D and 2D spectra. Thus, 1 was identified as 3\(\alpha\)-((E)-dodec-1-enyl)-4\(\beta\)-hydroxy-5\(\beta\)-methyldihydrofuran-2-one.

### Table 1. NMR data (CDCl\(_3\)) of 1 and 6. \(\delta\) in ppm, \(J\) in Hz.

| No. | \(\delta_H^a\) | \(\delta_C^b\) | \(\delta_H^c\) | \(\delta_C^d\) |
|-----|----------------|----------------|----------------|----------------|
| 1   | 132.4          |                |                |                |
| 2   | 175.6          | 6.61 (s)       |                | 109.3          |
| 3   | 3.19 (dd, \(J = 6.5, 4.7\)) | 52.7          |                | 146.5          |
| 4   | 4.23 (dd, \(J = 4.7, 4.5\)) | 74.6          |                | 143.8          |
| 5   | 4.64 (qd, \(J = 6.5, 4.5\)) | 78.1          | 6.79 (\(d, J = 8.0\)) | 114.2          |
| 6   | 1.39 (d, \(J = 6.5\)) | 13.8          | 6.68 (\(d, J = 8.0\)) | 121.7          |
| 7   | 5.37 (dd, \(J = 15.4, 6.5\)) | 120.9         | 2.62 (dd, \(J = 12.8, 5.0\)) | 35.9          |
| 8   | 5.72 (dt, \(J = 15.4, 7.4\)) | 136.7         | 1.85 (m)       | 44.1           |
| 9   | 2.04 (q, \(J = 7.4\)) | 32.6          | 3.51 (dd, \(J = 11.2, 6.4\)) | 60.6           |
| 10–15 | 1.24 (br s) |                | 29.0–31.9      |                |
| 16–17 | 1.24 (br s) |                | 22.7           |                |
| 18   | 0.86 (\(t, J = 6.6\)) |                | 14.1           |                |
Table 1. Cont.

| No. | δ_H^a | δ_C^b | δ_H^c | δ_C^d |
|-----|-------|-------|-------|-------|
| 1'  |       |       |       | 134.4 |
| 2'  | 6.61  |       | 111.4 |       |
| 3'  |       |       | 147.6 |       |
| 4'  |       |       | 145.7 |       |
| 5'  | 6.62  |       | 108.1 |       |
| 6'  | 6.57  |       | 121.9 |       |
| 7'  |       | 2.62  | 35.9  |       |
| 8'  | 1.85  | 3.51  | 60.5  |       |
| 9'  |       | 3.77  | 55.9  |       |
| OCH_3| 3.82  |       | 100.8 |       |
| OCH_3O| 5.90  |       |       |       |

Recorded at a 400 MHz; b 100 MHz; c 500 MHz; d 125 MHz.

Figure 2. Significant NOESY correlations (↔) of 1 and 11.

Compound 6 was a pale yellow amorphous solid, ([α]_D^20 = +20.2° (c = 0.42, CHCl_3)); it has a λ_max at 284.6 nm (log ε 3.27) in the ultraviolet (UV) spectrum and shows the presence of hydroxy (3310 cm⁻¹) and benzene (1605 and 1512 cm⁻¹) functionalities in its IR spectrum. The HREIMS data determined the molecular formula to be C_{20}H_{22}O_5 (m/z 342.1530 ([M]^+; calcd 342.1467)). The ^1H-NMR spectrum showed signals similar to those of matairesinol (7), such as the CH_2 group at δ_H 2.62 (1H, dd, J = 12.8, 5.0 Hz, Ha-7, Ha-7'), δ_H 2.72 (2H, dd, J = 12.8, 8.5 Hz, Hb-7, Hb-7'), δ_H 3.51 (2H, dd, J = 11.2, 6.4 Hz, Ha-9, Ha-9'), δ_H 3.77 (2H, dd, J = 11.2, 6.4 Hz, Hb-9, Hb-9') were assigned to H-7, H-7', H-9 and H-9', respectively. One set of the ABX system of aromatic protons exhibited at δ_H 6.61 (1H, 1H, s, H-2), δ_H 6.68 and 6.79 (each 1H, d, J = 8.0 Hz, H-6, H-5); the other set of aromatic protons showed at δ_H 6.61 (1H, s, H-2'), δ_H 6.57 and 6.62 (each 1H, d, J = 8.0 Hz, H-6', H-5'). The proton signals assignments are elucidation by HMBC technology. In addition, three functional groups attached on the different phenyl groups were revealed from the following ^1H-NMR signals: δ_H 5.90 (2H, s, methylene dioxide), 3.82 (3H, Ar-OMe, having a NOESY correlation to H-2).
and 5.66(1H, s, Ar-OH). The positive value of optical rotation could be inferred the trans-configuration between dibenzyl substituents on C-8 and C-8'. Based on the $^1$H- and $^{13}$C-NMR (Table 1), COSY, NOESY, HSQC and HMBC experiments, the structure of 6 was tentatively named as linderinol.

Compound 11 was a pale yellow amorphous solid, ([α]$^2_D \pm 0^\circ$ (c = 8.3, CH$_3$OH)). Its molecular formula was determined to be C$_{31}$H$_{28}$O$_{12}$ by HR-ESI-MS spectrometry (m/z 592.1576 ([Na]$^+$; calcd 592.5446). The IR spectrum exhibited bands at 3426 and 1651 cm$^{-1}$ due to a hydroxyl and a conjugated carbonyl group. The NMR signals of rhamnose were easily assigned by their characteristic multiplicities, especially on the unique proton signal of the methyl, which was up-field at δ$^H$ 0.78 (3H, d, $J = 6.3$ Hz), shielded by a C-ring, the aromatic ring of flavon [24]. An A$_2$X$_2$ coupling system at δ$_H$ 7.49 (2H, d, $J = 8.6$ Hz, H-5'''', -9''') and 6.84 (2H, d, $J = 8.6$ Hz, H-6''', -8'''), as well as two olefinic proton signals at δ$_H$ 6.25 and 7.53 (each 1H, d, $J = 16.0$ Hz) could be observed in the presence of an $E$-p-coumaroyl moiety. The H-4'' triplets (δ$_H$ 4.91, t, $J = 9.7$ Hz) in this compound appeared at a relatively low field with respect to the corresponding signal of afzelin [24]. Hence, this compound is esterified at this position. The apigenin group could be observed by NMR spectra, matching the literature [27], but the proton signal at H-3 (δ$_H$ 6.76, 1H, s) cannot be detected; moreover, a conspicuous difference of the carbon signal between C-3 of 11 (δ$^C$ 135.7) and C-3 of apigenin (δ$^C$ 103.2) was observed. By this evidence, we speculated that rhamnose connected on apigenin with a C-3-C-1'' linkage, just like the common afzelin; this speculation was certificated by 1- and 2-D NMR. A methoxy, with a resonance at δ$_H$ 3.85 (3H, s), correlated with C-4' (δ$^C$ 163.6) on the HMBC spectrum, indicating that C-4' was the position where it linked with a methoxy; furthermore, the significant NOE correlation on position 3' (δ$_H$ 7.14, 2H, d, $J = 8.8$) and a methoxy (δ$_H$ 3.85, 3H, s) proved this. The rhamnside and $E$-p-coumaroyl configurations were decided by the 1D-, 2D-NMR and comparison of the $^1$H- and $^{13}$C-NMR spectrum of compound 13 [24]. Based on the above deduction, 11 was designated to be a new compound 4'-O-methylkaempferol 3-O-α-L-(4''-$E$-p-coumaroyl)rhamnoside.

Compound 12 was a pale yellow amorphous solid, ([α]$^2_D \pm 0^\circ$ (c = 4.5, CH$_3$OH)). Its molecular formula was determined to be C$_{30}$H$_{26}$O$_{12}$ by HR-ESI-MS spectrometry (m/z 578.1416 ([Na]$^+$; calcd 578.1424). Together, a 2D technique predicted 12 as a combination with three units of p-coumaroyl, rhamnose and kaempferol derivative, such as in compound 13 [24]; the Z-configuration of C-2''' and C-3''' on the p-coumaroyl moiety was deduced by the smaller coupling constant (12.8 Hz), a higher shift of two olefinic proton signals (δ$_H$ 5.75 and 6.87) and a lower shift of H-5''' (δ$_H$ 7.66, d, $J = 8.6$ Hz), comparing to the corresponding protons in 11. Based on the above deduction, 12 was designated as a new compound, kaempferol 3-O-α-L-(4''-Z-p-coumaroyl)rhamnoside.

3-epilistenolide D$_1$ (2), 3-epilistenolide D$_2$ (3), (3Z,4α,5β)-3-(dodec-11-ynylidene)-4-hydroxy-5-methylbutanolide (4) and (3E,4β,5β)-3-(dodec-11-ynylidene)-4-hydroxy-5-methylbutanolide (5) were isolated as light yellow oils, whereas matairesinol (7), syringaresinol (8), (+)-pinoresinol (9), salicifoliol (10), 4''-p-coumaroylafzelin (13), catechin (14) and epicatechin (15) were obtained as pale yellow solids. The $^1$H and $^{13}$C NMR spectra of compounds 2–5, 7–10 and 13–15 were confirmed by comparison of their spectral data with the reported value from the literature.
2.2 Anti-Inflammatory Activity

NO, produced from L-arginine by NO synthase, has various biological actions, e.g., as a defense and regulatory molecule for homeostatic equilibrium [28]. However, in pathophysiologic conditions, such as inflammation, there is an increased production of NO by inducible NO synthase (iNOS) [29]. Macrophages have been expected to be an origin of inflammation, because they contain various chemical mediators that may be responsible for several inflammatory stages [30]. The inhibitory activity toward NO production, induced by lipopolysaccharides (LPS), by murine macrophage-derived RAW264.7 cells, was assayed. These compounds from L. akoensis were screened by anti-inflammatory activity in vitro with a decrease in nitrite of the LPS-stimulated production in RAW264.7 cells with IC₅₀ values of 4.1–413.8 µM (Table 2).

Table 2. Cell viability and in vitro decrease of nitrite of LPS-stimulated production in RAW264.7 cell activities of compound 1–15.

| Compound | Cytotoxicity IC₅₀ (µM) | Inhibition of NO production IC₅₀ (µM) |
|----------|------------------------|--------------------------------------|
| 1        | 78.0 ± 5.1             | 20.1 ± 0.3                           |
| 2        | 32.6 ± 0.5             | 4.1 ± 0.1                            |
| 3        | 27.7 ± 1.6             | 4.5 ± 0.1                            |
| 4        | 138.8 ± 2.8            | 21.7 ± 0.4                           |
| 5        | 142.8 ± 1.9            | 33.4 ± 1.0                           |
| 6        | >292.4                 | 196.0 ± 4.0                          |
| 7        | >279.3                 | 178.8 ± 12.1                         |
| 8        | >239.2                 | 49.7 ± 4.5                           |
| 9        | >279.3                 | 90.4 ± 8.6                           |
| 10       | >400.0                 | 311.6 ± 14.1                         |
| 11       | >84.5                  | 62.5 ± 2.2                           |
| 12       | >86.5                  | 67.9 ± 1.9                           |
| 13       | >86.5                  | 76.9 ± 7.3                           |
| 14       | >517.2                 | 413.8 ± 6.9                          |
| 15       | >517.2                 | 351.7 ± 37.4                         |
| indomethacin |                    | 182.9 ± 5.5                         |

Values are expressed as mean ± SD of three replicates.

3. Experimental Section

3.1. Chemicals

LPS (endotoxin from Escherichia coli, serotype 0127:B8), indomethacin, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3.2. General

UV spectra were obtained with a Shimadzu Pharmaspec-1700 UV-Visible spectrophotometer. Optical rotations were obtained with a Jasco P-1020 polarimeter. Infrared spectra were obtained with a
Shimadzu IRprestige-21 Fourier transform infrared spectrophotometer. 1D- and 2D-NMR spectra were recorded with a Bruker DRX-400 FT-NMR spectrometer. Mass spectrometric (HR-EI-MS and HR-ESI-MS) data were generated at the Mass Spectrometry Laboratory of the Chung Hsing University. Column chromatography was performed using Merck Si gel (30–65 µM), and TLC analysis was carried out using aluminum pre-coated Si plates; the spots were visualized using a UV lamp at $\lambda = 254$ nm. HPLC chromatograms were obtained with a Shimadzu LC-6A and a IOTA-2 RI-detector with a Phenomenex luna silica(2) 250 × 10 column.

3.3. Plant Material

*Lindera akoensis* was collected and identified by Dr. Yen-Hsueh Tseng (Department of Forestry, National Chung Hsing University) at Taichung, Taiwan, in July, 2008.

3.4. Extraction and Isolation

The materials were totally dried under dark in air. The dried aerial part of *L. akoensis* (5.9 kg) was cut into small pieces and soaked in 95% ethanol (60 liter, 7 days × 3). After filtration, the crude extract was concentrated and stored under vacuum to yield an brown thick paste (337.8 g) that was suspended in H$_2$O (1000 mL) and extracted with ethyl acetate (1000 mL, 3 times). The resulting ethyl acetate extract was concentrated to yield 127.8 g of a brown thick oil that was purified by 1900 g silica gel with a particle size 0.063–0.200 mm and an internal diameter of the column, 15 cm packed height, 25 cm chromatography, using a gradient of increasing polarity with *n*-hexane/ethyl acetate (99:1–0:100) as the mobile phase and separated into 21 fractions on the basis of TLC analysis for random isolation of compounds. Fraction 11 (5.08 g) was re-separated by chromatography and semi-preparative HPLC with 40% EtOAc in *n*-hexane to afford pure, butanolide 1 (6.1 mg, 0.00104‰), 2 (7.7 mg, 0.00131‰), 3 (7.8 mg, 0.00132‰) and 4 (2.1 mg, 0.00036‰) and 5 (1.6 mg, 0.00027‰). Fraction 15 (6.82 g) was re-separated by chromatography and semi-preparative HPLC with 50% EtOAc in *n*-hexane to afford pure lignans 7 (2.3 mg, 0.00039‰), 9 (1.5 mg, 0.00025‰) and 10 (1.2 mg, 0.00020‰). Fraction 16 (7.15 g) was re-separated by chromatography and semi-preparative HPLC with 60% EtOAc in *n*-hexane to afford pure lignans 6 (8.3 mg, 0.00141‰), 8 (15.8 mg, 0.00268‰), 11 (16.6 mg, 0.00281‰), 12 (8.9 mg, 0.00151‰), 13 (38.3 mg, 0.00649‰), 14 (62.3 mg, 0.01056‰) and 15 (2.2 mg, 0.00037‰). The flow of semi-preparative HPLC was 1.5 mL/min, the chromatograms of compounds showed on Figure 3.

3α-((E)-dodec-1-enyl)-4β-hydroxy-5β-methyldihydrofuran-2-one (1). Colorless oil; mp: 75.5–77.0 °C; $[\alpha]^{20}_D$ + 0° (c = 0.4, CHCl$_3$); HR-EI-MS m/z: 282.2198 [M]$^+$ (calcd for C$_{17}$H$_{30}$O$_3$, 282.2195); IR (KBr) $\lambda_{\text{max}}$: 3401, 1759, 1682, 1379 cm$^{-1}$; $^1$H-NMR and $^{13}$C-NMR (400/100 MHz, in CDCl$_3$): see Table 1.

Linderinol (6). Yellow amorphous solid; mp: 76.0–76.5°C; $[\alpha]^{20}_D$ + 20.2° (c = 0.42, CHCl$_3$); HR-EI-MS m/z: 342.1530 [M]$^+$ (calcd for C$_{20}$H$_{22}$O$_5$, 342.1467); UV max (CH$_3$OH): 253, 284 nm; IR (KBr) $\lambda_{\text{max}}$: 3310, 1605, 1512 cm$^{-1}$; $^1$H-NMR and $^{13}$C-NMR (500/125 MHz, in CDCl$_3$): see Table 1.

4′-O-methylkaempferol 3-O-α-L-(4″-E-p-coumaroyl) rhamnoside (11). Yellow amorphous solid; $[\alpha]^{20}_D$ ±0° (c = 8.3, CH$_3$OH); HR-ESI-MS m/z: 592.1576 [Na]$^+$ (calcd for C$_{31}$H$_{28}$O$_{12}$, 592.5446);
UV$_{\text{max}}$ (CH$_3$OH): 313, 277, 267, 247 nm; IR (KBr) $\lambda_{\text{max}}$: 3426, 2924, 1651, 1605, 1512, 1173 cm$^{-1}$; $^1$H-NMR and $^{13}$C-NMR (500/125 MHz, in CDCl$_3$): see Table 3.

Kaempferol 3-O-$\alpha$-L-(4''-Z-p-coumaroyl) rhamnoside (12). Yellow amorphous solid; [a]$_{2^\circ}$D $\pm$ 0° (c = 4.5, CH$_3$OH); HR-ESI-MS m/z: 578.1416 [Na]$^+$ (calcd for C$_{30}$H$_{26}$O$_{12}$, 578.1424); UV$_{\text{max}}$ (CH$_3$OH): 313, 277, 266, 247 nm; IR (KBr) $\lambda_{\text{max}}$: 3418, 2978, 1651, 1605, 1513, 1173 cm$^{-1}$; $^1$H-NMR and $^{13}$C-NMR (500/125 MHz, in CDCl$_3$): see Table 3.

Figure 3. The chromatograms of 1–15 on semi-preparative HPLC.
Table 3. NMR data (Methanol-\textit{d}_4) of 11 and 12. $\delta$ in ppm, $J$ in Hz.

| No. | $\delta_H$ $^a$ | $\delta_C$ $^b$ | $\delta_H$ $^a$ | $\delta_C$ $^b$ |
|-----|----------------|----------------|----------------|----------------|
| 2   | 159.1          |                | 159.6          |                |
| 3   | 135.7          |                | 135.8          |                |
| 4   | 179.6          |                | 179.9          |                |
| 4a  | 106.1          |                | 106.1          |                |
| 5   | 158.7          |                | 158.8          |                |
| 6   | 6.22, $d$, $J$ = 2.0 | 100.1        | 6.21, $d$, $J$ = 2.0 | 100.1        |
| 7   | 166.1          |                |                |                |
| 8   | 95.0           |                | 6.38, $d$, $J$ = 2.0 | 95.0           |
| 8a  | 163.3          |                | 163.4          |                |
| 1'  | 124.1          |                | 122.7          |                |
| 2'  | 7.84, $d$, $J$ = 8.8 | 132.0        | 7.73, $d$, $J$ = 8.5 | 132.1        |
| 3'  | 7.14, $d$, $J$ = 8.8 | 115.4        | 6.94, $d$, $J$ = 8.5 | 116.6        |
| 4'  | 163.6          |                | 161.8          |                |
| 5'  | 7.14, $d$, $J$ = 8.8 | 115.4        | 6.94, $d$, $J$ = 8.5 | 116.6        |
| 6'  | 7.84, $d$, $J$ = 8.8 | 132.0        | 7.73, $d$, $J$ = 8.5 | 132.1        |
| 1'' | 5.62, $br$ $s$ | 102.4         | 5.51, $d$, $J$ = 1.0 | 102.9        |
| 2'' | 4.23, $br$ $s$ | 71.9          | 4.23, $dd$, $J$ = 3.0, 1.0 | 72.0        |
| 3'' | 3.91, $dd$, $J$ = 9.7, 2.9 | 70.2        | 3.89, $dd$, $J$ = 9.7, 3.0 | 70.3        |
| 4'' | 4.91, $t$, $J$ = 9.7 | 74.9        | 4.90, $t$, $J$ = 9.7 | 74.6        |
| 5'' | 3.18, $m$      | 69.8          | 3.28, $m$      | 69.9          |
| 6'' | 0.78, $d$, $J$ = 6.3 | 17.8        | 0.78, $d$, $J$ = 6.3 | 17.8        |
| 1'''| 168.8          |                | 167.8          |                |
| 2'''| 6.25, $d$, $J$ = 16.0 | 115.3        | 6.75, $d$, $J$ = 12.8 | 116.0        |
| 3'''| 7.53, $d$, $J$ = 16.0 | 146.8        | 6.87, $d$, $J$ = 12.8 | 145.8        |
3.5. 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, St. Louis, MO, USA, 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).

3.6. 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. The cells were incubated with butanolides (0, 3.125, 6.25, 12.5, 25 and 50 µg/mL) 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, USA). Serum samples were diluted four times with distilled water and deproteinized by adding 1/20 volume of zinc sulfate (300 g/L) to a final concentration of 15 g/L. After centrifugation at 10,000×g for 5 min at room temperature, 100 µL supernatant was applied to a microtiter plate well, followed by 100 µL of Griess reagent. After 10 min of color development at room temperature, the absorbance was measured at 540 nm with a Micro-Reader. By using sodium nitrite to generate a standard curve, the concentration of nitrite was measured from absorbance at 540 nm.

3.7. 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 compounds 1–5 in the presence of 100 ng/mL LPS (lipopolysaccharide) for 24 h. After that, the cells were washed twice with DPBS and incubated with 100 µL of 0.5 mg/mL MTT for 3 h at 37 °C testing for cell viability. The medium was...
then discarded, and 100 µL dimethyl sulfoxide (DMSO) was added. After 30-min incubation, the absorbance at 570 nm was read using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

3.8. Statistical Analysis

IC₅₀ values were estimated using a non-linear regression algorithm (SigmaPlot 8.0; SPSS Inc., Chicago, IL, USA, 2002). Statistical evaluation was carried out by one-way analysis of variance (ANOVA followed by Scheffe’s multiple range tests).

4. Conclusions

These fifteen compounds 1–15 exhibited no significant cytotoxic activity. As to anti-inflammatory activity, compounds 2 and 3 are stronger than the other three, butanolides 1, 4 and 5. According to our previous study [26], the active site may result from the conjugation between the γ-lactone and olefinic functionalities despite the E- or Z-form. Although compounds 4 and 5 also possessed conjugation of γ-lactone and olefinic functionalities, it showed no significant active, due to the terminal acetylene group retarding the activity. Therefore, the butanolides that have saturated terminal or vinyl-terminal [24] were more active in anti-inflammatory than the acetylene-terminal group ones.

Comparing compounds 6–10, there is no significant difference on anti-inflammatory activity of the 8-8' linkage lignans 6 and 7, regardless of whether there is the presence of methoxy, γ-lactone or methylene dioxide groups; instead, the symmetry lignans 8 and 9 exhibited stronger anti-inflammatory activity than asymmetric ones. The number of methoxy group and the symmetry benzene ring may play an important role to affect anti-inflammatory activity.

Comparing flavonoids 11–15, there is no significant difference on anti-inflammatory activity of 11–13, regardless of whether there is the presence of methoxy, E- or Z-form of the p-coumaroyl group, but the flavonoids, which have a rhamnoside and p-coumaroyl group (11–13), exhibited stronger anti-inflammatory activity than catechin (14) and epicatechin (15).

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Conflict of Interest

The authors declare no conflict of interest.

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