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Anti-inflammatory Activities of New Succinic and Maleic Derivatives from the Fruiting Body of *Antrodia camphorata*

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Six new compounds, *trans*-3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]pyrrolidine-2,5-dione (**1**), *trans*-1-hydroxy-3-(4-hydroxyphenyl)-4-isobutylpyrrolidine-2,5-dione (**2**), *cis*-3-(4-hydroxyphenyl)-4-isobutyldihydrofuran-2,5-dione (**3**), 3-(4-hydroxyphenyl)-4-isobutyl-1*H*-pyrrole-2,5-dione (**4**), 3-(4-hydroxyphenyl)-4-isobutylfuran-2,5-dione (**5**), and dimethyl 2-(4-hydroxyphenyl)-3-isobutylmaleate (**6**), together with one known compound, 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]furan-2,5-dione (**7**), were isolated from the fruiting bodies of *Antrodia camphorata*. The structures of the compounds were elucidated by analysis of their spectroscopic data. To investigate the immunomodulatory potential of the compounds, RAW264.7 macrophage cells were treated with the compounds. Compound **1** significantly increased spontaneous TNF-α secretion from unstimulated RAW264.7 cells but suppressed IL-6 production [50% inhibition concentration value (IC_{50}) = 10 μg/mL] in LPS-stimulated cells. Compounds **3**, **4**, and **6** also suppressed IL-6 production with IC_{50} values of 17, 18, and 25 μg/mL, respectively, suggesting that these four compounds may have an anti-inflammatory effect on macrophage-mediated responses. Of the six compounds, compound **1** was the most effective, exerting both immunostimulatory and anti-inflammatory effects.

KEYWORDS: ***Antrodia camphorata*; succinic acid derivative; maleic acid derivative; macrophage; TNF-α; IL-6**

INTRODUCTION

*Antrodia camphorata*Wu, Ryvarden & Chang (Polyporaceae, Aphyllophorales) is native to Taiwan. Its fruiting body is a very rare and expensive mushroom that grows slowly in the wild and is difficult to cultivate in the greenhouse. The fruiting body of *A. camphorata* (= *Taiwanofungus camphorata*) has traditionally been used as an herbal medicine in Taiwan and is commonly known by the names "jang-jy" and "niu-chang-chih" (*1*). In the wild, it grows on the inner heartwood wall of *Cinnamomun kanehirai* Hay (Lauraceae), an endemic and endangered species in Taiwan. In traditional herbal medicine, *A. camphorata* fruiting bodies have been utilized as treatment for food and drug intoxications, diarrhea, abnormal pains, hypertension, itchy skin, and liver cancer (*2, 3*). The biological activities of the fruiting body (*4, 5*) and cultured mycelia (*6–8*) of *A. camphorata* have been studied. The wild-type fruiting body contains fatty acids, lignans, phenyl derivatives, sesquiterpenes, steroids, and triterpenoids (*1, 9–14*). The mycelium was studied by Nakamura et al., who purified maleic and succinic acid derivatives. Both the wild-type and mycelium possessed antiproliferative activity against a Lewis lung carcinoma (LLC) tumor cell line (*8*). The methanol extracts of *A. camphorata* also showed potential anti-inflammatory effects, and the effects seen with a solid-state culture were similar to those of the wild fruiting body (*15*). The first studies on the fruiting body of this fungus in a solid culture were achieved by Chen et al. (*16*), who evaluated five labdane-type diterpenes (including three new compounds)

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Table 1. ^{1}H NMR Data of Compounds **1**−**7** (400 MHz, **1**−**3** and **5**−**7** in $CDCl_3$, **4** in CD_3OD)

no.	1	2	3	4	5	6	7
1	7.94 br s						
2							
3	2.97 dt (5.2, 5.2)	3.68 d (5.0)	3.59 d (11.1)				
4	3.60 d (5.2)	2.91 m	3.17 td (11.1, 3.6)				
5							
1′	1.54 m	1.75−1.85 m 1.60m	1.37 m 1.70 m	2.48 d (7.6)	2.56 d (7.2)	2.14 d (7.2)	2.59 d (7.0)
2′	1.83 m	1.75−1.85 m	1.61 m	1.99 m	2.08 m	1.70 m	2.12 m
3′	0.71 d (6.4)	0.69 d (6.3)	0.91 d (6.5)	0.87 d (6.8)	0.91 d (6.7)	0.77 d (6.8)	0.94 d (6.8)
4′	0.89 d (6.4)	0.88 d (6.3)	0.97 d (6.5)	0.87 d (6.8)	0.91 d (6.7)	0.77 d (6.8)	0.94 d (6.8)
1″							
2″, 6″	7.10 d (8.8)	7.17 d (8.5)	7.20 d (8.6)	7.43 d (9.0)	7.54 d (8.8)	7.09 d (8.2)	7.50 d (8.8)
3″, 5″	6.88 d (8.8)	6.83 d (8.5)	6.75 d (8.6)	6.87 d (9.0)	6.94 d (8.8)	6.81 d (8.2)	7.02 d (8.8)
4″							
1‴	4.47 d (6.8)						4.57 d (6.6)
2‴	5.46 br t (6.8)						5.50 br t (6.6)
3‴							
4‴	1.72 s						1.76 s
5‴	1.78 s						1.81 s
1-OCH_3						3.70 s	
4-OCH_3						3.80 s	

in vitro for their neuroprotective activity. Recently, Chen et al. (*17*) have also reported anti-inflammatory benzenoid constituents from the fruiting body of wild-type *A. camphorata*. Interestingly, they found it contains an acetylene functionality. Here, we isolated and characterized three new succinic acid derivatives, three new maleic acid derivatives, and one previously known compound from the fruiting body of *A. camphorata* in a solid culture and studied the anti-inflammatory activity of their methanol extracts. To our knowledge, this is the first time maleic acid and succinic acid derivatives have been isolated from the fruiting body of *A. camphorata* in a solid culture.

MATERIALS AND METHODS

General Experimental Procedures. Melting points were determined with a Yanagimoto micromelting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 983G spectrophotometer. ^{1}H, ^{13}C, and DEPT spectra were acquired on a Bruker DMX-400 spectrometer, and two-dimensional NMR spectra were acquired on a Bruker DMX-500 spectrometer. EIMS, UV, and specific rotations were determined using a JEOL JMS-HX 300 spectrometer, a Hitachi S-3200 spectrometer, and a JASCO DIP-180 digital polarimeter, respectively. Extracts were initially fractionated on silica gel (Merck 70−230 mesh, 230−400 mesh, ASTM) and then purified with a semipreparative normal-phase HPLC column (250 × 10 mm, 7 μm, LiChrosorb Si 60) on an LDC Analytical-III system.

Plant Material. The solid cultural fruiting bodies of *A. camphorata* were identified and provided by Well Shine Biotechnology Development, Taipei, Taiwan. A voucher specimen was deposited at Well Shine Biotechnology Development Co. Ltd.

Extraction and Isolation. The fruiting bodies of solid culture *A. camphorata* (3.0 kg) were extracted with MeOH (12 L) by maceration at room temperature (4 days × 3). After removal of MeOH under vacuum, H_2O was added to bring the total volume to 1 L. This suspended phase was partitioned with EtOAc (1 L × 3). Evaporation of the combined EtOAc layers afforded black syrup (212 g). The EtOAc fraction (200 g) was chromatographed on a Si gel column (10 × 70 cm, Merk 70−230 mesh) using *n*-hexane, EtOAc, and MeOH of increasing polarity as eluent to obtain nine fractions: fraction 1 [8000 mL, *n*-hexane/EtOAc (19:1)], fraction 2 [7000 mL, *n*-hexane/EtOAc (9:1)], fraction 3 [6000 mL, *n*-hexane/EtOAc (8:2)], fraction 4 [10000 mL, *n*-hexane/EtOAc (7:3)], fraction 5 [8000 mL, *n*-hexane/EtOAc (1:1)], fraction 6 [9000 mL, *n*-hexane/EtOAc (1:3)], fraction 7 (8000 mL, EtOAc), fraction 8 [7000 mL, EtOAc/MeOH (1:1)], and fraction 9 (6000 mL, MeOH). HPLC of fraction 6 on a normal-phase column with *n*-hexane/EtOAc (4:1) as eluent, 4 mL/min, afforded **1** (12.5 mg),**2** (22.6 mg), **3** (6.8 mg), **4** (9.4 mg), **5** (15.0 mg), **6** (13.2 mg), and **7** (8.9 mg), respectively.

Table 2. ^{13}C Spectral Data of **1**−**6** (100 MHz, **1**−**3** and **5**−**7** in $CDCl_3$, **4** in CD_3OD)

no.	1	2	3	4	5	6	7
1						168.3	
2	179.2	172.0	173.3	172.6	166.4	137.5	166.4
3	48.3	49.7	53.9	138.7	140.0	140.1	139.8
4	53.8	44.2	47.5	138.3	140.2	169.4	140.2
5	177.4	173.0	174.1	173.5	165.5		165.4
1′	40.6	40.1	40.9	32.4	33.6	39.1	33.6
2′	25.6	25.0	26.3	28.1	27.9	27.4	27.9
3′	23.0	22.4	23.1	22.0	22.6	22.4	22.7
4′	21.4	20.7	20.6	22.0	22.6	22.4	22.7
1″	128.5	128.5	128.0	120.5	120.0	127.1	119.9
2″, 6″	128.7	129.3	129.5	131.0	131.3	130.4	131.1
3″, 5″	115.4	115.6	115.0	115.2	116.0	115.3	115.1
4″	158.6	156.8	156.8	158.8	158.2	155.5	160.9
1‴	64.8						65.0
2‴	119.4						118.7
3‴	138.5						139.1
4‴	18.2						18.2
5‴	25.8						25.2
1-OCH_3						52.4	
4-OCH_3						52.2	

***trans*-3-Isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]pyrrolidine-2,5-dione (1).** Colorless gum; $[\alpha]_D^{25}$ +24.0 (*c* 0.20, MeOH); IR ν_{max}^{film} cm^{-1} 3256, 2928, 2657, 1714, 1612, 1513, 1469; for ^{1}H NMR ($CDCl_3$, 400 MHz), see **Table 1**; for^{13}C NMR ($CDCl_3$, 100 MHz), see **Table 2**; EIMS 70 eV, *m*/*z* (rel intenstiy) 315 $[M]^+$ (1), 260 (9), 247 (75), 191 (100), 176 (15), 133 (61), 69 (67); HREIMS, *m*/*z* found, 315.1825; calcd for $C_{19}H_{25}NO_3$, 315.1828.

***trans*-1-Hydroxy-3-(4-hydroxyphenyl)-4-isobutylpyrrolidine-2,5-dione (2).** White solid; mp 189−193 °C; $[\alpha]_D^{25}$ +9.6 (*c* 0.11, MeOH); UV λ_{max}^{MeOH} nm (log ϵ) 221.0 (4.16), 275.0 (3.46), 283.0 (3.38); IR ν_{max}^{film} cm^{-1} 3349, 2929, 1716, 1608, 1510, 1460, 1214, 1116, 840; for ^{1}H NMR ($CDCl_3$, 400 MHz), see **Table 1**; for^{13}C NMR ($CDCl_3$, 100 MHz), see **Table 2**; EIMS 70 eV, *m*/*z* (rel intensity) 263 $[M]^+$ (28), 207 (71), 179 (63), 137 (46), 133 (100), 107 (35); HREIMS, *m*/*z* found, 263.1159; calcd for $C_{14}H_{17}NO_4$, 263.1153.

***cis*-3-(4-Hydroxyphenyl)-4-isobutyldihydrofuran-2,5-dione (3).** White solid; mp 184−187 °C; $[\alpha]_D^{25}$ +3.2 (*c* 0.03, MeOH); UV λ_{max}^{MeOH} nm (log ϵ) 229.0 (4.27), 276.0 (3.62), 286.0 (3.57); IR ν_{max}^{film} cm^{-1} 3339, 2955, 2919, 1830, 1785, 1664, 1598, 1506, 1449, 1214, 840; for ^{1}H NMR ($CDCl_3$, 400 MHz), see **Table 1**; for^{13}C NMR ($CDCl_3$, 100

MHz), see **Table 2**; EIMS 70 eV, *m/z* (rel intensity 248 $[M]^+$ (14), 220 (10), 176 (33), 133 (100), 107 (35); HREIMS, *m/z* found, 248.1050; calcd for $C_{14}H_{16}O_4$, 248.1044.

3-(4-Hydroxyphenyl)-4-isobutyl-1*H*-pyrrole-2,5-dione (4). Yellow needles; mp 199−201 °C; UV λ_{max}^{MeOH} nm (log ϵ) 231.0 (4.4), 269.0 (4.4), 360.0 (3.8); IR ν_{max}^{film} cm^{-1} 3401, 2956, 1723, 1611, 1513, 1434, 1269; for ^1H NMR (CD_3OD, 400 MHz), see **Table 1**; for ^{13}C NMR (CD_3OD, 100 MHz), see **Table 2**; EIMS 70 eV, *m/z* (rel intensity 245 $[M]^+$ (98), 203 (100), 132 (52), 131 (50); HREIMS, *m/z* found, 245.1041; calcd for $C_{14}H_{15}NO_3$, 245.1048.

3-(4-Hydroxyphenyl)-4-isobutylfuran-2,5-dione (5). Yellow oil; UV λ_{max}^{MeOH} nm (log ϵ): 227.0 (4.16), 358.0 (3.79); IR ν_{max}^{film} cm^{-1}: 3436, 1838, 1759, 1606, 1513, 1334, 1228, 1169, 923; For ^1H NMR ($CDCl_3$, 400 MHz), see **Table 1**; For ^{13}C NMR ($CDCl_3$, 100 MHz), see **Table 2**; EIMS 70 eV, *m/z* (rel intensity) 246 $[M]^+$ (3), 218 (2), 204 (5), 176 (10), 103 (21), 73 (44), 59 (100); HREIMS, *m/z* found, 246.0883; calcd for $C_{14}H_{14}O_4$, 246.0888.

Dimethyl 2-(4-Hydroxyphenyl)-3-isobutylmaleate (6). Colorless oil; UV λ_{max}^{MeOH} nm (log ϵ) 222.0 (4.3), 277.0 (3.9); IR ν_{max}^{max} cm^{-1} 3380, 3298, 2950, 1710, 1606, 1585, 1511; for ^1H NMR ($CDCl_3$, 400 MHz), see **Table 1**; for ^{13}C NMR ($CDCl_3$, 100 MHz), see **Table 2**; EIMS 70 eV, *m/z* (rel intensity) 292 $[M]^+$ (11), 260 (100), 232 (36), 217 (86), 190 (30), 173 (21), 131 (19); HREIMS, *m/z* found, 292.1300; calcd, for $C_{16}H_{20}O_5$, 292.1305.

3-Isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]furan-2,5-dione (7). Yellow oil; UV λ_{max}^{MeOH} nm (log ϵ) 227.0 (4.1), 258.0 (3.9), 275.0 (3.8), 355.0 (3.4); IR ν_{max}^{max} cm^{-1} 1763; for ^1H NMR ($CDCl_3$, 400 MHz), see **Table 1**; for ^{13}C NMR ($CDCl_3$, 100 MHz), see **Table 2**; EIMS 70 eV, *m/z* (rel intensity) 314 $[M]^+$ (100), 246 (95), 131 (97).

Cell Culture and Cell Viability Assay. The RAW264.7 cell line was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and cultured in DMEM containing 10% fetal bovine serum (FBS), 2 mM glutamine, 1% nonessential amino acid, and 1 mM sodium pyruvate. Cells were maintained in a humidified incubator at 37 °C in 5% CO_2. For all experiments, the cells were subjected to no more than 20 cell passages. To examine the effect of the fractions of *A. camphorata* on cell viability, cells were plated in 96-well plates at 10000 cells per well with DMEM containing 10% FBS. Cells were treated with various concentrations of extracts from *A. camphorata* in the presence or absence of 50 ng/mL LPS. Viable cell numbers were determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO) assay as described previously (*18*).

Determination of Cytokine Production. The cytokine levels in culture supernatants were measured by sandwich ELISA methods as described by Chen and Lin (*19*). Briefly, the anticytokine antibodies (PharMingen, San Diego, CA) were coated in the 96-well plates (Nunc, Roskilde, Denmark) after overnight incubation at 4 °C and having been blocked with 1% BSA−PBS buffer for 30 min. The samples and standards (recombinant mouse cytokines, PharMingen) were added to the 96-well plates for 2 h of incubation. The biotin-conjugated antibodies (biotinylated rat antimouse cytokine monoclonal antibodies, PharMingen) were added and incubated. After washing, streptavidin-conjugated peroxidase was added for 1 h. The substrate, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma), was added to each well for 20 min. The plates were read in a microplate autoreader (Microplate autoreader; Bio-Tek Instruments, Winooski, VT) at 405 nm.

Statistical Analysis. The data are expressed as the means ± standard deviation of three independent experiments. The significance of difference between each treatment was analyzed by unpaired Student's *t* test using Strategic Application Software (SAS Windows version 8.2; SAS Institute Inc., Cary, NC) throughout the study. Data are expressed as mean ± SD. Values at $p < 0.05$ are considered to be significant.

RESULTS AND DISCUSSION

Extraction of the fruiting bodies of *A. camphorata* in a solid culture with MeOH followed by liquid−liquid partition resulted in the localization of anti-inflammatory activity in the EtOAc fraction. Further fractionation on a silica gel column yielded fractions rich in anti-inflammatory activity. When cells were stimulated with LPS, the concentrations for 50% inhibition of IL-6 production by crude extract and EtOAc fraction were 42 and 30 μg/mL, respectively. The bioactive fraction was subjected to further chemical analysis to give six new compounds **1**−**6** and one known compound **7** (**Figure 1**) (*8*).

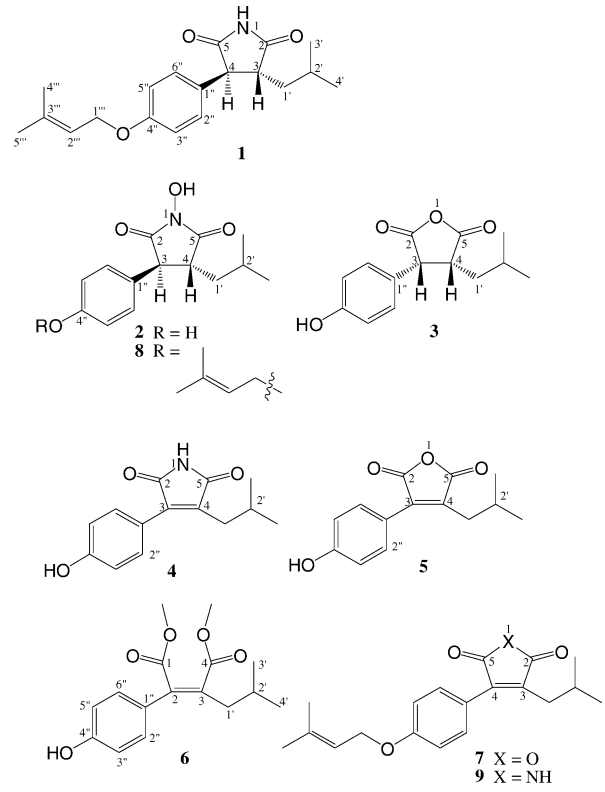


Figure 1. Structures of compounds **1**−**9**.

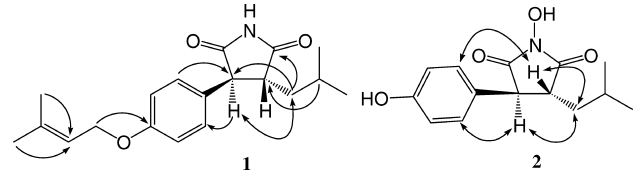
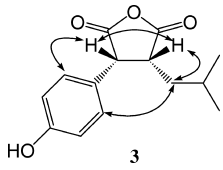


Figure 2. Key HMBC (→) and NOESY (↔) correlations of compounds **1** and **2**.

Compound **1** was isolated as a colorless gum. High-resolution mass spectrometry (HREIMS) revealed **1** to have the formula $C_{19}H_{25}NO_3$, and the IR spectrum indicated the presence of a succinimide moiety (3256 and 1714 cm^{-1}) group. The ^1H NMR spectrum (**Table 1**) showed the presence of NH of imide resonance at δ 7.94 (1H, br s), an isobutyl group at δ 0.71, 0.89 (3H each, $J = 6.4$ Hz), 1.54 (2H, m) and 1.83 (1H, m), an isoprenyl group attached on O atom of the phenoxyl group at δ 1.72, 1.78 (3H each, s), 4.47 (2H, d, $J = 6.8$ Hz), and 5.46 (1H, br t, $J = 6.8$ Hz, H-2‴), and a *p*-alkylphenoxy moiety at δ 6.88 and 7.10 (each 2H, $J = 8.8$ Hz), which was further confirmed by a COSY experiment. The HMBC experiment established the connection of **1** (**Figure 2**). The resonances at δ 3.60 (1H, d, $J = 5.2$ Hz) and 2.97 (1H, td, $J = 5.2$, 5.2 Hz) were assigned as H-4 and H-3, respectively, of the succinimide functionality by the COSY spectrum. A small coupling constant ($J = 5.2$ Hz) (*8*) together with NOESY correlations, H-4/H_2-1′ and H-2″/H-3, elucidated two substituents in trans configuration. The ^1H and ^{13}C NMR resonances are similar to those of **8** (*8*). The only difference between the two compounds is that



3

Figure 3. Key NOESY (↔) correlations of compound **3**.

compound **8** has one additional hydroxyl group on the N atom. Therefore, the structure of **1** can be defined as *trans*-3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]pyrrolidine-2,5-dione.

Compound **2** has the molecular formula $C_{14}H_{17}NO_4$ deduced from its HREIMS and ^{13}C NMR data (**Table 2**). The IR spectrum showed the presence of hydroxyl (3256 cm^{-1}) and maleimide carbonyl groups at 1716 cm^{-1} (*8*). The ^{13}C NMR and DEPT spectra showed signals of two methyl carbons, one methylene carbon, three methine carbons, and two carbonyl carbons, as well as one benzene ring. The ^1H NMR spectrum (**Table 1**) showed the presence of an isobutyl moiety [δ 0.69, 0.88 (each 3H, d, J = 6.3 Hz), 1.60, 1.75−1.85 (each 1H, m, H-1′) and 1.75−1.85 (1H, m, H-2)] and a *p*-alkylphenol moiety [δ 6.83, 7.17 (each 2H, d, J = 8.5 Hz)]. As in compound **1**, two methine protons of succinimide in compound **2** appeared at δ 2.91 (1H, m, H-4) and 3.68 (1H, d, J = 5.0 Hz, H-3). The consecutive protons were shown as follows: H-3 → H-4 → H-1′ → H-2′ → H-3′ (-4′), which was revealed from the COSY spectrum. The NOESY correlations (**Figure 2**), H-3/H-1′, H-4/H-2″ (-6″), and coupling constant elucidated H-3 and H-4 are in trans configuration. A comparison of the ^1H and ^{13}C NMR spectra of **2** and **8** (*8*) showed that the only difference is that an isoprenyl group in **8** disappears in **2**. On the basis of the above deduction, **2** was elucidated as a new compound named *trans*-1-hydroxy-3-(4-hydroxyphenyl)-4-isobutylpyrrolidine-2,5-dione. Assignment of the ^1H and ^{13}C NMR spectra of **2** was based on HSQC, HMBC, COSY, and NOESY.

Fourteen ^{13}C NMR resonances and exact mass spectrum data confirmed the molecular formula of **3** to be $C_{14}H_{16}O_4$. The IR spectrum revealed two succinic anhydride absorptions at 1830 and 1785 cm^{-1}. The ^1H NMR spectrum (**Table 1**) of **3** was similar to that of **2** and showed the presence of an isobutyl moiety at δ 0.91, 0.97 (each 3H), 1.37, 1.61, 1.70 (each 1H, m) and a para-substituted phenol AA′XX′ coupling system with signals at δ 7.20 (2H, d, J = 8.6 Hz) and 6.75 (2H, d, J = 8.6 Hz). The resonance at δ 3.17 (1H, td, J = 11.1, 3.3 Hz, H-4) and 3.59 (1H, d, J = 11.1 Hz, H-3) (*8*) together with NOESY correlation (**Figure 3**) disclosed that two protons connected on succinic anhydride are in the cis form. The HMBC spectrum showed that **3** had the same partial structure as **2**. Compound **3** was consequently defined as *cis*-3-(4-hydroxyphenyl)-4-isobutyldihydrofuran-2,5-dione.

Compound **4** has the molecular formula $C_{14}H_{15}NO_3$ as deduced from HREIMS and ^{13}C NMR data, which indicates eight indices of hydrogen deficiency (IHD). The IR spectrum showed an imide carbonyl absorption at 1723 cm^{-1}. Carbon signals for two methyl carbons, one methylene carbon, one sp^3 methine, one benzene ring, one olefinic group, and two carbonyl groups were shown in the ^{13}C NMR spectrum (**Table 2**). The ^1H NMR spectrum (**Table 1**) exhibited signals for an isobutyl moiety [δ 0.87 (6H, d, J = 6.8 Hz), 1.99 (1H, m), 2.48 (2H, m)] and a para-substitued phenol [δ 7.43 and 6.87 (each 2H, d, J = 9.0 Hz)]. The resonance at δ 7.43 (H-2″, -6″) correlated with C-3 (δ_C 138.7) and the cross peak of δ 2.48 (H-1′) coupling with C-3 and -4 (δ_C 138.7, 138.3) on HMBC spectrum and establishing the connections of C-1″−C-3 and C-1′−C-4. In addition, the UV λ_{max} absorption bands of **4** are very similar to those of **9** (*8*). We concluded that **4** has a similar base skeleton to **9**. The only difference is an additional isoprenyl group at the phenolic O atom in **9**. On the basis of the above deduction, **4** was elucidated as the new compound named 3-(4-hydroxyphenyl)-4-isobutyl-1*H*-pyrrole-2,5-dione. Assignment of the ^1H and ^{13}C NMR spectra of **4** was based on HSQC, HMBC, COSY, and NOESY.

Compound **5** was isolated as a yellow oil and has the molecular formula $C_{14}H_{14}O_4$ based on its exact mass and ^{13}C NMR spectrum. The IR spectrum revealed carbonyl absorptions of maleic anhydride at 1838 and 1759 cm^{-1}. Comparisons of ^1H and ^{13}C NMR data (**Tables 1** and **2**) and mass spectrum between **5** and **4** showed these two components have similar spectra, the only difference being that −NH− in **4** replaces −O− in **5**. By using HSQC, HMBC, COSY, and NOESY spectra, **5** was confirmed as 3-(4-hydroxyphenyl)-4-isobutylfuran-2,5-dione (*20*), its first isolation from nature. This compound is also an intermediate for the synthesis of camphorataanhydride A (**7**) (*8*).

Sixteen ^{13}C NMR resonance and exact mass spectrum data confirmed the molecular formula of **6** to be $C_{16}H_{20}O_5$. Seven IHD were provided from the formula. The IR spectrum revealed the presence of a hydroxyl group (3380 cm^{-1}) and a conjugated ester group (1710 cm^{-1}). The UV λ_{max} at 222.0 and 277.0 nm confirmed the conjugated skeleton. Two methyl, one methylene, one methine, one benzene, one olefinic, and two ester carbonyl groups were shown on ^{13}C NMR and DEPT spectra. The ^1H NMR data showed an AA′XX′ coupling system resonance at δ 7.09 (2H, d, J = 8.2 Hz) and 6.81 (2H, d, J = 8.2 Hz), indicating the existence of a para-substituted phenol moiety. Two methoxy groups at δ 3.70 and 3.80 correlating with δ_C 168.3 (C-1) and 169.4 (C-4), respectively, on the HMBC spectrum established the connection of two carbonyl methoxy groups. The resonance at δ 7.09 (H-2″, -6″) with the correlation with C-2 (δ_C 137.5) and the cross peak between δ 2.14 (H-1′) and C-2 and -3 (δ_C 137.5, 140.1) on the HMBC spectrum established the connections of C-2−C-1″ and C-1′−C-3, respectively. On the basis of the above deduction, **6** was elucidated as a new compound and named dimethyl 2-(4-hydroxyphenyl)-3-isobutylmaleate. Assignment of the ^1H and ^{13}C NMR spectra of **6** was based on HSQC, HMBC, COSY, and NOESY.

Macrophages are the first line of host defense against bacterial infection and cancer growth (*21, 22*). The induction of cytokine synthesis, such as tumor necrosis factor-α (TNF-α) from macrophages is one of the methods used to evaluate the activation of innate immunity (*23*). We evaluated the immunomodulatory effects of these new compounds by testing the in vitro production of cytokines in RAW264.7 cells treated with the compounds. As shown in **Table 3**, compound **1** significantly increased spontaneous TNF-α secretion by RAW264.7 cells without affecting cell viability, suggesting that compound **1** may have the potential to activate macrophages. TNF-α levels secreted by RAW264.7 cells stimulated by 0.5−5 μg/mL of compound **1** increased in a dose-dependent manner. Compounds **2**−**5** decreased the viability of RAW264.7 cells at doses over 5 μg/mL and had no significant effect on TNF-α production. Compound **6** had no effect on viability or spontaneous TNF-α production. These data suggest that compound **1** might be the active compound that stimulates macrophages to secret TNF-α without cell toxicity.

In addition, immunomodulatory effects of the new compound were evaluated by mitogen-activated macrophages. Activated macrophages secrete pro-inflammatory mediators such as TNF-α, IL-6, IL-1β, and nitric oxide (NO). The proper production of macrophage-derived mediators enhances the innate immune response (*24*), but an overproduction can result in acute phase endotoxemia and cause tissue injury, organ failure, shock, and even death (*23*). Macrophage cell lines, such as murine RAW264.7 and

Table 3. Effects of Isolates from *A. camphorata* on Viability and Spontaneous TNF-α Secretion in RAW264.7 Macrophage Cells[a]

	compound							
concn (μg/mL)	crude	EtOAc fraction	**1**	**2**	**3**	**4**	**5**	**6**
				Cell Viability (%)				
0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
0.5	100 ± 9	106 ± 8	103 ± 2	106 ± 7	91 ± 9	97 ± 13	101 ± 7	100 ± 6
1	101 ± 8	108 ± 9	101 ± 3	100 ± 5	87 ± 8	99 ± 4	102 ± 5	99 ± 7
2	105 ± 5	106 ± 7	108 ± 6	100 ± 5	80 ± 9*	97 ± 5	99 ± 6	102 ± 6
5	99 ± 8	95 ± 9	100 ± 10	94 ± 6	72 ± 11*	94 ± 7	95 ± 9	95 ± 8
10	99 ± 9	93 ± 11	99 ± 7	90 ± 4*	67 ± 15*	81 ± 8*	86 ± 8*	96 ± 11
				TNF-α Secretion (pg/mL)				
0	88 ± 45	88 ± 45	88 ± 45	88 ± 45	88 ± 45	88 ± 45	88 ± 45	88 ± 45
0.5	92 ± 35	96 ± 32	117 ± 31	108 ± 28	55 ± 2	91 ± 38	100 ± 26	100 ± 34
1	100 ± 36	120 ± 41	145 ± 44	97 ± 13	74 ± 7	83 ± 22	102 ± 30	114 ± 41
2	150 ± 30	143 ± 29	183 ± 89**	105 ± 23	68 ± 7	105 ± 13	90 ± 4	90 ± 14
5	179 ± 45	186 ± 41	212 ± 60*	96 ± 8	68 ± 26	118 ± 23	94 ± 19	110 ± 22
10	120 ± 60	120 ± 52	188 ± 41*	128 ± 70	77 ± 35	104 ± 25	106 ± 5	116 ± 4

[a] Cells were treated with or without isolates of *A. camphorata* for 48 h. Supernatants were collected for TNF-α assay, and cells were collected for viability analysis by MTT method. Data are means ± SD of three independent experiments with triplicates of each. *, $p < 0.05$; **, $0.05 < p > 0.1$; significantly different from the control (no extract treatment) group.

Table 4. Effects of Isolates from *A. camphorata* on Cell Viability and TNF-α and IL-6 Secretion in LPS-Stimulated RAW264.7 Macrophage Cells[a]

	compound							
concn (μg/mL)	crude	EtOAc fraction	**1**	**2**	**3**	**4**	**5**	**6**
				Cell Viability (%)				
0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
0.5	104 ± 5	106 ± 6	105 ± 3	110 ± 5*	105 ± 6	105 ± 6	106 ± 4*	108 ± 6
1	103 ± 7	102 ± 8	106 ± 4	111 ± 6*	104 ± 6	103 ± 5	105 ± 2*	107 ± 7
5	105 ± 6	107 ± 9	105 ± 3	111 ± 8*	99 ± 3	97 ± 5	102 ± 2	106 ± 9
10	102 ± 9	110 ± 7*	102 ± 4	109 ± 2*	91 ± 4*	92 ± 8	100 ± 3	102 ± 10
				TNF-α Secretion (ng/mL)				
0	3.81 ± 0.93	3.81 ± 0.93	3.81 ± 0.93	3.81 ± 0.93	3.81 ± 0.93	3.81 ± 0.93	3.81 ± 0.93	3.81 ± 0.93
0.5	3.71 ± 0.85	3.61 ± 0.69	3.69 ± 0.39	3.34 ± 0.58	3.17 ± 0.70	2.98 ± 0.24	3.44 ± 0.31	3.61 ± 0.22
1	3.83 ± 0.74	3.95 ± 1.07	3.92 ± 0.39	4.04 ± 0.46	3.64 ± 0.84	3.22 ± 0.35	4.06 ± 1.14	3.70 ± 0.50
5	3.95 ± 1.01	3.84 ± 0.89	3.68 ± 0.25	4.76 ± 0.65	3.88 ± 0.37	3.72 ± 0.23	4.67 ± 1.09	3.80 ± 0.53
10	3.35 ± 0.70	3.91 ± 0.98	3.65 ± 0.17	3.66 ± 0.67	3.90 ± 0.10	3.12 ± 0.61	3.63 ± 0.58	3.32 ± 0.30
				IL-6 Secretion (pg/mL)				
0	238 ± 86	238 ± 86	238 ± 86	238 ± 86	238 ± 86	238 ± 86	238 ± 86	238 ± 86
0.5	237 ± 110	229 ± 95	213 ± 88	216 ± 102	214 ± 82	194 ± 81	221 ± 102	235 ± 106
1	223 ± 95	220 ± 65	211 ± 99	224 ± 110	234 ± 109	257 ± 40	227 ± 94	215 ± 89
5	207 ± 92	210 ± 79	144 ± 64	210 ± 96	185 ± 76	195 ± 83	225 ± 68	209 ± 89
10	192 ± 57	185 ± 72	103 ± 61*	191 ± 68	147 ± 53**	169 ± 63**	215 ± 56	179 ± 91
IC_{50} (μg/mL)	42	30	10	54	17	18	96	25

[a] Cells were pretreated with isolates of *A. camphorata* for 30 min and then stimulated with 50 ng/mL LPS for 48 h. Supernatants were collected for TNF-α and IL-6 assays, and cells were collected for viability analysis by MTT method. Data are means ± SD as described under Materials ant Methods. *, $p < 0.05$; **, $0.05 < p < 0.1$; significantly different from the control (no extract treatment) group.

human THP-1, have been proposed as rapid in vitro screening methods to test the anti-inflammatory agents (*25*). In this study, LPS stimulation significantly increased TNF-α (3.81 ± 0.93 vs 0.05 ± 0.02 ng/mL) and IL-6 (238 ± 86 vs 48.5 ± 3.8 pg/mL) secretions (data not shown). The effect of pretreatment of new compounds from *A. camphorata* on TNF and IL-6 secretions by LPS-stimulated RAW264.7 cells is shown in **Table 4**. In general, these compounds did not affect the viability of RAW264.7 macrophages. However, at 10 μg/mL, compounds **2** and **5** increased cell viability, whereas compound **3** decreased cell viability. When cells were stimulated with LPS, TNF-α secretions were not significantly affected; however, IL-6 production was significantly decreased by compound **1** in a dose-dependent manner. The concentration required for 50% inhibition (IC_{50}) of IL-6 production by compound **1** was 10 μg/mL. Compounds **3**, **4**, and **6** also suppressed IL-6 production. They had IC_{50} values 17, 18, and 25 μg/mL, respectively. Compounds **2** and **5** had the least effect on IL-6 production. They had IC_{50} values of 54 and 96 μg/mL, respectively.

The wild-type fruiting body of *A. camphorata* is well-known as an effective and expensive folk remedy for many diseases. Anti-inflammatory activity of *A. camphorata* has been suggested to contribute to the prevention of neurodegenerative diseases through suppression of both the inducible nitric oxide synthetase and cyclooxygenase-2 expression in mouse microglia cell line (*15*). Furthermore, Rao et al. showed that methanol extracts from *A. camphorata* inhibited macrophage-mediated inflammatory mediators such as NO and TNF-α in LPS/IFNγ-activated mouse peritoneal macrophages (*26*). IL-6, a pro-inflammatory cytokine that is a useful marker of infection, is secreted by monocytes and macrophages in many infectious and inflammatory states, including cardiac surgery, cardiogenic shock, coronary bypass, and bacteria sepsis (*27–29*). The serum concentration of IL-6 has been reported to correlate with disease severity (*30*). IL-6 production from LPS-stimulated macrophages, from cell lines, and from primary cells was also significantly decreased by anti-inflammatory drugs, such as pyrrolidine dithiocarbamate (*24*). In the present study, compound **1** from the methanol extract of *A. camphorata* enhanced spontaneous TNF secretion, but decreased IL-6 production from LPS-activated macrophage without affecting cell viability, suggesting that compound **1** is the most effective compound of methanol

extracts from *A. camphorata*, exerting both immunostimulatory and anti-inflammatory effects.

In summary, we identified six new compounds, three succinic derivatives (compounds **1**−**3**) and three maleic derivatives (compound **4**−**6**), together with one known compound from the fruiting bodies of *A. camphorata*. Compound **1** significantly activated macrophages to secret TNF-α, indicating that it is a potential immunostimulator. Compounds **1**, **3**, **4**, and **6** significantly suppressed LPS-stimulated IL-6 from RAW264.7 macrophages. These data suggest that these four compounds may have an anti-inflammatory effect in macrophage-mediated responses. Among these compounds isolated from *A. camphorata*, compound **1** was the most effective, exerting both immunostimulatory and anti-inflammatory effects.

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