Article

Secoiridoids from the Seed of *Gonocaryum calleryanum* and Their Inhibitory Potential on LPS-Induced Tumor Necrosis Factor and Nitric Oxide Production

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Abstract: Three new secoiridoid constituents Gonocarin A-C (**1-3**) and a new derivative Gonocarin A monoacetate (**4**), along with two known lignins pinoresinol (**5**) and paulownin (**6**) were isolated from the seed of *Gonocaryum calleryanum* (Baill.) Becc. The structures of the new metabolites were determined on the basis of extensive spectroscopic analysis, particularly mass spectroscopy and 2D NMR (^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY) spectroscopy. When mouse macrophages RAW264.7 were treated with compounds **1-6** together with LPS -stimulated, a concentration-dependent inhibition of nitric oxide (NO) and tumor necrosis factor (TNF-α) productions were detected. The results confirmed that the *Gonocaryum calleryanumrrg* could be a potential anti-inflammatory agent.

Keywords: *Gonocaryum calleryanum*; secoiridoid; structure identification; anti-inflammatory.

1. Introduction

Gonocaryum Miq. plant is widely distributed in tropical and subtropical regions forests on coral rocks from Indonesia to Philippines (Luzon and Batan Islands) and to Taiwan (Hengchun Peninsula).[1] *Gonocaryum calleryanum* (Baill.) Becc. is the only species of *Gonocaryum* found in Kenting National Park in Taiwan. Its leaves are used in Philippine traditional folk medicine for treating stomach disease. [2] However, there are only few reports about chemical composition and biological activity of *Gonocaryum calleryanum*. Kaneko [3] and Chan [4] reported the isolation of secoiridoid glycosides, flavonoids and flavonoid glycosides from the leaves, branch, stem and root bark of this plant [5-10], but did not go any further to research its biological activity. The chemical composition of the seed part has never been analyzed.

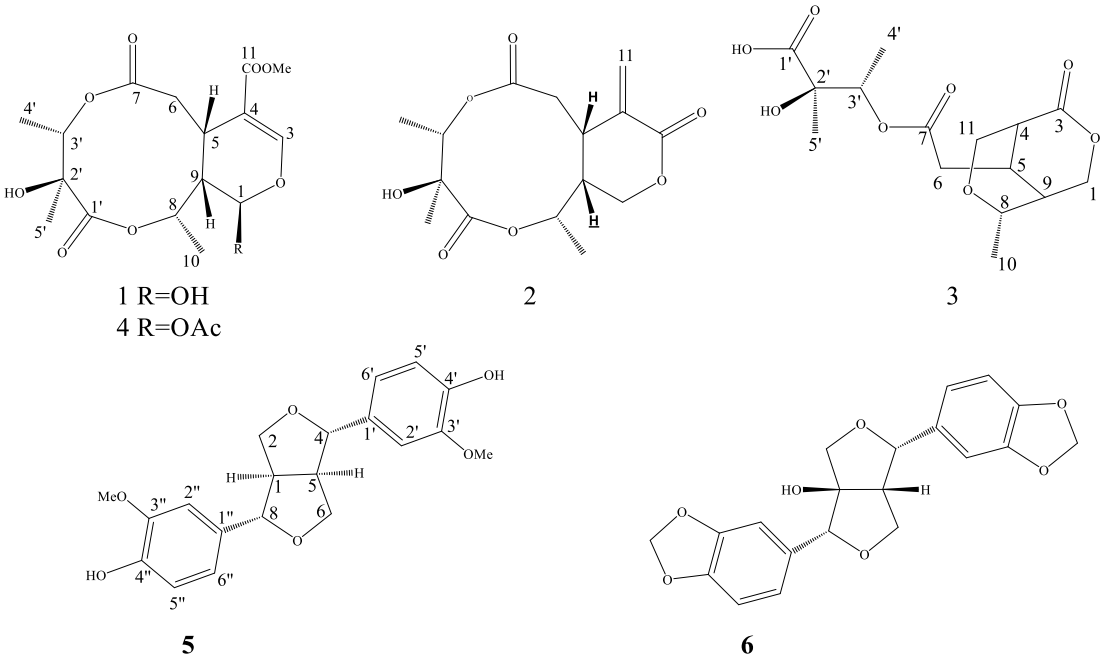

Figure 1. Chemical structures of compounds 1–6

2. Results and Discussion

The HR-ESI-MS of **1** revealed a pseudo-molecular-ion peak at m/z 381.1159 ([*M* + Na]$^{+}$), consistent with the molecular formula $C_{16}H_{22}O_9$, having six degrees of unsaturation. The IR spectrum displayed absorption bands diagnostic of OH (3456 cm^{-1}), ester (1738 cm^{-1}), and C=C bond (1635 cm^{-1}) functionalities. The ^{13}C NMR spectrum of **1** showed the signals of a ß- glucopyranosyl moiety, a trisubstituted double bond (δ_C 151.9 and 109.7), a carbomethoxyl group (δ_C 51.5 and 166.7) and a ketal carbon (δ_C 92.5). The presence of these partial structures suggested that **1** was an iridoid or secoiridoid. ^{1}H- and ^{13}C-NMR data (Tables 1) indicated the presence of one OMe unit(δ_H 3.74 s, δ_C 51.5 q), two lactones and ester group (δ_C 173.6 s, 175.7 s, 166.7 s) and one C=C bond (δ_H 7.52 s, δ_C 151.9 d, 109.7 s), accounted for 6 degrees of unsaturation and suggesting **2** additional rings. The ^{1}H-NMR, ^{13}C-NMR and DPET spectrums also revealed one CH_2 (δ_H 2.60 td, 3.10 td, δ_C 35.4 t,), three CHO groups (δ_H 5.88 br s, δ_C 92.5 d; δ_H 4.94 q, δ_C 72.9 d; δ_H 5.05 q, δ_C 73.2 d), two CH (δ_H 3.36 m, δ_C 30.6 d; δ_H 1.98 br d, δ_C 43.9 d), three additional Me (δ_H 1.46 d, δ_C 18.3 q; δ_H 1.33 d, δ_C 12.8 q; δ_H 1.35 s, δ_C 16.4 q) and one additional quaternary C (δ_C 74.5 s). The COSY correlations showed the connection from H-1 to H-9, H-5 to H-6 and H-9. The HMBC correlations of H-3 to C-4,C-11 and C-1, H-3 to C-1,C-5, and with the aid of ^{13}C-NMR spectrum indicating that the COOMe group is attached to C-4 and the OH group is attached to C-1, assuming the position of the atom O is between C-1/C-3. The HMBC and ^{13}C-NMR data also revealed the connections of H-6 to C-5, C-7 and C-9, H-3' to C-7, C-2', Me-5' to C-2', C-1', H-8 to C-1', C-9, and indicated the position of two lactones and the OH groups. The relative configuration of **1** was determined on the basis of NOESY experiment and the references. The NOESY data exhibited the connectivity between

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H-8 to H-5, H-9 and Me-10 to H-1. These findings and the references established the β-orientation of H-5, H-8 and H-9 and α -configuration of H-1, H-10. The stereochemical of C-2' and C-3' was determined by comparing the ^{1}H and ^{13}C NMR spectra using alkaline hydrolysis of Gonocaryoside A [3] [11]. It is speculated that if Gonocarin A undergoes alkaline hydrolysis, then it will obtain Kingiside aglycone and 2S, 3S angliceric acid[12-16]. Hence, the structure of Gonocarin A, one new natural compound, was established as **1**.

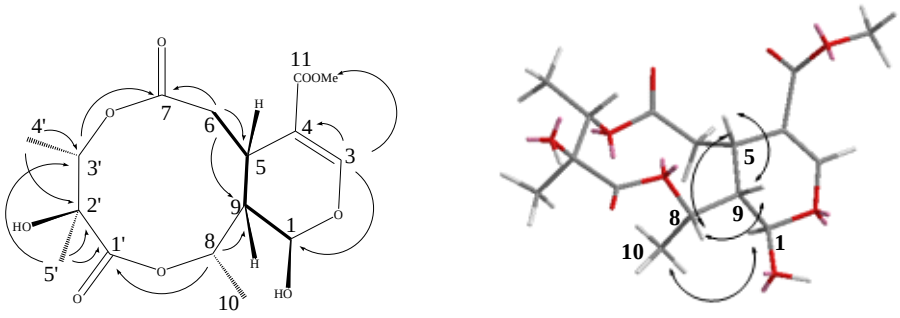

Figure 2. (a) The structure of metabolite **1** and selected ^{1}H-^{1}H COSY (−) and HMBC (→) correlations. (b) Selective NOESY correlations of **1**.

The HR-ESI-MS of **2** revealed a pseudo-molecular-ion peak at m/z 335.1105 ([*M* + Na]$^{+}$), consistent with the molecular formula $C_{15}H_{20}O_7$, having six degrees of unsaturation. The IR spectrum displayed absorption bands diagnostic of OH (3456 cm^{-1}),ester (1734 cm^{-1}), and C=C bond (1635 cm^{-1}) functionalities. The ^{1}H-NMR, ^{13}C-NMR (Table 1) and DPET spectrum indicated the presence of three Me (δ_H 1.23 s, δ_H 1.31 d, 1.38 d), one C=CH_2 (δ_H 5.68 s, δ_H 6.40 s; δ_C 128.8 t, 138.4 s), one CH_2 (δ_H 2.33 m, 2.70 t, δ_C 36.0 t), one CH_2O group (δ_H 4.64 d, δ_C 64.5 t), two CH (δ_H 3.48 d, δ_C 40.4 d; δ_H 2.30 m, δ_C 41.6 d), two CHO groups (δ_H 4.82 q, δ_C 73.8 d; δ_H 5.06 q, δ_C 73.5 d), three lactones (δ_C 171.6 s, 174.9 s, 163.7 s) and one quaternary C (δ_C 74.3 s). Accounting for 6 degrees of unsaturation, 2 additional rings were suggested. The COSY correlations showed the connection of from H-1 to H-9, from H-9 to H-5, from H-5 to H-6. The HMBC correlations of H-11 to C-4, C-5- and C-3 exhibited that the C=C was at C-4 and when the lactone was at C-3. The connections of HMBC spectrum also showed the correlations of Me-4' to C-3', C-2' and C-7, H-8 to C-9, C-1' and H- 5' to C-1', C-2' and C-3', indicating the assignment of the lactone and the OH groups at C-7, C-1' and C-2' and connectivity of HMBC to complete the plane structure of **2.** The relative configuration of **2** was determined on the basis of NOESY experiment. The NOESY data exhibits the connectivity between H-8 to H-5,H-9. These findings and the references established the β-orientation of H-5, H-8 and H-9 and α -configuration of Me-10. The stereo chemical of C-2' and C-3' was determined by comparing the ^{1}H and ^{13}C NMR spectra of Gonocarin A. Hence, the structure of newly discovered natural compound, Gonocarin B, was established as **2.**

The molecular formula $C_{15}H_{22}O_8$ (five degrees of unsaturation) of **3** was deduced from the HR-ESI-MS data (m/z 353.1209 ([*M* + Na]$^{+}$)). Its IR spectrum showed absorption bands

suggesting the functionalities of OH (3433 cm^{-1}) and ester (1738 cm^{-1}). The ^{1}H-NMR, ^{13}C-NMR and DEPT spectroscopic data (Table 1) indicated the presence of three CH_3, two OCH_2, two OCH and three CH and four quaternary C including one lactone, one ester group and one acid group. Accounting for 5 degrees of unsaturation, 2 additional rings were suggested. In comparison with Gonocarin B, two sets of signals (δH 2.70 d, δH 3.75 m; δC 48.9 d, 61.1 t) were found from ^{1}H-NMR and ^{13}C-NMR. According to the COSY correlations, it showed the connection of H-1 to H-9, H-5 to H-6, and H-4 to H-11. The HMBC correlations of H-1 to C-3, C-5, and C-9, H-4 to C-3, C-5, and C-11, H-6 to C-7, established lactones at C-3, the ester group at C-7 and the assignments of the right part of structure. The connections of HMBC spectrum also showed the correlations of H-3' to C-7, C-2', Me-4', and Me-5', Me-5' to C-1', C-2' and C-3', Me-4' to C-2' and C-3', which exhibited the location and the assignments of the left part of structure. From the above precise spectral data, it can be inferred that the oxygen-containing open ring at position C-8 of Gonocarin B is linked to the double bond on C-11. The NOESY spectrum showed the correlations of H-8 to H-5, H-4 to H-5, H-5 to H-9, H-8 to H9. The relative configuration of **3**, elucidated mainly from the nuclear Overhauser effect spectroscopy (NOESY) spectrum, which was compatible with that of **3** ascertained using molecular mechanics calculations (MM2). It is suggested to be the most stable conformations, as shown in Figure 3. These findings and the references established the β-orientation of H-4, H-5, H-8 and H-9 and α-configuration of Me-10. Hence, the structure of newly discovered natural compound, Gonocarin C, was established as **3.**

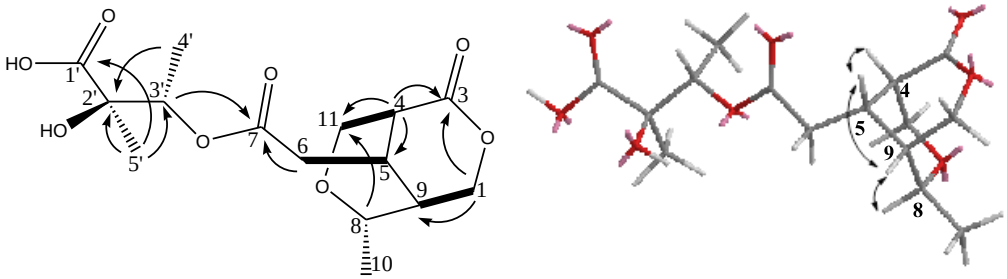

Figure 3. (a) The structure of metabolite **3** and selected ^{1}H-^{1}H COSY (−) and HMBC (→) correlations. (b) Selective NOESY correlations of **3**.

Table 1. ^{1}H-NMR and ^{13}C-NMR data for compounds **1–3**[a]

	1		2		3	
	No. δ_H	**δ_C**	**δ_H**	**δ_C**	**δ_H**	**δ_C**
1	5.88, 1H,br s	92.5	4.64, 2H, d *J*=12.0 Hz	64.5	4.61, 2H, d, *J*=11.6 Hz	65.1
3	7.52, 1H, s	151.9		163.7		172.3
4		109.7		138.4	2.70, 1H, m	48.9
5	3.36, 1H, m	30.6	3.48,1H, d *J*=12.4 Hz	40.4	2.88, 1H, m	35.9
6α	3.10, 1H, dd, *J*=15.2 , 2.8 Hz	35.4	2.70, 1H, t *J*=15.2 Hz	36.0	2.32, 1H, m	30.5
6β	2.60, 1H, dd *J*=15.2, 2.8 Hz		2.33, 1H, m		2.50, 1H, m	
7		173.6		171.6		171.8
8	4.94, 1H, q , *J*=4.8 Hz	72.9	4.82, 1H, q *J*=6.8 Hz	73.8	4.81, 1H, q *J*=6.4 Hz	74.2
9	1.98, 1H, br d *J*=1.6 Hz	43.9	2.30, 1H, m	41.6	2.32, 1H, m	43.1
10	1.46, 3H, d , *J*=6.8 Hz	18.3	1.38, 3H, d , *J*=6.8 Hz	18.6	1.38, 3H, d , *J*=6.4 Hz	18.7
11		166.7	5.68, 1H, s 6.40, 1H, s	128.8	3.75, 1H, m 3.92, 1H, m	61.1
OMe	3.74, 3H, s	51.5				
1'		175.7		174.9		174.8
2'		74.5		74.3		74.2
3'	5.05, 1H, q *J*=6.4 Hz	73.2	5.06, 1H, q *J*=6.8 Hz	73.5	5.05, 1H, q *J*=6.4 Hz	74.1
4'	1.33, 3H, d *J*=6.4 Hz	12.8	1.31, 3H, d *J*=6.8 Hz	12.7	1.35, 3H, m	12.7
5'	1.35, 3H, s	16.4	1.23, 3H, s	16.7	1.31, 3H, m	16.7

[a]Assignments aided by the HMQC, HMBC, and NOESY experiments; ^{1}H- and ^{13}C-NMR were measured at 400 and 100 MHz in CDCl3;.

3. Experimental Section

3.1. General

Prep. TLC: precoated silica-gel plates (SiO_2; silica gel 60 F-254, 1 mm; Merck). Column chromatography (CC): SiO_2 60 (Merck) or Sephadex LH-20 (Amersham Pharmacia Biotech AB,Sweden). HPLC: Hitachi system; LiChrospher_ Si 60 (5 mm, 250 – 10; Merck) for normal phase and LiChrospher_ 100 RP-18e (5 mm, 250 – 10; Merck) for reversed-phase. Optical rotations: Jasco-DIP-1000polarimeter. IR and UV Spectra: Hitachi-T-2001 and Hitachi-U-3210 spectrophotometers, resp. ^{1}H- and ^{13}C-NMR, COSY, HMQC, HMBC, and NOESY Experiments: Bruker-FT-300 spectrometer; chemical shifts d in ppm rel. to Me4Si as an internal standard, coupling constants J in Hz. EI-MS and HR-ESI-MS: Jeol-JMS-HX-110 mass spectrometer; in m/z (rel. %).

3.2. Plant Material

The seeds of plant *Gomocaryum calleryanum* was collected in south of Taiwan in July 2011.

3.3. Extraction and Isolation

The fresh seeds were dried and smashed and then 5.5 kg dry sample were collected. The dry sample was extracted with acetone (3 x 4 L) at r.t., and then the acetone extract was concentrated. The dark-brown crude extract (520 g) was partitioned between EtOAc and H_2O (1:1). The EtOAc layer (220 g) was subjected to CC (SiO_2, *n*-hexane/ EtOAc 20:1 ~ 1:1) and

got 11 fractions. *Fr. 11* was separated by reserved-phase HPLC (MeOH/H_2O/CH_3CN 60:35:5) and then further subjected to reserved-phase HPLC (MeOH/H_2O/CH_3CN 50:45:5): Gonocarin A (206.5 mg). *Fr*. 4, *Fr*. 6 and *Fr*. 7 were individually separated by CC (*Sephadex* LH-20, CH_2Cl_2/MeOH 1 : 1) and resulted in *Fr*. 4.3, *Fr*.6.4 and *Fr*.7.5. *Fr*. 4.3 was subject to reserved-phase HPLC (MeOH/H_2O/CH_3CN 60:35:5): Goncarin B (20.5 mg). *Fr*.6.4 and *Fr*. 7.5 were separately subjected to reserved-phase HPLC (MeOH/H_2O/CH_3CN 50:45:5): Gonocarin C (18.8 mg), Pinoresinol (3.0 mg) [17] [18] and Paulownin (1.7 mg). [19]

3.4 Reaction

Acetylation of **1** (100 mg) was treated with acetic anhydride / pyridine (1 : 1) and left at room temperature for 24 hours. Meanwhile, the work continued on the product with HPLC and resulted in the Gonocarin A monoacetate (**4**) 61 mg.

Gonocarin A (**1**): colorless oil. $[\alpha]_D$ = +299.4 (*c* 0.3, CH_2Cl_2). IR (CH_2Cl_2): 3456 (OH), 1738 (ester), 1635 (C=C) cm^{-1} . UV λ_{max} (MeOH): 242 nm. HR-ESI-MS: 381.1159 ($[M+Na]^+$, $C_{16}H_{22}NaO_9^+$; calc. 381.1161)

Gonocarin B (**2**): colorless oil. $[\alpha]_D$ = -51.1 (*c* 0.4, CH_2Cl_2). IR (CH_2Cl_2): 3456 (OH), 1734 (ester), 1635 (C=C) cm^{-1} . UV λ_{max} (MeOH): 242 nm. HR-ESI-MS: 335.1105 ($[M+Na]^+$, $C_{15}H_{20}NaO_7^+$; calc. 335.1107)

Gonocarin C (**3**): colorless oil. $[\alpha]_D$ = +100.5 (*c* 0.1, CH_2Cl_2). IR (CH_2Cl_2): 3433 (OH), 1738 (ester) cm^{-1} . UV λ_{max} (MeOH): 227 nm. HR-ESI-MS: 353.1209 ($[M+Na]^+$, $C_{15}H_{22}NaO_8^+$; calc. 353.1212)

Gonocarin A monoacetate (**4**): colorless oil. $[\alpha]_D$ = 319.0 (*c* 0.3, CH_2Cl_2). IR (CH_2Cl_2): 3456 (OH), 1738 (ester), 1635 (C=C) cm^{-1} . UV λ_{max} (MeOH): 241 nm. HR-ESI-MS: 423.1264 ($[M+Na]^+$, $C_{18}H_{24}NaO_{10}^+$; calc. 423.1267). ^1H NMR ($CDCl_3$): δ 7.38 (1H, s, H-3), 6.67 (1H, d, J = 10 Hz, H-l), 5.02 (1H, q, J = 6.8 Hz, H-3'), 4.83 (1H, q, H-8), 3.70 (3H, s, OMe), 3.40 (1H, dt, 12.0, 6.8 Hz H-5), 2.38(1H, m, H-6), 2.71(1H, m, H-6), 2.16 (3H, s, OAc), 2.14 (1H, m, H-9), 1.28 (3H, s, 5'-Me), 1.30 (3H, d, J = 6.8 Hz, 4'-Me), 1.50 (3H, d, J = 6.8 Hz, 10-Me); ^{13}C NMR (CDCl3): δ20.3 (C-10), 12.7(C-4'), 16.0(C-5'), 33.2(C-5), 36.1(C-6), 43.7(C-9), 73.3(C-3'), 75.2(C-8), 74.3(C-2'), 90.1(C-l), 111.2(C-4), 152.6(C-3), 165.7 and 51.5(COOMe), 171.3(C-7), 175.3(C-1'), 168.5 and 21.0(OAc).

3.5. Anti-Inflammatory Testing

3.5.1 Materials

Lipopolysaccharide (LPS; Escherichia coli O111:B4) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis , MO , USA). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffer saline (PBS) were obtained from Gibco, Invitrogen (Carlsbad, CA, USA). The TNF-α ELISA kit were obtained

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from Mouse TNF-α ELISA (Max Deluxe Sets; BioLegend, San Diego, CA). The MTS assay kit and NO assay kit were purchased from CellTiter 96 AQ Non-Radioactive Cell Proliferation Assay and Griess Reagent System (Promaga, Madison WI).

3.5.2 Cell Culture and Treatment

RAW 264.7 mouse macrophage cells were obtained from the Bioresources Collection and Research Center (Hsinchu, Taiwan; BCRC No. 60001). The macrophages were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactived fetal bovine serum (FBS) in a humidified atmosphere CO_2 incubator (5% CO_2 in air, ESCO, Singapore) at 37 °C. For the experiments, cells (1×10^5) were seeded in a culture plate with 24 wells and maintained within the incubator. RAW 264.7 macrophages were pre-treat with LPS (Escherichia coli O111:B4) and culture medium mixture (100 ng/mL) for 6 hours at 37 °C incubator. And then, the 6 compound (compounds **1-6**) were dissolved in dimethyl sulfoxide (DMSO) and added into LPS/medium mixture with final concentration 0.5, 1.0 and 2.0 μg/mL overnight. The positive and negative control were LPS/medium mixture and culture medium only.

3.5.3 MTS Assay

RAW 264.7 macrophages were treated as described. After overnight incubation, the culture medium were removed, and then cells were washed with PBS. MTS reagent (Promaga, Madison WI) was added 200 μL into each well for 1 hour at 37°C incubator. The absorbance was measured using a plate reader (BioTek, USA) at a wavelength of 490 nm.

3.5.4 Enzyme-linked Immunosorbent Assay Analysis

Raw 264.7 were cultured in 24-well plate with LPS/medium mixture for 6 hours, and then incubated with different compounds1-6 concentrations (0.5, 1 and 2 μg/mL) overnight. Supernatants were collected and measured using mouse TNF-α ELASA kit (Max Deluxe Sets; BioLegend, San Diego, CA) following manufacturer's protocol.

3.5.5 Nitric Oxide Assay

Supernatants from macrophages were stimulated with LPS/medium mixture for 6 hours and compounds **1-6** overnight. The amount of NO in the supernatants was detected by using the Griess Reagent System (Promaga, Madison WI) according to the manufacturer's protocol. Data calculations were performed using MS-Excel 2010 software.

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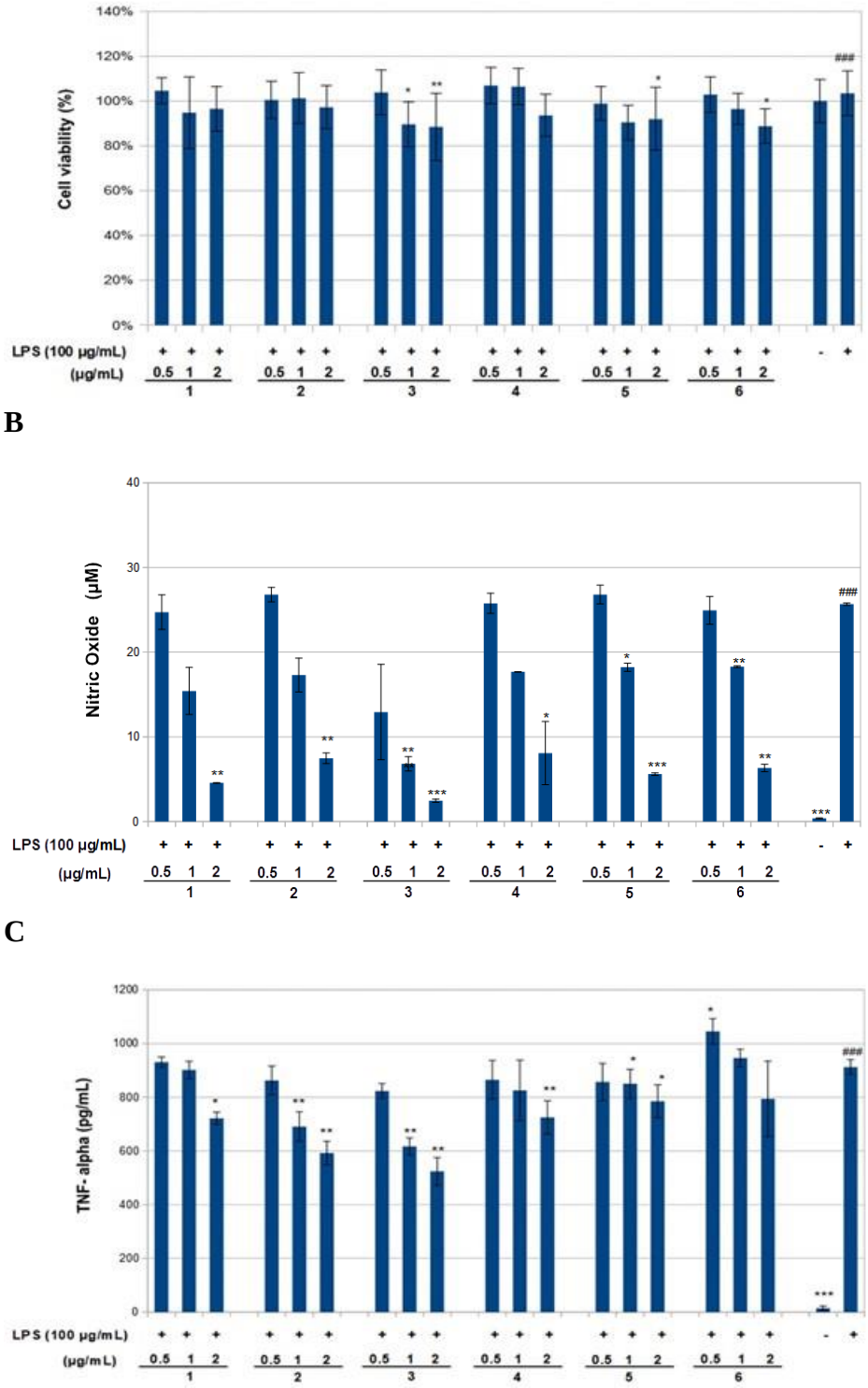

Figure 4. Cytotoxicity of the compounds 1-6 were isolated from the seed of *gonocaryum calleryanum* in RAW264.7 macrophage cells (A), and the effects of the compounds 1-6 on LPS induced NO (B) and TNF-α (C) productions of RAW264.7 macrophages. Cells were incubated and pretreated with LPS/medium mixture (100 ng/mL) for 6 hours at 37℃, the compound 1-6 were dissolved in DMSO and pre-mixed with 100 ng/mL LPS/medium. After 6 hours LPS pretreatment, the compounds mixture were added to each well with final concentration 0.5, 1.0 and 2.0 μg/mL. Cell viability assay was performed using MTS assay reagent kit. Nitric oxide concentration in the medium was determined using Griess Reagent

System. TNF-α level in the medium was determined using an ELISA kit. The data were presented as means ± SDs for three different experiments performed in triplicate. ### $p < 0.001$ control group as compared to LPS-treated group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ were compared with the LPS-alone group.

Supplementary Materials

The physical and spectroscopic data of compounds **1-6**, and NMR and MS spectra of compounds **1-6** are available online.

Acknowledgments

This research was supported by grants from the Ministry of Science and Technology (MOST 103-2320-B-276 -001 -), Taiwan, awarded to Y.-S. Lin.

Thanks to Forestry Research Institute, Council of Agriculture, Executive Yuan Hengchun Tropical Botanical Garden for providing plant sources.

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