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Anti-inflammatory sesquiterpene lactones from the flower of *Vernonia cinerea*

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ABSTRACT

Bioassay-guided fractionation of the hexane extract from the flowers of *Vernonia cinerea* (Asteraceae) led to the isolation of a new sesquiterpene lactone, 8α-hydroxyhirsutinolide (**2**), and a new naturally occurring derivative, 8α-hydroxyl-1-*O*-methylhirsutinolide (**3**), along with seven known compounds (**1** and **4**–**9**). The structures of the new compounds were determined by 1D and 2D NMR experiments and by comparison with the structure of compound **1**, whose relative stereochemistry was determined by X-ray analysis. The isolated compounds were evaluated for their cancer chemopreventive potential based on their ability to inhibit nitric oxide (NO) production and tumor necrosis factor alpha (TNF-α)-induced NF-κB activity. Compounds **1**, **2**, **4**, **5**, and **9** inhibited TNF-α-induced NF-κB activity with IC_{50} values of 3.1, 1.9, 0.6, 5.2, and 1.6 μM, respectively; compounds **4** and **6**–**9** exhibited significant NO inhibitory activity with IC_{50} values of 2.0, 1.5, 1.2, 2.7, and 2.4 μM, respectively.

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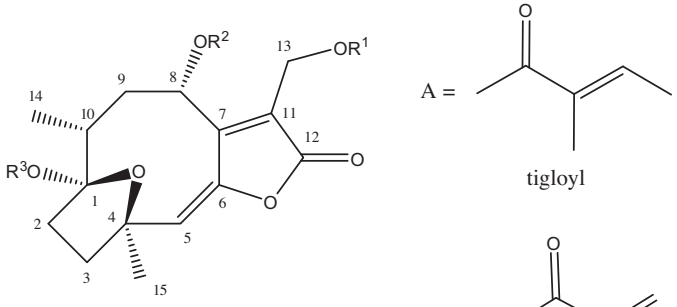
Vernonia cinerea Less (Asteraceae) is a perennial herbaceous plant mainly distributed in the tropical regions and commonly found in South-East Asia.[1,2] *V. cinerea* has traditionally been used for medicinal purposes to treat malaria, fever and liver diseases.[3,4]

There have been phytochemical reports of the isolation of diverse compounds from this species, including sesquiterpene lactones,[5,6] flavonoids,[7] and triterpenes.[8] Some of these compounds have been shown to have anticancer,[5,9] antimalarial,[6] and antifeedant biological activity.[10] However, no studies have been performed on the nitric oxide (NO) production and tumor necrosis factor alpha (TNF-α)-induced NF-κB activity.

As part of our continuing search for novel plant-derived cancer chemopreventive agents, the hexane-soluble extract of the flower of *V. cinerea* exhibited inhibitory effects on the tumor necrosis factor alpha (TNF-α)-induced NF-κB activity and lipopolysaccharide (LPS)-induced nitric oxide production using murine macrophage RAW 264.7 cells. This preliminary result encouraged us to further study *V. cinerea*, resulting in the isolation of two new sesquiterpene lactones, **2** and **3**, together with seven known compounds **1** and **4**–**9** (Fig. 1).[11] This Letter reports the isolation and structure elucidation of **2** and **3** as well as the inhibition on TNF-α-induced NF-κB



	R^1	R^2	R^3
1	H	A	H
2	H	H	H
3	H	H	CH_3
4	Ac	A	H
5	Ac	B	H
6	Ac	B	CH_3
7	Ac	A	CH_3
8	Ac	H	H
9	H	A	CH_3

Figure 1. Structures of compounds **1**–**9** isolated from the flower of *V. cinerea*.

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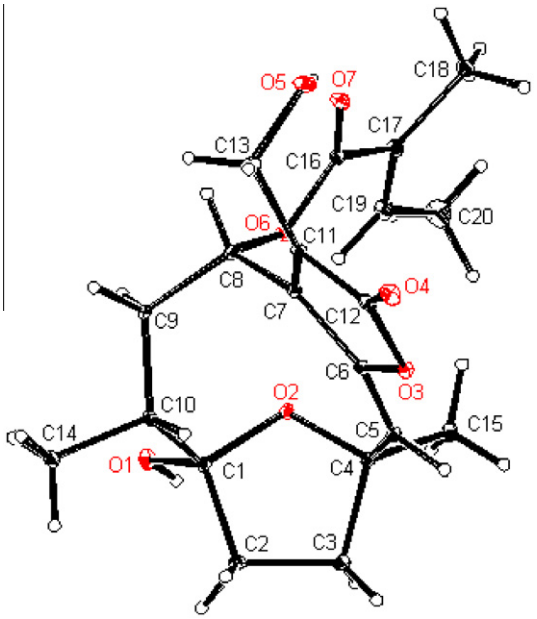

Figure 2. ORTEP drawing of compound **1** using 15% probability ellipsoids at 100 K.

activity and nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells.

Compound **1** was obtained as colorless needles and identified as a known compound, 8α-tigloyloxyhirsutinolide, which was previously isolated from *Vernonanthura pinguis*;[12] its structure was further confirmed by HSQC and HMBC analyses. The relative stereochemistry of **1** was established from its NOESY spectrum, which showed correlation peaks between H-8β and H-9β/H-13a, and between H-10β and H-2/H-9β, suggesting that the C-2–C-3 bridge was located on the same side (β face) with H-10β and H-9β and that the 1,4-epoxy ring was below the ten membered ring (C-1–C-10). X-ray crystallographic analysis[13] (Fig. 2) defined the relative configuration of **1** and gave results consistent with the NOESY data analysis. However, full spectroscopic and physicochemical assignments have been performed in this work for the first time.[14]

Compound **2** (Fig. 1) was obtained as a colorless oil and gave a molecular ion at *m/z* 297.1264 $[M+H]^+$ (calcd for $C_{15}H_{21}O_6$, 297.1267) in the HRESIMS, corresponding to an elemental formula of $C_{15}H_{20}O_6$. The UV spectrum showed an absorption maximum at 310 nm, indicating the presence of a conjugated lactone group. The IR absorption band at 1765 cm^{-1} and the ^{13}C NMR signals at [δ_C 168.0 (C-12), 152.5 (C-7), 146.0 (C-6), and 130.1 (C-11)] indicated the presence of a γ-lactone group.[5] The ^1H and ^{13}C NMR spectra displayed 15 carbon signals including a ketal quaternary carbon at δ_C 109.0 (C-1) and an oxygenated quaternary carbon δ_C 80.0 (C-4), as well as an olefinic, an oxymethylene, three methylenes, a methine, and an oxygenated methine carbon all indicative of a hirsutinolide-type sesquiterpene.[5] The NMR and HSQC spectra revealed two methyl groups at [δ_H 0.95 (d, J = 6.5 Hz)/δ_C 17.4 (CH_3-14) and δ_H 1.57 (s)/δ_C 27.0 (CH_3-15)], which showed correlation peaks with C-1/C-10 and C-4/C-5 in the HMBC spectrum, respectively, and that two methyls (CH_3-14 and CH_3-15) were attached at C-10 and C-4, respectively (Fig. 3). In addition, an oxygenated methylene proton at δ_H 4.33 (H-13a), 4.40 (H-13b) was observed in the ^1H NMR spectrum, which showed two- and three-bond HMBC correlation peaks with the γ-lactone group carbons (C-7, C-11, and C-12), which suggested that the oxymethylene group was connected to C-11 of the γ-lactone ring. The molecular formula and the 15 carbon signals in the ^{13}C NMR spectrum of **2** further indicated that the hydroxyl groups were attached at C-1, C-8 and C-13, respectively, compared to those of 8α-tigloyloxy-hirsutinolide-13-*O*-acetate (**4**).[12] The NOESY correlations between H-8β and H-9β/H-13a, and between H-10β and H-2_{endo}/H-9β suggested that **2** possessed an 8α oriented OH group, as in **1** (Fig. 3).[12] Thus, the structure of **2** was determined as a new compound, 8α-hydroxyhirsutinolide.[15]

Compound **3** (Fig. 1) was obtained as a colorless oil. The molecular formula was established as $C_{16}H_{22}O_6$ by HRESIMS, which was supported by ^{13}C NMR spectra. The ^1H and ^{13}C NMR spectra of **3** were similar to those of **2**, except for an additional methoxy group at δ_H 3.32 (3H, s)/δ_C 47.9. The HMBC spectral analysis displayed correlation peaks between the methoxy proton and a ketal carbon (C-1), which revealed that the methoxy group was connected to C-1 instead of the OH group in **2**. The configuration of **3** was same as that of compound **2**, as a result of the NOESY correlations. Therefore, compound **3** was elucidated as 8α-hydroxyl-1-*O*-methylhirsutinolide,[16] which has been mentioned previously in the literature,[17] but not as a natural compound.

The other seven isolates were identified as the known compounds, 8α-tigloyloxyhirsutinolide (**1**),[12] 8α-tigloyloxyhirsutinolide-13-*O*-acetate (**4**),[12] 8α-(2-methylacryloyloxy)-hirsutinolide-13-*O*-acetate (**5**),[12] 8α-(2-methylacryloyloxy)-1α-methoxyhirsutinolide-13-*O*-acetate (**6**),[16] vernolide-B (**7**),[5] hirsutinolide-13-*O*-acetate (**8**),[12] and vernolide-A (**9**)[5] by comparison of their physical and spectral data with published values.

The hexane-partition of the flower of *V. cinerea* exhibited potent inhibitory activity against TNF-α-induced NF-κB activity and NO

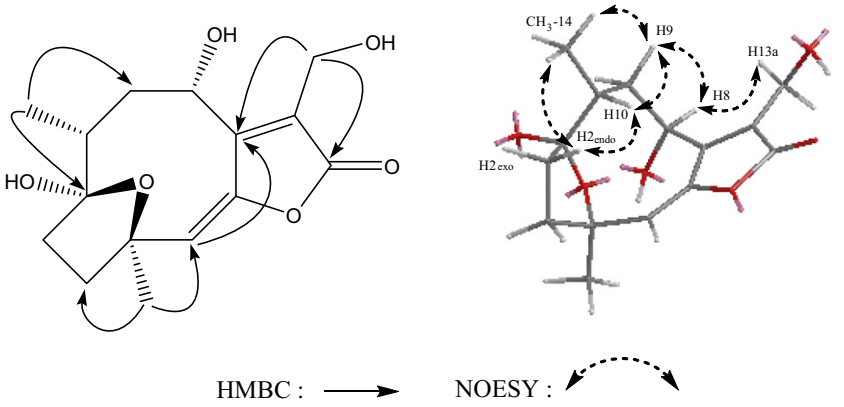

Figure 3. Important HMBC and NOESY correlations for compound **2**.

Table 1
^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data of compounds **2** and **3** (δ in ppm, J in Hz) in CD_3OD

Position	2		3	
	δ_H	δ_C	δ_H	δ_C
1		109.0		112.1
2_{endo}	2.03 m	35.8	2.12 m	38.0
2_{exo}	2.15 m		2.26 m	
3	2.20 m	37.5	2.26–2.29 m	38.2
4		80.0		80.3
5	5.99 s	123.0	6.00 s	123.3
6		146.0		147.0
7		152.5		153.0
8	5.27 (d, 6.5)	66.5	5.27 (d, 6.5)	66.5
9α	2.30 (dd, 12. 5, 12.5)	37.9	2.23 (dd, 12. 5, 12.5)	37.9
9β	1.88 (ddd, 12.5, 6.5, 1.6)		1.87 (ddd, 12.5, 6.5, 1.6)	
10	1.94 m	40.1	1.85 m	42.1
11		130.1		131.0
12		168.0		168.2
13a	4.33 (d, 13.2)	54.5	4.33 (d, 13.2)	54.6
13b	4.40 (d, 13.2)		4.43 (d, 13.2)	
14	0.95 (d, 6.6)	17.4	0.96 (d, 6.6)	16.9
15	1.57 s	27.0	1.63 s	25.9
OCH_3-1			3.32 s	47.9

Table 2
Inhibition effect of compounds **1**–**9** against the TNF-α-induced NF-κB activity and NO production in LPS-stimulated RAW 264.7 cells

Compounds	Nitrite assay			NF-κB		
	%Inhib.[a]	%Surv.[b]	IC_{50} (μM)	%Inhib.[c]	%Surv.[d]	IC_{50} (μM)
Hexane extract	63.3	149.3		62.3	113.0	
1	99.8	32.2	5.7 ± 0.4	98.6	87.6	3.1 ± 0.9
2	82.6	121.1	24.7 ± 3.0	65.6	100.0	1.9 ± 0.5
3	84.2	134.3	28.1 ± 1.3	33.4	91.9	—
4	98.0	31.1	2.0 ± 0.1	99.6	102.3	0.6 ± 0.1
5	99.2	31.1	6.6 ± 0.4	95.8	95.1	5.2 ± 1.9
6	98.8	6.3	1.5 ± 0.2	94.2	39.1	13.6 ± 2.5
7	99.0	5.4	1.2 ± 0.2	97.6	16.5	12.8 ± 2.0
8	99.3	18.0	2.7 ± 0.3	90.3	55.0	10.2 ± 1.3
9	99.8	15.6	2.4 ± 0.1	94.1	9.3	1.6 ± 0.5
L-NMMA[e]			25.1 ± 2.3			
TPCK[f]						3.8 ± 0.6
BAY-11[f]						2.0 ± 0.3

[a] % Inhibition of NO production at 50 μM.
[b] % Survival at concentration of 50 μM.
[c] % Inhibition of NF-κB at 50 μM.
[d] % Survival at concentration of 50 μM.
[e] Positive control for NO.
[f] Positive control for NF-κB.

production in LPS-stimulated RAW 264.7 cells with inhibition rates of 62.3% and 63.3%, respectively. Therefore, compounds **1**–**9** isolated from this extract were evaluated for their cancer chemopreventive potential based on their ability to inhibit TNF-α-induced NF-κB activity and NO production Table 1.[18,19]

NF-κB is a transcription factor that is associated with cell apoptosis, differentiation, and migration. Upon activation, it may promote cell proliferation and prevent cell death through anti-apoptotic factors.[20] Inhibition of NF-κB signaling has a potential application for either the treatment or prevention of cancer. Using stably-transfected human embryonic kidney cells 293 that express a NF-κB reporter, we found that incubation with compounds **1**–**9** led to inhibition with IC_{50} values ranging from 0.6 to 13.6 μM. Among the isolates, compounds **1**, **4,** and **5** showed significant inhibitory effects on TNF-α-induced NF-κB activity without cytotoxicity at 50 μM. Compound **9** also exhibited potent inhibitory activity with IC_{50} value of 1.6 μM, but cytotoxicity was observed at the same concentration. Compounds **6**–**8** showed moderate inhibition with IC_{50} values of 10.2–13.6 μM (Table 2), however, compound **3** was inactive. These results suggest that a hydroxyl at C-1 and a tigloyloxy group at C-8 in this molecule contribute to strong inhibitory effects on the TNF-α-induced NF-κB activity.

Nitric oxide (NO) is an inorganic gaseous molecule that is synthesized by the oxidation of L-arginine catalyzed by nitric oxide synthase (NOS) and is involved in a number of physiological and pathological processes in mammals.[21] In the NOS family, iNOS is expressed in a variety of cells including macrophages, endothelial cells, and smooth muscle cells in response to pro-inflammatory stimuli such as IL-1β, TNF-α, and LPS. NO plays an important role in the regulation of many physiological functions, such as host defense, neurotoxicity, and vasodilation.[18] However, excess production of NO has been implicated in immunological and inflammatory diseases including septic shock, rheumatoid arthritis, graft rejection, and diabetes.[22] Therefore, inhibition of NO increase is apparently an important therapeutic consideration in the development of anti-inflammatory and anticancer agents. As shown in Table 2, when treated with a fixed concentration of 50 μM, all compounds exhibited inhibitory activities against NO production with inhibition rates of 82.6–99.8%. Among the isolates, the sesquiterpene lactones **1** and **4**–**9**, which have alkenyl ester groups at C-8, exhibited significant NO inhibitory activity with IC_{50} values of 1.2–6.6 μM, which are much less than the positive control, L-N^G-monomethyl arginine citrate (25.1 μM). However, the cytotoxicity of these compounds was observed at 50 μM. In addition, compounds **2** and **3** showed potent inhibitory effects with IC_{50} values of 24.7 and 28.1 μM, respectively, and no cytotoxicity was detected at these concentrations. In particular, compound **2**, which has hydroxyl groups at C-1, C-8, and C-12, respectively, showed potent inhibitory effects without cytotoxicity with TNF-α-induced NF-κB activity as well as inhibiting NO production. In conclusion, the anti-inflammatory activity of sesquiterpene lactones isolated from the flower of *V. cinerea* in vitro can be attributed, at least in part, to inhibition of TNF-α-induced NF-κB activity and NO production. Since sesquiterpene lactones have potential anti-inflammatory and anticancer activity, more studies are required to further our understanding of the corresponding mechanisms of action.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.07.010.

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11. *Extraction and isolation*: The flowers of *V. cinerea* Less (Asteraceae) were collected from the Lampang Herb Conservation Club, Lampang Province, Thailand, in May 2011 and identified by comparison with the voucher specimen at the Forest Herbarium, Bangkok, Thailand. A voucher specimen (No. Vcf001) was deposited at the Natural Product Chemistry Laboratory, College of Pharmacy, University of Hawaii at Hilo. The flowers of *V. cinerea* (10 kg) were extracted with MeOH (3 × 40 L) at room temperature. The solvent was concentrated in vacuo to yield a MeOH extract (850 g), which was then suspended in distilled water (4 L) and fractionated with *n*-hexane (3 × 4 L), ethyl acetate (3 × 4 L), and *n*-butanol (3 × 4 L), successively. The hexane extracts (120 g) were subjected to silica gel column chromatography (CC; ϕ 15 cm; 230–400 mesh, 2.5 kg) using a gradient solvent system of hexane–ethyl acetate (100:1 to 0:100), to afford 14 fractions (H1−H14). Fraction H4 (78 g) was subjected to silica gel CC (ϕ 10 cm; 230–400 mesh, 1 kg), with hexane–ethyl acetate (100:0 to 1:1) as the solvent system, yielding seven subfractions (H4.1 to H4.7). Subfraction H4.1 (3.0 g) was chromatographed on a sephadex LH-20 gel (300 g) column and eluted with H_2O−MeOH (100:0 to 50:50), to afford three subfractions (H4.1.1 to H4.1.3). Subfraction H4.1.1 (0.2 g) was subjected to prep. HPLC (MeOH−H_2O/0.1% formic acid = 70:30) to yield **2** (2 mg, t_R 110 min) and **3** (1 mg, t_R 115 min). Fraction H8 (10 g) was subjected to silica gel CC (ϕ 8 cm; 230–400 mesh, 800 kg), with hexane–ethyl acetate (100:0 to 1:1) as the solvent system, yielding six subfractions (H8.1 to H8.6). Subfraction H8.4 (2.5 g) was chromatographed on a sephadex LH-20 gel (300 g) column and eluted with H_2O−MeOH (100:0 to 50:50), to afford five subfractions (H8.4.1 to H8.4.5). Subfraction H8.4.2 (0.4 g) was subjected to prep. HPLC (MeOH−H_2O/0.1% formic acid = 75:25) to yield **4** (52 mg, t_R 95 min), **5** (5 mg, t_R 100 min), and **1** (56 mg, t_R 108 min). Subfraction H8.4.3 (0.3 g) was subjected to prep. HPLC (MeOH−H_2O/0.1% formic acid = 75:25) to yield **8** (2 mg, t_R 120 min), **6** (3 mg, t_R 125 min), **7** (3 mg, t_R 128 min), and **9** (1 mg, t_R 130 min).
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13. X-ray crystal structure analysis of compound **1**: A crystal of compound **1** was ground to a sphere of radius 0.35 mm and mounted on a glass fiber. X-ray data were collected at 100 K using a Nonius Kappa CCD diffractometer to a maximum 2θ value of 72.6° using monochromatized Mo Kα radiation; 9301 unique reflections were collected. Crystal data for (**1**): $C_{20}H_{26}O_7$, M_r = 378.42, orthorhombic space group $P2_12_12_1$, a = 6.8259(1) Å, b = 15.1697(3) Å, c = 18.7843(4) Å, V = 1945.06(6) Å^3, Z = 4, ρ = 1.292 g/cm^3. The structure was solved by direct methods (SIR 2004). All non-H atoms were refined anisotropically and all H-atoms isotropically in the final full-matrix least-squares refinement cycles on F, which converged at R_1 = 0.0317, wR_2 = 0.0358 and GOF = 1.129 for 3217 reflections with $I > 5\sigma(I)$; a robust-resistant weighting scheme was used in the least-squares refinements of the 349 variables. The absolute configuration of the four stereogenic C-centers could not be determined unambiguously. X-ray coordinates of compound **1** have been deposited with the Cambridge Crystallographic Data Centre; deposition number: CCDC 888922.
14. 8α-tigloyloxyhirsutinolide (***1***): colorless needles (in $CHCl_3$:MeOH-1:1); $[\alpha]_D^{20}$ = +34.2° (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 285 (3.5) nm; CD: (*c* = 0.1, MeOH): 292 (+7.5); IR ν_{max} (KBr) 3330, 1760 cm^{-1}; ^{1}H NMR (400 MHz) δ 6.32 (1H, d, *J* = 7.6 Hz, H-8), 5.83 (1H, s, H-5), 4.61 (1H, d, *J* = 13.0 Hz, H-13b), 4.53 (1H, d, *J* = 13.0 Hz, H-13a), 2.09 (2H, m, H-2), 2.09 (2H, m, H-3), 1.85 (1H, m, H-10), 2.23 (1H, dd, *J* = 12.5, 12.5 Hz, H-9a), 1.87 (1H, ddd, *J* = 12.5, 7.6, 1.6 Hz, H-9b), 1.47 (3H, s, CH_3-15), 0.96 (3H, d, *J* = 6.8 Hz, CH_3-14), 7.07 (1H, q, *J* = 7.5 Hz, H-3′), 1.76 (3H, d, *J* = 6.8 Hz, CH_3-4′), 1.80 (3H, s, CH_3-5′); ^{13}C NMR (100 MHz) δ C-1 (108.2), C-2 (38.5), C-3 (38.6), C-4 (81.6), C-5 (125.6), C-6 (146.7), C-7 (146.4), C-8 (68.1), C-9 (36.0), C-10 (41.2), C-11 (133.7), C-12 (167.8), C-13 (54.3), C-14 (16.9), C-15 (28.1), C-1′ (168.2), C-2′ (128.2), C-3′ (139.3), C-4′ (14.4), C-5′ (11.9); ESIMS *m/z* 378 [M]$^+$.
15. 8α-Hydroxyhirsutinolide (***2***): colorless oil; $[\alpha]_D^{20}$ = +30.2° (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 285 (3.9) nm; CD: (*c* 0.1, MeOH): 292 (+6.1); IR ν_{max} (KBr) 3350, 1765 cm^{-1}; ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data, see Table 1; HRESIMS *m/z* 297.1264 [M+H]$^+$ (calcd for $C_{15}H_{21}O_6$, 297.1267).
16. 8α-Hydroxyl-1-*O*-methylhirsutinolide (***3***): colorless oil; $[\alpha]_D^{20}$ = +25.7° (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 285 (4.2) nm; CD: (*c* 0.1, MeOH): 292 (+9.6); IR ν_{max} (KBr) 3335, 1760 cm^{-1}; ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data, see Table 1; HRESIMS *m/z* 311.7806 [M+H]$^+$ (calcd for $C_{16}H_{23}O_6$, 311.7803).
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18. Tumor necrosis factor-α (TNF-α) activated nuclear factor-kappa B (NF-κB) assay: We employed human embryonic kidney cells 293 Panomic (Fremont, CA) for monitoring changes occurring along the NF-κB pathway.[23] Stable constructed cells were seeded into 96-well plates at 20 × 10^3 cells per well. Cells maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Co.; Carlsbad, CA), supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. After 48 h incubation, the medium was replaced and the cells were treated with various concentrations of test substances. TNF-α (Human, Recombinant, *E. coli*, Calbiochem, Gibbstown, NJ) was used as an activator at a concentration of 2 ng/ml (0.14 nM). The plate was incubated for 6 h. Spent medium was discarded and the cells were washed once with PBS. Cells were lysed using 50 μl (for 96-well plate) Reporter Lysis Buffer from Promega, by incubating for 5 min on a shaker, and stored at −80 °C. The luciferase assay was performed using the Luc assay system from Promega (Madison, WI). The gene product, luciferase enzyme, reacts with luciferase substrate, emitting light which was detected using a luminometer (LUMIstar Galaxy BMG). Data for NF-κB constructs are expressed as IC_{50} values (i.e., concentration required to inhibit TNF-activated NF-κB activity by 50%). As a positive control, two known NF-κB inhibitors were used: TPCK, IC_{50} = 3.8 μM and BAY-11, IC_{50} = 2.0 μM.
19. Inhibition of nitric oxide (NO) production in lipopolysaccharide (LPS)-activated murine macrophage RAW 264.7 cells (iNOp) assay: The level of nitrite, the stable end product of NO, was estimated as described previously.[24] Briefly, RAW 264.7 cells were seeded and incubated in 96-well culture plates at 37 °C, 5% CO_2 in a humidified air for 24 h. The cultured medium was replaced with phenol red-free medium containing various concentrations of compounds for 15 min prior to 1 μg/ml of LPS exposure for 20 h. The amount of nitrite in the culture media was measured by using Griess reagent. Under the same experimental conditions, SRB assays were performed to evaluate the cytotoxic effect of compounds toward RAW 264.7 cells. L-N^G-monomethyl arginine citrate (L-NMMA), as a positive control of this assay showed an IC_{50} value of 25.1 μM.
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