

Identification of a New Naphthalene and Its Derivatives from the Bulb of *Eleutherine americana* with Inhibitory Activity on Lipopolysaccharide-Induced Nitric Oxide Production

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A new naphthoquinone, (–)-3-[2-(acetyloxy)propyl]-2-hydroxy-8-methoxy-1,4-naphthoquinone (**1**) was isolated from the bulb of *Eleutherine americana* MERR. et HEYNE (Iridaceae) together with two known compounds, eleutherinol (**6**) and 1,5-dihydroxy-3-methylanthraquinone (**7**) which were found in this species for the first time. The other known compounds, (–)-isoeleutherin (**2**), (+)-eleutherin (**3**), (–)-hongconin (**4**), and (+)-dihydroeleutherinol (**5**) which were reported previously from this species, were also isolated in the present study. Compounds **2**–**6** exhibited potent inhibitory activity on nitric oxide production in RAW 264.7 lipopolysaccharide-activated mouse macrophage cells with IC₅₀ values of 7.7, 11.4, 19.8, 21.7, and 34.4 μM, respectively, whereas the other two compounds, **1** and **7**, were inactive. The structure of compound **1** was elucidated by spectroscopic data analysis including 1D and 2D NMR experiments.

Key words *Eleutherine americana*; Iridaceae; naphthoquinone; nitric oxide inhibitor

Eleutherine species (Iridaceae) are herbal plants distributed in South America and Southeast Asia. The bulb of this plant has been used as a folk medicine for coronary vasodilating,¹⁾ prothrombin decreasing,²⁾ antifertility,³⁾ and wound-healing activities.⁴⁾ Previous phytochemical studies on the bulb of *E. americana* have resulted in the isolation of various types of naphthoquinones and anthraquinones.^{5–8)} Some of these compounds were found to have diverse biological activities such as coronary vasodilating,¹⁾ topoisomerase II inhibitory,⁷⁾ human immuno deficiency virus (HIV) inhibitory,⁷⁾ and antifungal activity.⁸⁾

During our screening procedure to find new inducible nitric oxide synthase (iNOS) inhibitory agents from higher plants, the methanol extract (60 g) of the bulb of *E. americana* exhibited considerable inhibitory activity with an IC₅₀ value of 20 μg/ml. Therefore, it was subjected to detailed phytochemical laboratory investigation, affording eight compounds including a new naphthoquinone (**1**).

Compound **1** gave a molecular ion peak at *m/z* 305.1042 [M+H]⁺ in the high resolution ESI-mass spectrometry corresponding to the elemental formula, C₁₆H₁₆O₆. The IR spectrum showed absorption bands at 3680 cm⁻¹ for one or more hydroxyl groups and 1738 cm⁻¹ for an ester carbonyl functionality.⁹⁾ The UV spectrum of **1** exhibited absorption maxima at 225 and 276 nm, indicating the presence of benzene ring(s).⁹⁾ The ¹H- and ¹³C-NMR spectra of **1** showed signals for a 1,2,3-trisubstituted aromatic ring system at δ_H 7.80 (1H, dd, *J*=8.0, 0.8 Hz)/δ_C 120.0 (C-5), 7.71 (1H, t, *J*=8.0 Hz)/136.7 (C-6), and 7.24 (1H, dd, *J*=8.0, 0.8 Hz)/116.9 (C-7) and two conjugated carbonyl groups at δ_C 184.4 (C-4) and 179.8 (C-1). In the HMBC experiment of **1**, these signals showed two bond and three bond correlations of H-5/C-4, C-8a, H-6/C-4a, C-7, C-8, and H-7/C-5, C-8, C-8a, indicating the presence of a 1,4-naphthoquinone skeleton in **1**. A signal for an aromatic methoxy group appeared at δ_H 4.03 and was assigned to C-8 according to the three bond

connectivity with C-8 in the HMBC experiment. The methyl functionality of an acetyl group was observed at δ_H 1.96 (3H, s, COCH₃)/δ_C 21.5. The carbonyl carbon of the acetyl group was resonated at δ_C 170.9 (COCH₃). The position of the acetyl group was assigned at C-2' of a propyl group {δ_H 1.28 (3H, d, *J*=6.5 Hz)/δ_C 20.3 (C-3'), 2.86 (2H, d, *J*=6.5 Hz)/29.7 (C-1'), and 5.21 (1H, sext, *J*=6.5 Hz)/69.8 (C-2')} by the three bond HMBC correlation between H-2' and COCH₃. C-1' of the propyl group was positioned at C-3 according to the HMBC correlations of H-1'/C-2, C-3, C-4. One oxygenated carbon at δ_C 154.8 was assigned at C-2 which contained a hydroxyl group, by the three bond HMBC correlation between H-1' and C-2. Compound **1** had a structure similar to that of the known naphthoquinone, maderone,¹⁰⁾ except for the absence of two methoxy groups. Further detailed analysis of ¹H–¹H COSY, ¹H–¹³C HSQC,

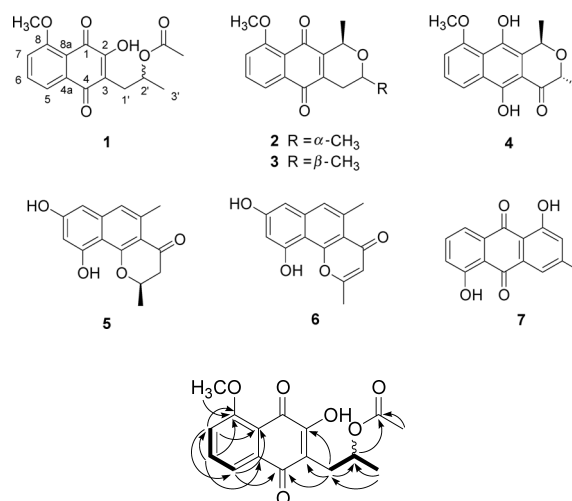


Fig. 1. Important ¹H–¹H COSY (—) and ¹H–¹³C HMBC (---) Correlations of Compound **1**

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Table 1. Inhibitory Effects of Compounds 1—7 on NO Production in RAW264.7 LPS-Activated Mouse Macrophage Cells

Compounds ^{a)}	1	2	3	4	5	6	7	L-NMMA ^{b)}
IC ₅₀ (μM)	>50	7.7	11.4	19.8	21.7	34.4	>50	19.7

a) These compounds exhibited no cytotoxicity at 20 μg/ml. b) *N*^ω-monomethyl-L-arginine (L-NMMA) was used as a positive control.

and ¹H–¹³C HMBC NMR data (Fig. 1) allowed unambiguous assignments for all of the ¹H- and ¹³C-NMR signals of **1**. The circular dichroism (CD) data ($\Delta\epsilon_{243} -0.7$, $\Delta\epsilon_{268} -0.6$, $\Delta\epsilon_{304} -0.2$, $\Delta\epsilon_{367} +0.9$, $\Delta\epsilon_{433} -0.4$) and optical rotation value $\{[\alpha]_D -1.4$ ($c=0.1$, CHCl₃) $\}$ of **1** were obtained. However, there has not been any report on the CD data for any other similar structures to compare with those of **1** to determine the configuration at C-2' of **1**. As a result, it was concluded **1** was a new compound, namely, (–)-3-[2-(acetyloxy)propyl]-2-hydroxy-8-methoxy-1,4-naphthoquinone.

The six known compounds **2**–**7**, were identified as (–)-isoeleutherin,^{6,13} (+)-eleutherin,^{6,11} (–)-hongconin,^{6,11} (+)-dihydroeleutherinol,¹² eleutherinol,¹³ and 1,5-dihydroxy-3-methylanthraquinone,¹⁴ respectively, by physical and spectroscopic methods as well as by comparison of their data with those of published values. To the best of our knowledge, compounds **6** and **7** were found from this plant for the first time in this study. Moreover, there has not been any report on compound **7** from the family Iridaceae previously.

The isolates **1**–**7** were evaluated for their inhibitory activity on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated mouse macrophage RAW264.7 cells. Compounds **2**–**6** exhibited potent inhibitory activity with IC₅₀ values of 7.7, 11.4, 19.8, 21.7, and 34.4 μM, respectively, as shown in Table 1. Furthermore, these compounds did not show cytotoxicity at the test concentration of 20 μg/ml, which was indicative of their NO production inhibitory activity without cytotoxicity. Compounds **2** and **3** were more potent than the positive control, *N*^ω-monomethyl-L-arginine (L-NMMA) which demonstrated an IC₅₀ value of 19.7 μM. Both of the potent compounds **2** and **3** had a naphthoquinone with a pyran ring in each structure. Compounds **4**–**6**, naphthalenes with lactone rings, were also active. However, compound **1**, the naphthoquinone with an open side chain and compound **7** which had an anthraquinone moiety, did not show any activity in the present study. Therefore, we assume that naphthoquinone or naphthalene with a pyran ring or a lactone ring was probably important for the inhibitory activity against the NO production. For instance, Liu *et al.* reported that β-lapachone, the naphthalene with a pyran ring, showed the anti-inflammatory activity, inhibiting expression and function of iNOS in rat alveolar macrophages and aortic rings.¹⁵ An inhibitor of the pathological conditions related to NO can be considered as a therapeutic agent for inflammatory and carcinogenesis diseases.^{16,17} To the best of our knowledge, this is the first report on the evaluation of compounds obtained from *E. americana* for their NO production inhibitory activity.

Experimental

General Optical rotations were measured with a JASCO P-1010 polarimeter at 25 °C. CD measurements were performed using a JASCO J-715 CD/ORD spectropolarimeter. UV and IR spectra were recorded on a Hitachi U-3000 spectrophotometer and a Bio-Rad FTS 135 FT-IR spectrometer, respectively. 1D and 2D NMR experiments were performed on a Varian Unity

INOVA 400 MHz FT-NMR instrument with tetramethylsilane (TMS) as internal standard. EI-MS and ESI-MS were obtained on a JEOL JMS-SX102A and Thermo Finnigan LCQ DECA XP mass spectrometer, respectively. Silica gel (230–400 mesh, Merck, Germany) was used for column chromatography. Thin-layer chromatographic (TLC) analysis was performed on Kieselgel 60 F₂₅₄ (silica gel, 0.25 mm layer thickness, Merck, Germany) with visualization under UV light (254, 365 nm) and 10% (v/v) sulfuric acid spray followed by heating (120 °C, 5 min).

Plant Material The bulb of *Eleutherine americana* MERR. *et* HEYNE (Iridaceae) was collected in Batu Herba Medica Centre, East Java, Indonesia, in May 2005 and was identified by one of the authors, Prof. Adam Wiryawan (Brawijaya University, Malang 65145, Indonesia). A voucher specimen (No. EA254) has been deposited at the Batu Herba Medica Centre.

Extraction and Isolation The bulb of *E. americana* (900 g) was extracted with MeOH (10×21) overnight at room temperature. The extracts were concentrated *in vacuo* at 40 °C, to afford a MeOH-soluble residue (60 g). The MeOH extracts (60 g) were separated by vacuum liquid column chromatography (φ 15 cm; silica gel 230–400 mesh, 500 g) using gradient mixtures of MeOH in CH₂Cl₂ (0→10%) as mobile phases, affording 10 fractions (F-I–F-X). Compounds **3** (1 g, 0.11% w/w), **2** (0.8 g, 0.089% w/w), and **6** (2 g, 0.22%) were isolated from fraction F-II, F-III, and F-VII, respectively, by precipitation in MeOH. Fraction II (3 g), eluted with 100% CH₂Cl₂ from the first separation, was subjected to flash silica gel column chromatography (φ 3.5 cm; 230–400 mesh, 80 g) with *n*-hexane–EtOAc (100:1→1:1) as solvent system, providing twenty fractions. The third fraction (260 mg) from this column was subjected again to flash silica gel column chromatography (φ 2 cm; 230–400 mesh, 15 g), using *n*-hexane–EtOAc (50:1) as eluent, affording **7** (2 mg, 0.00022% w/w). Fraction III (11 g), eluted with 0.2% MeOH in CHCl₃ from the first separation, was chromatographed to flash silica gel column chromatography (φ 4.5 cm; 230–400 mesh, 200 g) with a gradient of MeOH in CH₂Cl₂ (0→2%) as mobile phases, affording **1** (10 mg, 0.0011% w/w). Fraction IV (4.6 g), eluted with 0.5% MeOH in CHCl₃ from the first separation, was separated by flash silica gel column chromatography (φ 4 cm; 230–400 mesh, 100 g) with a gradient of *n*-hexane–EtOAc–CH₂Cl₂ (10:1:0→1:1:1) as a mobile phase, affording **4** (2 mg, 0.00022% w/w) and **5** (300 mg, 0.033% w/w).

3-[2-(2*R*)-2-(acetyloxy)propyl]-2-hydroxy-8-methoxy-1,4-naphthoquinone (**1**): Red orange powder; $[\alpha]_D^{25} -1.4$ ($c=0.1$, CHCl₃); CD ($c=1$ mm, MeOH): $\Delta\epsilon_{243} -0.7$, $\Delta\epsilon_{268} -0.6$, $\Delta\epsilon_{304} -0.2$, $\Delta\epsilon_{367} +0.9$, $\Delta\epsilon_{433} -0.4$. UV (MeOH): λ_{max} (log ϵ)=225 (4.28), 276 (4.22) nm; IR (film): ν_{max} =3680, 3246, 2986, 2931, 1738, 1660, 1640, 1473, 1245, 1120 cm⁻¹; ¹H-NMR (CDCl₃, 400 MHz) δ : 7.80 (1H, dd, $J=8.0$, 0.8 Hz, H-5), 7.71 (1H, t, $J=8.0$ Hz, H-6), 7.24 (1H, dd, $J=8.0$, 0.8 Hz, H-7), 5.21 (1H, sext, $J=6.5$ Hz, H-2'), 4.03 (3H, s, 8-OCH₃), 2.86 (2H, d, $J=6.5$ Hz, H-1'), 1.96 (3H, s, COCH₃), 1.28 (3H, d, $J=6.5$ Hz, H-3'); ¹³C-NMR (CDCl₃, 100 MHz) δ : 184.4 (s, C-4), 179.8 (s, C-1), 170.9 (s, COCH₃), 160.4 (s, C-8), 154.8 (s, C-2), 136.7 (d, C-6), 135.3 (s, C-4a), 120.0 (d, C-5), 117.9 (s, C-3), 117.1 (s, C-8a), 116.9 (d, C-7), 69.8 (d, C-2'), 56.7 (q, 8-OCH₃), 29.7 (t, C-1'), 21.5 (q, COCH₃), 20.3 (q, C-3'); LR-ESI-MS (positive mode) m/z : (rel. int.) 305 [M+H]⁺ (100), 287 (15), 273 (20), 245 (65), 231 (10), 209 (10); HR-ESI-MS (positive mode) m/z : 305.1042 [M+H]⁺ (Calcd for C₁₆H₁₆O₆, 305.1025).

(–)-Isoeleutherin (**2**): $[\alpha]_D^{25} -52.7$ ($c=0.22$, CHCl₃); literature value: –46, CHCl₃.¹¹

(+)-Eleutherin (**3**): $[\alpha]_D^{25} +378$ ($c=0.30$, CHCl₃) ($c=0.3$, CHCl₃); literature value: +346, CHCl₃.¹¹

(–)-Hongconin (**4**): $[\alpha]_D^{25} -13.4$ ($c=0.39$, CHCl₃); literature value: –26.0, $c=1.94$, CHCl₃.¹¹

(+)-Dihydroeleutherinol (**5**): $[\alpha]_D^{25} +8.8$ ($c=0.26$, MeOH).

Measurement of NO Production on LPS-Stimulated Macrophage Cells Measurement of NO formation by iNOS was performed in cultured RAW 264.7 macrophage cells. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with penicillin–streptomycin and 10% fetal bovine serum (FBS) at 37 °C, in 5% CO₂ of humidified air. To evaluate the inhibitory activity of test materials on LPS-induced NO

production, the cells in 10% FBS-DMEM without phenol red were plated in 24-well plates (5×10^5 cells/ml), and then incubated for 24 h. After incubation, the cells were washed with PBS, replaced with new media, and then incubated in the medium with $1 \mu\text{g/ml}$ of LPS in the presence or absence of test samples. After an additional 20 h incubation, the media were collected and analyzed for nitrite accumulation as an indicator of NO production by the Griess reaction. Briefly, $180 \mu\text{l}$ of Griess reagent [0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in H_2O and 1% sulfanilamide in 5% H_3PO_4] was added to $100 \mu\text{l}$ of each supernatant from LPS or sample-treated cells in 96-well plates. The absorbance was measured at 540 nm using a microplate reader, and nitrite concentration was determined by comparison with a sodium nitrite standard curve. The percentage inhibition was expressed as $[1 - (\text{NO level of test samples} / \text{NO level of vehicle-treated control})] \times 100$. The IC_{50} value, the sample concentration resulting in 50% inhibition of NO production, was determined using non-linear regression analysis (% inhibition versus concentration). *N*^ω-monomethyl-L-arginine (L-NMMA) was used as a positive control. All compounds were also tested for cytotoxicity using MTT assay.¹⁸⁾

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