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Corilagin with Inhibitory Activity against NO Production from *Euphorbia supina*

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Abstract – Bioactivity-guided investigation on whole plant of *Euphorbia supina*, using LPS-induced Raw264.7 cells, led to the identificaiton of a tannin which was not reported from this plant along with four known constituents (quercetin, astragalin, juglanin and methyl gallate). The structure of the tannin was determined as corilagin by the interpretation of NMR (1D and 2D) and MS spectroscopic data. All the isolates were tested for the inhibitory activity against NO production in LPS-induced Raw264.7 cells. Among the tested isolates, corilagin was found to be the most active compound.
Keywords – *Euphorbia supina,* Euphorbiaceae, Corilagin, Tannin, Nitric oxide

Introduction

Euphorbia supina Raf. belongs to the family Euphorbiaceae and its whole plant has been used traditionally for the treatment of inflammatory and allergic diseases in Korea. Previous studies on this plant reported the presence of flavonoids as constituents of this plant, as well as anti-oxidative, anti-viral and peroxynitrite-scavenging activity of flavonoids.[1] As part of our ongoing search for biologically active constituents from medicinal plants, *E. supina* was selected for the follow-up isolation work since its MeOH extract showed NO inhibitory activity (IC_{50} 15.1 μg/ml) in LPS-induced Raw264.7 cells. Bioactivity-guided separation on the MeOH extract of *E. supina* led to the isolation of a gallic acid derivative (methyl gallate), three flavonoids (quercetin, astragalin and juglanin) and a new constituent in this plant, corilagin. All the isolates were tested in LPS-induced Raw264.7 cells.

Experimental

General experimental procedures – NMR spectra were recorded on a Brucker DMX-800 MHz FT-NMR spectrometer with the tetramethylsilane as an internal standard. HRESIMS and LC-MS/MS were performed with on a Waters Q-Tof Premier spectrometer (Micromass UK Ltd., Manchester, UK) and API4000 triple quadrupole mass spectrometer (AB Sciex, Foster city, CA), respectively. Sephadex LH-20 (25 - 100 μm, Sigma-Aldrich, Steinheim, Germany), silica gel (230 - 400 mesh, SiliCycle Inc., Quebec, Canada) were used for column chromatography. TLC was performed on precoated Kiesel-gel 60 F_{254} (0.25 mm, Merck, Darmstadt, Germany) and Kiesel-gel 60 RP-$18F_{254s}$ (0.25 mm, Merck, Steinheim, Germany).

Plant materials – Whole plants of *E. supina* (4.3 kg) were collected in Chungbuk province, Korea in May 2011. These plant samples were identified by one of authors. A voucher specimen (DUCYW-0009) has been deposited at College of Pharmacy, Dongguk University, Seoul, Republic of Korea.

Extraction and isolation – Dried *E. supina* (4.3 kg) were pulverized and extracted with methanol at the room temperature three times (each 8 L) to obtain 520 g of solid extract. The methanol extract was suspended in H_2O and then partitioned with *n*-hexane, chloroform, ethyl acetate, *n*-butanol (BuOH). All the fractions, *n*-hexane, chloroform, ethyl acetate and BuOH-soluble fractions, were tested in LPS induced RAW 264.7 cells and it was found the

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BuOH-soluble fraction potently inhibited NO production with IC_{50} value 7.53 μg/ml. The BuOH-soluble extract (ESB) (4.5 g) was subjected to a column chromatography using a silica gel (500 g) eluting with gradient mixtures of chloroform: methanol (20 : 1 to 1 : 1) to give 13 fractions (ESB1 - ESB13). Fraction ESB2 (74.5 mg) was subjected to Sephadex LH-20, eluted with MeOH (100%), and afforded nine fractions (ESB2S1 - ESB2S9). Quercetin (**1**) (1 mg) was precipitated from ESB2S2 fraction. Fraction ESB3 (68.1 mg) was chromatographed on a medium pressure liquid chromatography (MPLC) column of RP C-18 (3.5 × 40 cm, 40C18-PREP), eluted with 50% MeOH and afforded five fractions (ESB3R1 - ESB3R5). Fraction ESB3R3 (19mg) was subjected to Sephadex LH-20 eluted with MeOH (100%) and afforded five fractions (ESB3R3S1 - ESB3R3S5). Astragalin (**2**) (2 mg) was precipitated from ESB3R3S2 fraction. Fraction ESB5 (194 mg) was chromatographed on medium pressure liquid chromatography (MPLC) column of RP C-18 (3.5 × 40 cm, 40C 18-PREP), eluted with 50% - 100% MeOH and afforded seven fractions (ESB5R1 - ESB5R7). Fraction ESB5R6 (52 mg) was subjected to Sephadex LH-20 eluted with MeOH (100%) and afforded five fractions (ESB5R6S1 - ESB5R6S5). Juglanin (**3**) (4.4 mg) was precipitated from ESB5R6S3 fraction. Fraction ESB4 (52.7 mg) was subjected to Sephadex LH-20 eluted with MeOH (100%) and afforded nine fractions (ESB4S1 - ESB4S9). Methyl gallate (**4**) (7 mg) was precipitated from ESB4S2 fraction. Corilagin (**5**) (18 mg) was precipitated from ESB9S2 fraction.

Quercetin (1) – ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.66 (1H, d, J = 1.2 Hz, H-2'), 7.53 (1H, dd, J = 8.6, 1.2 Hz, H-6'), 6.88 (1H, d, J = 8.6 Hz, H-5'), 6.38 (1H, d, J = 2.0 Hz, H-8), 6.17 (1H, d, J = 2.0 Hz, H-6); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 175.8 (C-4), 163.9 (C-7), 160.7 (C-5), 156.1 (C-9), 147.7 (C-4'), 146.8 (C-2), 145.0 (C-3'), 135.7 (C-3), 121.9 (C-1'), 120.1 (C-6'), 115.6 (C-5'), 115.2 (C-2'), 103.0 (C-10), 98.2 (C-6), 93.3 (C-8); ESI-MS*: m/z* 301 $[M-H]^-$

Astragalin (2) – ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.99 (2H, d, J = 8.8 Hz, H-2', 6'), 6.85 (2H, d, J = 8.8 Hz, H-3', 5') 6.01 (1H, brs, H-8), 5.91 (1H, brs, H-6) 5.35 (1H, d, J = 7.2 Hz, H-1''), 3.57 - 3.08 (glucose protons); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 176.1 (C-4), 160.9 (C-5), 159.9 (C-4'), 156.9 (C-9), 156.6 (C-2), 132.8 (C-3), 130.6 (C-2', 6'), 121.0 (C-1'), 115.1 (C-3', 5'), 103.8 (C-10), 101.4 (C-1''), 98.9 (C-6), 93.8 (C-8), 77.4 (C-5''), 76.5 (C-3''), 74.2 (C-2''), 69.8 (C-4'') 62.8 (C-6''); ESI-MS*: m/z* 447 $[M-H]^-$

Juglanin (3) – ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.02 (2H, d, J = 8.8 Hz, H-2', 6'), 6.89 (2H, d, J = 8.8 Hz, H-3', 5'), 6.44 (1H, d, J = 1.6 Hz, H-8), 6.20 (1H, d, J = 1.6 Hz, H-6), 5.62 (1H, d, J =0.8 Hz, H-1''), 4.15 (1H, d, J = 2.4 Hz, H-5''), 3.75 – 3.16 (arabinose protons); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 177.6 (C-4), 164.5 (C-7), 161.2 (C-5), 160.0 (C-4'), 156.7 (C-9), 156.4 (C-2), 133.4 (C-3), 130.9 (C-2'), 130.7 (C-6'), 120.7(C-1'), 115.4 (C-3', 5'), 108.0 (C-1''), 103.9 (C-10), 98.7 (C-6), 93.7 (C-8), 86.3 (C-4''), 82.1 (C-2''), 77.1 (C-3''), 60.4 (C-5''); ESI-MS*: m/z* 417 $[M-H]^-$

Methyl gallate (4) – ^{1}H NMR (acetone-d_6, 400 MHz) δ 7.11 (2H, s, H-2', 6'), 3.78 (-OCH_3, s, H-1); ESI-MS*: m/z* 183 $[M-H]^-$

Corilagin (5) – $[\alpha]^{25}_D$ –37.9 (c 0.1, DMSO), ^{1}H NMR (DMSO-d_6, 800 MHz) δ 6.98 (2H, s, H-2', H-6'), 6.53 (1H, s, H-6'''), 6.43 (1H, s, H-6''), 6.17 (1H, d, J = 7.5 Hz, H-1), 4.58 (1H, brs, H-3), 4.36 (1H, t, J = 8.1 Hz, H-5), 4.27 (1H, d, J = 3.0 Hz, H-4), 4.21 (1H, dd, J = 10.8, 7.7 Hz, H-6), 3.99 (1H, dd, J = 10.8, 10.2 Hz, H-6), 3.88 (1H, d, J = 7.5 Hz, H-2) ppm; ^{13}CNMR (DMSO-d_6, 200 MHz) δ 167.1 (C-7''), 166.9 (C-7'''), 164.8 (C-7'), 145.6 (C-3'), 145.6 (C-5'), 143.7 (C-3''), 143.9 (C-3'''), 143.9 (C-5'''), 143.7 (C-5''), 139.0 (C-4'), 135.8 (C-4''), 135.4 (C-4'''), 123.9 (C-2''), 123.1 (C-2'''), 118.7 (C-1'), 116.1 (C-1''), 115.9 (C-1'''), 109.0 (C-2'), 109.0 (C-6'), 106.9 (C-6''), 105.9 (C-6'''), 92.2 (C-1), 77.5 (C-3), 76.4 (C-5), 71.7 (C-2), 63.9 (C-6), 62.2 (C-4); HRESIMS*: m/z* 633.0728 $[M-H]^-$ (calcd for $C_{20}H_{25}O_{23}$, 633.0787). CD (c 1.97 × 10^{-4} M, MeOH) λ_{max} (Δε) 239 (−1.83), 264 (0), 284 (−1.08) nm

LC-MS/MS analysis – Test sample was analyzed using an API4000 triple quadrupole mass spectrometer (AB Sciex, Foster city, CA) in the multiple reaction monitoring (MRM) mode with electrospray ionization interface used to negative ions ($[M-H]^-$). The turbo ion-spay interface was operated in negative ion mode at −4500 V and 400ºC. The mass transition used for test sample was *m/z* 633.1 → 301.0 (collision energy, −50 eV), respectively. Quadrupoles Q1 and Q3 were set to unit resolution. The analytical data were processed using Analyst software (Version 1.5.1; Applied Biosystems).

Measurement of nitric oxide (NO) production – NO production was assayed by measuring nitrite in supernatants of cultured RAW 264.7 cells.[2] Cells (1 × 10^6/mL) were seeded in 96 well culture plates. After pre-incubation of RAW 264.7 cells for 18 hours, cells were pretreated with test samples (5 μg/ml) for 30 min and then stimulated LPS (500 ng/mL) for 24 hours. The supernatant was mixed with an equal volume of Griess reagent and incubated at room temperature for 5 min. The concentration of nitrite was measured at 570 nm.

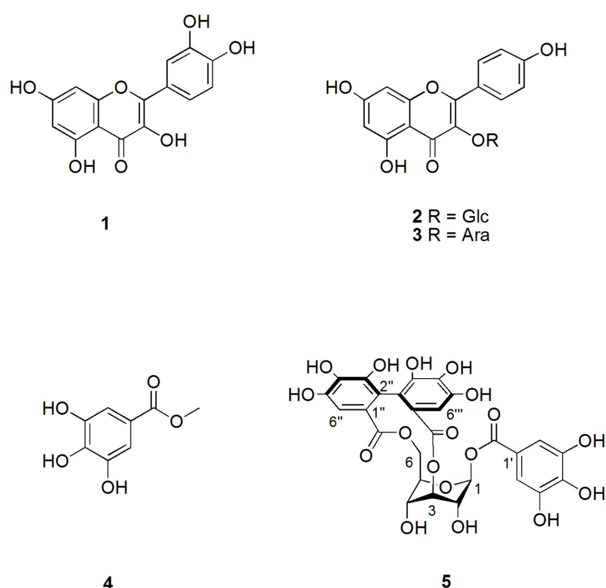


Fig. 1. Structures of compounds **1** - **5**.

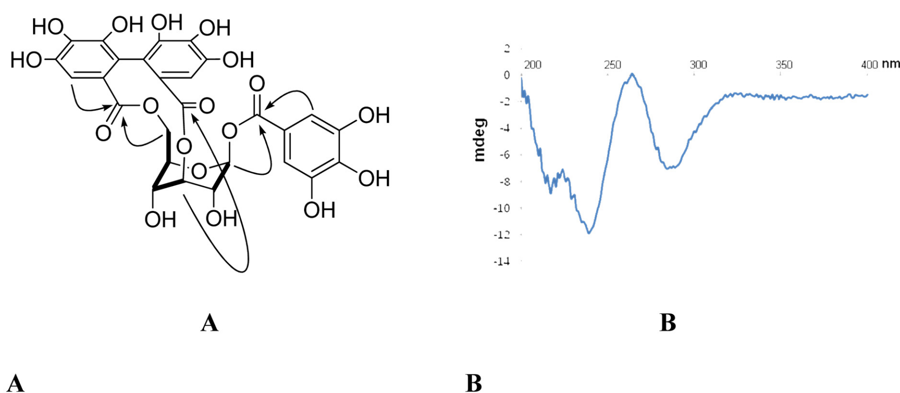
Table 1. Effects of compounds on production of NO in LPS-induced RAW 264.7 cells. The cells (1×10^5 cells/ml) were pretreated with compounds (5 μg/ml) 30 min prior to stimulation with LPS (500 ng/ml) 24 hours after stimulation, the NO level of the supernatants was measured by Griess reagent. Statistical signicance: $p < 0.05$, as compared to the LPS treated group. Signicanct differences between treated groups were determined using the Dunnett's t-test. Values shown are the mean S.E. of duplicate determinations from three separate experiments. 1: quercetin, 2: astragalin, 3: juglanin, 4: methyl gallate, 5: corilagin

Group	Nitric oxide (μM) ± S.D
Normal	0.01 ± 0.48
LPS (500 ng/ml)	29.81 ± 3.44
LPS + compound **1** (5 μg/ml)	24.32 ± 2.23
LPS + compound **2** (5 μg/ml)	27.64 ± 1.89
LPS + compound **3** (5 μg/ml)	23.50 ± 2.12
LPS + compound **4** (5 μg/ml)	15.28 ± 1.42*
LPS + compound **5** (5 μg/ml)	12.08 ± 1.16*

Result and Discussion

Compounds **1** - **5** were identified as quercetin (**1**), astragalin (**2**), juglanin (**3**), and methyl gallate (**4**),[3] the known constituents from this plant, by comparison with the published values. ^{1}H NMR spectra displayed two singlet peaks at δ_H 6.53 (1H, s) and 6.43 (1H, s) assignable to a hexahydroxydiphenoyl (HHDP) group, one singlet peak at δ_H 6.98 (2H, s) from a galloyl group, and seven peaks in the range δ_H 6.17-3.84 corresponding to a glucose unit. MS fragments at *mz* 301 ($[M-H]^-$-332, loss of a galloyl glucose) and *mz* 463 ($[M-H]^-$-170, loss of a galloyl group) supported the presence of a HHDP and a galloyl group in MS spectrum. Anomeric proton at δ_H 6.17 with a coupling constant of 7.5 Hz indicated this glucose was β-confomer. The location of the HHDP and the galloyl group was established by the HMBC correlations (Fig. 2). Long range couplings were observed between H-6 (δ_H 4.21 and 3.99) and δ_C 167.1, as well as H-3 (δ_H 4.58) and δ_C 166.9, suggesting that the HHDP was linked to C-3 and C-6. The remaining galloyl group was connected to C-1 via an ester linkage by the observed HMBC correlation of H-1 (δ_H 6.17) to δ_C 164.8. Based on all the data and the published values,[4] this compound was elucidated as corilagin. Furthermore, the stereochemistry of corilagin was confirmed as shown in Fig. 1 by CD measurement and comparison with the literature.[5] Corilagin (**5**) was isolated for the first time from this plant.

The isolated compounds **1** - **5** were tested in LPS-induced NO production assay using Raw 264.7 cells and two compounds were found to inhibit NO production, significantly, in this assay system (Table 1). The known structure of this plant, methyl gallate, was known to possess the inhibitory activity against NO production[6-8]



A B

Fig. 2. Key HMBC correlations and ^{1}H-^{1}H COSY of corilagin (A); CD spectrum of corilagin (B).

and in the present study, similarly, the level of NO was decreased by the treatment of this compound. Of the isolates, corilagin demonstrated to inhibit potently NO production in LPS-induced Raw 264.7 cells, which was consistent with the previous studies.[9]

In the current investigation, it was found that corilagin with inhibitory activity against NO production in LPS-induced Raw 264.7 cells, in part, may be responsible for anti-inflammatory activity of this plant.

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