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Phenolic Amides from the Fruits of *Tribulus terrestris* and Their Inhibitory Effects on the Production of Nitric Oxide

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Tribulus terrestris L. (Zygophyllaceae) is an annual plant widely distributed in warm temperate and tropical regions of southern Asia, Africa, parts of Eastern Europe, and Australia. The fruits of *T. terrestris* have a long history of use in traditional Chinese medicines for the treatment of high blood pressure, eye problems, edema, abdominal distention, sexual dysfunction and veiling.[1,6] This plant is also used as an aphrodisiac, diuretic and nervine in Ayurveda, and in Unani, another system of Indian medicine.[2] Preparation containing steroidal glycosides of *T. terrestris* extracts are used in contemporary medicines and food supplements as a component of drugs effective in treating impotence (Tribulus Terrestris in Canada and TRIB-650 in USA) and cardiovascular diseases (Xin-nao-shu-tong in China).[3,4]

Phytochemical studies of this plant have yielded several steroidal saponins, flavonoids, phenolic amides, and alkaloids.[5-15] The excessive production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) has been implicated in many inflammatory responses such as vasodilation, rheumatoid arthritis and chronic or acute inflammatory conditions.[16,17] Therefore, inhibitors of NO production in macrophages might be of therapeutic benefit in various inflammatory diseases.[18,19] As a part of our continuing phytochemical investigation of natural product-derived inhibitors of NO production, we examined the chemical composition of the fruits of *T. terrestris*. In our present research, we launched a further study of its chemical constituents, which led to the isolation of a new phenolic amide, together with four known compounds. Their structures were characterized on the basis of spectroscopic methods, including extensive 2D-NMR and MS spectrometry. This paper reports on the isolation, structure elucidation of these phenolic amide analogues, in addition to evaluation of their ability to inhibit lipopolysaccharide (LPS)-induced NO production in RAW264.7 cells. The aqueous ethanolic extract of the dried fruits of *T. terrestris* was subjected to Sephadex LH-20, reversed-phase and silica gel column chromatography to afford compounds **1-5** (Fig. 1).

Tribulusamide D (**1**) was isolated as a colorless powder. The molecular formula $C_{17}H_{15}NO_5$ was determined from

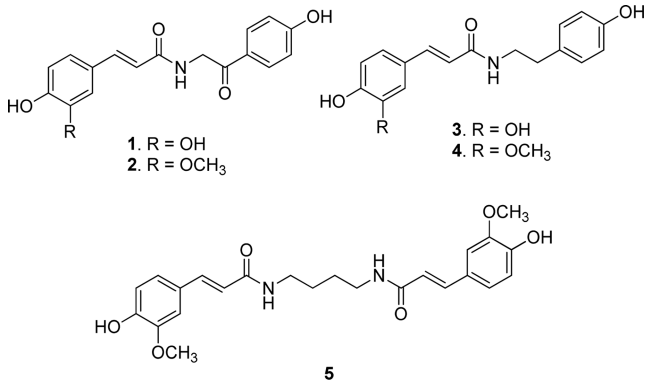

Figure 1. Chemical structures of compounds **1-5**.

the quasimolecular ion peak observed using electrospray ionization (ESI)-MS and HRESI-MS measurement at *m/z* 314.1017 $[M+H]^+$ (calcd for $C_{17}H_{16}NO_5$, 314.1023), suggesting 11 degrees of unsaturation of the molecules. The UV spectra of **1** with an absorption at 219, 242, 290, and 319 nm were similar to those of terrestriamide (**2**), and were characteristic of 3,4-dioxygenated cinnamic acid derivatives.[9,20] Its IR spectrum indicated the presence of hydroxyl (3316 cm^{-1}) and amide groups (1653 cm^{-1}). The ^1H NMR spectrum of **1** revealed an ABX spin system at δ_H 6.99 (1H, d, J = 2.1 Hz), 6.88 (1H, d, J = 8.4, 2.1 Hz), and 6.76 (1H, d, J = 8.4 Hz) for H-2, H-6, and H-5, respectively in the caffeic acid moiety. A downfield doublet at δ 7.27 (1H, d, J = 16.1 Hz) was assigned to the H-7 on the caffeic acid moiety showing *trans*-coupling with H-8 olefinic proton, which appeared as a doublet at δ 6.52 (1H, d, J = 16.1 Hz). As expected, the ^{13}C NMR spectrum of **1** showed carbon signals that were classified as *trans*-caffeoyl unit at δ_C 126.8 (C-1), 114.3 (C-2), 146.0 (C-3), 147.9 (C-4), 116.2 (C-5), 121.0 (C-6), 140.0 (C-7), 118.6 (C-8), 166.2 (C-9). Also, ^1H, ^{13}C, and HSQC spectra of **1** displayed 1,4-disubstituted aromatic ring signals [δ_H 7.90 (2H, d, J = 8.4 Hz) and 6.88 (2H, d, J = 8.4 Hz), and δ_C 115.8, 127.0, 130.9, and 162.8], a methylene signal [δ_H 4.65 (2H, d, J = 5.6 Hz) and δ_C 45.9], a ketonic carbon signal at δ_C 193.9 (C-7'), amide proton signal at δ_H 8.29 (1H, t, J = 5.6 Hz), and three hydroxyl groups at δ_H 10.44 (1H, s), 9.39 (1H, s), and 9.16 (1H, s). The ^1H and ^{13}C NMR spectra of

[a]These authors contributed equally to this work.

Table 1. The NMR spectroscopic data for compound **1**[a]

Position	δ_H mult., (*J* Hz)	δ_C mult.
1		126.8 s
2	6.99 d (2.1)	114.3 d
3		146.0 s
4		147.9 s
5	6.76 d (8.4)	116.2 d
6	6.88 d (8.4, 2.1)	121.0 d
7	7.27 d (16.1)	140.0 d
8	6.52 d (16.1)	118.6 d
9		166.2 s
1'		127.0 s
2'	7.90 d (8.4)	130.9 d
3'	6.88 d (8.4)	115.8 d
4'		162.8 s
5'	6.88 d (8.4)	115.8 d
6'	7.90 d (8.4)	130.9 d
7'		193.9 s
8'	4.65 d (5.6)	45.9 t
NH	8.29 t (5.6)	
OH	10.44 s	
	9.39 s	
	9.16 s	

[a]Measured at 700 and 175 MHz; obtained in DMSO-d_6 with TMS as an internal standard. The assignments were based on HSQC and HMBC experiments.

compound **1** (Tables 1) were very similar to those of terrestriamide (**2**), and these findings suggested that **1** is also a phenolic amide derivative.

Comparison of the NMR data of **1** with those of the known compound terrestriamide (**2**) showed that the only difference between the two compounds was that the methoxyl group at C-3 in **2** was replaced by a hydroxyl group in **1**. The connectivity of these units was established on the basis of HMBC correlations (Fig. 2) such as H-7 (δ 7.27) with C-2 (δ 114.3), C-6 (δ 121.0), and C-9 (δ 166.2); amide proton (δ 8.29) with C-9 (δ 166.2), C-8' (δ 45.9), and C-7' (δ 193.9); and H-2'/H-6' (δ 7.90) with C-7' (δ 193.9). Consequently, compound **1** was determined to be (*E*)-3-(3,4-dihydroxyphenyl)-*N*-[2-(4-hydroxyphenyl)-2-oxoethyl]-prop-2-enamide, a new compound named as tribulusamide D.

In addition, the known compounds were identified as terrestriamide (**2**),[12,14] *N-trans*-caffeoyl tyramine (**3**),[21,22] *N-trans*-feruloyl tyramine (**4**),[21,23] and terrestribisamide (**5**)[9] by comparing their spectroscopic data with those reported in the literature.

We examined the inhibitory effects of all compounds (**1-5**) isolated from *T. terrestris* on LPS-induced NO generation in

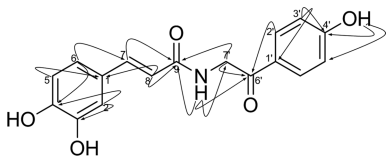

Figure 2. Key HMBC (H → C) correlations of compound **1**.

Table 2. Inhibitory effects of compounds **1-5** on the LPS-induced NO production RAW264.7 cells

Compound	IC_{50} value (μM)	MTT assay IC_{50} (μM)
1	83.4 ± 0.8[a]	> 100
2	38.6 ± 0.4	> 100
3	14.7 ± 0.3	> 100
4	42.3 ± 0.6	> 100
5	> 100	> 100
P.C.[b]	25.3 ± 0.3	

[a]The experiments were repeated in triplicate, and the values were expressed as mean ± standard deviation. [b]*N*-Monomethyl-L-arginine (L-NMMA) was used as a positive control.

RAW264.7 cells. Using the Griess method,[24] the concentration required to inhibit the production of NO by 50% (IC_{50} value) was calculated on the basis of the concentrations of nitrite released into the culture media. Among them, compounds **1-4** were found to inhibit NO production in LPS-stimulated RAW264.7 cells, with IC_{50} values ranging between 14.7 and 83.4 μM (Table 2). All isolates were not cytotoxic by themselves at concentrations up to 100 μM against RAW264.7 cells in the MTT assay,[24] which suggested that the inhibitory activity against NO production in LPS-activated RAW264.7 cells was not due to the cytotoxicity of tested compounds.

All isolates have a diaryl skeleton, which is reminiscent of diarylheptanoid and curcumin derivatives. Natural diarylheptanoid and curcumin derivatives were reported to show inhibitory effects on LPS-induced NO production in RAW264.7 cells.[25-29] Also, phenolic amide analogues from the aerial parts of *Celtis africana* exhibited significant anti-inflammatory activity in carrageenan-induced paw edema in rats.[30] On the basis of our results and those published in previous reports, phenolic amide derivatives isolated from *T. terrestris* could be potential candidates for development as an anti-inflammatory agent that exerts its therapeutic effects through inhibition of NO production.

Experimental Section

General Procedures. UV and IR spectra were obtained using a Shimadzu UV-1650PC and a Jasco FT/IR-4100 spectrometer, respectively. 1D and 2D NMR spectra were measured on a Bruker Ascend 700 MHz NMR spectrometer with tetramethylsilane as an internal standard, and chemical shifts expressed in terms of ä values. Electrospray ionization (ESI) mass spectra were obtained on a LTQ Orbitrap XL (Thermo Scientific) mass spectrometer. Preparative HPLC was performed using a Shimadzu system (LC-8A pump and SPD-20A UV/VIS detector) and a YMC-Pack ODS A column (250 × 20 mm i.d.), using a mixed solvent system of methanol-water at a flow rate of 8 mL/min. MPLC (Combi Flash RF, Teledyne ISCO) separations were performed using a RediSep Rf C_{18} column (130 g C18 Reverse Phase) with a flow rate of 75 mL/min. Open column chromatography was performed using a silica gel (Kieselgel 60, 70-230 mesh,

Merck), Sephadex LH-20 (18-111 mM, GE Healthcare), and thin layer chromatography (TLC) was performed using a pre-coated silica gel 60 F_{254} (0.25 mm, Merck). RAW264.7 murine macrophage cell lines were obtained from American type culture collection (ATCC). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), 1% penicillin/streptomycin were purchased from JBI (WelGENE, USA). LPS (*E. coli* 0111:B4), and cell dissociation solution were obtained from Sigma (USA). Dimethylsulfoxide (DMSO) was obtained from Duchefa Biochemie (The Netherlands). Trypan blue stain 4% was purchased from Invitrogen (USA). Griess reagents (Reagent A: 0.1% *N*-1-Naphthylethylene diamine dihydrochloride/Reagent B: 1% sulfanilamide, Merck) and sodium nitrite were obtained from Merck (Germany). ELISA was performed using a SPECTRAmax system (USA). All other chemicals and reagents were analytical grade.

Plant Material. The dried fruits of *T. terrestris* were purchased from Kyungdong Oriental Herbal Store, Seoul, South Korea, on September 2011; and were identified by Professor Ok Pyo Zee (College of Pharmacy, Sungkyunkwan University). A voucher specimen (G46) was deposited at the Natural Products Research Laboratory, Gyeonggi Institute of Science & Technology Promotion.

Extraction and Isolation. The dried and ground fruits (5 kg) of *T. terrestris* were extracted with EtOH (3 × 18 L) at room temperature. After filtration and evaporation of the solvent *in vacuo*, the EtOH extract was suspended in distilled water and then partitioned, in turn, with CH_2Cl_2, EtOAc and *n*-BuOH. The EtOAc fraction showed a potent inhibitory activity (IC_{50} < 25 μg/mL) on NO production in RAW264.7 cells, and it was subjected to further isolation. The EtOAc extract (8 g) was subjected to column chromatography over silica gel ($CHCl_3$/MeOH, 100:0 to 50:50) to yield 13 fractions (G46-51-1 ~ 13). Fraction G46-51-7 (1.5 g) was chromatographed over Sephadex LH-20 ($CHCl_3$/MeOH = 1:1) and purified using HPLC (30% methanol, Shimadzu system, YMC ODS H-80, 250 × 20 mm i.d., flow rate 8 mL/min) to yield compounds 1 (17 mg), 3 (97 mg), and 5 (2.5 mg). Fraction G46-51-5 was subjected to MPLC on RP-18, eluted with acetonitrile/water (10-100% acetonitrile, then acetonitrile/acetone 1:1, CombiFlash system, RediSep Rf, 130 g C18 Reverse Phase, flow rate 75 mL/min), chromatographed over silica gel ($CHCl_3$/EtOAc = 10:1), and finally purified by semi-preparative HPLC (25-40 % methanol, Shimadzu system, YMC ODS H-80, 250 × 20 mm i.d., flow rate 8 mL/min), to yield compounds **2** (70 mg) and **4** (27 mg).

Determination of NO Production.[24,31] The concentration of nitrite in the medium was measured as an indicator of NO production according to the Griess reaction. Briefly, RAW 264.7 cells were seeded in 96-well plates at 4×10^5 cells/mL for 24 hours with 10% FBS DMEM. The cells were stimulated with final concentrations of 0.5 μg/mL LPS without negative control. After 1 h, the wells were treated with samples of different concentrations or were left untreated (negative control). The plates were then incubated for 24 hours at 37 °C in 5% CO_2. Following incubation, the inhibition of NO release was assessed by quantification of nitrite (NO_2^-), one of the final products of NO oxidation, in the 50 μL of the cell supernatant by using the 50 μL mixture of Griess reagents. The mixture was incubated for 10 min at room temperature. Absorbance at 540 nm was measured using an ELISA reader, and the results were compared with a calibration curve using sodium nitrite as the standard. The percentages of NO inhibition were calculated as follows:

$$\% \text{ Inhibition} = 100 - \frac{\text{concentration of } NO_2 \text{ in the sample}}{\text{concentration of } NO_2 \text{ in LPS treated wells}} \times 100.$$

MTT Assay for Cell Viability.[24] Cell viability was assessed by MTT colorimetric assay. RAW264.7 cells were treated with MTT (5 mg/mL) in serum-free DMEM. After a 3 h incubation at 37 °C and 5% CO_2, 100 μL of 100% DMSO was added to all the wells to dissolve the insoluble purple formazan product into a colored solution. Absorbance was measured at 540 nm using an ELISA reader.

Tribulusamide D (1). Colorless powder; IR ν_{max} cm^{-1}: 3414, 3316, 2911, 2592, 1679, 1653, 1524, 1453, 1238, 1167, 1124; UV (MeOH) λ_{max} nm (log ε): 219 (4.12), 242 (2.29), 290 (4.53), 319 (3.66); ^1H (700 MHz) and ^{13}C (175 MHz) NMR spectroscopic data, see Table 1; ESI-MS (positive mode) *m/z* 312 $[M-H]^-$; HRESIMS (negative mode) *m/z* 314.1017 (calcd for $C_{17}H_{16}NO_5$, 314.1023).

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Supporting Information. IR, HRESI-MS, 1D and 2D NMR spectra of compound **1**, ^1H and ^{13}C NMR data of compounds **2-5** are available as Supporting Information.

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