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Anti-inflammatory constituents from *Perilla frutescens* on lipopolysaccharide-stimulated RAW264.7 cells

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ABSTRACT

Perilla frutescens (L.) Britt. (Lamiaceae) is a traditionally medicinal herb in East Asian countries to treat various diseases. In present study, the low-polarity constituents of the aerial parts of *P. frutescens* were investigated and their anti-inflammatory effect on lipopolysaccharide (LPS)-stimulated RAW264.7 cells were assayed. Three new furanoid monoterpenoids, named as frutescenones A – C (**1–3**), together with thirteen known compounds (**4–16**) were isolated and identified on the basis of extensive spectroscopic analysis and a single-crystal X-ray diffraction study. Among these components, **1** is an unusual monoterpenoid with 2,3′-bifuran skeleton, and **3** is a rare perillaketone-adenine hybrid heterodimer, while the revised NMR arrangements of **4** were reported at the first time. Furthermore, monoterpenoid **4** and alkaloid **15** showed remarkably inhibitory effect on the production of inflammatory mediator (NO) and pro-inflammatory cytokines (TNF-α and/or IL-6) in LPS-stimulated RAW264.7 cells.

1. Introduction

Perilla frutescens (L.) Britt. is an aromatic annual herb belonging to the family Lamiaceae that is widely distributed throughout many Asian countries [1]. It is commonly known as “Zisu” in China, where it receives widespread used as folk medicine, predominantly in the treatment of cold, headache, cough, poisoning from fish and crabs [2]. More than 100 constituents, including monoterpenoids, sesquiterpenoids, triterpenoids, flavonoids, polyphenols, fatty acids, phytosterols, etc., have been reported from the stems, leaves and seeds of the title plant, and most of them are contributed to its medical benefits such as anti-allergic, anti-inflammatory, anti-oxidant, anticancer, antibacterial, or antidepressant effects [1].

Inflammation is a natural biological response to injury or infection in the human body. Inhibition of the production of inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and interleukin 1β (IL-1β), and inflammatory mediator such as nitric oxide (NO) and prostaglandin E2 (PGE2), serves as a key mechanism in the control of inflammation [3]. Recent studies for the anti-inflammatory activity of *P. frutescens* extract or components revealed the low-polarity fraction containing perillaldehyde- and/or perillaketone-type monoterpenoids to have more potential activity [4–7]. In our continuing search for bioactive and diverse ingredients from medicinal plants [8–10], we investigated the petroleum ether (PE)-soluble constituents of this plant and resulted in the isolation of 16 metabolites, including three new furanoid monoterpenoids, named frutescenones A – C (**1–3**), along with thirteen known compounds (**4–16**) (Fig. 1). Among them, frutescenone A (**1**) is the first example of perillaketone-type monoterpenoid with 2,3′-bifuran framework, whereas frutescenone C (**3**) is a rare perillaketone-adenine hybrid heterodimer. A perillaketone-type monoterpenoid, 9-hydroxyisoegomaketone (**4**) and an indole alkaloid, indole-3-carboxaldehyde (**15**), showed markedly anti-inflammatory effect by inhibiting the production of NO, TNF-α and/or IL-6 in LPS-stimulated RAW264.7 cells.

2. Experimental

2.1. General experimental procedures

Optical rotations were determined on a Perkin-Elmer 341

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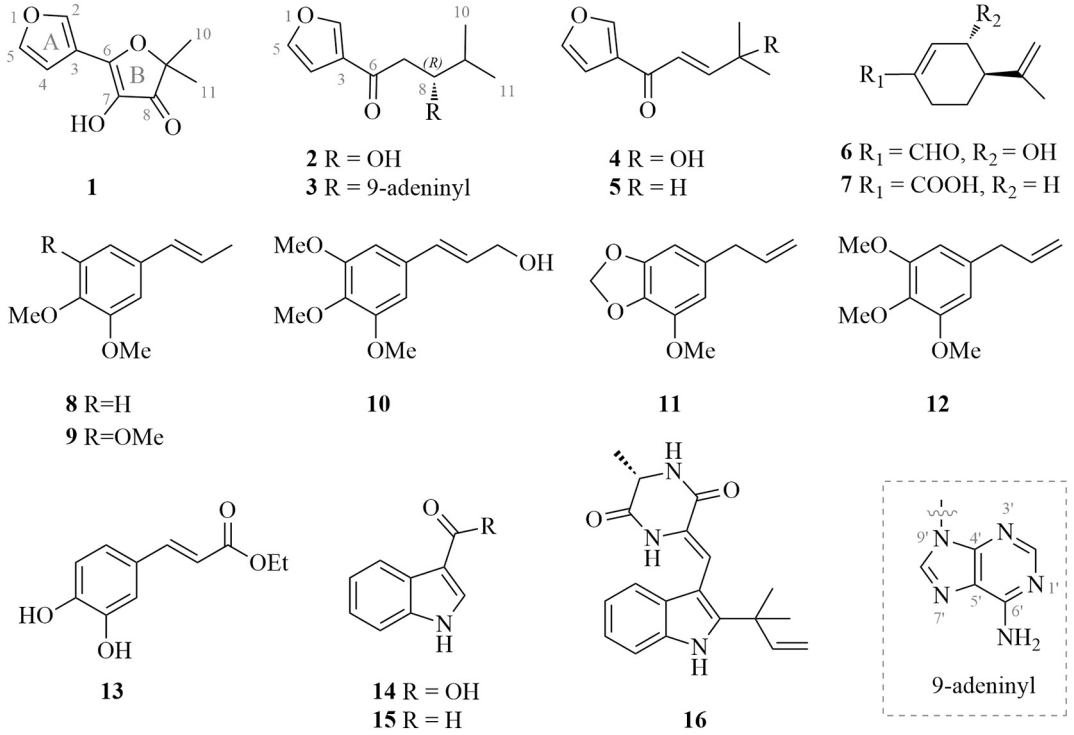

Fig. 1. Structures of isolated compounds **1–16** from *P. frutescens*.

polarimeter (Perkin-Elmer, Wellesley, MA, USA). UV spectra were recorded on a Varian Cary 50 spectrophotometer. IRs were run on a Thermo Nicolet JS5 spectrophotometer. NMR spectra were measured on a Bruker DRX-500 spectrometer (Bruker Biospin AG, Fällanden, Germany). Chemical shifts (δ) are reported in ppm with tetramethylsilane (TMS) as reference and coupling constants (J) in Hz. Electrospray ionization mass (ESI-MS) and electron impact mass (EI-MS) data were acquired using a Waters/Micromass Q-TOF-Ultima Global mass spectrometer (Waters, Milford, MA, USA) or a Finnigan-MAT-95 mass spectrometer (Finnigan MAT, San Jose, CA, USA). Silica gel (200–300, 300–400 mesh, Qingdao Haiyang Chemical Co. Ltd., Qingdao, China), Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden), RP-18 reversed-phase silica gel (ODS, 150–200 mesh, Fuji Silysia Chemical Ltd., Aichi, Japan), and MCI Gel CHP20P (Mitsubishi Chemical, Tokyo, Japan) were used for column chromatography (CC). Silica gel HSGF254 (Yantai Jiangyou Guijiao Kaifa Co., Yantai, China) was used for TLC. Semi-preparative HPLC was run on a Waters HPLC system (Waters) with Waters-2545-HPLC pump, Waters-2489 detector, and Xbridge-C_{18} or YMC-C_{18} column (5 μm, i.d. 10 mm × 250 mm).

Lipopolysaccharide (LPS, *Escherichia coli* O55: B5), dimethyl sulfoxide (DMSO), *N*-[naphthyl]ethylene -diamine dihydrochloride, 3,3′,5,5′-tetramethylbenzidine (TMB) and sulfanilamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). Mouse TNF-α and IL-6 ELISA kits were purchased from BD Biosciences Pharmingen (San Diego, CA, USA).

2.2. Plant material

The aerial parts of *P. frutescens* were collected from Yongshun County, Hunan Province of China, in August of 2015, and authenticated by Prof. Y.-H. Zheng, one of the authors. A voucher specimen (No. 20150815) was deposited with the Herbarium of Shanghai Institution of Materia Medica, Chinese Academy of Sciences, China.

2.3. Extraction and isolation

The dried and powdered aerial parts of *P. frutescens* (5.5 kg) were extracted with 95% EtOH (20 L × 3) at room temperature to afford a crude extract (430 g), which was partitioned successively among PE (3 × 2.5 L), EtOAc (3 × 2.5 L) and BuOH (3 × 2.5 L) with water. The PE layer (220 g) was subjected to column chromatography (CC) of MCI Gel CHP-20P (30, 50, 65, 75, 85 and 95% EtOH in H_2O) to give fractions P1–6. Fr. P3 (1.3 g) was fractioned on CC of Sephadex LH-20 (MeOH) to give frs. P3A – C. Fr. P3B (250 mg) was further separated on CC of silica gel (PE - acetone, 50: 1 to 1: 5) to afford compounds **1** (12 mg), **6** (20 mg) and **13** (6 mg). Fr. P3C (700 mg) furnished **3** (4 mg), **8** (5 mg) and **12** (12 mg) after purification of an ODS CC (MeOH in H_2O, 40–100%) and semipreparative HPLC (41% CH_3OH in H_2O, 4 mL/min). Fr. P4 (13 g) was chromatographed over CC of silica gel (PE - acetone, 50: 1 to 10: 1) to yield frs. P4A – E. Fr. P4A (1.5 g) was purified by continuous CC of silica gel (PE - acetone, 25: 1) to yield **4** (50 mg) and **11** (23 mg). Fr. P4B (4 g) was separated by sequential CCs of Sephadex LH-20 (MeOH), ODS (MeOH in H_2O, 20–100%) and silica gel (PE - acetone, 40: 1–10: 1) to offer **2** (40 mg) and **5** (800 mg). Similarly, compounds **14** (6 mg), **16** (5 mg) and **9** (9 mg) were acquired from fr. P4C (5 g). By similar separating procedures, compounds **15** (5 mg), **10** (8 mg) and **7** (6 mg) were obtained from fr. P5.

Frutescenone A (**1**): Colorless crystals (MeOH); UV (MeOH) λ_{max} (log ε): 328 (3.68) nm; IR (KBr) ν_{max} 3234, 1697, 1629, 1339, 1188, 1116 cm^{-1}; ^1H and ^{13}C NMR data see Table 1; HREIMS *m/z* 194.0579 $[M]^+$ (calcd for $C_{10}H_{10}O_4$, 194.0574).

Single crystals of **1** were grown by slow evaporation method using methanol as solvent at room temperature. A suitable crystal (0.15 × 0.12 × 0.11 mm^3) was selected and mounted on a Bruker APEX-II CCD diffractometer using Mo-Kα (0.71073 Å) radiation for the crystal. The crystal was kept at 173 K during data collection. Using Olex2 [11], the structure was solved with the ShelXT [12] structure solution program using Intrinsic Phasing and refined with the ShelXL [13] refinement package using Least Squares minimisation. Crystal Data for **1** ($C_{10}H_{10}O_4$, M = 194.18 g/mol): triclinic, space group P-1 (no. 2), a = 5.4534(16) Å, b = 8.247(2) Å, c = 10.800(3) Å,