Effects of Angelica Oil and the Isolated Butylphthalides on Glutamate-induced Neurotoxicity in PC12 Cells
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1. General experimental procedures

NMR spectra were obtained using a Bruker-AVIIIHD-600 spectrometer with the solvent peaks used as references. HRESIMS were measured with a Waters Synapt G2 Q-TOF HDMS instrument. Column chromatography was performed with silica gel (200–300 mesh, Yantai Institute of Chemical Technology, Yantai, China) and Sephadex LH-20 (Amersham Pharmacia Biotech AB, Uppsala, Sweden). RP-HPLC separations were performed using an Agilent 1220 instrument with a Kromasil (250 × 10 mm) preparative column packed with C$_{18}$ (5 μm). NP-HPLC separations were performed using a Cometro 6000 instrument with a Kromasil (250 × 10 mm) preparative column packed with silica (5 μm).

PC12 cells were purchased from KeyGEN BioTECH (Nanjing, China). Dulbecco’s Modified Eagles Medium (DMEM) was purchased from Gibco (Grand Island, NY, USA). Calf serum (CS) was purchased from Zhejiang Tianhang Biological Technology Co. Ltd. (Zhejiang, China). L-Glutamic acid, 3-(4,5-Dimethythiazol-2-yl)-2,5-dipheny-tetrazolium bromide (MTT), and trypsase were obtained from Sigma Chemical Co. Ltd. (St Louis, MO, USA). Nimodipine was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Peking, China). Penicillin and streptomycin were purchased from North China Pharmaceutical Group Corporation (Hebei, China).

2. Plant material

The roots of *A. sinensis* were purchased from Sichuan Neautus Traditional Chinese Medicine Co., Ltd (Chengdu, China) in June 2014 and identified by Prof. Min Li (Chengdu University of TCM, Chengdu, China). A voucher specimen (AS20140607) was deposited at State Key Laboratory Breeding Base of Systematic Research, Development and Utilization of Chinese Medicine Resources, Chengdu University of TCM.

3. Extraction and isolation

The roots of *A. sinensis* (60 kg) were subjected to hydrodistillation for 10 h using a big modified Clevenger-type apparatus with a water-cooled oil receiver to obtain the essential oil (180 g). After dried with anhydrous sodium sulfate, the oil was subjected to silica gel column chromatography using a gradient elution of petroleum ether/EtOAc (1:0–0:1, v/v) to give 15 major fractions (F$_1$−F$_{15}$) based on TLC analysis. Fraction F$_4$ (4.2 g) was further separated by column chromatography over silica gel with a gradient elution of petroleum ether/EtOAc
Fraction F$_{4,5}$ was purified via preparative TLC (petroleum ether/EtOAc, 12:1) followed by normal phase (NP) semipreparative HPLC (n-hexane/EtOH, 100:1) purification to afford 9 (7.4 mg). Separation of F$_{4,6}$ by Sephadex LH-20 (petroleum ether/CHCl$_3$/MeOH, 5:5:1) gave seven subfractions (F$_{4,6a}$−F$_{4,6g}$). Compound 7 (6.5 mg) was crystallized from F$_{4,6c}$ and recrystallized in n-hexane. Fractions F$_{4,6e}$ and F$_{4,6f}$ were purified separately by preparative TLC (petroleum ether/EtOAc, 3:1 and 1:1) to afford 5 (10 mg) and 3 (12 mg). Fraction F5 (2 g) was separated over silica gel column chromatography eluted with a gradient elution of petroleum ether/Me$_2$CO (1:0−1:1, v/v) to give eight subfractions (F$_{5,1}$−F$_{5,8}$). Separation of F$_{5,2}$ by repeated silica gel column chromatography afforded 4 (45.0 mg). Fraction F$_{5,3}$ was further purified by preparative TLC (petroleum ether/EtOAc, 15:1) followed by NP semipreparative HPLC (n-hexane/EtOH, 350:1) purification to afford 1 (1.8 mg) and 8 (3.2 mg). Separation of F$_{5,4}$ using preparative TLC (petroleum ether/EtOAc, 12:1), NP semipreparative HPLC (n-hexane/EtOH, 400:1), and reverse phase (RP) semipreparative HPLC (80% MeOH in H$_2$O) successively afforded 2 (1.0 mg) and 6 (3.0 mg).

(+)-(R)-3-Butyl-7-hydroxy-3-methoxypthalide (1): White powder; $[\alpha]_{20}^{D}$ +41.9 (c 0.03, MeOH); $^1$H and $^{13}$C NMR data see Table 1; HRESIMS m/z: 259.0945 [M+Na]$^+$ (calcd. for C$_{13}$H$_{16}$O$_4$Na, 259.0946).

Senkyunolide I-6,7-acetonide (2): Colorless oil; $^1$H and $^{13}$C NMR data see Table 1; HRESIMS m/z: 287.1262 [M+Na]$^+$ (calcd. for C$_{15}$H$_{20}$O$_4$Na, 287.1259), 551.2617 [2M+Na]$^+$ (calcd. for C$_{30}$H$_{40}$O$_8$Na, 551.2621).

4. PC12 cell viability assay

PC12 cells were maintained in high glucose DMEM medium supplemented with 10% CS, 100 U/mL penicillin and streptomycin in a humidified incubator at 37 °C under an atmosphere of 95% air and 5% CO$_2$. The cells were seeded in 96-well plates at a density of 1.5 × 10$^4$ cells per well and cultured for 24 h. The cultured cells were preincubated with different concentrations of angelica essential oil (3.125, 6.25, 12.5, 25, 50, 100 μg/mL) or the isolated butylphthalides (12.5, 25, 50, 100, 200, 400 μM), then exposed to glutamate (30 mM). After incubation for an additional 24 h, MTT (0.5 mg/mL) was added to the medium and incubated for 4 h. Then, the MTT was removed carefully, and the formazan crystals were dissolved with 150 μL DMSO. Absorbance was measured on a microplate reader at 570 nm. Statistical significance between two groups was determined using one-way or two-way analysis of variance (ANOVA) and a p-value of <0.05 was considered as significant.