SHORT REPORT

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Effects of Angelica Oil and the Isolated Butylphthalides on Glutamate-induced Neurotoxicity in PC12 Cells

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Abstract: *Angelica sinensis* contains a large amount of essential oil (angelica oil), which is rich in phthalide derivatives with a lot of bioactivities. In vitro activity screening of angelica oil from the roots of *A. sinensis* found that it had concentration-dependent effect on glutamate-induced injury in PC12 cells. Further phytochemical investigation on this angelica oil led to the isolation of nine butylphthalides (**1**–**9**) including two new compounds (**1** and **2**). Their structures were elucidated by extensive spectroscopic analyses. It is noteworthy that most of the isolated butylphthalides also displayed protective activity at low concentrations and cytotoxicity at high concentrations. These results imply that angelica oil and its main chemical components have protective effect for injured neurons only in appropriate concentration range.

Keywords: *Angelica sinensis*; essential oil; butylphthalides; concentration-dependent effect; glutamate-induced injury; PC12 cells.

1. Plant Source

Angelica sinensis (Oliv.) Diels is a widely used traditional Chinese medicine with medical and edible dual purpose. Pharmacological researches have shown that the extract of the roots of *A. sinensis* exerted a neuroprotective activity against a variety of cell injuries, including amyloid *β*-peptide-induced neuronal death in Neuro 2A cells [1], glutamate excitotoxicity in primary rat cortical cells [2], and β-amyloid-induced neurotoxicity in cultured cortical neurons [3].

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.

2. Previous Studies

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The characteristic constituents of *A. sinensis* and its oil are various alkylphthalides. Since 1970s, 24 alkylphthalide monomers and 20 dimers have been isolated from *A. sinensis*, but their pharmacological activities have been little investigated except for *Z*-ligustilide and *Z*-butylidenephthalide [4]. The former accounting for 45−60% of angelica oil [5] was reported to have protective activity against neurotoxicity in mice brains and hydrogen peroxide-induced injury in PC12 cells in the concentration range of 0.1−5.0 μg/mL [6-9]. The latter could also attenuate neurotoxicity by reducing the release of various proinflammatory molecules from activated microglia [10].

3. Present Study

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 essential oil (180 g). Column chromatographic separations of this oil afforded nine butylphthalides (**1**–**9**, Figure 1) including two new ones (**1** and **2**) (Detailed extraction and isolation process see Supporting Information).

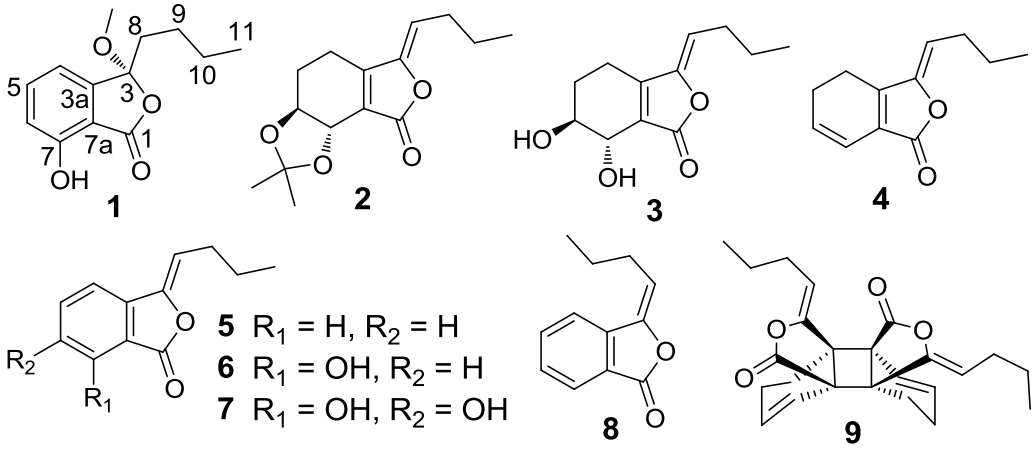


Figure 1. Chemical structures of butylphthalides **1**–**9**

Compound **1** was obtained as a white powder. The molecular formula, $C_{13}H_{16}O_4$, was established by an HRESIMS ion at *m/z* 259.0945 $[M+Na]^+$ (calcd. for $C_{13}H_{16}O_4Na$, 259.0946), corresponding to six degrees of unsaturation. The ^{1}H NMR spectrum of **1** in $CDCl_3$ (Table 1) showed resonances attributable to a 1,2,3-trisubstituted phenyl ring [δ_H 6.99 (1H, d, J = 7.8 Hz, H-4), 7.59 (1H, t, J = 7.8 Hz, H-5), and 6.94 (1H, d, J = 7.8 Hz, H-6)], a *n*-butyl unit [δ_H 2.15 (H-8a), 2.01 (H-8b), 1.41 (H-9a), 1.18 (H-9b), 1.30 (H_2-10), and 0.86 (d, J = 7.2 Hz, H_3-11)], and a methoxy group. Analysis of the COSY data led to the confirmation of the above two discrete proton spin-systems, H-4−H-6 and H_2-8−H_3-11. The ^{13}C NMR spectrum of **1** showed 13 carbon signals corresponding to the above units and five additional quaternary carbons including an ester carbonyl (δ_C 170.1), three aromatic quaternary carbons (δ_C 156.5, 146.9, and 112.8), and a double-oxygenated carbon (δ_C 113.1). The above spectroscopic data suggested that compound **1** is likely a NBP (3-*n*-butylphthalide) [11] with substitutions of a hydroxy and a methoxy group. The location of the methoxy group at C-3 was determined by HMBC correlations of *OMe*-3 with C-3 and of H_2-8 with C-3 and C-3a, which was consistent with the chemical shifts of C-3 (δ_C 113.1) and the methoxy (δ_H 3.10). In addition, HMBC correlations of H-4 with C-3 and C-6, of H-5 with C-3a and C-7, and of H-6 with C-4 and C-7a, indicated the hydroxy group being located at C-7. The absolute configuration of **1** was established by comparison of specific rotation between **1** and similar phthalide analogues. Since the specific rotation $\{[\alpha]^{20}_D$ +41.9 (MeOH)$\}$ of **1** was consistent with that of (*R*)-3-demethylpurpurester A [12], but opposite that of (*S*)-purpurester A [11], 3*R*-configuration was assigned for **1**. Therefore, compound **1** was determined to be (+)-(*R*)-3-butyl-7-hydroxy-3-methoxyphthalide.

The spectroscopic data of compound **2** suggested that it was another NBP analogue. Its molecular formula was deduced to be $C_{15}H_{20}O_4$ on the basis of HRESIMS data. The NMR spectra of **2** (Table 1) resembled those of the co-occurring senkyunolide I (**3**) [13] except that resonances for an additional isopropylidene unit [δ_H 1.24 (3H, s) and 1.34 (1H, s), δ_C 109.7, 28.1, and 26.3] were observed in the spectra of **2**. Meanwhile, acid hydrolysis of **2** in diluted hydrochloric acid liberated senkyunolide I. Therefore, compound **2** was determined to be senkyunolide I-6,7-acetonide. This isolate might be not a natural product in angelica oil, since it is likely to be produced by reaction of senkyunolide I with the solvent during the isolation process. Although no obvious product was generated as indicated by TLC when acetone solution of senkyunolide I was stirred at room temperature for 24 h, compound **2** was obtained in low yield after this solution was further refluxed for 24 h. Thus, compound **2** was deduced to be an artifact.

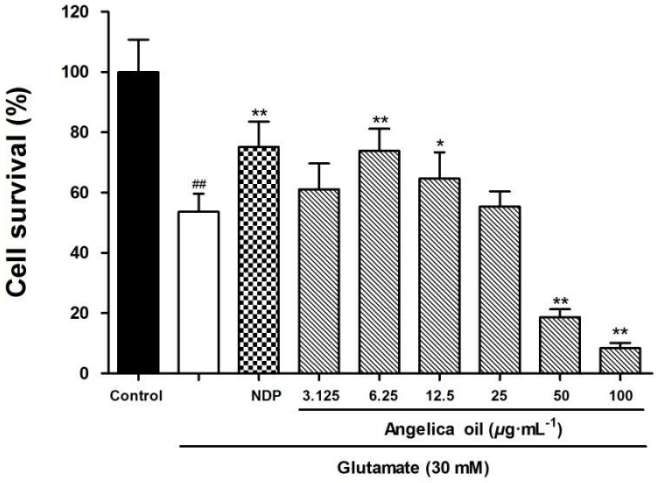
The known compounds were identified by comparison of spectroscopic data with those reported in the literature as senkyunolide I (**3**) [13], *Z*-ligustilide (**4**) [14], *Z*-butylidenephthalide (**5**) [15], 3-butylidene-7-hydroxyphthalide (**6**) [16], (*Z*)-3-butylidene-6,7-dihydroxyphthalide (**7**) [17], (*E*)-3-butylidenephthalide (**8**) [18], and *endo-Z,Z′*-(3a.7a′,7a.3a′)-diligustilide (**9**) [19].

Table 1. NMR Data (δ) for Compounds **1** and **2** [a]

No.	**1** [b]		**2** [c]	
	δ_H	δ_C	δ_H	δ_C
1	–	170.1	–	168.4
3	–	113.1	–	154.3
3a	–	146.9	–	149.2
4	6.99 d (7.8)	117.4	2.52 m	17.1
5	7.59 t (7.8)	137.4	2.19 m, 1.88 m	26.0
6	6.94 d (7.8)	114.2	4.56 ddd (6.0, 4.8, 2.4)	73.7
7	–	156.5	4.84 d (6.0)	68.6
7a	–	112.8	–	125.5
8	2.15 ddd (13.8, 12.0, 4.8) 2.01 ddd (13.8, 12.0, 4.8)	38.4	5.48 t (7.8)	113.5
9	1.41 m, 1.18 m	25.3	2.33 m	28.7
10	1.30 m	22.7	1.51 m	22.9
11	0.86 d (7.2)	14.0	0.95 t (7.2)	14.0
1′				109.7
MeO–3	3.10 s	51.6		
Me–1′			1.24, 1.34 (s)	26.3, 28.1

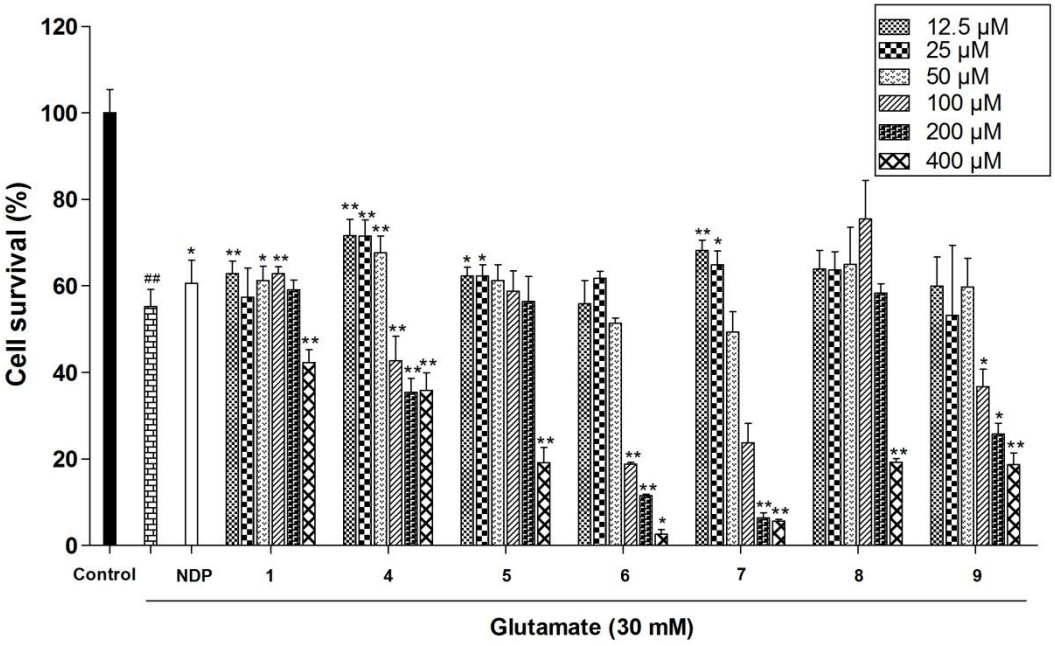
[a] Data were measured at 600 MHz for ^{1}H and 150 MHz for ^{13}C, respectively. [b] Data were measured in $CDCl_3$. [c] Data were measured in CD_3COCD_3.

The neuroprotective activity of angelica essential oil and the isolated butylphthalides was assayed according to the previous literatures [20,21]. The results showed that angelica oil significantly protected the injured PC12 cells at low concentrations of 6.25 μg/mL and 12.5 μg/mL, with their cell survival rates rising to 73.87 ± 7.29% ($p < 0.01$) and 64.66 ± 8.74% ($p < 0.05$) from 53.64 ± 5.60%, respectively (Figure 2). The protection of 6.25 μg/mL of angelica oil was equivalent to that of 10 μM of nimodipine (NDP). However, angelica oil at 50 μg/mL and 100 μg/mL impelled PC12 cells to death rather than promoted their repair. At the highest concentration of 100 μg/mL, the survival rate was only 8.37 ± 1.74%. Although angelica oil is widely used in medicated diets and health products, a previous study reported that angelica oil at high concentration had acute toxicity, and the oil of roots is more toxic than the oil of leaves [20]. The present study also suggests that angelica oil at high concentrations may have neurotoxicity for human neurons.



Data represent mean ± S.D. (n = 6). $^{\#\#}p < 0.01$ vs. control group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. glutamate group.

Figure 2. Effect of angelica oil on glutamate-induced neurotoxicity in PC12 cells



Data represent mean ± S.D. (n = 6). $^{\#\#}p < 0.01$ vs. control group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. glutamate group.

Figure 3. Effects of butylphthalides on glutamate-induced neurotoxicity in PC12 cells

The isolated butylphthalides from the angelica oil except for the artifact (**2**) were further assayed for their effects on glutamate-induced cytotoxicity in PC12 cells. It is worth mentioning that the effect tendency of the butylphthalides at different concentrations was consistent with that of angelica oil (Figure 3), except for compound **3** that did not show obvious effect at all six concentrations. The new compound **1** at concentrations of 12.5, 50, and 100 μM showed significant inhibition of glutamate-induced cytotoxicity in PC12 cells ($p < 0.01$ or $p < 0.05$). In contrast, the cell viability decreased significantly when the concentration rose to 400 μM ($p < 0.01$). *Z*-Ligustilide (**4**) and *Z*-butylidenephthalide (**5**), two characteristic phthalides in the essential oil of several Umbelliferae plants, have been reported to have neuroprotective effect [6-9, 22]. Consistent with these previous reports, the present results showed that **4**

and **5** significantly attenuated PC12 cell death caused by glutamate at low concentrations. The cell viabilities of **4** at 12.5, 25, and 50 μM rose to 71.62 ± 3.80%, 71.56 ± 3.74%, and 67.64 ± 3.93% from 55.20 ± 3.95% ($p < 0.01$), respectively. Instead of increasing the cell viabilities, 100, 200, and 400 μM of **4** decreased the cell viabilities markedly ($p < 0.01$). Similar to compound **1**, only the highest concentration of **5** (400 μM) could promote PC12 cell death. Compound **7** also showed protective activity for damaged PC12 cells at concentrations of 12.5 and 25 μM, but the cell viability began to drop drastically when the concentration exceeded 50 μM. The cell viabilities of 200 μM and 400 μM groups fell to just 6.35 ± 1.12% ($p < 0.01$) and 5.61 ± 0.40% ($p < 0.05$), respectively. In addition, pretreatment with the other butylphthalides (**6**, **8**, and **9**) at low concentrations resulted in a weak increase in cell viability, but with no significant difference ($p > 0.05$), while their cytotoxicities were conspicuous at high concentrations (100, 200, and 400 μM of **6** and **9**, and 400 μM of **8**).

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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