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

Lu-Si Liu\textsuperscript{1,2}, Cheng Peng*\textsuperscript{1,2}, Qin-Mei Zhou\textsuperscript{1,2}, Liang Xiong\textsuperscript{1,2}, Li Guo\textsuperscript{1,2}, Ya-Nan Wang\textsuperscript{3} and Ou Dai*\textsuperscript{1,2}

\textsuperscript{1}State Key Laboratory Breeding Base of Systematic Research, Development and Utilization of Chinese Medicine Resources, Chengdu University of Traditional Chinese Medicine, Chengdu 611137, China
\textsuperscript{2}School of Pharmacy, Chengdu University of Traditional Chinese Medicine, Chengdu 611137, China
\textsuperscript{3}State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China

(Received August 01, 2016; Revised October 20, 2016; Accepted October 26, 2016)

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 \textit{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. © 2016 ACG Publications. All rights reserved.

1. Plant Source

\textit{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 \textit{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 \textit{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

* Corresponding author: E-mail addresses: pengchengchengdu@126.com, Phone: +86-028-61800018 (C. Peng); E-Mail: oudai1123@hotmail.com, Phone: +86-028-61800045 (O. Dai).
Angelica oil and the isolated butylphthalides

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-butyldienephthalide [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](image)

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_{4}Na, 259.0946), corresponding to six degrees of unsaturation. The 1H NMR spectrum of 1 in CDCl3 (Table 1) showed resonances attributable to a 1,2,3-trisubstituted phenyl ring [δH 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 [δH 2.15 (H-8a), 2.01 (H-8b), 1.41 (H-9a), 1.18 (H-9b), 1.30 (H-10), and 0.86 (d, J = 7.2 Hz, H$_2$-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$_2$-11. The 13C NMR spectrum of 1 showed 13 carbon signals corresponding to the above units and five additional quaternary carbons including an ester carbonyl (δC 170.1), three aromatic quaternary carbons (δC 156.5, 146.9, and 112.8), and a double-oxygenated carbon (δC 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 (δC 113.1) and the methoxy (δH 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 ([α]$_D^{20}$ +41.9 (MeOH)) of 1 was consistent with that of (R)-3-demethylpurpurester A [12], but opposite that of (S)-purpurester A [11], 3R-configuration was assigned for 1. Therefore, compound 1 was determined to be (+)-(R)-3-butyl-7-hydroxy-3-methoxyphthalide.
Although angelica oil is widely used in medicated diets and health products, a previous study reported rising to 73.8% (p < 0.01) and 64.6% ± 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.

The spectroscopic data of compound 2 suggested that it was another NBP analogue. Its molecular formula was deduced to be C_{10}H_{12}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-butylidene-7-hydroxyphthalide (5) [15], 3-butylidene-7-hydroxyphthalide (6) [16], (Z)-3-butylidene-6,7-dihydroxyphthalide (7) [17], (E)-3-butylidene-7-phthalide (8) [18], and endo-Z,Z’-(3a.7a,7a.3a’)-diligustilide (9) [19].

### Table 1. NMR Data (δ) for Compounds 1 and 2

<table>
<thead>
<tr>
<th>No.</th>
<th>δ_H</th>
<th>δ_C</th>
<th>δ_H</th>
<th>δ_C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>170.1</td>
<td>–</td>
<td>168.4</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>113.1</td>
<td>–</td>
<td>154.3</td>
</tr>
<tr>
<td>3a</td>
<td>–</td>
<td>146.9</td>
<td>–</td>
<td>149.2</td>
</tr>
<tr>
<td>4</td>
<td>6.99 d (7.8)</td>
<td>117.4</td>
<td>2.52 m</td>
<td>17.1</td>
</tr>
<tr>
<td>5</td>
<td>7.59 t (7.8)</td>
<td>137.4</td>
<td>2.19 m, 1.88 m</td>
<td>26.0</td>
</tr>
<tr>
<td>6</td>
<td>6.94 d (7.8)</td>
<td>114.2</td>
<td>4.56 ddd (6.0, 4.8, 2.4)</td>
<td>73.7</td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>156.5</td>
<td>4.84 d (6.0)</td>
<td>68.6</td>
</tr>
<tr>
<td>7a</td>
<td>–</td>
<td>112.8</td>
<td>–</td>
<td>125.5</td>
</tr>
<tr>
<td>8</td>
<td>2.15 ddd (13.8, 12.0, 4.8)</td>
<td>38.4</td>
<td>5.48 t (7.8)</td>
<td>113.5</td>
</tr>
<tr>
<td></td>
<td>2.01 ddd (13.8, 12.0, 4.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.41 m, 1.18 m</td>
<td>25.3</td>
<td>2.33 m</td>
<td>28.7</td>
</tr>
<tr>
<td>10</td>
<td>1.30 m</td>
<td>22.7</td>
<td>1.51 m</td>
<td>22.9</td>
</tr>
<tr>
<td>11</td>
<td>0.86 d (7.2)</td>
<td>14.0</td>
<td>0.95 t (7.2)</td>
<td>14.0</td>
</tr>
<tr>
<td>1’</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>109.7</td>
</tr>
<tr>
<td>MeO–3</td>
<td>3.10 s</td>
<td>51.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Me–1’</td>
<td></td>
<td></td>
<td>1.24, 1.34 (s)</td>
<td></td>
</tr>
</tbody>
</table>

*Data were measured at 600 MHz for ^1^H and 150 MHz for ^13^C, respectively. ^* Data were measured in CDCl_3. ^ 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.
Angelica oil and the isolated butylphthalides

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

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).

Acknowledgments

The Project of Youth Technological Innovation Research Team of Sichuan Province (grant No. 2015TD0028) and the Fundamental Research Funds for the Central Universities (grant No. 2016ZX350012) are acknowledged.

Supporting Information

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

References

Angelica oil and the isolated butylphthalides


© 2016 ACG Publications.