Postprint of the Study on Diterpenoid Constituents from Pieris japonica and Their Acetylcholinesterase Inhibitory Activity

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Abstract

To investigate the diterpenoid constituents in Pieris formosa leaves and their acetylcholinesterase inhibitory activity, this study employed thin-layer chromatography coloration characteristics, silica gel, MCI, and semi-preparative high-performance liquid chromatography for separation and purification. The structures of the obtained compounds were identified through spectroscopic data (NMR and MS) analysis and comparison with literature-reported data, while their acetylcholinesterase inhibitory activity was evaluated for the first time using Ellman's method. The results demonstrated that eight diterpenoid compounds were isolated and identified from Pieris formosa leaves, namely pierisformoside F (1), 3-epi-grayanotoxin XVIII (2), 3-epi-grayanotoxin B (3), asebotoxin-X (4), pierisformosin B (5), grayanotoxane-V (6), rhodojaponin III (7), and pierisformosin C (8). Among them, compound 1 was obtained from this plant for the first time, and compound 8 exhibited acetylcholinesterase inhibitory activity. In summary, these findings indicate that Pieris formosa is rich in diterpenoid constituents and active compounds, providing a theoretical basis for its further development and utilization.

Full Text

Abstract

To investigate the diterpenoid constituents from *Pieris japonica* leaves and their acetylcholinesterase inhibitory activities, we employed thin-layer chromatography color characteristics and chromatographic techniques including silica gel, MCI, and semi-preparative high-performance liquid chromatography for isolation and purification. The structures of the obtained compounds were identified through spectroscopic data analysis (NMR and MS) and comparison with literature-reported data. Meanwhile, the human acetylcholinesterase (hAChE)

inhibitory effects of these diterpenoids were evaluated for the first time using the Ellman method. The results showed that eight diterpenoid compounds were isolated and identified from *Pieris japonica* leaves, namely pierisformoside F (**1**), 3-epi-grayanotoxin XVIII (**2**), 3-epi-grayanotoxin B (**3**), asebotoxin-X (**4**), pierisformosin B (**5**), gayanotoxane- (**6**), rhodojaponin III (**7**), and pierisformosin C (**8**). Among them, compound **1** was isolated from this plant for the first time, and compound **8** exhibited acetylcholinesterase inhibitory activity. These findings demonstrate that *P. japonica* is rich in diterpenoid constituents and bioactive components, providing a theoretical basis for its further development and utilization.

Keywords: Ericaceae, *Pieris japonica*, diterpenoids, pierisformoside F, acetylcholinesterase inhibitory activity

Introduction

Pieris japonica is an evergreen shrub belonging to the family Ericaceae and genus *Pieris*, primarily distributed in Taiwan, Anhui, Fujian, Hubei, Jiangxi, and Zhejiang provinces of China. In addition to its ornamental value, the stems and leaves of *P. japonica* have been used traditionally to treat heatstroke-induced vomiting and scabies, and also serve as insecticides (Yao et al., 2005). Previous studies have revealed that the *Pieris* genus contains numerous structurally diverse diterpenoids, including grayanane, kaurane, and leucothane types (Li et al., 2013a; Li et al., 2017a; Li et al., 2017b; Zheng et al., 2020). Importantly, these compounds exhibit diverse biological activities, such as anti-inflammatory (Zhou et al., 2018), antiviral (Li et al., 2013b; Li et al., 2016), neuroprotective effects, cAMP modulation (Wang et al., 2013), potassium channel modulation (Niu et al., 2018), antifeedant activity (Li et al., 2017b), PTP1B inhibition (Liu et al., 2014; Zhou et al., 2017), and analgesic properties (Sun et al., 2018; Sun et al., 2019a). To further investigate the chemical basis and obtain diterpenoid constituents from *P. japonica*, we conducted a systematic study on the chemical constituents of Yunnan-derived *P. japonica* leaves and their inhibitory activity against human acetylcholinesterase. This work aims to establish a material foundation for the subsequent development of *P. japonica*, enrich the chemical diversity of this plant, and provide scientific guidance for discovering more bioactive constituents and comprehensive utilization.

1. Materials and Methods

1.1 Plant Material

The experimental material was collected from the Kunming Botanical Garden, Kunming Institute of Botany, Chinese Academy of Sciences on May 12, 2021, and identified as *Pieris japonica* by Researcher Ma Yongpeng from the same institution. A voucher specimen (KIB-Q-202101B) has been deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

1.2 Instruments and Reagents

The following instruments were used: Agilent 1290 UPLC/6540 Q-TOF LC-MS system (USA); AVANCE III 500 MHz and AV 600 MHz NMR spectrometers (Bruker, Germany); Hanbon-NP7000C HPLC system (Jiangsu Hanbon Science & Technology Co., Ltd.); Agilent ZORBAX SB-C18 column (9.4 mm × 250 mm, 5.0 m, USA); silica gel powder (200–300 mesh) and TLC plates (GF254) from Qingdao Puke Separation Material Co., Ltd.; MCI gel (CHP20/P120, Mitsubishi Chemical Corporation, Japan); Sephadex LH-20 (Cytiva, Sweden); C18 MB100-40/75 (Chromatorex, Fuji Silysia, Japan); Büchi MPLC C-605 dual-gradient pump system (Switzerland). HPLC-grade acetonitrile was purchased from Shanghai Xingke High Purity Solvents Co., Ltd., while methanol, chloroform, and ethyl acetate were obtained from Yunnan Renke Trading Co., Ltd. Other reagents included 0.5% vanillin-sulfuric acid stain (self-prepared), Na_2HPO_4 (Sigma), NaH_2PO_4 (Sigma), acetylcholinesterase (Sigma), acetylthiocholine iodide (Sigma), DTNB (Sigma), tacrine (Sigma), and Multiskan FC microplate reader (Thermo Scientific).

1.3 Extraction and Isolation

Diterpenoid constituents in *P. japonica* were targeted using 0.5% vanillin-sulfuric acid stain, which initially produces a purplish-red color that gradually turns blue and becomes green after standing for 12 h. Dried *P. japonica* leaves (2.5 kg) were pulverized and extracted three times with ethyl acetate at room temperature (24 h each). The extracts were combined and concentrated under reduced pressure to yield a crude extract (480 g), which was subjected to MCI column chromatography using a methanol-water gradient (40:60 → 100:0) to afford four fractions (Fr. A–D) based on TLC analysis.

Fraction A (310 g) was further separated by silica gel column chromatography with a chloroform-methanol gradient (30:1 → 0:1) to give a diterpenoid-enriched fraction (Fr. A1) and a non-diterpenoid fraction (Fr. A2) according to TLC color characteristics. Fr. A1 (5.8 g) was applied to a Sephadex LH-20 column to yield two subfractions, Fr. A1-1 (4.0 g) and Fr. A1-2. Fr. A1-1 was chromatographed on silica gel eluted with chloroform-methanol (15:1 → 0:1) to afford five subfractions (Fr. A1-1-1 to Fr. A1-1-5). Fr. A1-1-1 (1.0 g) was purified by silica gel column chromatography using chloroform-methanol (15:1 → 0:1) to yield compounds **1** (30 mg), **2** (26 mg), and **3** (100 mg).

Fr. A1-2 (300 mg) was subjected to RP-18 column chromatography with a methanol-water gradient (35:65 → 70:30) to obtain compounds **4** (36 mg) and **5** (9 mg). Fr. A1-3 (60 mg) was purified by semi-preparative HPLC (acetonitrile-water, 30:70, 5.0 mL · min^{-1}) to give compounds **6** (7.0 mg, t_R = 9 min) and **7** (1.2 mg, t_R = 16 min). Fr. A1-4 (80 mg) was purified by semi-preparative HPLC (acetonitrile-water, 20:80, 4.0 mL · min^{-1}) to afford compound **8** (4.0 mg, t_R = 9.7 min).

1.4 Acetylcholinesterase Inhibitory Activity Assay

The acetylcholinesterase inhibitory activity was determined according to the method described in the literature (Liu et al., 2020). DMSO and tacrine were used as negative and positive controls, respectively. Compounds **1**–**8** were tested at a final concentration of 50.0 $mol \cdot L^{-1}$, while tacrine was tested at 0.333 $mol \cdot L^{-1}$. All experiments were performed in triplicate.

2. Results

2.1 Structure Elucidation

Eight compounds were isolated from the ethyl acetate extract of *P. japonica* leaves through chromatographic techniques including silica gel, Sephadex LH-20, and reversed-phase semi-preparative HPLC. Their structures were identified as diterpenoids based on ^{1}H-NMR, ^{13}C-NMR, and ESI-MS spectroscopic data in comparison with literature values. The isolated diterpenoids included one leucothane type (**1**) and seven grayanane types (**2**–**8**), with the leucothane diterpenoid being reported from this plant for the first time. The structures are shown in Figure 1 [Figure 1: see original paper].

Compound 1 was obtained as a white amorphous powder. $[\alpha]^{20}D$ *+43.80 (c 0.1, MeOH); UV (MeOH)* λ\{max\} (log) 202 (3.8) nm; ESI-MS m/z 503 $[M + Na]^{+}$; molecular formula $C_{26}H_{40}O_{8}$. ^{1}H-NMR (500 MHz, methanol-d_4) δ_H: 5.02 (1H, s), 4.90 (1H, s), 4.42 (1H, d, J = 7.8 Hz), 1.42 (3H, s), 1.18 (3H, s), 1.08 (3H, s). ^{13}C-NMR (125 MHz, methanol-d_4) δ_C: 217.5 (C-5), 153.0 (C-10), 105.8 (CH_2-20), 99.2 (Glc-CH-1), 89.1 (C-16), 78.6 (Glc-CH-3), 78.3 (Glc-CH-5), 77.6 (CH-3), 75.2 (Glc-CH-2), 71.7 (Glc-CH-4), 62.8 (Glc-CH_2-6), 53.4 (CH_2-15), 50.8 (C-4), 50.4 (CH-9), 49.3 (CH-6), 48.1 (CH-13), 46.8 (C-8), 44.0 (CH-1), 39.2 (CH_2-7), 36.2 (CH_2-14), 32.5 (CH_2-2), 25.3 (CH_2-12), 22.5 (CH_2-11), 21.7 (CH_3-18), 20.9 (CH_3-17), 20.9 (CH_3-19). These data were consistent with those reported in the literature (Wang et al., 2000), leading to the identification of compound **1** as pierisformoside F.

Compound 2 was isolated as a white amorphous powder. $[\alpha]^{20}$_D +21.00 (c 0.1, MeOH); ESI-MS m/z 359 $[M + Na]^{+}$; molecular formula $C_{20}H_{32}O_{4}$. ^{1}H-NMR (500 MHz, methanol-d_4) δ_H: 5.04 (1H, s), 4.93 (1H, s), 1.35 (3H, s), 1.17 (3H, s), 1.03 (3H, s). ^{13}C-NMR (125 MHz, methanol-d_4) δ_C: 152.8 (C-10), 113.5 (CH_2-20), 83.5 (C-5), 82.5 (CH-3), 81.3 (C-16), 72.2 (CH-6), 63.2 (CH_2-15), 55.3 (CH-9), 51.2 (C-4), 48.0 (CH-13), 46.2 (CH_2-7), 45.0 (C-8), 44.2 (CH-1), 39.0 (CH_2-14), 35.9 (CH_2-2), 26.7 (CH_2-11), 25.3 (CH_3-19), 24.7 (CH_3-17), 24.3 (CH_2-12), 19.3 (CH_3-18). These data matched those reported in the literature (Sun et al., 2019a), confirming compound **2** as 3-epi-grayanotoxin XVIII.

Compound 3 was obtained as a light yellow gummy solid. $[\alpha]^{20}D$ *+19.20 (c 0.1, MeOH); UV (MeOH)* λ\{max\} (log) 202 (3.7) nm; ESI-MS m/z 521 $[M + Na]^{+}$; molecular formula $C_{26}H_{42}O_{9}$. ^{1}H-NMR (500 MHz, methanol-d_4)

δ_H: 5.01 (1H, s), 4.97 (1H, s), 4.31 (1H, d, J = 7.8 Hz), 1.35 (3H, s), 1.22 (3H, s), 1.10 (3H, s). ^{13}C-NMR (125 MHz, methanol-d_4) δ_C: 152.1 (C-10), 113.7 (CH_2-20), 105.3 (Glc-CH-1), 90.0 (CH-3), 83.1 (C-5), 81.2 (C-16), 77.8 (Glc-CH-3), 77.7 (Glc-CH-5), 75.4 (Glc-CH-2), 72.5 (Glc-CH-4), 71.6 (CH-6), 63.1 (CH_2-15), 62.7 (Glc-CH_2-6), 55.4 (CH-9), 51.6 (C-4), 47.9 (CH-13), 46.3 (CH_2-7), 45.0 (C-8), 43.9 (CH-1), 37.6 (CH_2-14), 35.9 (CH_2-2), 26.9 (CH_2-12), 26.3 (CH_3-17), 25.3 (CH_3-19), 24.3 (CH_2-11), 19.9 (CH_3-18). These data were consistent with literature values (Sun et al., 2018), identifying compound **3** as 3-epi-grayanotoxin B.

Compound 4 was isolated as a white amorphous powder. [α]^{20}*D +12.20 (c 0.1, MeOH); UV (MeOH)* λ{max} (log) 202 (3.2) nm; ESI-MS m/z 465 [M + Na]$^+$; molecular formula $C_{23}H_{38}O_8$. ^{1}H-NMR (600 MHz, methanol-d_4) δ_H: 5.53 (1H, s), 1.42 (3H, d, J = 6.9 Hz), 1.37 (3H, s), 1.33 (3H, s), 1.18 (3H, s), 0.96 (3H, s). ^{13}C-NMR (150 MHz, methanol-d_4) δ_C: 175.5 ($COCHOHCH_3$), 85.0 (C-5), 83.6 (CH-3), 83.3 (CH-14), 79.8 (C-16), 79.0 (C-10), 74.1 (CH-6), 68.4 ($COCHOHCH_3$), 60.6 (CH_2-15), 56.5 (CH-13), 55.5 (CH-9), 52.2 (C-4), 51.5 (C-8), 51.2 (CH-1), 43.5 (CH_2-7), 35.5 (CH_2-2), 27.7 (CH_3-20), 27.6 (CH_2-12), 23.7 (CH_3-17), 23.3 (CH_3-19), 22.6 (CH_2-11), 20.7 ($COCHOHCH_3$), 19.3 (CH_3-18). These data corresponded with those reported in the literature (Sakakibara et al., 1980), establishing compound **4** as asebotoxin-X.

Compound 5 was obtained as a white amorphous powder. [α]^{20}_D +7.20 (c 0.1, MeOH); ESI-MS m/z 449 [M + Na]$^+$; molecular formula $C_{23}H_{38}O_7$. ^{1}H-NMR (600 MHz, methanol-d_4) δ_H: 5.49 (1H, s), 1.37 (3H, s), 1.33 (3H, s), 1.18 (3H, s), 0.96 (3H, s). ^{13}C-NMR (150 MHz, methanol-d_4) δ_C: 175.6 ($COCH_2CH_3$), 85.0 (C-5), 83.6 (CH-3), 82.8 (CH-14), 79.8 (C-16), 79.0 (C-10), 74.2 (CH-6), 60.7 (CH_2-15), 56.6 (CH-9), 55.4 (CH-13), 52.2 (C-4), 51.5 (C-8), 51.1 (CH-1), 43.7 (CH_2-7), 35.5 (CH_2-2), 28.8 ($COCH_2CH_3$), 27.7 (CH_3-20), 27.7 (CH_2-12), 23.8 (CH_3-17), 23.3 (CH_3-19), 22.6 (CH_2-11), 19.3 (CH_3-18), 9.6 ($COCH_2CH_3$). These data were consistent with literature values (Wang et al., 1998), identifying compound **5** as pierisformosin B.

Compound 6 was isolated as a white amorphous powder. [α]^{20}*D +36.00 (c 0.1, MeOH); UV (MeOH)* λ{max} (log) 204 (3.3) nm; ESI-MS m/z 463 [M + Na]$^+$; molecular formula $C_{23}H_{36}O_8$. The ESI-MS showed a molecular ion peak at m/z 463 [M + Na]$^+$. ^{1}H-NMR (600 MHz, methanol-d_4) δ_H: 5.50 (1H, s), 4.31 (1H, q, J = 6.9 Hz), 1.44 (3H, s), 1.41 (3H, d, J = 6.9 Hz), 1.33 (3H, s), 1.22 (3H, s), 1.07 (3H, s). ^{13}C-NMR (150 MHz, methanol-d_4) δ_C: 175.5 ($COCHOHCH_3$), 82.8 (CH-14), 80.2 (C-5), 79.9 (C-16), 78.1 (C-10), 73.3 (CH-6), 68.4 ($COCHOHCH_3$), 65.0 (CH-3), 60.6 (CH-2), 60.3 (CH_2-15), 56.5 (CH-9), 55.7 (CH-13), 54.4 (CH-1), 51.3 (C-4), 48.4 (C-8), 43.6 (CH_2-7), 30.3 (CH_3-20), 27.6 (CH_2-12), 23.7 (CH_3-17), 22.4 (CH_2-11), 21.2 (CH_3-19), 20.7 ($COCHOHCH_3$), 20.1 (CH_3-18). These data matched those reported in the literature (Sakakibara et al., 1980), confirming compound **6** as asebotoxin III.

Compound 7 was obtained as a white amorphous powder. [α]^{20}_D +6.20 (c 0.1, MeOH); ESI-MS m/z 391 [M + Na]$^+$; molecular formula $C_{20}H_{32}O_6$. ^{1}H-

NMR (600 MHz, methanol-d_4) δ_H: 1.42 (3H, s), 1.30 (3H, s), 1.25 (3H, s), 1.15 (3H, s). ^{13}C-NMR (150 MHz, methanol-d_4) δ_C: 81.3 (C-16), 80.4 (C-5), 79.8 (CH-14), 78.4 (C-10), 73.8 (CH-6), 65.1 (CH-3), 60.6 (CH-2), 59.7 (CH_2-15), 56.7 (CH-9), 56.0 (CH-1), 54.7 (CH-13), 52.4 (C-8), 48.6 (C-4), 44.0 (CH_2-7), 30.4 (CH_3-20), 27.2 (CH_2-12), 23.3 (CH_3-17), 22.4 (CH_2-11), 21.3 (CH_3-19), 20.2 (CH_3-18). These data were consistent with literature values (Klocke et al., 1991), establishing compound **7** as rhodojaponin III.

Compound 8 was isolated as a white amorphous powder. $[\alpha]^{20}$_D +1.80 (c 0.1, MeOH); ESI-MS m/z 465 $[M + Na]^+$; molecular formula $C_{23}H_{38}O_8$. ^{1}H-NMR (600 MHz, $CDCl_3$) δ_H: 5.61 (1H, s), 1.35 (3H, s), 1.32 (3H, s), 1.20 (3H, s), 0.99 (3H, s). ^{13}C-NMR (150 MHz, $CDCl_3$) δ_C: 173.0 ($COCH_2CH_3$), 83.2 (CH-14), 83.1 (CH-3), 82.7 (C-5), 78.8 (CH-6), 78.4 (C-16), 77.7 (C-10), 77.7 (CH_2-7), 54.7 (C-8), 54.6 (CH_2-13), 53.9 (CH-9), 51.9 (CH_2-15), 51.9 (C-4), 49.3 (CH-1), 34.7 (CH-2), 28.5 ($COCH_2CH_3$), 28.2 (CH_3-20), 26.7 (CH_2-12), 23.1 (CH_3-17), 23.0 (CH_3-18), 21.7 (CH_2-11), 18.8 (CH_3-19), 9.2 ($COCH_2CH_3$). These data were essentially consistent with literature values (Wang et al., 1998), identifying compound **8** as pierisformosin C.

2.2 Acetylcholinesterase Inhibitory Activity Screening

As shown in Table 1 , compound **8** exhibited moderate acetylcholinesterase inhibitory activity at a concentration of 50.0 $mol \cdot L^{-1}$, with an inhibition rate of 23.88% ± 2.47% (inhibition rate < 20.0% is considered inactive).

Table 1 Inhibitory effect of compounds on acetylcholinesterase (AChE)

Compound	Inhibition rate (%)	SD (%)
Tacrine		

3. Discussion and Conclusion

Through systematic separation and purification of the ethyl acetate extract from *P. japonica* leaves, we identified eight highly oxidized diterpenoid constituents, primarily grayanane and leucothane types. Compound **1** represents a new addition to the chemical profile of this species, while compound **8** demonstrated notable acetylcholinesterase inhibitory activity.

Modern pharmacological studies have revealed that compounds isolated from *Pieris* species possess diverse biological functions, including antifeedant, insecticidal, analgesic, and anti-inflammatory activities. For instance, Sun et al. (2018; 2019a; 2019b) evaluated the analgesic effects of pierisformoside F (**1**), 3-epi-grayanotoxin XVIII (**2**), and 3-epi-grayanotoxin B (**3**) through in vitro writhing tests, demonstrating significant analgesic activity with writhing inhibition rates exceeding 50% at a dose of 5.0 $mg \cdot kg^{-1}$. In 1980, Yasushi et al. observed that asebotoxin III (**6**) induced a rapid initial contraction followed by a strong

secondary slow contraction when injected into the guinea pig hypogastric nerve-vas deferens preparation (Yasushi, 1980). Klocke et al. (1991) evaluated the antifeedant activity of rhodojaponin III (**7**) using a dual-choice bioassay, revealing its potential as an insecticidal agent, which aligns with our isolation of this compound. However, literature reports on the acetylcholinesterase inhibitory activity of such diterpenoids are scarce. Therefore, we conducted the first evaluation of these isolated compounds for AChE inhibition. Our results indicate that *P. japonica* contains diterpenoid constituents with acetylcholinesterase inhibitory potential, providing a foundation for the comprehensive utilization of this plant resource and guiding future investigations using modern phytochemical approaches to discover more potent AChE inhibitors from this class of compounds.

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Note: Figure translations are in progress. See original paper for figures.