A new flavonol C-glycoside and a rare bioactive lignanamide from Piper wallichii Miq. Hand.-Mazz

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[ABSTRACT] This study was conducted to investigate the chemical constituents of Piper wallichii (Miq.) Hand.-Mazz. and evaluate their biological activity. Compounds were isolated by various column chromatographic methods, and their structures were elucidated on the basis of physical characteristics and spectral data. The 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-scavenging activity and acetylcholinesterase (AChE)-inhibitory activity of the compounds were evaluated. Five compounds were obtained and identified as 8-C-β-D-glucopyranosylkaempferol-3-O-β-D-glucopyranoside (1), 1, 2-dihydro-6,8-dimethoxy-7-hydroxy-1-(3,5-dimethoxy-4-hydroxyphenyl)-N₁,N₂-bis-[2-(4-hydroxyphenyl)ethyl]-2, 3-naphthalene dicarboxamide (2), goniothalactam (3), aristololactam A IIIa (4) and piperlonguminine (5). Compound 1 was a new flavonol C-glycoside, 2 was a rare lignanamide, which was isolated from the family Piperaceae for the first time, and compound 3 was isolated from this plant for the first time. Among them, 2 showed potent DPPH-scavenging activity, with IC₅₀ of 31.38 ± 0.97 μmol·L⁻¹; Compounds 2, 3, and 4 showed AChE inhibitory activity at 100 μmol·L⁻¹, with inhibition rates of 28.57% ± 1.47%, 18.48% ± 2.41% and 17.4% ± 3.03%, respectively.

[KEY WORDS] Piper wallichii; Flavonol C-glycoside; Lignanamide; DPPH scavenging activity; AChE inhibitory activity

Introduction

Piper wallichii (Miq.) Hand.-Mazz (Piperaceae) is mainly distributed in the south of China, including Hubei, Hunan, and Guangxi Provinces. The stem of P. wallichii is used for the treatment of rheumatic pain and lumbocural pain [1]. Some neolignans, lignans, and alkaloids have been isolated from this plant [2, 3]. Previous investigation in this laboratory found that the aqueous extract of P. wallichii possessed in vivo anti-AD activity (data not shown). Phytochemical investigation led to the isolation of ten compounds, including four benzoic acid derivatives, three neolignans, and three amides. Only 4-hydroxy-3,5-dimethoxy-benzoic acid showed potent DPPH scavenging activity, and none of them showed anti-AChE activity [4]. In a continuing screening for the bioactive constituents in P. wallichii, five compounds were isolated. In this article, the structural elucidation, the DPPH scavenging activities and AChE inhibitory activities of these five compounds are described.

Results and Discussion

Compound 1 was obtained as yellow amorphous powder. It showed a blue color when reacted with 5% FeCl₃. The molecular formula of 1 was determined to be C₂₇H₂₉O₁₆ on the basis of HR-ESI-MS data (m/z 611.1617[M + H]⁺, calcd. 611.1611 2 for C₂₇H₂₉O₁₆; m/z 633.1435[M + Na]⁺, calcd. 633.1432 2 for C₂₇H₃₀NaO₁₆). UV λmax (H₂O) nm (log ε): 347 (3.62) and 266 (3.77), indicating 1 was a flavonoid. The IR spectrum of 1 at 3 384, 1 655, 1 610, 1 561, 1 509, and 1 440 cm⁻¹ indicated the presence of the hydroxyl group, carbonyl group and benzene ring. In the ¹H-NMR spectrum (Table 1), the signals at δ 6.85 (2H, d, J = 7.0 Hz) and 8.24 (2H, d, J = 7.0 Hz) indicated the presence of an AA'BB' aromatic ring. In the ¹H-NMR spectrum (Table 1), the signals at δ 6.85 (2H, d, J = 7.0 Hz) and 8.24 (2H, d, J = 7.0 Hz) indicated the presence of an AA'BB' aromatic ring. An isolated aromatic proton at δ 6.19 (1H, s) suggested a...
penta-substituted aromatic ring. The signal at δ 12.74 (1H, s) indicated the presence of a hydroxyl group which was located at C-5 of the flavonoid. In the 13C NMR spectrum (Table 1), the signal at δ 177.6 indicated a carbonyl carbon, the signals at δ 160.4, 160.3, 155.6, and 156.1 were oxygen-connected aromatic carbons. In the HMBC spectrum, the aromatic proton at δ 6.85 correlated with δ 115.5, 121.4, 131.7, and 160.3, respectively. The anomeric proton at δ 5.46 (1H, d, J = 6.0 Hz) of one sugar moiety correlated with the carbon at δ 133.1, indicating this sugar moiety was connected with C-3 of a flavonol. It was confirmed to be O-connected β-D-glucopyranose based on the coupling constant of the anomeric proton and the carbon signals of the sugar which were same as those in the literature [7]. In addition, another anomeric proton at δ 4.72 had HMBC correlations with the carbons at δ 70.7, 81.8, 104.7, and 155.6, indicating that this sugar was located at C-8 of the flavonol. It was confirmed to be a C-connected β-D-glucopyranose according to the coupling constant of the anomeric carbon and the carbon signals of the sugar which were similar with those of C-connected glucopyranose in the literature [5]. According to the above analysis, I was identified as 8-C-β-D-glucopyranosyl-5,7,4′-trihydroxyflavonol-3-O-β-D-gluco-pyranoside, i.e., 8-C-β-D-glucopyranosylkaempferol-3-O-β-D-glucopyranoside (Fig. 1). A structural isomer of I, 6-C-glycosylkaempferol-3-O-glucoside, was first isolated from the leaves of Japomorilirion osense (Petrosaviaceae) in 2005 [6].

By comparison of the NMR data with the literature, four known compounds (Fig. 1) were identified as 1, 2-dihydro-6, 8-dimethoxy-7-hydroxy-1-(3, 5-dimethoxy-4-hydroxyphenyl)-N3,N4-bis-[2-(4-hydroxyphenyl) ethyl]-2, 3-naphthalene dicarboxamide (2) [7], goniothalactam (3) [8], aristololactam A IIIa (4) [3, 8], and piperlongumine (5) [9]. Compounds 2 and 3 were identified for the first time in P. wallichii. Although 2 is a known alkaloid, this type of lignanamide with a phenylidihyronaphthalene skeleton is rare in nature. To date, they have only been isolated from Cannabis sativa (Cannabidaceae) [10], Procelia macrocarpa (Proceliaceae) [11], and Commelina communis (Commelinaceae) [7]. This was the fourth report that it occurred in nature, and it was present in a relatively high amount in P. wallichii.

Compounds 1-5 were tested for their DPPH scavenging activity at five concentrations between 1.56–25 μmol·L−1. Interestingly, it was found for the first time that the rare lignanamide 2 showed potent DPPH-scavenging activity in a dose-dependent manner (Fig. 2), with an IC50 value of 31.38 ± 0.97 μmol·L−1, similar to that of vitamin E [IC50 22.41 ± 0.31 μmol·L−1]. The other four compounds had no DPPH scavenging activity. In the assay for AChE inhibitory activity at the concentrations of 1, 10, and 100 μmol·L−1, only 2, 3, and 4 displayed weak anti-AChE activity at 100 μmol·L−1, with inhibition rates of 28.57% ± 1.47%, 18.48% ± 2.41%, and 17.4% ± 3.03%, respectively, compared with the positive control galanthamine hydrobromide which showed 24.17% ± 4.20%, 79.81% ± 5.63%, and 106.99% ± 10.07% inhibition at 1, 10, and 100 μmol·L−1, respectively. Compounds 1 and 5 had no anti-AChE activity.

**Experimental**

**Apparatus and reagents**

NMR spectra: Bruker AV-600 spectrometer, with TMS as an internal standard. HR-MS spectra: ThermoFinnigan LTQ-Orbitrap XL mass spectrometer (ThermoFinnigan, D-Bremen, Germany). UV spectra: TU-1800 UV spectrophotometer (Beijing Purkinje General Instrument Co., China). IR spectrum: NEXUS 470 Fourier-transform infrared spectrometer ( Nicolet Co., USA). Column chromatography (CC): silica gel (200–300 mesh; Qingdao Marine Chemical Co., Qingdao, China); Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, USA); polyamide (100–200 mesh; Zhejiang Siging Chemical Co., Taizhou, China); AB-8 resin (Changzhou Bao’en Chemical Co., Hebei, China); ODS-C18 (75 μm, YMC Co., Japan). TLC: Merck silica gel 60 F 254, RP-18 F254, or polyamide membrane; visualization under UV 365 nm and by spraying with 5% H2SO4 or 5% FeCl3 reagent, or reacting with iodine vapor. AChE from electric eel (type VI-S, lyophilized powder, 265 U/mg solid), acetylthiocholine iodide (ATCI), 5, 5′-dithio-bis(2-nitrobenzoic) acid (DTNB), 1, 1-diphenyl-2-picyryldiazyl (DPPH), and vitamin E (VE) were purchased from Sigma-Aldrich. Galanthamine hydrobromide was purchased from Aladdin Chemistry, China. All other reagents were of analytical grade. Microplate reader (model 680 UV, Bio-Rad) was used in the microplate assay.

**Plant material**

Dried stems of P. wallichii were purchased in Jinan, Shandong Province, China in February 2007 and identified by Prof. Lan Xiang, Institute of Pharmacognosy, School of Pharmaceutical Sciences, Shandong University. A voucher specimen (No. 2007-2-15) was deposited in the Institute of Pharmacognosy.

**Extraction and isolation**

Dried stems of P. wallichii (18 kg) were refluxed (3 × 6 L) with water (1 h each time). The concentrated syrup was extracted subsequently with petroleum ether, EtOAc, and n-BuOH. The n-BuOH extract (156.83 g) was passed through an AB-8 macroporous resin column and eluted with water and EtOH (30%–95%). The 30% EtOH eluate (47.5 g) was subjected to polyamide chromatography (60–100 mesh, 400 g) column chromatography and eluted with water and gradient EtOH (20%–95%). The resulting Fr. 30–36 was separated by repeated Rp-C18 and sephadex LH-20 column chromatography to afford 1 (10 mg). The EtOAc extract (37.95 g) was separated by silica gel (100–200 mesh, 1.52 kg) chromatography, eluted with CHCl3–(CH3)2CO (1 : 0–1 : 1) to afford ten fractions (Fr.1–10). (1) Fr. 5 (4.6 g) was separated by silica gel (solvent: CHCl3–MeOH, 9 : 1–1 : 1) to afford 37 fractions. The resulting Frs. 1–28 (1.53 g) was subjected to silica gel
chromatography (200–300 mesh, 81.28 g) and eluted with petroleum ether-(CH$_3$)$_2$CO (9 : 1–1 : 1) to afford 17 fractions. The resulting Frs. 5–17 were separated by Rp-C$_{18}$ (solvent: MeOH–H$_2$O, 3 : 7) to yield 42 fractions. The resulting Frs. 5–23 was purified by Sephadex LH-20 (solvent: MeOH) column chromatography to yield 3 (8 mg). (2) Fr. 7 (2 g) was subjected to silica gel chromatography (200-300 mesh) (solvent: petroleum ether-(CH$_3$)$_2$CO, 9 : 1–1 : 1) to obtain 64 fractions. The resulting Fr. 21-30 yielded 4 (6 mg); The resulting Fr. 44-64 (1.63 g) was separated by Rp-C$_{18}$ (MeOH–H$_2$O, 1 : 1) to afford 79 fractions. The resulting Frs. 5–10 yielded 2 (70 mg).

**DPPH scavenging activity**

The assay for DPPH scavenging activity was performed according to the reported method [12]. Briefly, the mixture of 0.1 mL of the sample (1, 0.5, 0.25, 0.125, and 0.062 5 mmol·L$^{-1}$, respectively) and 3.9 mL of DPPH solution (25 μg·mL$^{-1}$) was shaken vigorously, and kept at r.t. in the dark for 30 min prior to the absorbance determination at 515 nm. Vitamin E was used as the positive control.

\[
\text{DPPH scavenging activity (\%) = } \left[ 1 - \frac{\text{DPPH}^*_{T=30}}{\text{DPPH}^*_{T=0}} \right] \times 100
\]

Where \( \text{DPPH}^*_{T=0} \) is the initial concentration of DPPH$^*$, and \( \text{DPPH}^*_{T=30} \) is the concentration of DPPH$^*$ at 30 min. The experiment was undertaken in triplicate, and the results were expressed as \( \bar{X} \pm s \). The IC$_{50}$ value was obtained by interpolation from the linear regression analysis, which represented the concentration of sample that decreased the initial DPPH$^*$ concentration by 50%.

**AChE inhibitory activity**

A microplate assay was employed for the measurement of AChE inhibitory activity based on Ellman’s method [13]. Briefly, 25 μL of 15 mmol·L$^{-1}$ ATCI in deionized water, 125 μL of 3 mmol·L$^{-1}$ DTNB in buffer C (50 mmol·L$^{-1}$ Tris-HCl, pH 8.0; containing 0.1 mol·L$^{-1}$ NaCl and 0.02 mol·L$^{-1}$ MgCl$_2$·6H$_2$O), 50 μL of buffer B (50 mmol·L$^{-1}$ Tris-HCl, pH 8; containing 0.1% bovine serum albumin), and 25 μL of sample [compound 1 and positive control galanthamine hydrobromide dissolved in water, and compounds 2–5 dissolved in MeOH were diluted ten times with buffer A (50 mmol·L$^{-1}$ Tris-HCl, pH 8) to afford concentrations of 0.01, 0.1, and 1 mmol·L$^{-1}$, respectively] which were added to the 96-well plate, the absorbance was measured at 405 nm every 25 s, five times. Thereafter, 25 μL of 0.22 U·mL$^{-1}$ of AChE (diluted with buffer B) was added, the absorbance was read again every 25 s, eight times. The percentage inhibition was calculated by comparing the rates of reaction for the samples relative to the blank (in which 25 μL of MeOH or distilled water in buffer A replaced 25 μL of samples) using the following equation:

\[
\text{Inhibition (\%) = } \left[ 1 - \frac{(K_{\text{blank}+\text{AChE}} - K_{\text{sample}})}{(K_{\text{blank}+\text{AChE}} - K_{\text{blank}})} \right] \times 100
\]

Where \( K_{\text{blank}+\text{AChE}} \) and \( K_{\text{sample}+\text{AChE}} \) are the reaction rates (or slopes of absorbance) after adding AChE to the wells of blank and samples, while \( K_{\text{blank}} \) and \( K_{\text{sample}} \) are the reaction rates (or slopes of absorbance) before adding AChE. The experiment was done in triplicate, and the results were expressed as \( \bar{X} \pm s \).

**Compound 1** Yellow amorphous powder. It showed a dark brown color under UV 365 nm and a blue color when sprayed with 5% FeCl$_3$ reagent [\( R_f = 0.5 \), developed with MeOH–H$_2$O (35 : 65) on polyamide membrane]. UV \( \lambda_{max} \) (H$_2$O) nm
Fig. 2 DPPH scavenging activity of compound 2 and positive control VE (n = 3)

Table 1 \(^1\)H NMR and \(^{13}\)C NMR data of compound 1 (J in Hz, \(\delta\))

<table>
<thead>
<tr>
<th>No.</th>
<th>(^1)H NMR (600 MHz, D(2)O + CD(3)OD)</th>
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</tr>
</thead>
</table>
| 2   | 3.17–3.49 (m)                          | 3.13–3.55 (m)                     | 171.0 (C-2a), 167.9 (C-3a), 156.04 (C-4\(a\)), 156.01 (C-4\(b\)), 147.9 (C-3\(b\), 5\(b\)), 147.6 (C-6), 145.8 (C-8), 141.3 (C-7), 134.5 (C-4\(a\)), 134.2 (C-1\(a\)), 132.4 (C-4), 130.0 (C-1\(b\), 1\(a\)), 129.9 (C-2\(b\), 2\(a\), 6\(b\), 6\(a\)), 127.0 (C-3), 124.5 (C-8a), 123.1 (C-4a), 115.5 (C-3\(a\), 5\(a\)), 115.4 (C-3\(a\), 5\(b\)), 108.0 (C-5), 105.7 (C-2\(b\), 6), 60.0 (8-OMe), 56.4 (3\(a\)-OMe), 56.3 (5\(a\)-OMe), 55.3 (6-OMe), 47.8 (C-2), 41.6 (C-\(2\)), 41.4 (C-\(a\)), 40.4 (C-1), 34.8 (C-\(b\)), and 34.7 (C-\(b\)). It was identified as 1, 2-dihydro-6, 8-dimethoxy-7-hydroxy-1-(3, 5-dimethoxy-4-hydroxyphenyl) -N\(^1\), N\(^2\)-bis-[2-(4-hydroxy-phenyl)ethyl]-2, 3-naphthalene dicarboxamide by analysis of the 1D-NMR and 2D-NMR spectra and comparison of the NMR data with the literature. 

Compound 2 White amorphous powder (MeOH). It showed blue fluorescence under UV 365 nm, and yellow when reacting with iodine vapor. 

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Compounds 2 White amorphous powder (MeOH). It showed blue fluorescence under UV 365 nm, and yellow when reacting with iodine vapor.
ESI-MS: m/z 282.5 [M + H]+. It was identified as aristololactam A IIIa by comparison of the NMR data with the literature [3, 10].

**Compound 5** White needle crystals (MeOH). It showed blue fluorescence under UV 365 nm [Rf = 0.29, developed with MeOH–H2O (75 : 25) on Rp-8 F254 plate]. It was identified as piperlonguminine by comparison of the 1H NMR data with the literature [11].

**Conclusions**

8-β-β-D-glucopyranosylkaempferol-3-O-β-D-glucopyranoside (1), 1,2-dihydro-6, 8-dimethoxy-7-hydroxy-1-(3, 5- dimethoxy-4-hydroxyphenyl)-N1,N2-bis-[2-(4-hydroxy-phenyl)ethyl]-2, 3-naphthalene dicarboxamide (2), goniothalactam (3), aristololactam A IIIa (4), and piperlonguminine (5) were isolated from the stem of *P. wallichii*. Compound 1 was a new flavonol C-glycoside, 2 was a rare lignanamide which was isolated from the family Piperaceae for the first time, and compound 3 was isolated from this plant for the first time. All of the compounds were evaluated for their DPPH-scavenging activity and AChE inhibitory activity. Among them, only 2 showed potent DPPH-scavenging activity, compounds 2-4 showed weak AChE inhibitory activities. Further intensive *in vitro* or *in vivo* bioassay on the active compounds is necessary in the future.

**References**
