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Original Article

Perillanolides A and B, new monoterpene glycosides from the leaves of *Perilla frutescens*

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ABSTRACT

Two new monoterpene glycosides, perillanolides A and B, together with a known compound reported from the genus *Perilla* for the first time were isolated and characterized from the leaves of *Perilla frutescens* (L.) Britton, Lamiaceae, a garnish and colorant for foods as well as commonly used for traditional medicine. The structures of the isolated compounds were elucidated on the basis of extensive spectroscopic evidences derived from nuclear magnetic resonance experiments, mass spectrometry and by comparing their physical and spectroscopic data of literature. These compounds, together with the previously isolated secondary metabolites of this species, were investigated for their inhibitory effects on xanthine oxidase *in vitro*. Of the compounds, luteolin showed the strongest inhibitory activity with an IC_{50} value of 2.18 μM. Esculetin and scutellarein moderately inhibited the enzyme, while perillanolides A and B, and 4-(3,4-dihydroxybenzoyloxymethyl)phenyl-*O*-β-D-glucopyranoside exerted weak activities.

Introduction

Perilla frutescens (L.) Britton, a member of the mint family Lamiaceae, is widely distributed worldwide, especially in eastern Asia (Heci, 2001). As an edible crop, the cultivation of *P. frutescens* has more than 2000 years of history in China as well as in other Asian countries (Lee and Kim, 2007). In addition to its edible usages, *P. frutescens* leaf is commonly used in traditional medicines for relieving exterior syndrome and cold-dispelling, promoting the circulation of Qi, and harmonizing the stomach (Yu et al., 2016). Pharmacological research has been reported indicating that *P. frutescens* leaf possesses anti-allergic (Shin et al., 2000), anti-inflammatory (Makino et al., 2002), anti-oxidative (Zekonis et al., 2008), anticancer (Wang et al., 2013), antibacterial (Choi et al., 2010), and antidepressant activities (Takeda et al., 2002). Other physiological effects of *P. frutescens* leaf in several diseases have also been recently described in the literatures. For example, ultraviolet radiation-induced extracellular matrix damage in human dermal fibroblasts and hairless mice were significantly protected by *P. frutescens* leaves extract (Bae et al., 2017). Water-extracted *P. frutescens* leaves and stems increased the level of leukemia inhibitory factor (LIF), a major cytokine regulating endometrial receptivity, and LIF receptor in human endometrial Ishikawa cells (Kim et al., 2016).

In our searching for xanthine oxidase (XO) inhibitors from edible and medical Chinese natural plants, we found the aqueous extracts of *P. frutescens* leaves showed XO inhibitory activity for the first time. Bioactivity-guided fractionation led to the isolation of caffeic acid, vinyl caffeate, rosmarinic acid, methyl rosmarinate, and apigenin with XO inhibitory activity (Huo et al., 2015). Furthermore, twelve compounds were also isolated from the aqueous extract of *P. frutescens* leaf (Huo et al., 2016). Our continued interest in discovering new compounds from this source led us to isolate two new monoterpene glycosides, named perillanolides A (**1**) and B (**2**), together with a known compound, 4-(3,4-dihydroxybenzoyloxymethyl)phenyl-*O*-β-D-glucopyranoside (**3**). The structures of the isolated compounds were determined by spectroscopic methods, including 1D and 2D NMR, HR-ESI-MS, and chemical modification. Here, we also report the evaluation of XO inhibitory activities of the compounds isolated by us from this plant.

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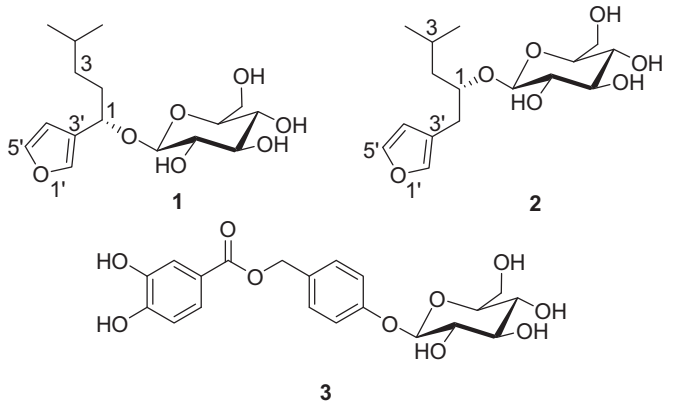

Materials and methods

General experimental procedures

The experimental procedures, including HPLC, MS and NMR instrumentation, were previously described (Gao et al., 2017). Allopurinol, XO, and xanthine were bought from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Compounds loliolide, isololiolide (Kimura and Maki, 2002), dehydrovomifoliol (Park et al., 2011), *trans*-*p*-hydroxycinnamic acid (Fu et al., 2011), esculetin (Zhang et al., 2014), luteolin, scutellarein, scutellarin, *p*-hydroxybenzaldehyde (Liang et al., 2015), negletein (Yoon et al., 2011), *p*-hydroxyacetophenone (Feng et al., 2013), and sericoside (Ponou et al., 2010) were isolated and identified from *P. frutescens* leaves in our laboratory.

Plant material

The raw materials of *Perilla frutescens* (L.) Britton, Lamiaceae, leaves were collected from Changchun suburb, Jilin Province of China, in October 2011. Botanical authentication was performed by Bao-Min Feng, College of Life Science and Technology, Dalian University. A voucher specimen (ZS20111001) was deposited at the Institute of Phytochemistry, Jilin Academy of Chinese Medicine Sciences.

Extraction and isolation

The air-dried leaves of *P. frutescens* (4 kg) were extracted three times with boiling water for 2 h and then filtered through a two-layer mesh. The water filtrates were concentrated under reduced pressure to afford a dark brown residue (583 g). The extract was suspended in water (2 l) and partitioned with *n*-butanol (2 l × 4 times), followed by concentration to yield 125 g the *n*-butanol extract. Part of the *n*-butanol extract (60 g) was suspended in water (4 l) and then passed through a open column chromatography on HPD 600 macroporous resin and successively eluted with water, 20% ethanol, 50% ethanol, and 70% ethanol to give 20% ethanol eluted part (14.3 g), 50% ethanol eluted part (27.5 g), and 70% ethanol eluted part (8.0 g).

The 20% ethanol eluted part (4 g) was chromatographed further on a RP-18 silica gel column and eluted with a gradient increasing methanol (30–100%) in water to yield eight fractions (Frs. 20-1–20-8) on the basis of TLC analyses. Compounds **1** (2.97 mg, R_t = 99 min) and **2** (2.71 mg, R_t = 108 min) were obtained from Fr. 20-7 by preparative HPLC employing MeOH/H_2O (55:45) as the mobile phase at a flow rate of 2.0 ml/min. The fraction eluted with 50% ethanol (15 g) was fractionated by column chromatography on silica gel eluting with a solvent system ($CHCl_3$:MeOH = 40:1, 19:1, 9:1, 8:2) to obtain six fractions (Frs. 50-1–50-6). Fr. 50-1 was separated chromatographically on a RP-18 silica gel column with a solvent system (MeOH:H_2O = 5:5, 10:0) to give subfractions, which were purified by preparative HPLC using MeOH:H_2O (40:60) as the mobile phase at a flow rate of 2.0 ml/min to yield compound **3** (9.65 mg, R_t = 79 min).

Spectral data

Perillanolide A (**1**): colorless gum; $[\alpha]_D^{20}$ −21.7 (*c* 0.23, MeOH); UV (MeOH) λ_{max} (log ε): 202 (3.72) nm; ^{1}H (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) see Table 1; HR-ESI-MS *m*/*z* 353.1565 $[M+Na]^+$ (calcd. for $C_{16}H_{26}O_7Na$, 353.1570).

Perillanolide B (**2**): colorless gum; $[\alpha]_D^{20}$ −46.7 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε): 202 (3.94) nm; ^{1}H (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) see Table 1; HR-ESI-MS *m*/*z* 353.1600 $[M+Na]^+$ (calcd. for $C_{16}H_{26}O_7Na$, 353.1570).

Acidic hydrolysis and sugar identification

Compounds **1** and **2** (each 1 mg) were acidic hydrolyzed with 1 ml of 7% HCl heated at 60 °C for 2 h. After cooling, the reaction mixture was neutralized with an Amberlite IRA400 column, and the eluate was concentrated. The sugar residues obtained from the hydrolysis were dissolved in anhydrous pyridine (0.5 ml) and L-cysteine methyl ester hydrochloride (2 mg) was added. The mixture was stirred at 60 °C for 1.5 h. After the reaction mixture was dried *in vacuo*, the residue was trimethylsilylated with 1-trimethylsilylimidazole (0.1 ml) for 2 h. The mixture was partitioned between hexane and H_2O (1 ml each), and organic layer (1 μl) was analyzed by GC–MS. Identification of D-glucopyranosides for **1** and **2** was detected in each case by co-injection of hydrolysate with standard silylated samples, giving single peaks at D-glucopyranose (13.5 and 13.5 min) for **1** and **2**, respectively. Retention times of authentic samples treated in the same way with 1-trimethylsilylimidazole in pyridine were D-glucopyranose (13.5 min).

XO inhibitory activity assay and XO inhibitory mode of action assay

The XO inhibition assays and the inhibitory mode assay of the isolated compounds were performed according to the method modified by our group (Liu et al., 2016). An overview about the effects of these substances on XO activity is given in Table 2.

Results and discussion

Compound **1** was isolated as a colorless gum, and showed positive Liebermann–Burchard reaction, suggesting it to be a glycoside. The molecular formula was determined to be $C_{16}H_{26}O_7$ on the basis of the quasimolecular ion peak at *m*/*z* 353.1565 $[M+Na]^+$ in the positive HR-ESI-MS (calcd. for $C_{16}H_{26}O_7Na$, 353.1570), which was further supported by the ^{1}H NMR and ^{13}C NMR spectral data (Table 1). The ^{13}C NMR and DEPT spectra revealed 16 carbon signals due to two methyl carbons, three methylene carbons, ten methine carbons, and one nonprotonated carbon, of which ten carbons were assigned to the aglycone part including two methyl carbons at δ_C 22.9 and 23.0, two methylene carbons at δ_C 35.4 and 35.8, two methine carbons (one oxygenated) at δ_C 29.1 and 72.6, and three methine carbons at δ_C 110.0, 142.5, 144.7 and one quaternary carbon at δ_C 126.6 belonging to a furan ring. These spectral data implied that **1** was to be a monoterpene glycoside. The ^{1}H NMR spectrum of the aglycone moiety showed signals for two methyl protons at δ_H 0.87 (3H, d, *J* = 6.7 Hz) and 0.87 (3H, d, *J* = 6.7 Hz), two groups of methylene protons at δ_H 1.71 (1H, m) and 1.86 (1H, m), and 1.10 (1H, m) and 1.27 (1H, m), one methine proton at δ_H 1.53 (1H, m), one oxygen-substituted proton at δ_H 4.83 (1H, t,

Table 1
^{1}H NMR and ^{13}C NMR spectral data of compounds **1** and **2** (500 and 125 MHz, CD_3OD, δ ppm, J in Hz).

Position	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
Aglycone				
1	4.83, t, (7.5)	72.6 (d)	2.69, dd (14.7, 4.6) 2.64, dd (14.7, 6.6)	30.5 (d)
2	1.86, m 1.71, m	35.4 (t)	4.01, m	77.4 (d)
3	1.27, m 1.10, m	35.8 (t)	1.44, m 1.22, m	44.8 (t)
4	1.53, m	29.1 (d)	1.84, m	25.2 (d)
5	0.87, d (6.7)	23.0 (q)	0.87, d (6.7)	23.8 (q)
6	0.87, d (6.7)	22.9 (q)	0.87, d (6.7)	22.5 (q)
2′	7.51, br s	142.5 (d)	7.36, br s	141.6 (d)
3′		126.6 (s)		122.4 (s)
4′	6.47, d (1.7)	110.0 (d)	6.42, d (1.6)	112.9 (d)
5′	7.46, d (1.7)	144.7 (d)	7.38, d (1.6)	143.7 (d)
Sugar				
1″	4.16, d (7.4)	100.5 (d)	4.40, d (7.8)	102.4 (d)
2″	3.21, m	75.1 (d)	3.18, m	75.3 (d)
3″	3.24, m	78.1 (d)	3.35, m	78.3 (d)
4″	3.25, m	71.9 (d)	3.31, m	71.9 (d)
5″	3.14, m	77.9 (d)	3.24, m	77.8 (d)
6″	3.87, dd (11.8, 2.3) 3.66, dd (11.8, 6.1)	63.0 (t)	3.86, dd (11.7, 2.4) 3.68, dd (11.7, 5.4)	63.0 (t)

Table 2
Xanthine oxidase inhibitory activities of the isolated compounds.

Compounds	Concentration (μM)	Inhibition ratio (%)	IC_{50} (μM)	Mode of inhibition
Perillanolide A (**1**)	200	1.10 ± 0.08	>200	–
Perillanolide B (**2**)	200	1.46 ± 0.13	>200	–
4-(3,4-Dihydroxybenzoyloxymethyl) phenyl-*O*-β-D-glucopyranoside (**3**)	200	35.46 ± 0.59	>200	–
Loliolide	200	14.53 ± 1.47	>200	–
Isololiolide	200	1.23 ± 0.32	>200	–
Dehydrovomifoliol	200	1.45 ± 1.08	>200	–
trans-*p*-Hydroxycinnamic acid	200	10.52 ± 1.00	>200	–
Esculetin	100 50 25 5	78.85 ± 0.23 64.40 ± 0.75 44.39 ± 1.60 9.96 ± 2.36	32.56	Competitive
Luteolin	5 4 3 2	66.07 ± 0.29 62.21 ± 0.28 56.46 ± 1.44 49.37 ± 1.06	2.18	Competitive
Scutellarein	100 75 50 25	65.96 ± 0.19 60.59 ± 0.43 51.71 ± 0.56 30.05 ± 1.04	48.66	Mixed
Scutellarin	200	40.06 ± 1.23	>200	–
p-Hydroxybenzaldehyde	200	10.11 ± 0.78	>200	–
Negletein	200	40.91 ± 0.65	>200	–
p-Hydroxyacetophenone	200	8.54 ± 0.94	>200	–
Sericoside	200	1.05 ± 0.24	>200	–
Allopurinol	–	–	2.07	Competitive

J = 7.5 Hz), and 3-furanyl ring protons at 6.47 (1H, d, J = 1.7 Hz), 7.46 (1H, d, J = 1.7 Hz) and 7.51 (1H, br s). Inspection of the DQFCOSY and HMBC correlations allowed assemble of the aliphatic chain and furanyl ring starting from the methyl signals at δ_H 0.87. The aglycone moiety of **1** was proposed to be 1-(3′-furanyl)-4-methyl-pentan-1-ol, which was similar to perillaketone, the most abundant volatile compound from *P. frutescens* leaves (Bassoli et al., 2013). The carbonyl carbon in the structure of perillaketone was absent in that of **1**, instead, signals for an oxymethine ($\delta_{H/C}$ 4.83/72.6) were observed. The DQFCOSY correlations from H-1 (δ_H 4.83) to H-2a (δ_H 1.86) and H-2b (δ_H 1.71) as well as the HMBC correlations from H-1 (δ_H 4.83) to C-2 (δ_C 35.4), C-3′ (δ_C 122.4), C-3 (δ_C 35.8), C-2′ (δ_C 141.6), and C-4′ (δ_C 110.0) supported the above deduction (Fig. 1). The remaining ^{1}H NMR and ^{13}C NMR spectral data were ascribed to the sugar moiety. Using the anomeric proton at

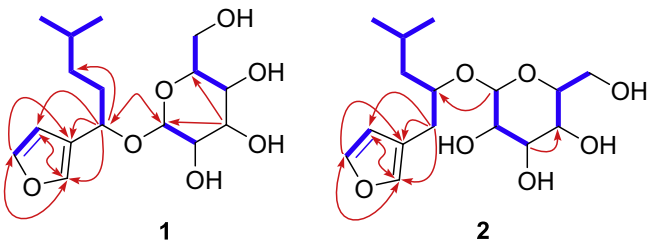

Fig. 1. Key DQFCOSY (—) and HMBC (→) correlations of compounds **1** and **2**.

δ_H 4.16 (1H, d, J = 7.4 Hz) as a starting point, analysis of DQFCOSY experiment allowed the identification, in sequence, of four oxymethine groups and one oxymethylene group. The sugar moiety was identified as β-glucose in the pyranose form, on the basis of large

couplings observed for all of the oxymethine protons implying their axial position. The position of glucose was unambiguously established by an HMBC experiment, in which a long-rang correlation was observed between the H-1″ (δ_H 4.16) of D-glucose and the C-1 (δ_C 72.6) of aglycone (Fig. 1). Acid hydrolysis of **1** afforded β-D-glucopyranose, which was identified by GC analysis with authentic sugars. To figure out the configuration of the only chiral center (C-1) for the aglycone moiety of **1**, the optical rotation of the aglycone was experimentally recorded ($[\alpha]_D^{20}$ −30.0, *c* 0.10, MeOH). By comparison with that of (*S*)-1-(3′-furyl)-3-buten-1-ol ($[\alpha]_D^{25}$ −30.7, *c* 1.72, CH_2Cl_2) (Bierstedt et al., 2001), the *S*-configuration at C-1 was tentatively deduced to this compound. Thus, the structure of **1** was confirmed as (*S*)-1-(3′-furanyl)-4-methyl-pentan-1-*O*-β-D-glucopyranoside and the compound was named perillanolide A.

Compound **2** was isolated as a colorless gum. It showed a quasimolecular ion peak at *m*/*z* 353.1600 $[M+Na]^+$ in the positive HR-ESI-MS. A comparative analysis of ^1H and ^{13}C NMR spectral data of **2** (Table 1) with those of **1** suggested the presence of an aliphatic chain, a furanyl ring, and a glucose moiety. However, the nuanced spectral data and the different retention time in the preparative-HPLC indicated the distinct construction of the two compounds. Detailed analysis of the above NMR spectral data as well as 2D NMR correlations (Fig. 1), the significant difference between **1** and **2**, lied in the glycosylation site. The DQFCOSY correlations from H-2 (δ_H 4.01) to H-1a (δ_H 2.69), H-1b (δ_H 2.64), H-3a (δ_H 1.44), and H-3b (δ_H 1.22) as well as HMBC correlations from H-1a (δ_H 2.69) and H-1b (δ_H 2.64) to C-2 (δ_C 77.4), C-3 (δ_C 44.8), C-2′ (δ_C 141.6), C-3′ (δ_C 122.4), and C-4′ (δ_C 112.9), from the anomeric proton at δ_H 4.40 (1H, d, *J* = 7.8 Hz) to the oxymethine C-2 (δ_C 77.4) supported the glycosylation site to be C-2 (Fig. 1). Acid hydrolysis of **2** afforded β-D-glucopyranose and aglycone. By comparison of the aglycone optical rotation ($[\alpha]_D^{25}$ −50.0, *c* 0.10, MeOH) with that of (*R*)-methyl 3-hydroxyhexanoate ($[\alpha]_D^{rt}$ −23.3, *c* 1.16, $CHCl_3$) (Jiang et al., 2010) and (*S*)-ethyl 4-hydroxy-5-phenylpentanoate ($[\alpha]_D^{25}$ +14.5, *c* 1.00, $CHCl_3$) (Kotkar et al., 2007), the *R*-configuration at C-2 was tentatively deduced to this compound. The structure of **2** was thus determined as (*R*)-1-(3′-furanyl)-4-methyl-pentan-2-*O*-β-D-glucopyranoside and a trivial name perillanolide B was assigned.

Compound **3** was identified by comparing the ^1H and ^{13}C NMR, as well as MS spectra with that reported in the literature. It was determined to be 4-(3,4-dihydroxybenzoyloxymethyl)phenyl-*O*-β-D-glucopyranoside (**3**) (Nakatani and Kikuzaki, 1987).

In this study, compounds **1**–**3** and twelve previously isolated compounds were evaluated for XO inhibitory activity. Allopurinol was used as a positive control. When xanthine was added to the mixture of XO and increasing concentrations of esculetin, luteolin, scutellarein, or allopurinol, the production of uric acid showed a concentration-dependent decrease compared to control. Luteolin significantly inhibited XO activity with an IC_{50} value of 2.18 μM, which is close to allopurinol's IC_{50} value of 2.07 μM. IC_{50} values for esculetin and scutellarein were 32.56 μM and 48.66 μM, respectively, indicating weak inhibition of XO activity. The IC_{50} value for other compounds was too high to determine. The Lineweaver–Burk plots were performed to understand the enzyme inhibition modes of esculetin, luteolin, and scutellarein. Esculetin and luteolin exhibited competitive inhibitions, while a mixed inhibition mode was observed for scutellarein.

Conclusions

Phytochemical investigation of the leaves of *P. frutescens* led to the isolation of two new monoterpene and a known compound. These compounds, together with the previously isolated secondary metabolites of this species, were investigated for their inhibitory effects on xanthine oxidase *in vitro*. Of the compounds, luteolin showed the strongest inhibitory activity with an IC_{50} value of 2.18 μM. Esculetin and scutellarein moderately inhibited the enzyme, while perillanolides A (**1**) and B (**2**), and 4-(3,4-dihydroxybenzoyloxymethyl)phenyl-*O*-β-D-glucopyranoside (**3**) exerted weak activities. The obtained results were benefit for the subsequent researches of genus *Perilla*.

Authors' contributions

YL, XHL, GLL, and WJG performed the extraction, isolation, and elucidation of the constituents. SZ and XYF were involved in the biological evaluations. HG confirmed all the isolation and structural elucidation procedures. WW designed the study, supervised the laboratory work, and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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