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C-Glycosylflavones from the Leaves and Flowers of *Gentiana algida* in Japan

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Abstract *Gentiana algida* is a major alpine plant in Japan and is known as traditional medicinal plant. Four *C*-glycosylflavones, isoorientin 4′-*O*-glucoside, isoorientin, isovitexin 4′-*O*-glucoside and isovitexin were isolated from the leaves and flowers of *G. algida*. The latter two compounds were reported from this species for the first time. Although many plant species generally possess different flavonoids in their leaves and flowers, those of *G. algida* were substantially uniform.

Key words: chemical ecology, chemical profiling, flavonoids, *Gentiana algida*, Gentianaceae, *C*-glycosylflavones.

Introduction

The genus *Gentiana* consists of ca. 360 species (Mabberley, 2017), and 14 species are growing in Japan (Toyokuni and Yamazaki, 1993). Among them, arctic gentian *Gentiana algida* Pall. (Fig. 1) is distributed in north circumpolar area of Asia and North America, and the plant is a common alpine species in Japan. Japanese name of the plant 'Tōyaku-rindō' means medicinal gentian, because it is used as traditional medicine in some countries such as China and Mongolia. In Daisetsu Mountains, Hokkaido, Japan, *G. algida* f. *igarashii* (Miyabe et Kudô) Toyok. (Fig. 1) occurs with shorter stems, wider and thicker leaves and larger flowers (Toyokuni and Yamazaki, 1993).

Gentians including *G. algida* produce bioactive compounds such as secoiridoids, xanthones, aromatic acids and flavonoids (Butayarov *et al.*, 1993; Lin *et al.*, 1997; Yang *et al.*, 2013; Mustafa *et al.*, 2015; Pan *et al.*, 2016). In particular, the roots of *G. lutea* L., *G. scabra* Bunge and *G. triflora* Pall. contain secoiridoid, gentiopicroside that is active ingredients of stomach medicine. As for *G. algida*, seven secoiridoids, gentiopicroside, sweroside, 2′-(2,3-dihydroxybenzoyl)sweroside, trifloroside, amplexine 1-glucoside, gelidoside (rindoside) and algiolide A (Tan *et al.*, 1996; Tanaka *et al.*, 2015), two xanthones, 1,3,5,8-tetrahydroxyxanthone and 1,3,4,5,8-pentahydroxyxanthone (Butayarov *et al.*, 1993), and five flavonoids, isoorientin, isoorientinm 4′-*O*-glucoside, orientin, swertisin and santin (5,7-dihydroxy-3,6,4′-trimethoxyflavone) (Hostetmann-Kaldas *et al.*, 1981; Tadzhibaev *et al.*, 1992; Tan *et al.*, 1996; Tozhiboev *et al.*, 2011; Yang *et al.*, 2013) have been reported. Previous studies have suggested that geographical variation of flavonoids occur in *G. algida* (Hostettmann-Kaldas *et al.*, 1981; Tadzhibaev *et al.*, 1992; Tan *et al.*, 1996), however, that of Japanese population has not been surveyed. As flavonoids of another Japanese *Gentiana* species, three *C*-glycosylflavones, isoorientin, isoorientin 4′-*O*-glucoside and apigenin *C*-hexoside-*O*-hexoside, were reported from the leaves of *G. triflora* var. *japonica*

Fig. 1. *Gentiana algida* f. *algida* (left) and *G. algida* f. *igarashii* (right).

(Kusn.) Hara (Sasaki *et al.*, 2015). Anthocyanins were isolated and characterized from the blue or pink flowers of *G. makinoi* Kusn. (Goto *et al.*, 1982), *G. scabra* var. *buergeri* (Miq.) Maxim. (Ueno *et al.*, 1969), *G. triflora* (Hosokawa *et al.*, 1995; Yoshida *et al.*, 2000) and *G. zollingeri* Fawc. (Yoshitama *et al.*, 1980). Although the flower color of *G. algida* is varied from white, greenish-white and bluish-white, flavonoid profiling in the flowers of *G. algida* has not been carried out to date. The aim of the present survey is to reveal the flavonoid composition in the leaves and flowers of *G. algida* in Japan.

Materials and Methods

Plant materials

The plants of *Gentiana algida* f. *algida* was collected from Mt. Norikura, 3000 m elev., Hida Mountains, Nagano Prefecture, Japan in September 2005 and August 2006, Mt. Akaishi-dake, 2730 m elev., Akaishi Mountains, Yamanashi Prefecture, Japan in July and September 2014, and Mt. Gassan, 1800 m elev., Dewa Mountains, Yamagata Prefecture, Japan in August 2012. On the other hand, the plants of *G. algida* f. *igarashii* was collected from Mt. Aka-dake, 2000 m elev., Daisetsu Mountains, Hokkaido, Japan in August 2012. Plant collections were carried out under the permissions of each prefecture, the Ministry of Environment, the Ministry of Agriculture, Forestry and Fisheries, the Agency for Cultural Affairs, Japan, Tokushu Tokai Paper Co., Ltd. and Gassan Shrine. Voucher specimens were deposited in the herbarium of the Department of Botany, National Museum of Nature and Science, Japan (TNS).

Extraction and separation

Fresh leaves (30.7 g) and flowers (3.8 g) of *G. algida* were extracted with MeOH and FM (formic acid/ MeOH = 8 : 92), respectively. The concentrated extracts were applied to preparative paper chromatography using solvent systems: BAW (*n*-BuOH/HOAc/H_2O = 4 : 1 : 5, upper phase) and 15% HOAc. The compounds were purified by Sephadex LH-20 column chromatography (solvent system: 70% MeOH).

Qualitative and quantitative HPLC analysis of flavonoids

Fresh leaves (0.2 g) and flowers (0.1 g) of each

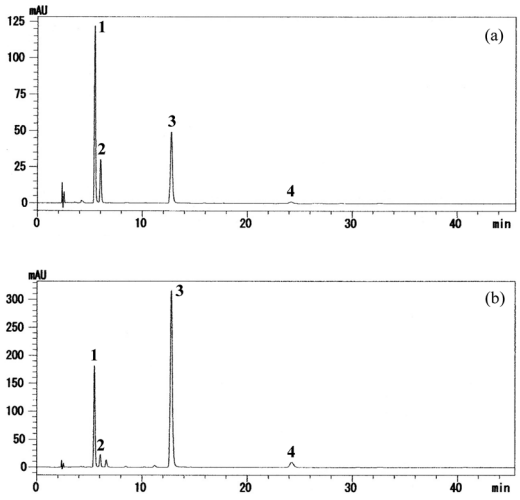

Fig. 2. HPLC patterns of MeOH and FM extracts from *Gentiana algida* in Japan. (a) Leaves. (b) Flowers. **1**: Isoorientin 4′-*O*-glucoside, **2**: Isovitexin 4′-*O*-glucoside, **3**: Isoorientin and **4**: Isovitexin.

population were extracted with 4 mL MeOH and 1 mL of FM, respectively. High performance liquid chromatography (HPLC) separation of the extracts filtered with GL Chromatodisk 13N (0.45 µm pore size, GL Sciences, Inc., Japan) was performed with a Shimadzu Prominence HPLC system using a SunShell C18 column (2.6 µm particle material, I.D. 4.6 × 100 mm, ChromaNik Technologies Inc., Japan) at a flow rate of 0.4 mL min^{-1}, detection wavelength at 190–700 nm and H_3PO_4/HOAc/MeCN/H_2O (3 : 8 : 5 : 84) as eluent. Injection volume was 1 µL. HPLC chromatograms of flavonoids (at 350 nm) are shown in Fig. 2.

Liquid chromatograph-mass spectra (LC-MS)

LC-MS were measured with a Shimadzu LC-MS system using an Inertsil ODS-4 (3 µm particle material, I.D. 2.1 × 100 mm, GL Sciences Inc.), at a flow-rate of 0.2 mL min^{-1}, eluting with HCOOH/MeCN/H_2O (1 : 15 : 84), injection: 3 µL, ESI^+ 4.5 kV, ESI^- 3.5 kV, 250°C.

Identification of flavonoids

In this research, four flavones were isolated and identified from the leaves and flowers of *G. algida* f. *algida* and f. *igarashii*. Flavonoids **1**–**4** were characterized by UV spectroscopy according to Mabry *et al.* (1970) and LC-MS. In the cases of **1** and **2**, their chemical structures were estimated by ^1H and ^{13}C NMR. Flavonoids **3** and **4** were identified by HPLC comparisons with authentic specimens of isoorientin from the leaves of *Japonolirion osense* Nakai (Iwashina *et al.*, 2005) and isovitexin from the flowers of *Iris ensata* Thunb. (Iwashina *et al.*, 1996), respectively. UV, LC-MS, ^1H and ^{13}C NMR data of the isolated compounds are as follows.

Isoorientin 4′-*O*-glucoside (**1**). White powder. UV: λmax (nm) MeOH 273, 334; + NaOMe 270, 280sh, 382 (dec.); + $AlCl_3$ 282, 295sh, 350, 388sh; + $AlCl_3$/HCl 284, 294sh, 344, 385sh; + NaOAc 268sh, 276, 380; + NaOAc/H_3BO_3 273, 339. HPLC: Rt 4.9 min. LC-MS: *m/z* 611 $[M+H]^+$, 609 $[M-H]^-$ (luteolin + 2 mol glucose). ^1H NMR (600 MHz, pyridine-d_5): δ_H 14.20 (1H, *s*, 5-OH), 7.82 (1H, *d*, *J* = 2.3 Hz, H-2′), 7.65 (1H, *d*, *J* = 8.7 Hz, H-5′), 7.37 (1H, *dd*, *J* = 2.3 and 8.6 Hz, H-6′), 6.87 (1H, *s*, H-3), 6.78 (1H, *s*, H-8), 5.81 (1H, *d*, *J* = 9.8 Hz, 6-*C*-glucosyl H-1), 5.70 (1H, *d*, *J* = 7.7 Hz, 4′-glucosyl H-1), 5.26 (1H, *t*, *J* = 9.1 Hz, 6-*C*-glucosyl H-2), 4.61 (1H, *dd*, *J* = 2.1 and 12.1 Hz, 6-*C*-glucosyl H-6a), 4.58 (1H, *dd*, *J* = 2.3 and 12.0 Hz, 4′-glucosyl H-6a), 4.47 (1H, *m*, 6-*C*-glucosyl H-4), 4.45 (1H, *m*, 4′-glucosyl H-6b), 4.44 (1H, *m*, 6-*C*-glucosyl H-3), 4.40 (1H, *m*, 4′-glucosyl H-3), 4.39 (1H, *m*, 6-*C*-glucosyl H-6b), 4.33 (1H, *t*, *J* = 9.1 Hz, 4′-glucosyl H-2), 4.29 (1H, *t*, *J* = 8.9 Hz, 4′-glucosyl H-4), 4.20 (1H, *m*, 6-*C*-glucosyl H-5), 4.20 (1H, *m*, 4′-glucosyl H-5). ^{13}C NMR (150 MHz, pyridine-d_5): (luteolin) δ_C 163.9 (C-2), 104.9 (C-3), 182.9 (C-4), 162.1 (C-5), 110.2 (C-6), 165.0 (C-7), 94.6 (C-8), 157.4 (C-9), 104.8 (C-10), 126.1 (C-1′), 115.1 (C-2′), 149.0 (C-3′), 150.1 (C-4′), 117.3 (C-5′), 118.8 (C-6′); (6-*C*-glucose) δ_C 75.3 (C-1), 72.6 (C-2), 80.7 (C-3), 72.0 (C-4), 83.0 (C-5), 62.2 (C-6); (4′-glucose) δ_C 102.8 (C-1), 74.8 (C-2), 78.2 (C-3), 71.3 (C-4), 79.1 (C-5), 62.3 (C-6).

Isovitexin 4′-*O*-glucoside (**2**). White powder. UV: λmax (nm) MeOH 273, 323; + NaOMe 279,

372 (dec.); +$AlCl_3$ 281, 292, 337, 390sh; +$AlCl_3$/HCl 266sh, 285, 293, 336, 385sh; +NaOAc 279, 373; +NaOAc/H_3BO_3 274, 328. HPLC: Rt 5.1 min. LC-MS: *m/z* 595 $[M+H]^+$, 593 $[M-H]^-$ (apigenin + 2 mol glucose). ^1H NMR (600 MHz, pyridine-d_5): δ_H 14.19 (1H, *s*, 5-OH), 7.80 (2H, *d*, *J* = 8.9 Hz, H-2′,6′), 7.42 (2H, *d*, *J* = 8.9 Hz, H-3′,5′), 6.85 (1H, *s*, H-8), 6.83 (1H, *s*, H-3), 5.82 (1H, *d*, *J* = 9.8 Hz, 6-*C*-glucosyl H-1), 5.73 (1H, *d*, *J* = 7.6 Hz, 4′-glucosyl H-1), 5.27 (1H, *t*, *J* = 9.1 Hz, 6-*C*-glucosyl H-2), 4.63 (1H, *dd*, *J* = 2.1 and 12.1 Hz, 6-*C*-glucosyl H-6a), 4.58 (1H, *dd*, *J* = 2.0 and 11.8 Hz, 4′-glucosyl H-6a) 4.48 (1H, *m*, 6-*C*-glucosyl H-4), 4.46 (1H, *m*, 6-*C*-glucosyl H-3), 4.46 (1H, *m*, 4′-glucosyl H-6b), 4.43 (1H, *m*, 4′-glucosyl H-3), 4.39 (1H, *m*, 6-*C*-glucosyl H-6b), 4.38 (1H, *m*, 4′-glucosyl H-2), 4.32 (1H, *t*, *J* = 9.0 Hz, 4′-glucosyl H-4), 4.25 (1H, *m*, 4′-glucosyl H-5), 4.20 (1H, *m*, 6-*C*-glucosyl H-5). ^{13}C NMR (150 MHz, pyridine-d_5): (apigenin) δ_C 163.6 (C-2), 104.8 (C-3), 182.9 (C-4), 162.1 (C-5), 110.3 (C-6), 165.1 (C-7), 94.7 (C-8), 157.4 (C-9), 104.7 (C-10), 124.9 (C-1′), 128.4 (C-2′, 6′), 117.2 (C-3′,5′), 161.2 (C-4′); (6-*C*-glucose) δ_C 75.3 (C-1), 72.6 (C-2), 80.7 (C-3), 72.1 (C-4), 83.0 (C-5), 62.4 (C-6); (4′-glucose) δ_C 101.6 (C-1), 74.8 (C-2), 78.4 (C-3), 71.3 (C-4), 79.1 (C-5), 63.0 (C-6).

Isoorientin (Luteolin 6-*C*-glucoside) (**3**). White powder. UV: λmax (nm) MeOH 254, 269, 349; +NaOMe 269, 280sh, 334sh, 410 (inc.); +$AlCl_3$ 275, 337, 424; +$AlCl_3$/HCl 276, 296sh, 361, 385; +NaOAc 273, 327, 397; +NaOAc/H_3BO_3 269, 383, 430sh. HPLC: Rt 14.4 min. LC-MS: *m/z* 449 $[M+H]^+$, 447 $[M-H]^-$ (luteolin + 1 mol glucose).

Isovitexin (Apigenin 6-*C*-glucoside) (**4**). Pale yellow solution. UV: λmax (nm) MeOH 271, 332; +NaOMe 278, 331, 396 (inc.); +$AlCl_3$ 279, 303, 349, 378; +$AlCl_3$/HCl 280, 302, 343, 380; +NaOAc 278, 307, 389; +NaOAc/H_3BO_3 273, 338. HPLC: Rt 27.5 min. LC-MS: *m/z* 433 $[M+H]^+$, 431 $[M-H]^-$ (apigenin + 1 mol glucose).

Results

In this survey, four flavonoids were found and isolated from the flowers and leaves of *Gentiana algida*. Of their compounds, **3** and **4** were identified as isoorientin and isovitexin by UV spectral properties, LC-MS, and TLC and HPLC comparisons with authentic samples. On the other hand, **1** and **2** were identified by UV spectra, LC-MS, and mainly ^1H and ^{13}C NMR.

The presence of free 5-, 7- and 3′-hydroxyl and a substituted 4′-hydroxyl groups of **1** was shown by UV spectral analysis according to Mabry *et al.* (1970). Furthermore, estimation of **1** was performed by NMR survey. The proton and carbon signals were assigned by ^1H-^1H COSY, ^1H-^1H NOESY, HSQC and HMBC. ^1H NMR of **1** showed the presence of five aromatic protons, i.e. H-2′ at δ_H 7.82 (*d*, *J* = 2.3 Hz), H-5′ at δ_H 7.65 (*d*, *J* = 8.7 Hz), H-6′ at δ_H 7.37 (*dd*, *J* = 2.3 and 8.6 Hz), H-3 at δ_H 6.87 (*s*) and H-6 or H-8 at δ_H 6.78 (*s*), and two glucosyl anomeric protons at δ_H 5.81 (*d*, *J* = 9.8 Hz) and 5.70 (*d*, *J* = 7.7 Hz). Their coupling constants showed that both glucoses are in *β*-pyranose form. Of their anomeric protons, since δ_H 5.81 proton signal correlated with carbon signal at δ_C 75.3 by HSQC and showed coupling constant, *J* = 9.8 Hz, it was cleared that it is *C*-glycosyl anomeric proton (Markham and Geiger, 1994). On the other hand, since proton signal at δ_H 6.78 correlated with C-7 at δ_C 165.0, C-9 at δ_C 157.4 and C-10 at δ_C 104.8 by HMBC, this was shown to be H-8 but not H-6. Moreover, another anomeric proton, δ_H 5.70 correlated C-4′ at δ_C 150.1 by HMBC. Thus, **1** was identified as luteolin 6-*C*-*β*-glucopyranosyl-4′-*O*-*β*-glucopyranoside (isoorientin 4′-*O*-glucoside, Fig. 3).

The presence of free 5- and 7-hydroxyl and a substituted 4′-hydroxyl groups was shown by UV spectral survey of **2**. ^1H NMR of **2** showed the presence of six aromatic protons, H-2′,6′ at δ_H 7.80 (*d*, *J* = 8.9 Hz), H-3′,5′ at δ_H 7.42 (*d*, *J* = 8.9 Hz), H-3 at δ_H 6.83 (*s*), and H-6 or H-8 at δ_H 6.85 (*s*), together with two anomeric protons at δ_H 5.82 (*d*, *J* = 9.8 Hz) and 5.73 (*d*, *J* = 7.6 Hz).

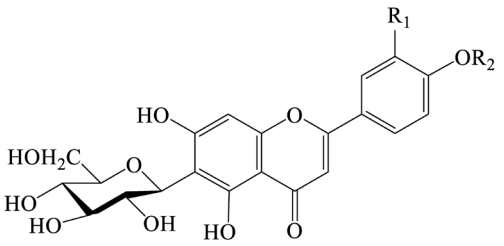

Fig. 3. Chemical structures of *C*-glycosylflavones from *Gentiana algida.*
1: Isoorientin 4′-*O*-glucoside (R_1 = OH, R_2 = glucosyl), **2**: Isovitexin 4′-*O*-glucoside (R_1 = H, R_2 = glucosyl), **3**: Isoorientin (R_1 = OH, R_2 = H), and **4**: Isovitexin ($R_1 = R_2$ = H).

Their coupling constants showed that the sugars are in *β*-pyranose form. Since former anomeric proton signal correlated with carbon signal at δ_C 75.3 by HSQC and indicated coupling constant, J = 9.8 Hz, it was shown to be *C*-glycosyl anomeric proton. A proton signal at δ_H 6.85 correlated with C-7 at δ_C 165.1, C-9 at δ_C 157.4 and C-10 at δ_C 104.7 by HMBC, this was shown to be H-8 but not H-6. Moreover, anomeric proton signal at δ_H 5.73 correlated with C-4′ at δ_C 161.2 by HMBC. Thus, **2** was identified as apigenin 6-*C*-*β*-glucopyranosyl-4′-*O*-*β*-glucopyranoside (isovitexin 4′-*O*-glucoside, Fig. 3).

Discussion

Major flavonoids in the Gentianaceae family are *C*-glycosylflavones (Iwashina, 2018). Four flavone *C*-glycosides, isoorientin 4′-*O*-glucoside (**1**), isovitexin 4′-*O*-glucoside (**2**), isoorientin (**3**) and isovitexin (**4**) were isolated from the leaves and flowers of *G. algida* in Japan. Although **1** and **3** were previously reported from this species (Hostettmann-Kaldas *et al.*, 1981), other compounds (**2** and **4**) were isolated for the first time. Orientin which is an isomer of isoorientin was found in this species (Tan *et al.*, 1996). However, it could not be isolated in this survey. Recently, **1** and **3** were isolated from the leaves of *G. triflora* in Japan (Sasaki *et al.*, 2015). Interestingly, the HPLC pattern of leaf flavonoids in *G. triflora* was similar to that in *G. algida*. Many previous studies on chemical compounds of gentians were carried out using aerial parts or roots. However, since flowers and leaves were not separately extracted, the peculiarities of the flavonoids in the leaves and flowers cannot be determined.

The flavonoid composition of the leaves and flowers of *G. algida* was approximately the same within all populations including Mt. Aka-dake (f. *igarashii*). Plant collections in Mt. Norikura were carried out in 2005 and 2006. Howerer, there was no difference in flavonoid composition between two years. Furthermore, the leaves sampled from Mt. Akaishi-dake in July and September 2014 also showed the same flavonoid pattern. The content of **3** in the flowers was relatively higher than that in the leaves. Leaf flavonoids are generally different from those of flowers in most plants. However, the major flavonoids in the leaves and flowers of *G. algida* was qualitatively the same.

Luteolin-type flavonoids, *ortho*-dihydroxylated compounds, were main components in alpine species *G. algida* in Japan. High accumulation of their flavonoids was similar to other alpine plants such as *Campanula lasiocarpa* Cham. (Campanulaceae) (Murai *et al.*, 2014) and *Pedicularis chamissonis* Steven (Orobanchaceae) (Murai and Iwashina, 2015) in Japan. Such compounds may act as anti-stress compounds against UV radiation and oxidative stress in harsh alpine environment. In this survey, we also detected some anthocyanins, that were presumed as delphinidin type by HPLC with photodiode array detector (data not shown). Although the anthocyanins could not be identified because of their small amounts, they might be contributed to the bluish lines in the flowers (Fig. 1).

Further studies (e.g. chemical comparisons with continental populations and other gentians) are required for the comprehensive understanding of flavonoid distribution in *G. algida*.

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