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Flavonoids from Two Alpine *Campanula* Species in Japan

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Abstract Two alpine *Campanula* species, *C. lasiocarpa* and *C. chamissonis*, were surveyed for flavonoids in their leaves. Two major flavones, 7-*O*-gentiobioside and 7-*O*-glucoside of luteolin, and two minor flavones, apigenin 7-*O*-glucoside and luteolin were isolated from *C. lasiocarpa*. On the other hand, 3-*O*-galactoside, 3-*O*-glucoside and 3-*O*-glucuronide of quercetin, and 3-*O*-galactoside and 3-*O*-glucoside of kaempferol were isolated from *C. chamissonis*. These species are distantly-related from the standpoint of chemotaxonomy. In addition, ecological significance of them was also described.

Key words: *Campanula chamissonis*, *Campanula lasiocarpa*, chemical ecology, chemotaxonomy, flavonoids.

Introduction

The genus *Campanula* consists of ca. 300 species that is distributed in temperate to tropical zone, and is famous as bellflowers (Mabberley, 2008). Of their species, *C. lasiocarpa* Cham. and *C. chamissonis* Al.Fedr. exclusively occur in alpine habitats in Japan (Fig. 1). Since both species possess similar morphology and growth environment, they were classified as the same section within the genus *Campanula* (Okazaki, 1997). In recent years, gene phylogeny, however, revealed that they were divided into different well-supported clades each other (Roquet *et al.*, 2008; Wendling *et al.*, 2011).

In the late 20th century, 45 *Campanula* species have been investigated for their flavonoids by Teslov's and Dzhumyrko's groups (e.g., Dzhumyrko *et al.*, 1969; Teslov and Blinova, 1972, 1974; Dzhumyrko, 1973, 1985; Teslov, 2000). Over 50 flavonoids were isolated from *Campanula* species by the studies mentioned above, and some of them exhibited antioxidant activity (Touafek *et al.*, 2011).

On the other hand, flavonoids are one of the most effective UV-absorbing compounds that are synthesized in plants (Caldwell *et al.*, 1983). These compounds can absorb both UV-B (280–320 nm) and UV-A (320–400 nm) regions. Hashiba *et al.* (2006) have been surveyed the foliar flavonoid composition of *Campanula punctata* Lam. in relation to its UV-absorbing property using coastal (higher intensities of UV-B) and inland (lower intensities of UV-B) populations in Suzaki Peninsula, Shizuoka Prefecture, Japan. The work concluded that there was little relationship between UV-intensities and flavonoid contents in the leaves of *C. punctata* sampled from coastal and inland populations. However, the intensity of UV radiation markedly increases at high altitude (Shibata, 1992; Murai *et al.*, 2009). Plants that occupy the

Fig. 1. *Campanula lasiocarpa* (left) and *C. chamissonis* (right).

highland area are thought to be adaptive to several environmental stresses (e.g., intense UV-B and cold) by accumulating UV-absorbing compounds such as flavonoids and phenolic acids (Spitaler *et al.*, 2006; Murai *et al.*, 2009).

In this study, we analyzed two alpine *Campanula* species for their flavonoids, and describe the chemotaxonomical and ecological significance of their compounds.

Materials and Methods

Plant materials

Campanula lasiocarpa was collected from Mt. Norikura, Hida Mountains, Nagano Prefecture, Japan in September 2005, August 2006 and August 2011, Mt. Chokai, Asahi Mountains, Yamagata Prefecture and Mt. Aka-dake, Daisetsu Mountains, Hokkaido, Japan in August 2012. On the other hand, *Campanula chamissonis* was collected from Mt. Norikura and Mt. Hoken-dake, Kiso Mountains, Nagano Prefecture, Japan in July–August 2011, Mt. Gassan, Dewa Mountains, Yamagata Prefecture and Mt. Shari-dake, Shiretoko Peninsula Range, Hokkaido, Japan in August 2012. Plant collections were carried out under the permissions of Nagano, Yamagata and Hokkaido Prefectures, and the permission of the Ministry of Environment, Japan (No. 050823005, No. 060719001, No. 110624004, No. 120720004 and No. 120620003). Voucher specimens were deposited in the Herbarium of National Museum of Nature and Science, Japan (TNS).

Extraction and separation

Fresh leaves of *C. lasiocarpa* (1.2 g), *C. chamissonis* (2.1 g) were extracted with MeOH. 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), and further isolated by preparative HPLC using Inertsil ODS-4 column (5 μm particle material, I.D. 10 × 250 mm, GL Sciences Inc., Japan), at flow-rate: 3.0 ml min^{-1}, detection: 350 nm, and eluent: MeCN/H_2O/HCOOH (18:81:1).

Quantitative HPLC analysis of flavonoids

Fresh leaves (0.2 g) of each population in nature and the plants grown in Tsukuba, Ibaraki Prefecture, Japan were extracted with 4 ml MeOH. After filtration with Maisyoridisc H-13-5 (Tosoh), the extracts were analyzed using a Shimadzu HPLC system with *L*-column2 ODS (3 μm particle material, I.D. 3.0 × 150 mm, Chemicals Evaluation and Research Institute, Japan), at flow-rate: 0.3 ml min^{-1}, detection: 190–400 nm and eluents: MeCN/H_2O/H_3PO_4 (16:84:0.2), injection: 2 μl.

Liquid chromatograph-mass spectra (LC-MS)

LC-MS were measured with Shimadzu

LC-MS system using 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 (1:20:79), injection: 3 μl, ESI$^+$ 4.5 kV, ESI$^-$ 3.5 kV, 250°C.

Identification of compounds

In this research, nine flavonoids from the leaves of *C. lasiocarpa* and *C. chamissonis* were identified. Flavonoids **1**, **2**, **5–7** were characterized by UV spectroscopy according to Mabry *et al.* (1970), LC-MS, HPLC comparisons with authentic standards and characterization of acid hydrolysates (in 12% HCl, 100°C, 30 min). In the case of flavonoid **1**, its chemical structure was further estimated by ^{1}H and ^{13}C NMR. Flavonoids **3**, **4**, **8** and **9** were minor compounds and identified by HPLC comparison with authentic specimens and LC-MS. UV, acid hydrolysis, LC-MS, ^{1}H and ^{13}C NMR data of the isolated compounds are as follows.

Luteolin 7-*O*-gentiobioside (**1**). Pale yellow powder. UV: λmax (nm) MeOH 256, 266, 350; + NaOMe 266, 389 (inc.); + $AlCl_3$ 273, 427; + $AlCl_3$/HCl 267sh, 270, 295, 362, 382sh; + NaOAc 260sh, 402; + NaOAc/H_3BO_3 258, 373. HPLC: Rt 7.7 min. Acid hydrolysis: luteolin and glucose. LC-MS: *m/z* 611 $[M+H]^+$, 609 $[M-H]^-$ (luteolin + 2 mol glucose), 449 $[M-162+H]^+$ (luteolin + 1 mol glucose), and 287 $[M-324+H]^+$, 285 $[M-324-H]^-$ (luteolin). ^{1}H NMR (600 MHz, pyridine-d_5): δ 13.64 (1H, *s*, 5-OH), 8.02 (1H, *d*, J = 2.2 Hz, H-2′), 7.60 (1H, *dd*, J = 2.2 and 8.4 Hz, H-6′), 7.43 (1H, *d*, J = 8.3 Hz, H-5′), 7.07 (1H, *d*, J = 2.1 Hz, H-8), 6.91 (1H, *s*, H-3), 6.91 (1H, *d*, J = 2.2 Hz, H-6), 5.76 (1H, *d*, J = 7.7 Hz, *i*-glucosyl H-1), 5.09 (1H, *d*, J = 7.7 Hz, *t*-glucosyl H-1), 4.83 (1H, *d*, J = 10.5 Hz, *i*-glucosyl H-6a), 4.55 (1H, *dd*, J = 2.1 and 11.8 Hz, *t*-glucosyl H-6a) 4.41 (1H, *m*, *t*-glucosyl H-3), 4.40 (1H, *m*, *i*-glucosyl H-6b), 4.39 (1H, *m*, *i*-glucosyl H-4), 4.38 (1H, *m*, *i*-glucosyl H-3), 4.36 (1H, *m*, *t*-glucosyl H-6b), 4.29 (1H, *m*, *i*-glucosyl H-2), 4.28 (1H, *m*, *t*-glucosyl H-4), 4.27 (1H, *m*, *t*-glucosyl H-5), 4.17 (1H, *t*, J = 16.5 Hz, *t*-glucosyl H-2), 3.95 (1H, *m*, *i*-glucosyl H-5). ^{13}C NMR (150 MHz, pyridine-d_5): δ (luteolin) 162.5 (C-2), 104.1 (C-3), 182.9 (C-4), 164.1 (C-5), 100.8 (C-6), 165.5 (C-7), 95.6 (C-8), 157.9 (C-9), 106.6 (C-10), 122.6 (C-1′), 117.0 (C-2′), 147.7 (C-3′), 152.0 (C-4′), 114.7 (C-5′), 120.0 (C-6′); δ (*i*-glucose) 102.1 (C-1), 74.7 (C-2), 77.5 (C-3), 70.8 (C-4), 78.6 (C-5), 69.9 (C-6); δ (*t*-glucose) 105.6 (C-1), 75.2 (C-2), 78.2 (C-3), 71.7 (C-4), 78.4 (C-5), 62.7 (C-6).

Luteolin 7-*O*-glucoside (**2**). Pale yellow powder. UV: λmax (nm) MeOH 255, 266, 348; + NaOMe 266, 389 (inc.); + $AlCl_3$ 273, 427; + $AlCl_3$/HCl 266sh, 272, 294, 360, 383sh; + NaOAc 259, 402; + NaOAc/H_3BO_3 259, 372. HPLC: Rt 13.8 min. Acid hydrolysis: luteolin and glucose. LC-MS: *m/z* 449 $[M+H]^+$, 447 $[M-H]^-$ (luteolin + 1 mol glucose) and 287 $[M-162+H]^+$ (luteolin).

Apigenin 7-*O*-glucoside (**3**). HPLC: Rt 24.7 min. LC-MS: *m/z* 433 $[M+H]^+$, 431 $[M-H]^-$ (apigenin + 1 mol glucose) and 271 $[M-162+H]^+$, 269 $[M-162-H]^-$ (apigenin).

Luteolin (**4**). HPLC: Rt 43.7 min. LC-MS: *m/z* 287 $[M+H]^+$, 285 $[M-H]^-$ (luteolin).

Quercetin 3-*O*-galactoside (**5**). UV: λmax (nm) MeOH 257, 266sh, 360; + NaOMe 273, 329, 412 (inc.); + $AlCl_3$ 274, 435; + $AlCl_3$/HCl 269, 299, 365sh, 405sh; + NaOAc 274, 325, 391; + NaOAc/H_3BO_3 262, 381. HPLC: Rt 12.1 min. Acid hydrolysis: quercetin and galactose. LC-MS: *m/z* 465 $[M+H]^+$, 463 $[M-H]^-$ (quercetin + 1 mol galactose) and 303 $[M-162+H]^+$, 301 $[M-162-H]^-$ (quercetin).

Quercetin 3-*O*-glucoside (**6**). UV: λmax (nm) MeOH 257, 267sh, 357; + NaOMe 272, 330, 409 (inc.); + $AlCl_3$ 270, 434; + $AlCl_3$/HCl 270, 300, 365sh, 400sh; + NaOAc 273, 325, 400; + NaOAc/H_3BO_3 262, 297, 378. HPLC: Rt 13.0 min. Acid hydrolysis: quercetin and glucose. LC-MS: *m/z* 465 $[M+H]^+$, 463 $[M-H]^-$ (quercetin + 1 mol glucose) and 303 $[M-162+H]^+$ (quercetin).

Quercetin 3-*O*-glucuronide (**7**). UV: λmax (nm) MeOH 257, 267sh, 359; + NaOMe 273, 329, 408 (inc.); + $AlCl_3$ 271, 420; + $AlCl_3$/HCl

269, 300, 363sh, 396sh; +NaOAc 273, 325, 391; $+NaOAc/H_3BO_3$ 262, 296, 379. HPLC: Rt 13.2 min. Acid hydrolysis: quercetin and glucuronic acid. LC-MS: *m/z* 479 $[M+H]^+$, 477 $[M-H]^-$ (quercetin + 1 mol glucuronic acid) and 303 $[M-176+H]^+$ (quercetin).

Kaempferol 3-*O*-galactoside (**8**). HPLC: Rt 17.6 min. Acid hydrolysis: kaempferol and galactose. LC-MS: *m/z* 449 $[M+H]^+$, 447 $[M-H]^-$ (kaempferol+1 mol galactose) and 287 $[M-162+H]^+$, 285 $[M-162-H]^-$ (kaempferol).

Kaempferol 3-*O*-glucoside (**9**). HPLC: Rt 21.1 min. Acid hydrolysis: kaempferol and glucose. LC-MS: *m/z* 449 $[M+H]^+$, 447 $[M-H]^-$ (kaempferol + 1 mol glucose) and 287 $[M-162+H]^+$, 285 $[M-162-H]^-$ (kaempferol).

Results and Discussion

Flavonoid composition

Two major flavones, luteolin 7-*O*-gentiobioside (**1**) and luteolin 7-*O*-glucoside (**2**), and two minor flavones, apigenin 7-*O*-glucoside (**3**) and luteolin (**4**) were isolated from the leaves of *C. lasiocarpa* (Fig. 2). On the other hand, three major flavonols, quercetin 3-*O*-galactoside (**5**), quercetin 3-*O*-glucoside (**6**) and quercetin 3-*O*-glucuronide (**7**), and two minor flavonols, kaempferol 3-*O*-galactoside (**8**) and kaempferol

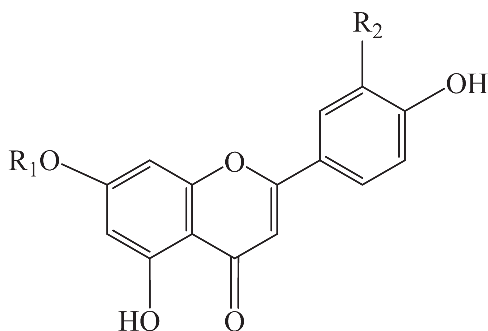

Fig. 2. Chemical structures of flavones from *Campanula lasiocarpa*. **1**: luteolin 7-*O*-gentiobioside (R_1 = gentiobiosyl, R_2 = OH), **2**: luteolin 7-*O*-glucoside (R_1 = glucosyl, R_2 = OH), **3**: apigenin 7-*O*-glucoside (R_1 = glucosyl, R_2 = H), **4**: luteolin (R_1 = H, R_2 = OH).

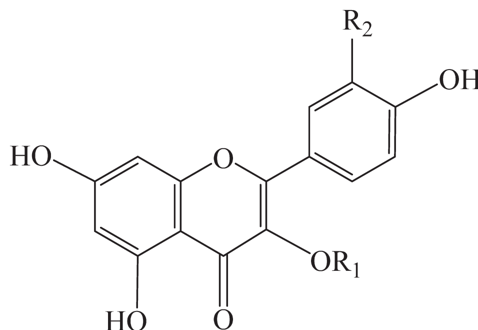

Fig. 3. Chemical structures of flavonols from *Campanula chamissonis*.
5: quercetin 3-*O*-galactoside (R_1 = galactosyl, R_2 = OH), **6**: quercetin 3-*O*-glucoside (R_1 = glucosyl, R_2 = OH), **7**: quercetin 3-*O*-glucuronide (R_1 = glucuronyl, R_2 = OH), **8**: kaempferol 3-*O*-galactoside (R_1 = galactosyl, R_2 = H), **9**: kaempferol 3-*O*-glucoside (R_1 = glucosyl, R_2 = H).

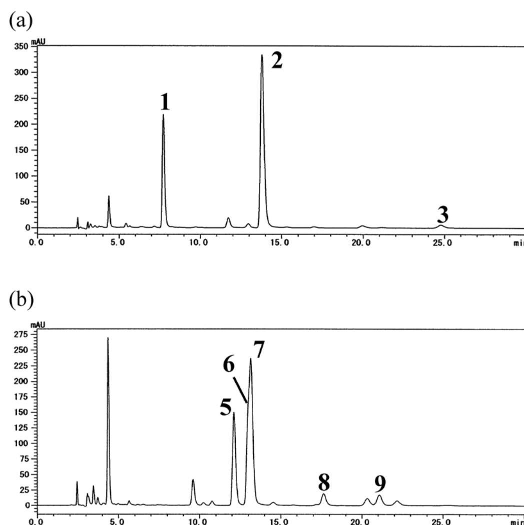

Fig. 4. HPLC patterns of MeOH extracts from two alpine *Campanula* species in Japan.
(a) *C. lasiocarpa*. (b) *C. chamissonis*. **1**: luteolin 7-*O*-gentiobioside, **2**: luteolin 7-*O*-glucoside, **3**: apigenin 7-*O*-glucoside, **5**: quercetin 3-*O*-galactoside, **6**: quercetin 3-*O*-glucoside, **7**: quercetin 3-*O*-glucuronide, **8**: kaempferol 3-*O*-galactoside, **9**: kaempferol 7-*O*-glucoside. Peak of **4** appears at 43.7 min of *C. lasiocarpa* (a).

3-*O*-glucoside (**9**) were found in *C. chamissonis* (Fig. 3). MeOH extracts from each species of all populations were analyzed by HPLC (Fig. 4). Geographical chemical variation of *C. chamissonis* was not observed among the plants collected from Mts. Norikura, Hoken-dake, Gassan and Shari-dake. *C. lasiocarpa* collected from Mts. Norikura, Chokai and Aka-dake also showed the same flavonoid composition. Furthermore, HPLC profiles of *C. lasiocarpa* in Mt. Norikura in 2005, 2006 and 2011 showed the same pattern. These results suggested that flavonoids are stable chemotaxonomic markers in both species.

Flavonoids and phylogeny of the genus Campanula

In this study, four flavones and five flavonols were isolated from *C. lasiocarpa* and *C. chamissonis*, respectively. Though these flavonoids have previously been reported from the aerial parts of *Campanula* species (Dzhumyrko, 1973, 1974; Teslov and Blinova, 1974; Teslov, 1976), the flavonoid composition of *C. lasiocarpa* and *C. chamissonis* were elucidated for the first time. Dzhumyrko (1985) suggested that flavones and flavonols are alternatively distributed in the subsection level within the genus *Campanula*. Both species surveyed in this study have been previously classified into the section *Rapunculus* (Okazaki, 1997), but the flavonoid composition of them was clearly different from each other. The flavonoid composition of *C. chamissonis* was similar to that of *C. puncutata*, which is distributed in Japan and contains quercetin and kaempferol glycosides (Hashiba *et al.*, 2006).

Recently, ITS and *trnL-F* data revealed that *C. chamissonis* belongs to the *Campanula* s. str. clade along with *C. punctata* (Roquet *et al.*, 2008), whereas *C. lasiocarpa* belongs to the *Rapunculus* clade as before (Wendling *et al.*, 2011). *C. lasiocarpa* and *C. chamissonis* are morphologically-resemble, and are growing in similar environmental condition in alpine zone. Our chemical data also suggested that both species are not closely-related within the genus *Campanula* in addition to the gene phylogeny mentioned above. Furthermore, four species belonging to the *Campanula* s. str. contained flavonol glycosides, whereas nine species belonging to the *Rapunculus* contained flavone glycosides.

Ecological significance

C. lasiocarpa and *C. chamissonis* mainly contained luteolin and quercetin glycosides, respectively. They are B-ring *ortho*-dihydroxy lated flavonoids, which have been reported as efficient antioxidants in flavonoid compounds (Rice-Evans *et al.*, 1996; Pietta, 2000). Several plants growing in higher altitude synthesize such B-ring *ortho*-dihydroxy lated flavonoids (Spitaler *et al.*, 2006; Murai *et al.*, 2009; Murai and Iwashina, 2010). In the present study, we collected *C. chamissonis* at ca. 2,600 m elevation in Mt. Norikura and then cultivated it in lowland, Tsukuba city (25 m alt.) with the same method as described by Murai and Iwashina (2010). Interestingly, total flavonoid content of *C. chamissonis* at alpine habitat (n = 3) was 1.57 times higher than that of the plants cultivated at low altitude (n = 3) by HPLC analysis. These compounds may play an important role in protection against several environmental stresses in high altitudes. Further studies (e.g., physiological experiment) using alpine plants are required for the understanding of their functions.

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