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Phenolic Compounds in the Leaves of *Pedicularis chamissonis* in Japan

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Abstract We analyzed phenolic compounds in the leaves of *Pedicularis chamissonis* which were obtained from a wide range of Japan. Acteoside and luteolin 7-*O*-glucuronide were found as major compounds in the all plants from various populations. Luteolin 7-*O*-glucoside was additionally found in the plants sampled from in the southern part of their habitats. Fujii *et al.* (2013) divided the plants into two species, i.e., *P. chamissonis* (Northern type) and *P. japonica* (Central Honshu type) based on DNA, morphological and ecological data. Our chemical data seem to support this classification from the view point of chemotaxonomy. In addition, ecological significance of the isolated compounds is also described.

Key words: chemical ecology, chemotaxonomy, flavonoids, *Pedicularis chamissonis*, *Pedicularis japonica*, phenylpropanoid.

Introduction

The genus *Pedicularis* (Orobanchaceae) consists of ca. 600 species that is mainly distributed in mountain area of Central and East Asia (Mabberley, 2008). Of their species, *P. chamissonis* Steven is a perennial herb and widely distributed in subalpine and alpine zone of Japan. The plant shows a variety of morphological features, and the diversity resulting in several intraspecific taxa (Yamazaki, 1993). Fujii *et al.* (1997) have reported that the two major clades of the species, i.e., Central Honshu clade and Northern clade by gene phylogeny using chloroplast DNA. Subsequently, they suggested that the plants should be treated Central Honshu type as *P. japonica* and Northern type as *P. chamissonis* from the evidence obtained of genetic, morphological and ecology data (Fujii *et al.*, 2013).

Phenolic compounds such as flavonoids and phenylpropanoids are known as UV protectants, antioxidants and so on. The intensity of UV radiation markedly increases at high altitude (Shibata 1992; Murai *et al.*, 2009). Plants that occupy the 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 phenylpropanoids (Spitaler *et al.*, 2006; Murai *et al.*, 2009, 2015). In addition, these compounds are also used as chemical markers of plant species. Some studies have been carried out on phenolics of *Pedicularis* (Yoshitama *et al.*, 1980; Zimin and Zhongjian, 1991; Di *et al.*, 2004; Chu *et al.*, 2007), but the number of related study is limited.

In this study, we analyzed *P. chamissonis* growing in Central Japan to Hokkaido for their phenolic compounds, and describe the chemotaxonomical and ecological significance of their compounds.

Materials and Methods

Plant materials

Pedicularis chamissonis was collected from Nagano to Hokkaido (S1–S8), Japan (Fig. 1),

Fig. 1. Collection sites of *Pedicularis chamissonis*. S1: Mt. Hoken-dake. S2: Mt. Norikura-dake. S3: Mt. Arakawa-naka-dake. S4: Mt. Jizo-dake. S5: Mt. Gassan. S6: Mt. Chokai. S7: Mt. Hayachine. S8: Mt. Shari-dake.

i.e., S1: Mt. Hoken-dake, Kiso Mountains, Nagano Prefecture, S2: Mt. Norikura, Hida Mountains, Nagano Prefecture, S3: Mt. Arakawa-naka-dake, Akaishi Mountains, Shizuoka Prefecture, S4: Mt. Jizo-dake, Akaishi Mountains, Yamanashi Prefecture, S5: Mt. Gassan, Dewa Mountains, Yamagata Prefecture, S6: Mt. Chokai-san, Dewa Mountains, Yamagata Prefecture, S7: Mt. Hayachine, Iwate Prefecture, and S8: Mt. Shari-dake, Hokkaido, in summer of 2011-2014. Plant collections were carried out under the permissions of each prefecture, the Ministry of Environment, the Ministry of Agriculture, Forestry and Fisheries, and the Agency for Cultural Affairs, Japan, and Tokushu Tokai Paper Co., Ltd. and Dewa Sanzan Shrine. Voucher specimens were deposited in the Herbarium of National Museum of Nature and Science, Japan (TNS).

Extraction and separation

Fresh leaves of each individual of *P. chamissonis* (1.5 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).

Quantitative HPLC analysis of phenolic compounds

Fresh leaves (0.2 g) from three individuals of each population 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* (5 μm particle material, I.D. 6.0 × 150 mm, Chemicals Evaluation and Research Institute, Japan), at flow-rate: 1.0 mL min^{-1}, detection: 190–400 nm and eluents: MeCN/H_2O/H_3PO_4 (20 : 80 : 0.2), injection: 10 μL.

Liquid chromatograph-mass spectra (LC-MS)

LC-MS were measured with Shimadzu LC-MS system using *L-column2 ODS* (3 μm particle material, I.D. 2.1 × 100 mm, Chemicals Evaluation and Research Institute), 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, one phenylpropanoid, acteoside (**1**) (Fig. 3), and two flavonoids, 7-*O*-glucoside (**2**) and 7-*O*-glucuronide (**3**) of luteolin (Fig. 4), were identified by UV spectroscopy (Mabry *et al.*, 1970), acid hydrolysis, LC-MS and HPLC comparisons with authentic standards from the leaves of *Plantago* spp. (Murai *et al.*, 2008, 2009). UV, acid hydrolysis, HPLC and LC-MS data of the isolated compounds are as follows.

Acteoside (verbascoside) (**1**). White powder. UV: λmax (nm) MeOH 292, 330; + NaOMe 380; + $AlCl_3$ 300, 361; + $AlCl_3$/HCl 292, 330; + NaOAc 290, 340; + NaOAc/H_3BO_3 295, 355.

Fig. 2. *Pedicularis chamissonis* in Mt. Norikura, Nagano Prefecutre (left) and Mt. Chokai, Yamagata Prefecture (right).

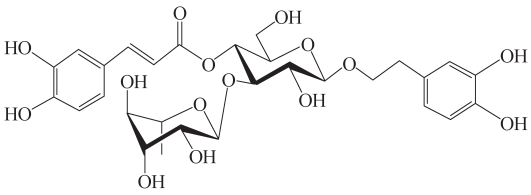

Fig. 3. Chemical structure of acteoside from *Pedicularis chamissonis.*

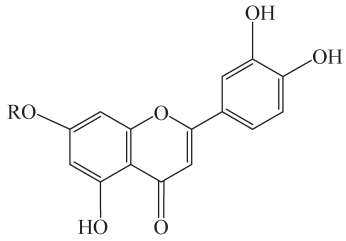

Fig. 4. Chemical structures of flavones from *Pedicularis chamissonis*. **2**: luteolin 7-*O*-glucoside (R = glucosyl), **3**: luteolin 7-*O*-glucuronide (R = glucuronyl).

HPLC: Rt 7.4 min. LC-MS: *m/z* 647 $[M + Na]^+$, 623 $[M - H]^-$.

Luteolin 7-*O*-glucoside (**2**). Pale yellow powder. UV: λmax (nm) MeOH 255, 267, 343; + NaOMe 267, 385 (inc.); + $AlCl_3$ 273, 426; + $AlCl_3$/HCl 262sh, 276, 297 362, 392sh; + NaOAc 260, 400; + $NaOAc/H_3BO_3$ 260, 373. HPLC: Rt 8.0 min. Acid hydrolysis: luteolin and glucose. LC-MS: *m/z* 449 $[M + H]^+$, 447 $[M - H]^-$ (luteolin + 1 mol glucose), and 287 $[M - 162 + H]^+$, 285 $[M - 162 - H]^-$ (luteolin).

Luteolin 7-*O*-glucuronide (**3**). Pale yellow powder. UV: λmax (nm) MeOH 256, 268, 348; + NaOMe 266, 389 (inc.); + $AlCl_3$ 274, 296sh, 427; + $AlCl_3$/HCl 263sh, 272, 295, 364, 386sh; + NaOAc 260, 401; + $NaOAc/H_3BO_3$ 260, 373. HPLC: Rt 8.6 min. Acid hydrolysis: luteolin and glucuronic acid. LC-MS: *m/z* 463 $[M + H]^+$, 461 $[M - H]^+$ (luteolin + 1 mol glucuronic acid), and 287 $[M - 176 + H]^+$, 285 $[M - 176 - H]^-$ (luteolin).

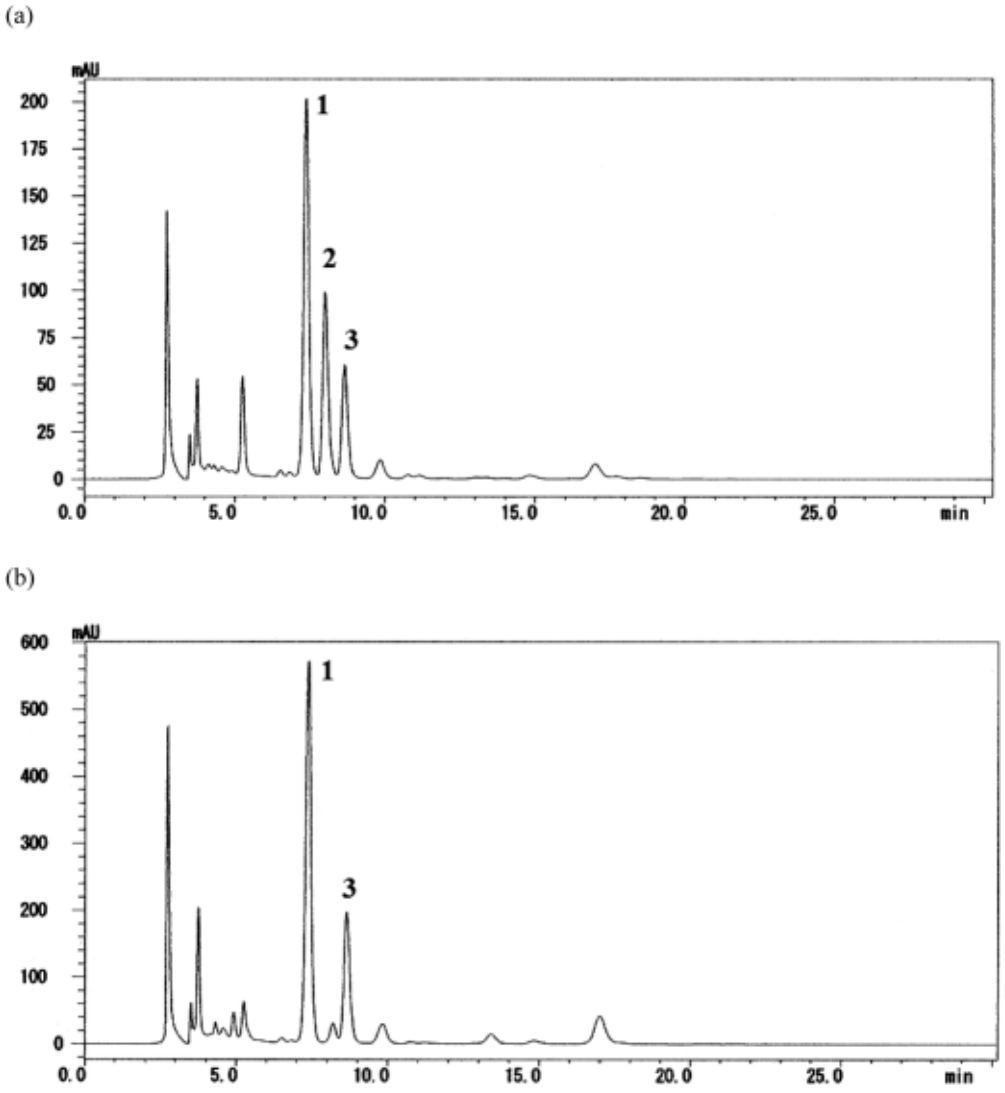

Fig. 5. HPLC patterns of MeOH extracts from *Pedicularis chamissonis*. (a) Central Honshu type. (b) Northern type. **1**: acteoside, **2**: luteolin 7-*O*-glucoside, **3**: luteolin 7-*O*-glucuronide.

Results and Discussion

The composition of phenolic compounds

One phenylpropanoid, acteoside (**1**) and two flavones, luteolin 7-*O*-glucoside (**2**) and 7-*O*-glucuronide (**3**) were isolated from the leaves of *P. chamissonis*. However, the presence or absence of **2** is different between the northern and southern populations (Fig. 5; Table 1). Compound **2** was exclusively produced by the plants sampled from S1 (Mt. Hoken-dake) to S5 (Mt. Gassan), while the other populations locating in the more northern part of Japan lacked this compound. The other peaks on HPLC chromatograms (Fig. 5) were presumed as other phenylpropanoids and flavonoids from their UV spectra obtained by photo diode array HPLC detector, and they were not identified in this study. The concentration of these minor compounds was varied in low level among populations.

Table 1. Phenolic compounds and DNA type of *Pedicularis chamissonis*

Population	Phenolic compounds			DNA type*
	1	2	3	
Mt. Hoken-dake (S1)	+	+	+	CHT
Mt. Norikura-dake (S2)	+	+	+	CHT
Mt. Arakawa-naka-dake (S3)	+	+	+	CHT
Mt. Jizo-dake (S4)	+	+	+	CHT
Mt. Gassan (S5)	+	+	+	CHT/NT
Mt. Chokai (S6)	+	−	+	CHT/NT
Mt. Hayachine (S7)	+	−	+	NT
Mt. Shari-dake (S8)	+	−	+	NT

1: acteoside, 2: luteolin 7-*O*-glucoside, 3: luteolin 7-*O*-glucuronide. + : presence. − : absence.

*According to Fujii *et al.* (2013). CHT: Central Honshu type, NT: Northern type.

Flavonoids and phylogeny of Pedicularis chamissonis

Flavonoids are more stable chemotaxonomic marker than phenylpropanoids among phenolic compounds (Sahin *et al.*, 2007). Two chemotypes (presense or absence of **2**) were found in *P. chamissonis* of Japan. These chemotypes seem to support the classification by Fujii *et al.* (2013) that divided *P. chamissonis* in Japan into *P. japonica* (Central Honshu type) and *P. chamissonis* (Northern type). According to Fujii *et al.* (2013), two species, *P. japonica* and *P. chamissonis* are both distributed in Mt. Gassan and Mt. Chokai. In the present study, we collected the plants from their large population in each mountain and another chemotype (species) was not detected in HPLC analysis, but some plants at the other populations in those mountains might possess the opposite chemotype.

Ecological significance

In this study, acteoside and luteolin glycosides were isolated from *Pedicularis chamissonis*. Both phenolics are *ortho*-dihydroxylated phenolics that are strong antioxidants among related compounds (Rice-Evans *et al.*, 1996; Pietta, 2000; Nishibe, 2002). Interestingly, several plants growing in higher altitudes synthesize such *ortho*-dihydroxylated phenolics (Spitaler *et al.*, 2006; Murai and Iwashina, 2010; Murai *et al.*, 2009, 2015). These compounds may play an important role in protection against several environmental stresses (e.g. intense UV light and cold) in high altitudes. Further studies, e.g. physiological experiment, using alpine plants are required for the understanding of their functions.

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