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Phenolic Compounds from *Artemisia kitadakensis* Endemic to Japan

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Abstract Phenolic compounds in the leaves of *Artemisia kitadakensis*, a rare and endangered alpine species endemic to Japan, were surveyed. Two phenylpropanoids, chlorogenic acid and isochlorogenic acid A, and five flavonoids, luteolin 7-*O*-glucoside, 7-*O*-glucuronide and 7-*O*-pentosylhexoside, chrysoeriol 7-*O*-glucuronide and quercetin 3-*O*-glucoside, were isolated from *A. kitadakensis* leaves. HPLC analysis revealed that the overall content of bioactive phenolic compounds decreased when plants were cultivated in a glasshouse, compared to plants grown in their natural habitat. This suggests that some of these phenolics may act as anti-stress compounds in the harsh alpine environment. Insights into the glasshouse cultivation of *A. kitadakensis* are also discussed.

Keywords: *Artemisia kitadakensis*, Asteraceae, chemical ecology, chemical profiling, phenolic compounds.

Introduction

The genus *Artemisia* consists of 540 species (Mabberley, 2017), and 30 species are growing in Japan (Koyama, 1995). Among them, *A. kitadakensis* H.Hara et Kitam. (Fig. 1) is distributed only in a few sites in the alpine zone (2600–3000 m elev.) of Akaishi Mountains, Central Japan. Owing to its limited distribution, the plant has been listed as endangered species (EN) by the Ministry of the Environment, Japan (2015).

Artemisia species are rich sources of bioactive natural products such as terpenoids and flavonoids (Tan, *et al.*, 1998). Recently, flavonoid profiling has been carried out using Japanese *Artemisia* species (Uehara *et al.*, 2018). However, chemical constituents of *A. kitadakensis* have not been surveyed to date. On the other hand, the significant decreases of phenolic compounds have been observed in some common Japanese alpine plants, *Geum calthifolium* var. *nipponicum* (F.Bolle) Ohwi (Rosaceae) and *Campanula*

Fig. 1. *Artemisia kitadakensis* in Mt. Kita-dake, Yamanashi Prefecture.

chamissonis Fed. (Campanulaceae) cultivated in lowland (Murai and Iwashina, 2010; Murai *et al.*, 2014). To clarify the biological properties concerning anti-stress components of *A. kitadakensis*, the variation of phenolic compounds under cultivation was also surveyed.

Materials and Methods

Plant materials

Artemisia kitadakensis was collected from Mt. Kita-dake, 2960 m elev., Yamanashi Prefecture, Japan in July 2015 and September 2019, and Mt. Senmai-dake, 2860 m elev., Shizuoka Prefecture, Japan in August 2015. Plant collections were carried out under the permissions of Yamanashi Prefecture, the Ministry of the Environment, Japan, and Tokushu Tokai Paper Co., Ltd. Voucher specimens were deposited in the herbarium of National Museum of Nature and Science, Japan (TNS).

Plant cultivation

A. kitadakensis sampled from Mt. Kita-dake was cultivated in a glasshouse of Tsukuba Botanical Garden (ca. 25 m elev.), National Museum Nature and Science, Tsukuba City, Ibaraki Prefecture, Japan. Plants were grown for two months at a photosynthetic photon flux density of ca. 150 μmol photons $m^{-2} s^{-1}$ during the daytime, and the temperatures regulated between 18℃ (night) and 25℃ (daytime). Relative humidity was kept around 50–60%. Mature leaves were randomly selected and sampled for chemical analysis. This experiment was repeated using three individuals. In addition to chemical analysis, propagation of the endangered species, *A. kitadakensis* was successful by division under the same growth conditions.

Extraction and separation of phenolic compounds

Fresh leaves (6.1 g) of *A. kitadakensis* were extracted with MeOH. The concentrated extracts were cleaned up by InertSep C18 (10 g/ 60 mL, GL Sciences Inc., Japan), and then applied to Preparative HPLC with an Inertsil ODS-4 column [5 μm particle material, I.D. 10 × 250 mm (GL Sciences Inc.)] using solvent system: HCOOH/MeCN/H_2O = 1 : 18 : 81. The compounds were purified by Sephadex LH-20 column chromatography (solvent system: 80% MeOH).

Quantitative HPLC analysis of phenolic compounds

Fresh leaves (0.2 g) from cultivation and natural habitats were extracted with 4 mL MeOH. High performance liquid chromatography

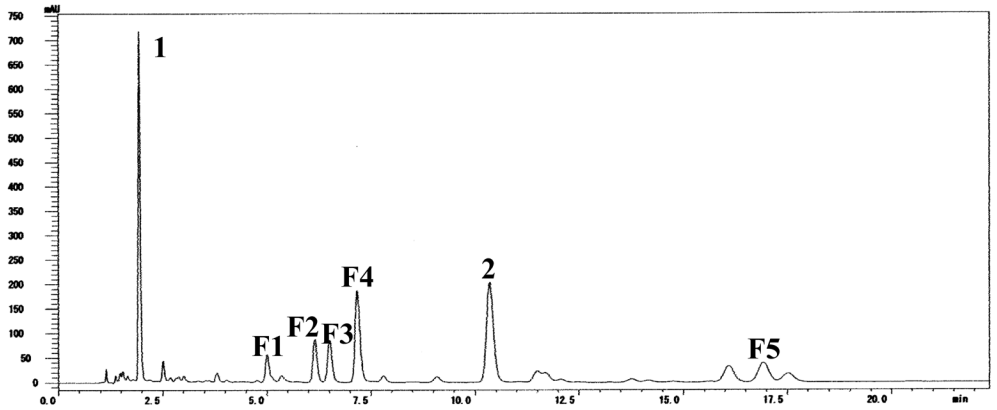

Fig. 2. HPLC chromatogram of MeOH extracts from *Artemisia kitadakensis*. **1**: chlorogenic acid, **2**: isochlorogenic acid A, **F1**: luteolin 7-*O*-pentosylhexoside, **F2**: quercetin 3-*O*-glucoside, **F3**: luteolin 7-*O*-glucoside, **F4**: luteolin 7-*O*-glucuronide and **F5**: chrysoeriol 7-*O*-glucuronide. Detection: 340 nm.

(HPLC) separation of the extracts filtered with GL Chromatodisk 13N (0.45 µm pore size, GL Sciences, Inc.) 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 0.8 mL min^{-1}, detection wavelength at 190–700 nm and MeCN/H_2O/H_3PO_4 (15 : 85 : 0.2) as eluent. Injection volume was 2 µL. HPLC chromatogram of phenolic compounds is shown in Fig. 2.

Liquid chromatograph-mass spectra (LC-MS)

LC-MS was 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 : 20 : 79), injection: 3 µL, ESI^+ 4.5 kV, ESI^- 3.5 kV, 250°C.

Identification of compounds

In this research, two phenylpropanoids (**1** and **2**) (Fig. 3a) and five flavonoids (**F1–F5**) (Fig. 3b) were isolated from the leaves of *A. kitadakensis*. Phenylpropanoids were characterized by UV spectroscopy, LC-MS and HPLC comparisons with authentic specimens. In the case of isolated flavonoids, their chemical structures were further estimated by UV spectroscopy according to Mabry *et al.* (1970) and characterization of acid hydrolysates (in 12% HCl, 100°C, 30 min). Chemical data of the isolated compounds are as follows.

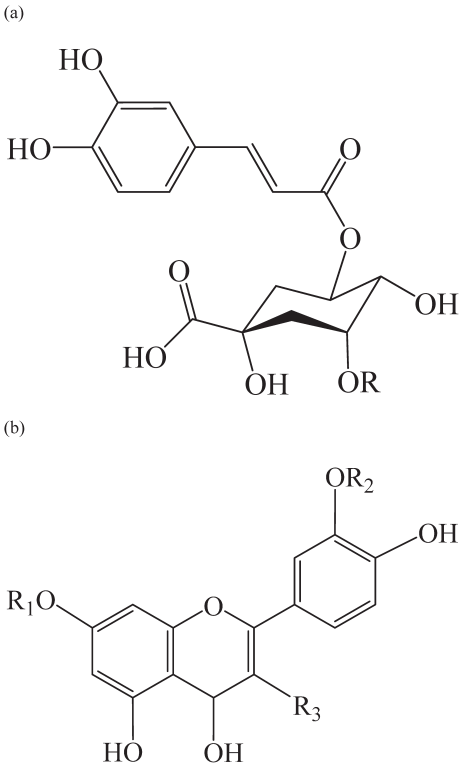

Fig. 3. Chemical structures of phenolic compounds from *Artemisia kitadakensis*.
(a) **1**: chlorogenic acid (R = H), **2**: isochlorogenic acid A (R = caffeoyl). (b) **F1**: luteolin 7-*O*-pentosylhexoside (R_1 = pentosylhexosyl, $R_2 = R_3 = H$), **F2**: quercetin 3-*O*-glucoside ($R_1 = R_2 = H$, R_3 = glucosyl), **F3**: luteolin 7-*O*-glucoside (R_1 = glucosyl, $R_2 = R_3 = H$), **F4**: luteolin 7-*O*-glucoronide (R_1 = glucuronyl, $R_2 = R_3 = H$), **F5**: chrysoeriol 7-*O*-glucoside (R_1 = glucosyl, $R_2 = CH_3$, $R_3 = H$).

Chlorogenic acid (3-Caffeolylquinic acid) (**1**). Clear solution. UV: λmax (nm) MeOH 300sh, 329. HPLC: Rt 1.9 min. LC-MS: *m/z* 355 $[M + H]^+$, 353 $[M - H]^-$.

Isochlorogenic acid A (3,5-Dicaffeolylquinic acid) (**2**). Clear solution. UV: λmax (nm) MeOH 301sh, 329. HPLC: Rt 10.3 min. LC-MS: *m/z* 515 $[M - H]^-$.

Luteolin 7-*O*-pentosylhexoside (**F1**). Pale yellow solution. UV: λmax (nm) MeOH 255, 265, 347; + NaOMe 267, 388 (inc.); + $AlCl_3$ 273, 421; + $AlCl_3$/HCl 273, 295sh, 357sh, 387sh; + NaOAc 263, 402; + NaOAc/H_3BO_3 261, 372. HPLC: Rt 5.0 min. Acid hydrolysis: luteolin. LC-MS: *m/z* 581 $[M + H]^+$, 579 $[M - H]^-$ (luteolin + 1 mol hexose + 1 mol pentose), 449 $[M + H]^+$ (luteolin + 1 mol hexose), 287 $[M + H]^+$ (luteolin).

Quercetin 3-*O*-glucoside (**F2**). Pale yellow solution. UV: λmax (nm) MeOH 256, 266sh, 357; + NaOMe 273, 325, 410 (inc.); + $AlCl_3$ 274, 303sh, 415; + $AlCl_3$/HCl 270, 298sh, 362sh, 399sh; + NaOAc 272, 324, 396; + NaOAc/H_3BO_3 262, 302sh, 379. HPLC: Rt 6.1 min. Acid hydrysis: quercetin and glucose. LC-MS: *m/z* 465 $[M + H]^+$, 463 $[M - H]^-$ (quer-

cetin + 1 mol glucose), 303 $[M+H]^+$ (quercetin).

Luteolin 7-*O*-glucoside (**F3**). Pale yellow solution. UV: λmax (nm) MeOH 255, 268sh, 348; + NaOMe 265, 301sh, 389 (inc.); + $AlCl_3$ 273, 297sh, 409; + $AlCl_3$/HCl 266sh, 274, 295sh, 360sh, 388sh; + NaOAc 260, 365sh, 401; + NaOAc/H_3BO_3 260, 372. HPLC: Rt 6.5 min. Acid hydrolysis: luteolin and glucose. LC-MS: *m/z* 449 $[M+H]^+$, 447 $[M-H]^-$ (luteolin + 1 mol glucose), 287 $[M+H]^+$ (luteolin).

Luteolin 7-*O*-glucuronide (**F4**). Pale yellow solution. UV: λmax (nm) MeOH 255, 266sh, 348; + NaOMe 266, 302sh, 390 (inc.); + $AlCl_3$ 273, 302sh, 415; + $AlCl_3$/HCl 272, 296sh, 362sh, 387sh; + NaOAc 260, 403; + NaOAc/H_3BO_3 260, 373. HPLC: Rt 7.1 min. Acid hydrolysis: luteolin and glucuronic acid. LC-MS: *m/z* 463 $[M+H]^+$, 461 $[M-H]^-$ (luteolin + 1 mol glucuronic acid), 287 $[M+H]^+$ (luteolin).

Chrysoeriol 7-*O*-glucuronide (**F5**). Pale yellow solution. UV: λmax (nm) MeOH 267, 343; + NaOMe 262, 277sh, 393 (inc.); + $AlCl_3$ 263sh, 272, 298sh, 359sh, 385sh; + $AlCl_3$/HCl 260sh, 276, 296sh, 351, 382sh; + NaOAc 259, 406; + NaOAc/H_3BO_3 266, 348. HPLC: Rt 16.8 min. Acid hydrolysis: chrysoeriol and glucuronic acid. LC-MS: *m/z* 477 $[M+H]^+$, 475 $[M-H]^-$ (chrysoeriol + 1 mol glucuronic acid), 301 $[M+H]^+$ (chrysoeriol).

Results and Discussion

Two phenylpropanoids, chlorogenic acid (**1**) and isochlorogenic acid A (**2**), and five flavonoids, luteolin 7-*O*-pentosylhexoside (**F1**), quercetin 3-*O*-glucoside (**F2**), luteolin 7-*O*-glucoside (**F3**) luteolin 7-*O*-glucuronide (**F4**) and chrysoeriol 7-*O*-glucuronide (**F5**), were isolated from the leaves of *A. kitadakensis*. The amount of luteolin 7-*O*-pentosylhexoside (**F1**) was too small to identify the sugars. Though chlorogenic acid (**1**), quercetin 3-*O*-glucoside (**F2**) and luteolin 7-*O*-glucoside (**F3**) were common compounds in *Artemisia* species (Tan *et al.*, 1998; Iwashina, 2018; Uehara *et al.*, 2018), other compounds were isolated from Japanese *Artemisia* species for the first time. Chrysoeriol 7-*O*-glucuronide (**F5**) is a relatively rare flavone glycoside, but it was reported from *A. judaica* L. which is distributed from north Africa to Middle East (Saleh *et al.*, 1987). Furthermore, HPLC survey of MeOH extracts was carried out using a different eluent: MeCN/H_2O/H_3PO_4 (25 : 75 : 0.2). Flavone aglycones, luteolin and jaceosidin which were inferred from their UV spectra and retention times, were detected at trace levels. The phenolic composition in the leaves of *A. kitadakensis* was approximately the same within each population (Mt. Kita-dake and Mt. Senmai-dake). Plant collections in Mt. Kita-dake were carried out in 2015 and 2019, but there was no difference in phenolics composition between two years.

Main phenolics in the leaves of *A. kitadakensis* were *ortho*-dihydroxylated compounds. This accumulation pattern was quite similar to other alpine species such as *Campanula chamissonis*, *C. lasiocarpa* Cham. (Campanulaceae) (Murai *et al.*, 2014) and *Pedicularis chamissonis* Steven (Orobanchaceae) (Murai and Iwashina, 2015) in Japan. Such phenolics may act as anti-stress compounds against UV radiation and oxidative stresses in harsh alpine environment. Interestingly, total phenolic content of *A. kitadakensis* at alpine habitat (Mt. Kita-dake, n = 3) was 1.8 times higher than that of plants cultivated in a glasshouse (Tsukuba Botanical Garden, n = 3) by HPLC analysis. This quantitative variation of phenolic compounds between natural habitat (alpine zone) and cultivation site (lowland) was previously observed in *Geum calthifolium* var. *nipponicum* and *C. chamissonis*, which are widely distributed in the Japanese alpine zone (Murai and Iwashina, 2010; Murai *et al.*, 2014). It is also to be noted that the glasshouse cultivation of *A. kitadakensis* led to a 66% decrease in leaf flavonoid content, compared to natural habitat.

The synthesis of phenolic compounds is affected by several environmental factors, e.g. low temperature and UV radiation (Dixon and Paiva, 1995). Phenolics play important roles in chemical defenses of plants (Lattanzio *et al.*,

2006). In the present study, low temperature and UV radiation were almost excluded under glasshouse conditions. Because of the lower concentrations of phenolics, the plants cultivated in glasshouse may become vulnerable to some environmental stresses such as insect pests and pathogen attacks (Mazza *et al.*, 1999; Lattanzio *et al.*, 2006). Further studies (e.g. physiological experiment) are required for understanding the actual functions of phenolic compounds in *A. kitadakensis*.

Acknowledgments

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