Bull. Natl. Mus. Nat. Sci., Ser. B, 47(2), pp. 109–114, May 21, 2021

Phenolic Compounds from Three Alpine *Anaphalis* Species in Japan and Bhutan

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(Received 9 February 2021; accepted 24 March 2021)

Abstract Phenolic compounds of *Anaphalis alpicola* in Japan, and *A. nepalensis* and *A. cooperi* in Bhutan, were surveyed. Three phenylpropanoids (chlorogenic acid, isochlorogenic acid A, and isochlorogenic acid C) were isolated together with several flavonoids. A novel bioactive compound chlorogenic acid, and its derivative isochlorogenic acid A, were the main components in *Anaphalis* leaves. The flavonoid composition of *A. alpicola* was similar to that of *A. cooperi*. In addition, the overall content of phenolic compounds in the leaves of *A. alpicola* decreased when plants were cultivated in a glasshouse, compared to plants grown in their natural habitat. Here, we describe the chemotaxonomic and ecological significance of the isolated compounds.

Keywords: alpine plants, Asteraceae, chemical ecology, chemical profiling.

Introduction

The genus *Anaphalis* (Asteraceae), comprising ca. 110 species (Mabberley, 2017), is mainly distributed in Asia. Three species are found in Japan, including *A. alpicola* Makino (Fig. 1), which is endemic to Japan and distributed in alpine zones from Hokkaido to Central Honshu (Koyama, 1995). In Bhutan, two Himalayan species, *A. nepalensis* (Spreng.) Hand.-Mazz. and *A. cooperi* Grierson & Spring. (Fig. 1), are widely distributed. *Anaphalis cooperi* is endemic to Bhutan (Grierson and Long, 2001).

Anaphalis species are often used as traditional medicinal plants. Previous phytochemical investigations of the genus have led to the identification of a variety of compounds such as flavonoids and triterpenes; several new and rare flavonoids have been reported (Ikramov *et al.*, 1986; Wollenweber *et al.*, 1993; Wu *et al.*, 2003; Ren *et al.*, 2008, 2009; Joshi *et al.*, 2009; Abdullaeva *et al.*, 2016). However, a limited number of *Anaphalis* species were used for the chemical analyses, and little is known about the composition of a major class of the phenolic compound, phenylpropanoid.

On the other hand, plants that occupy alpine regions are thought to adapt to several environmental stresses (e.g., intense UV-B and cold) by accumulating UV-absorbing and antioxidative phenolics such as phenylpropanoids and flavonoids (Spitaler *et al.*, 2008; Murai *et al.*, 2009; Murai *et al.*, 2015).

In the present study, we surveyed the phenolic compounds of alpine *Anaphalis* species that grow in harsh environments, and compared the compositions in the plants distributed in two geographically distant regions (Japan and Bhutan).

Fig. 1. *Anaphalis alpicola* in Japan (left), *A. nepalensis* (center) and *A. cooperi* (right) in Bhutan.

Materials and Methods

Plant materials

Anaphalis alpicola was collected from Mt. Koakaishi-dake, 3,025 m elev., Shizuoka Prefecture, Japan in July 2014 and Mt. Kita-dake, 2,850 m elev., Yamanashi Prefecture, Japan in September 2017. Plant collections of *A. alpicola* were carried out under the permissions of Shizuoka and Yamanashi Prefectures, 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). The Himalayan species, *A. nepalensis* and *A. cooperi*, were collected from Dagala trek, 3,900–4,020 m elev., Thimphu, Bhutan in August 2019. Voucher specimen was deposited in the National Herbarium (THIM) of National Biodiversity Centre, Bhutan. Furthermore, a Japanese endemic species, *A. alpicola* was cultivated in a glasshouse at Tsukuba Botanical Garden, Tsukuba, Ibaraki Prefecture, Japan under the same growth conditions according to the methods of Murai (2020).

Extraction and separation

The dried aerial parts of *A. alpicola* (0.8 g), *A. nepalensis* (1.1 g) and *A. cooperi* (1.9 g) were extracted repeatedly with MeOH. The concentrated extracts were cleaned up by InertSep C18 (5 g/20 mL, GL Sciences Inc., Japan), and then applied to preparative high-performance liquid chromatography (HPLC) with InertSustain AQ-C18 column [5 µm particle material, I.D. 10 × 250 mm (GL Sciences Inc.)] using solvent systems: HCOOH/MeCN/H_2O = 1 : 20 : 79. The separated compounds were purified by Sephadex LH-20 column chromatography (solvent system: 80% MeOH).

HPLC analysis of phenolic compounds

To compare the composition of phenolic compounds, the dried leaves (0.1 g) of each *Anaphalis* species were extracted with 2 mL MeOH. In addition, to survey and compare the variation of phenolic compounds in the leaves of *A. alpicola* cultivated in the natural habitat as well as in a glasshouse at lower altitude, fresh leaves (0.1 g) were extracted with 2 mL MeOH. 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 PFP&C18 column [2.6 µm particle material, I.D. 4.6 mm × 100 mm (ChromaNik Technologies Inc., Japan)] at a flow rate of 0.8 mL min^{-1}, detection wavelength at 190–700 nm and H_3PO_4/MeCN/H_2O (0.2 : 15 : 85) as the eluent. Injection volume was 2 µL. HPLC chromatograms of phenolic compounds (at 340 nm) in the dried leaves of each species are shown in Fig. 2. The amount of each phenolic compound was estimated by the absor-

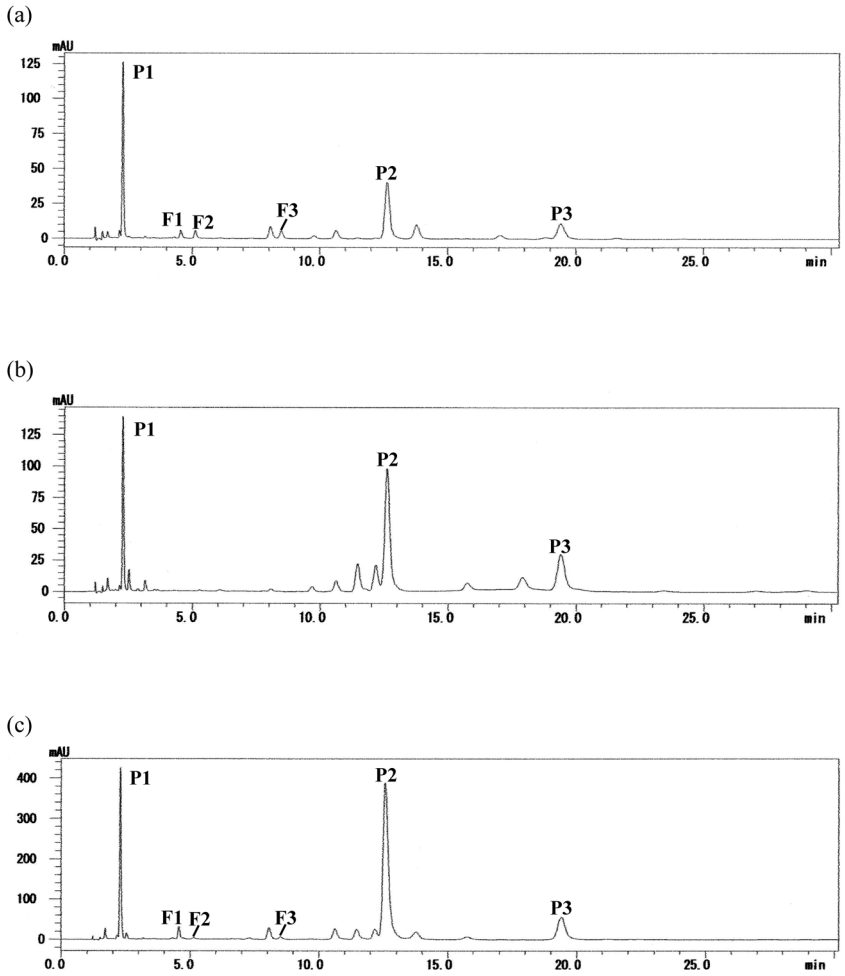

Fig. 2. HPLC chromatograms of MeOH extracts from the leaves of three *Anaphalis* species. (a) *A. alpicola* (Japan). (b) *A. nepalensis* (Bhutan). (c) *A. cooperi* (Bhutan). **P1**: chlorogenic acid (2.3 min), **P2**: isochlorogenic acid A (12.6 min), **P3**: isochlorogenic acid C (19.4 min), **F1**: quercetagetin 7-*O*-glucoside (4.6 min), **F2**: 6-hydroxyluteolin 7-*O*-glucoside (5.1 min), and **F3**: scutellarein 7-*O*-glucoside (8.5 min).

bance at 340 nm (mAU) of HPLC.

Liquid chromatograph-mass spectra (LC-MS)

LC-MS measurement of the isolated compounds was performed with a Shimadzu LC-MS system using a SunShell C18 column [2.6 µm particle material, I.D. 2.1 mm × 100 mm (ChromaNik Technologies Inc., Japan)] at a flow-rate of 0.2 mL min^{-1}, eluting with HCOOH/MeCN/H_2O (1 : 15 : 84), injection: 2 µL, ESI^+ 4.5 kV, ESI^- 3.5 kV, 250°C.

Identification of compounds

In this survey, three phenylpropanoids (**P1**–**P3**) and four flavonoids (**F1**–**F4**) were isolated from the aerial parts of three alpine *Anaphalis* species. Phenylpropanoids were characterized by UV spectroscopy, LC-MS, and HPLC comparisons with authentic samples of chlorogenic acid (MP Biomedicals, USA), isochlorogenic acid A and isochlorogenic acid C (ChemFaces, China). In the case of isolated flavonoids, their chemical structures were elucidated by UV spectroscopy according to Mabry *et al.* (1970), acid hydrolysates (in 12% HCl, 100°C, 30 min), LC-MS, and

HPLC comparisons with authentic samples of quercetagetin 7-*O*-glucoside (MedChemExpress, USA), 6-hydroxyluteolin 7-*O*-glucoside, and scutellarein 7-*O*-glucoside from the leaves of *Plantago asiatica* L. (Plantaginaceae) (Murai *et al.*, 2009), and quercetin 3-*O*-glucoside from the aerial parts of *Osyris alba* L. (Santalaceae) (Iwashina *et al.*, 2008) (TNS collection number **77-5**). Chemical data of the isolated compounds are as follows.

Chlorogenic acid (3-Caffeolylquinic acid) (**P1**). White powder. UV: λmax (nm) MeOH 300sh, 330. HPLC: Retention time (Rt) (min) 2.3. LC-MS: *m/z* 355 $[M+H]^+$, 353 $[M-H]^-$.

Isochlorogenic acid A (3,5-Dicaffeolylquinic acid) (**P2**). Clear solution. UV: λmax (nm) MeOH 301sh, 331. HPLC: Rt (min) 12.6. LC-MS: *m/z* 515 $[M-H]^-$.

Isochlorogenic acid C (4,5-Dicaffeolylquinic acid) (**P3**). Clear solution. UV: λmax (nm) MeOH 302sh, 331. HPLC: Rt (min) 19.4. LC-MS: *m/z* 515 $[M-H]^-$.

Quercetagetin 7-*O*-glucoside (**F1**). Pale yellow solution. UV: λmax (nm) MeOH 260, 277sh, 361; +NaOMe 298, 375 (dec.); +$AlCl_3$ 282, 450; +$AlCl_3$/HCl 271, 393, 425sh; +NaOAc 259, 290sh, 370, 420sh; +NaOAc/H_3BO_3 271, 280sh, 376. HPLC: Rt (min) 4.6. Acid hydrolysis: quercetagetin and glucose. LC-MS: *m/z* 481 $[M+H]^+$, 479 $[M-H]^-$ (quercetagetin + 1 mol glucose), 319 $[M-162+H]^+$ (quercetagetin).

6-Hydroxyluteolin 7-*O*-glucoside (**F2**). Pale yellow solution. UV: λmax (nm) MeOH 255, 284, 344; +NaOMe 263, 304, 392 (inc.); +$AlCl_3$ 274, 301, 421; +$AlCl_3$/HCl 261, 296, 370; +NaOAc 260, 302, 392; +NaOAc/H_3BO_3 262, 287, 357. HPLC: Rt (min) 5.1. Acid hydrolysis: 6-hydroxyluteolin. LC-MS: *m/z* 465 $[M+H]^+$, 463 $[M-H]^-$ (6-hydroxyluteolin + 1 mol glucose), 303 $[M-162+H]^+$ (6-hydroxyluteolin).

Scutellarein 7-*O*-glucoside (**F3**). Clear solution. UV: λmax (nm) MeOH 285, 334; +NaOMe 311, 372 (inc.); +$AlCl_3$ 302, 365; +$AlCl_3$/HCl 301, 359; +NaOAc 312, 381; +NaOAc/H_3BO_3 292, 331. HPLC: Rt (min) 8.5. Acid hydrolysis: scutellarein. LC-MS: *m/z* 465 $[M+H]^+$, 463 $[M-H]^-$.

Quercetin 3-*O*-glucoside (**F4**). Pale yellow solution. UV: λmax (nm) MeOH 256, 267sh, 356; +NaOMe 273, 331, 409 (inc.); +$AlCl_3$ 274, 425; +$AlCl_3$/HCl 269, 299sh, 362sh, 400; +NaOAc 273, 327, 394; +NaOAc/H_3BO_3 261, 297sh, 377. HPLC: Rt (min) 7.9. Acid hydrolysis: quercetin and glucose. LC-MS: *m/z* 465 $[M+H]^+$, 463 $[M-H]^-$ (quercetin + 1 mol glucose), 303 $[M-162+H]^+$ (quercetin).

Results and Discussion

Three phenylpropanoids, chlorogenic acid (**1**), isochlorogenic acid A (**2**) (Fig. 3) and isochlorogenic acid C (**3**), and four flavonoids, quercetagetin 7-*O*-glucoside (**F1**), 6-hydroxyluteolin 7-*O*-glucoside (**F2**), scutellarein 7-*O*-glucoside (**F3**) and quercetin 3-*O*-glucoside (**F4**) (Fig. 4) were isolated from the aerial parts of three *Anaphalis* species.

Compared to flavonoids, Asteraceae plants often contain phenylpropanoids predominantly in their leaves. The same trend was observed in *Anaphalis* species in this study. HPLC analysis revealed that the main components in leaves of *Anaphalis* species were chlorogenic acid (**1**) and isochlorogenic acid A (**2**). Chlorogenic acid and derivatives are well known as bioactive compounds isolated from coffee. In particular, chlorogenic acid is effective for several chronic and

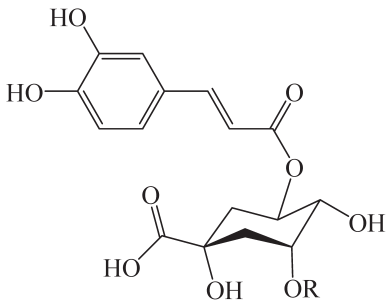

Fig. 3. Chemical structures of main phenylpropanoids in the leaves of three *Anaphalis* species. **1**: chlorogenic acid (R = H) and **2**: isochlorogenic acid A (R = caffeoyl).

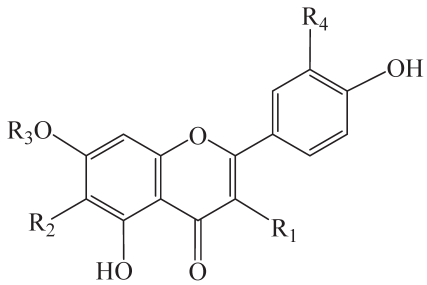

Fig. 4. Chemical structures of flavonoids identified from three *Anaphalis* species. **F1**: quercetagetin 7-*O*-glucoside ($R_1 = R_2 = OH$, R_3 = glucosyl, $R_4 = OH$), **F2**: 6-hydroxyluteolin 7-*O*-glucoside ($R_1 = H$, $R_2 = OH$, R_3 = glucosyl, $R_4 = OH$), **F3**: scutellarein 7-*O*-glucoside ($R_1 = H$, $R_2 = OH$, R_3 = glucosyl, $R_4 = H$), and **F4**: quercetin 3-*O*-glucoside (R_1 = glucosyl, $R_2 = R_3 = H$, $R_4 = OH$).

degenerative diseases, such as cancer, cardiovascular disorders, diabetes, and Parkinson's disease (Clifford *et al.*, 2017). Moreover, chlorogenic acid possesses strong antioxidant activity, and may play important roles in plant cells. Recently, chlorogenic acid has been isolated from the leaves of several alpine plants, e.g., *Campanula chamissonis* Fed. (Campanulaceae) (Murai *et al.*, 2014) and *Artemisia kitadakensis* H. Hara et Kitam. (Asteraceae) (Murai, 2020), and from the flowers of Himalayan black pea *Thermopsis barbata* Royle (Fabaceae) (Murai *et al.*, 2017).

The presence of glycosides of quercetagetin, 6-hydroxyluteolin and scutellarein were reported from the *Anaphalis* genus for the first time. HPLC analysis revealed that **F1**–**F3** were leaf components accumulated in *A. alpicola* (Japan) and *A. cooperi* (Bhutan). The flavonoid composition of the two species distributed in two different regions was similar. These flavonoids were not found from *A. nepalensis* in this study. *A. alpicola* was formerly identified as *A. lactea* Maxim. (Shimizu, 1982). However, the flavonoid composition of *A. alpicola* was different from that of *A. lactea* as reported by Ren *et al.* (2008). Most of the studies on chemical components of the *Anaphalis* genus were carried out using whole plants or aerial parts, and they did not distinguish between leaves and flowers. **F4** was not found in the leaf extracts of the three *Anaphalis* species, but an additional HPLC survey (data not shown) detected it in flower extracts.

Furthermore, the total phenolic content in the leaves of *A. alpicola* in the alpine habitat was 2.4 times higher than that of plants cultivated in the glasshouse according to the HPLC analysis ($P < 0.05$, *t*-test). This quantitative variation of phenolic compounds between a natural habitat (alpine zone) and a cultivation site (lowland) was previously observed in some Japanese alpine species (Murai and Iwashina, 2010; Murai *et al.*, 2014; Murai, 2020). In particular, three flavonoids (**F1**–**F3**) in the leaves of three *Anaphalis* species were detected at trace levels from the plants grown in the glasshouse. Since major environmental stresses such as low temperature and UV radiation were almost excluded under glasshouse conditions, such flavonoids were barely produced in *Anaphalis* leaves. Some of isolated phenolic compounds may act as anti-stress compounds against cold, UV light and oxidative stresses in harsh alpine environments. Further studies (e.g., gene phylogeny and physiological experiments) are required for a comprehensive understanding of the distribution and functions of phenolics in *Anaphalis* species.

Acknowledgments

This work was supported by the integrated research entitled "Biological Properties of Biodiversity Hotspots in Japan" and "Discretionary expenses of Director General" of the National Museum of Nature and Science.

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