Bull. Natl. Mus. Nat. Sci., Ser. B, 43(3), pp. 87–91, August 22, 2017

Flower Pigments of Black Pea *Thermopsis barbata* (Fabaceae) in Bhutan

Yoshinori Murai[1, *], Rinchen Yangzom[2], Choki Gyeltshen[2], Kencho Dorji[2], Choki Wangmo[2] and Tsukasa Iwashina[1]

[1] Department of Botany, National Museum of Nature and Science,
Amakubo 4–1–1, Tsukuba, Ibaraki 305–0005, Japan
[2] National Biodiversity Centre, Ministry of Agriculture and Forests,
Post Box 875 Serbithang, Thimphu, Bhutan
*E-mail: murai@kahaku.go.jp

(Received 14, May 2017; accepted 28, June 2017)

Abstract Flower pigments of Black Pea (*Thermopsis barbata*) sampled from alpine zone in western Bhutan were surveyed. Major anthocyanins of the blackish flowers were delphinidin 3-*O*-glucoside, cyanidin 3-*O*-glucoside and petunidin 3-*O*-glucoside together with some minor anthocyanins and other flavonoids. Interestingly, chlorogenic acid was also found from the flowers. Contribution of each compound to flower coloration and other ecological functions are discussed.

Key words: anthocyanins, black flower, chlorogenic acid, *Thermopsis barbata*, Fabaceae.

Introduction

The genera *Thermopsis* belongs to the family Fabaceae (Leguminosae) and consists of 23 species in East Asia and North America (Mabberley, 2008). *Thermopsis barbata* Royle (Fig. 1) is distributed from south-western China to Kashmir in high montane meadows at ca. 3000–4250 m above sea level. The flowers is 2–3 cm length and unique blackish purple color and the leaves are covered with silvery hairs.

There are several flowers called as 'black flower' in plant kingdoms. Some studies on blackish (dark purple or dark red) flower coloration have been carried out using some ornamental cultivars of pansy (*Viola tricolor* L.) (Violaceae), tulip (*Tulipa gesneriana* L.) (Liliaceae), hollyhock (*Alcea rosea* L.) (Malvaceae), dahlia (*Dahlia variabilis* Hort.) (Asteraceae), chocolate cosmos (*Cosmos atrosanguineus* (Hook.) Voss) (Asteraceae) (Shibata and Ishikura, 1960; Takeda and Hayashi, 1965; Hosaka *et al.*, 2012; Amamiya and Iwashina, 2016; Deguchi *et al.*, 2016). However, the number of related studies on blackish flowers of wild species is limited, and no information is available for phenolic compounds in the dark purple flowers of *T. barbata*.

On the other hand, alpine plants grow under severe environmental conditions (e.g., intense UV-B and cold) and they tend to accumulate efficient antioxidant and UV-absorbing compounds in their flowers and leaves (Spitaler *et al.*, 2006; Murai and Iwashina, 2010, 2015, 2016; Murai *et al.*, 2014).

The aim of this study was to reveal the phenolic compounds in the flowers of *T. barbata*, and discuss the flower coloration and chemical property related to environmental conditions.

Materials and Methods

Plant materials

Thermopsis barbata was collected from Jomolhari Trek near Jangothang (3708 m a.s.l.),

Thimphu, in early July 2015. Voucher specimen was deposited in the National Herbarium (THIM) of National Biodiversity Centre, Bhutan.

Extraction and separation

Dried flowers of *T. barbata* (1.2 g) were extracted with MAW (MeOH/HOAc/HCOOH = 75 : 5 : 20). The concentrated extracts were applied to preparative paper chromatography (PPC) 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 eluted with MAW2 (MeOH/HOAc/H_2O = 70 : 5 : 25).

HPLC analysis

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, I.D. 4.6 mm × 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 : 3 : 86) as eluent. Injection volume was 1 μL. HPLC chromatograms of anthocyanins and other compounds are shown in Fig. 2. To identify each compound, HPLC comparison with authentic samples of delphinidin 3-*O*-glucoside, petunidin 3-*O*-glucoside (Tokiwa Phytochemical Co., Ltd., Japan), cyanidin 3-*O*-glucoside (Murai *et al.*, 2008), quercetin 3-*O*-glucoside (Murai *et al.*, 2014), luteolin 7-*O*-rutinoside (Iwashina and Kadota, 1999) and chlorogenic acid (MP Biomedicals, LLC, France) were carried out. The retention times and UV-Vis spectra of **A1**–**A3**, **O1**, **O3** and **O4** were identical with those of authentic specimens.

LC-MS analysis

Liquid chromatography-mass spectrometry (LC-MS) was performed with the Shimadzu LCMS-2010EV system using Inertsil ODS-4 column [HP 3 μm, I.D. 2.1 mm × 100 mm (GL Sci-

Fig. 1. *Thermopsis barbata*.

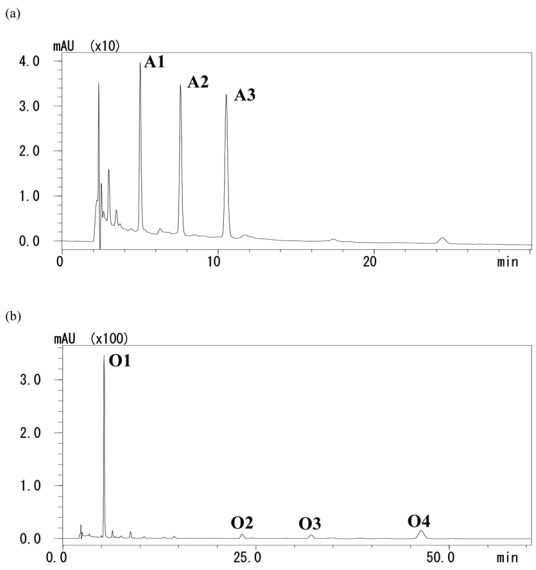

Fig. 2. HPLC chromatogram of MAW extracts from *Thermopsis barbata*.
(a) **A1**: Delphinidin 3-*O*-glucoside, **A2**: Cyanidin 3-*O*-glucoside, **A3**: Petunidin 3-*O*-glucoside. Detection: 530 nm.
(b) **O1**: Chlorogenic acid, **O2**: Caffeic acid derivative, **O3**: Quercetin 3-*O*-glucoside, **O4**: Luteolin 7-*O*-rutinoside. Detection: 350 nm.

ences Inc., Japan)] at a flow rate of 0.2 mL min^{-1}, detection wavelength at 190–700 nm, and HCOOH/MeCN/H_2O (5 : 10 : 80) as eluent. Injection volume was 3 μL. ESI^+ 4.5 kV, ESI^- 3.5 kV, 250℃.

UV-Vis absorption spectra

UV-Vis absorption spectra of the isolated compounds were measured by a Shimadzu UV-2600 UV-Vis Spectrophotometer (Shimadzu Corporation, Japan) (anthocyanins: 220–700 nm, other phenolics: 220–500 nm).

Identification of compounds

The isolated compounds were identified by UV-Vis spectra, LC-MS, characterization of acid hydrolysates (anthocyanidins) and HPLC comparisons with authentic specimens. Caffeic acid derivative (**O2**) was extrapolated from its UV spectra by UV-Vis spectrophotometer and HPLC photodiode array detector.

In this survey, three anthocyanins (**A1**–**A3**), two phenolic acids (**O1** and **O2**) and two flavonoids (**O3** and **O4**) were found in the flowers of *T. barbata*. Chemical data of the isolated compounds are as follows.

Delphinidin 3-*O*-glucoside (**A1**). Purple solution. UV: λmax (nm) 0.01%HCl–MeOH 277, 540; + $AlCl_3$ 323, 580. LC-MS: *m/z* 465 $[M]^+$ (delphinidin + 1 mol glucose), 303 $[M\text{-}162]^+$ (delphinidin). HPLC: *Rt* 5.0 min. Acid hydrolysate: delphinidin.

Cyanidin 3-*O*-glucoside (**A2**). Red solution. UV: λmax (nm) 0.01%HCl–MeOH 281, 528; + $AlCl_3$ 313, 569. LC-MS: *m/z* 449 $[M]^+$ (cyanidin + 1 mol glucose), 287 $[M\text{-}162]^+$ (cyanidin). HPLC: *Rt* 7.6 min. Acid hydrolysate: cyanidin.

Petunidin 3-*O*-glucoside (**A3**). Purple solution. UV: λmax (nm) 0.01%HCl–MeOH 278, 538; + $AlCl_3$ 313, 586. LC-MS: *m/z* 479 $[M]^+$ (petunidin + 1 mol glucose), 317 $[M\text{-}162]^+$ (petunidin). HPLC: *Rt* 10.5 min. Acid hydrolysate: petunidin.

Chlorogenic acid (**O1**). UV: λmax (nm) MeOH 299sh, 325. HPLC: *Rt* 5.3 min. LC-MS: *m/z* 355 $[M+H]^+$, 353 $[M-H]^-$.

Caffeic acid derivative (**O2**). UV: λmax (nm) MeOH 298sh, 326. HPLC: *Rt* 23.2 min. LC-MS: *m/z* 367 $[M-H]^-$.

Quercetin 3-*O*-glucoside (**O3**). Pale yellow solution. UV: λmax (nm) MeOH 255, 268sh, 355. HPLC: *Rt* 32.1 min. LC-MS: *m/z* 463 $[M-H]^-$ (quercetin + 1 mol glucose) and 303 $[M-162+H]^+$ (quercetin).

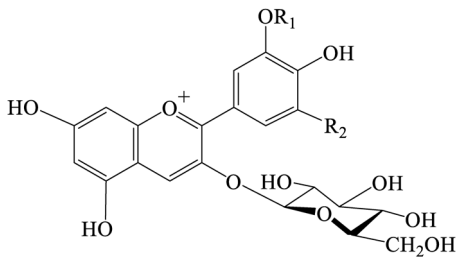

Fig. 3. Chemical structures of anthocyanins from *Thermopsis barbata.*
A1: Delphinidin 3-*O*-glucoside (R_1 = H, R_2 = OH), **A2**: Cyanidin 3-*O*-glucoside (R_1 = R_2 = H), **A3**: petunidin 3-*O*-glucoside (R_1 = CH_3, R_2 = OH).

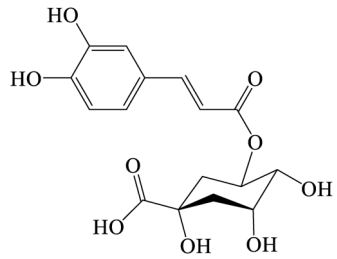

Fig. 4. Chemical structure of chlorogenic acid (**O1**) from *Thermopsis barbata*.

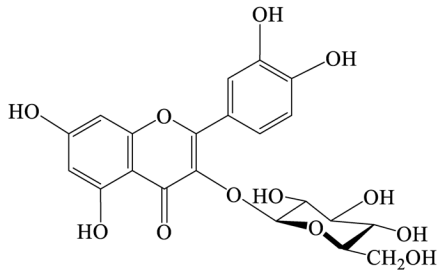

Fig. 5. Chemical structure of quercetin 3-*O*-glucoside (**O3**) from *Thermopsis barbata*.

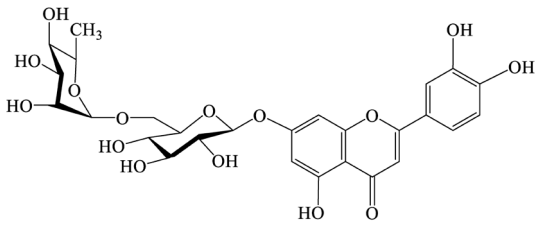

Fig. 6. Chemical structure of luteolin 7-*O*-rutinoside (**O4**) from *Thermopsis barbata*.

Luteolin 7-*O*-rutinoside (**O4**). Pale yellow solution. UV: λmax (nm) MeOH 256, 266sh, 349. HPLC: *Rt* 46.4 min. LC-MS: *m/z* 595 $[M+H]^+$, 593 $[M-H]^-$ (luteolin + 1 mol glucose + 1 mol rhamnose) and 287 $[M-308+H]^+$ (luteolin).

Results and Discussion

Blackish coloration and anthocyanins from Thermopsis barbata

The present study simultaneously revealed the composition of anthocyanins, other flavonoids and phenolic acids in the flowers of *Thermopsis barbata* for the first time. The major anthocyanins responsible for the blackish color were 3-*O*-glucosides of delphinidin (**A1**), cyanidin (**A2**) and petunidin (**A3**) (Figs. 2 and 3). When the black coloration occurs, there is broad and strong absorption in the visible region. The combination of delphinidin and cyanidin covers wide range of visible light. Cyanidin 3-*O*-rutinoside and delphinidin 3-*O*-rutinoside were major anthocyanins in the dark purple perianth-bottoms of some tulip cultivars (Nakayama *et al.*, 2004).

The glycosylation pattern of each anthocyanin from *T. barbata* was simple compared with previous reports. In the case of blackish flowers of wild species, the dark purple flowers of *Lisianthius nigrescens* Cham. et Schlecht. (Gentianaceae) contain delphinidin 3-*O*-rhamnosyl-(1→6)-galactoside and delphinidin 3-*O*-rhamnosyl-(1→6)-galactoside-5-*O*-glucoside (Markham *et al.*, 2004). Furthermore, dark red flowers of *Pulsatilla cernua* (Thunb.) Bercht. et C.Presl (Ranunculaceae) contained complicatedly acylated anthocyanin, pelargonidin 3-[2″-(2‴-*E*-caffeoly-*β*-D-glucopyranosyl)-*β*-D-galactopyranoside] (Yoshitama *et al.*, 1998).

Other flavonoids sometimes affect flower colorations as co-pigment substances. However, *L. nigrescencs* and *P. cernua* barely contain other flavonoids. *T. barbata* also does not contain such substances (Fig. 2b). Though chlorogenic acid (Fig. 4) was found in the flowers of *T. barbata*, the compound has obstructed co-pigmentation in hydrangea flower (Takeda *et al.*, 1985). These results suggests that the possibility of co-pigment effect in the blackish coloration of *T. barabata* is low. Anthocyanins and other flavonoids, i.e., flavone and flavonol, are share the crucial steps of biosynthetic pathway. The synthesis of anthocyanins may have priority over other flavonoids in such species. Further investigations, e.g., evaluation of color using wild species and the relationship with pollinators, are needed.

Other ecological significance

The flowers of *T. barbata* contained chlorogenic acid (**O1**) as major phenolic compound. Chlorogenic acid has been reported as an efficient antioxidant. In addition, two minor compounds, quercetin 3-*O*-glucoside (**O3**) and luteolin 7-*O*-rutinoside (**O4**) (Figs. 5 and 6) in the flowers of *T. barbata*, are also thought to be strong antioxidant (Rice-Evans *et al.*, 1996; Pietta, 2000). Recent numerous studies showed that several plants growing in higher altitudes synthesize antioxidant and UV-absorbing substances including phenolic acids and flavonoids (Spitaler *et al.*, 2006; Murai and Iwashina, 2010, 2015; Murai *et al.*, 2009, 2014, 2015, 2016). We preliminary reported chlorogenic acid from alpine *Campanula* species (Murai *et al.* 2014). UV radiation cases not only direct physiological damages but indirect oxidation damages to plant cells and tissues, and such oxidation stresses are also caused by cold and other environmental factors. Flower is the most crucial reproductive organ of plants. The compound might play an important role in protection against several environmental stresses in high altitudes.

Acknowledgements

This work was partly supported by JSPS KAKENHI Grant Numbers JP15H02914, JP16K18830.

References

Amamiya, K. and Iwashina, T. 2016. Qualitative and quantitative analysis of flower pigments in chocolate cosmos, *Cosmos atrosanguineus*, and its hydrids. Natural Product Communications 11: 77–78.

Deguchi, A., Tatsuzawa, F., Hosokawa, M., Doi, M. and Ohno, S. 2016. Quantitative evaluation of the contribution of four major anthocyanins to black flower coloring of dahlia petals. The Horticulture Journal 85: 340–350.

Hosaka, H., Mizuno, T. and Iwashina, T. 2012. Flavonoid pigments and color expression in the flowers of black hollyhock (*Alcea rosae* 'Nigra'). Bulletin of the National Museum of Nature and Science, Series B 38: 69–75.

Iwashina, T. and Kadota, Y. 1999. Flavonoids from *Schmalhausenia nidulans* (Compositae): a taxon endemic to the Tien Sian Mountains. Biochemical Systematics and Ecology 27: 97–98.

Mabberley, D. J. 2008. Mabberley's Plant-book, third ed. Cambridge University Press, Cambridge, pp. 143.

Markham, K. R., Bloor, S. J., Nicholson, R., Rivera, R., Shemluck, M., Kevan, P. G. and Michener, C. 2004. Black flower coloration in wild *Lisianthius nigrescens*: its chemistry and ecological consequences. Zeitschrift für Naturforschung 59c: 625–630.

Murai, Y. and Iwashina, T. 2010. Flavonol glucuronides from *Geum calthifolium* var. *nipponicum* and *Sieversia pentapetala* (Rosaceae). Biochemical Systematics and Ecology 38: 1081–1082.

Murai, Y. and Iwashina, T. 2015. Phenolic compounds in the leaves of *Pedicularis chamissonis* in Japan. Bulletin of the National Museum of Nature and Science, Series B 41: 131–136.

Murai, Y. and Iwashina, T. 2016. Phenolic compounds from *Sanguisorba obtusa* endemic to Japan. Bulletin of the National Museum of Nature and Science, Series B 42: 143–147.

Murai, Y., Kitajima, J. and Iwashina, T. 2014. Flavonoids from two alpine *Campanula* species in Japan. Bulletin of the National Museum of Nature and Science, Series B 40: 113–118.

Murai, Y., Kokubugata, G., Yokota, M., Kitajima, J. and Iwashina, T. 2008. Flavonoids and anthocyanins from six *Cassytha* taxa (Lauraceae). Biochemical Systematics and Ecology 36: 745–748.

Murai, Y., Takemura, S., Takeda, K., Kitajima, J. and Iwashina, T. 2009. Altitudinal variation of UV-absorbing compounds in *Plantago asiatica*. Biochemical Systematics and Ecology 37: 378–384.

Nakayama, M., Okada, M., Taya-Kizu, M., Urashima, O., Kan, Y., Fukui, Y. and Koshioka, M. 2004. Coloration and anthocyanin profile in tulip flowers. Japan Agriculture Research Quarterly 38: 185–190.

Pietta, P.-G. 2000. Flavonoids as antioxidants. Journal of Natural Products 63: 1035–1042.

Rice-Evans, C. A., Miller, N. J. and Paganga, G. 1996. Structure-antioxidant activity relationships and phenolic acids. Free Radical Biology and Medicine 20: 933–956.

Shibata, M. and Ishikura, N. 1960. Paper chromatographic survey of anthocyanin in tulip-flowers. I. Japanese Journal of Botany 17: 230–238.

Spitaler, R., Schlorhaufer, P. D., Ellmerer, E. P., Merfort, I., Bortenschlager, S., Stuppner, H. and Zidorn, C. 2006. Altitudinal variation of secondary metabolite profiles in flowering heads of *Arnica montana* cv. ARBO. Phytochemistry 67: 409–417.

Takeda, K. and Hayashi, K. 1965. Studies on anthocyanins, XLVIII. Crystallization and some properties of the genuine anthocyanin inherent to the deep violet color of pansy. Proceedings of Japan Academy 41: 449–454.

Takeda, K., Kariuda, M. and Itoi, H. 1985. Blueing of sepal colour of *Hydrangea macrophylla*. Phytochemistry 24: 2251–2254.

Yoshitama, K., Saeki, A., Iwata, T., Ishikura, N. and Yahara, S. 1998. An acylated pelargonidin diglycoside from *Pulsatilla cernua*. Phytochemistry 47: 105–107.