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Flavonol Glycosides from the Whitish Flowers of *Primula alpicola* and *P. sikkimensis* var. *hopeana* in Bhutan

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Abstract Five flavonols were isolated from the whitish flowers of *Primula alpicola* and *P. sikkimensis* var. *hopeana* which belong to the section Sikkimensis in Bhutan. Flavonoid composition of two *Primula* species were qualitatively the same and they were identified as quercetin 3-*O*-xylosyl-(1→2)-[rhamnosyl-(1→6)-glucoside] (**1**), quercetin 3-*O*-rutinoside (**2**), quercetin 3-*O*-glucoside (**3**), kaempferol 3-*O*-rutinoside (**4**) and isorhamnetin 3-*O*-rutinoside (**5**). Compound **1** which might characterize in these species was reported from the family Primulaceae for the first time. Although gossypetin has been reported as principle yellow pigment in *Primula* species, it was not found in the whitish flowers of two *Primula* taxa.

Key words: flavonol, flower color expression, *Primula alpicola*, *Primula sikkimensis* var. *hopeana*, quercetin, section Sikkimensis.

Introduction

The genus *Primula* is widely distributed in the Northern Hemisphere and consists of ca. 500 species. In this genus, Himalaya-Hengduan and its adjacent regions have been considered to represent the modern diversification center (Richards, 1993). The species of section Sikkimensis having campanulate flowers are mainly distributed in the Himalayas and western China. Their flowers are colorful from purple, violet, yellow to white (Aitken *et al.*, 1999).

Some flavonoids have been reported from the flowers of *Primula* species (e.g. Karl *et al.*, 1981; Saito *et al.*, 1990; Hashimoto *et al.*, 2015; Apel *et al.*, 2017). In section Sikkimensis, flower flavonoids have been reported to understand the color expression and their systematics (Harborne, 1968, 1969). According to Harborne (1968, 1969), yellow pigment of four *Primula* species, i.e. *P. alpicola* (W.W.Smith) Stapf, *P. florindae* Kingdon-Ward, *P. sikkimensis* Hook.f. and *P. waltonii* Watt ex I.B.Balfour has been identified as gossypetin (3,5,7,8,3′,4′-hexahydroxyflavone) but not carotenoid (Harborne, 1968). Thus, it was suggested that carotenoids are replaced to gossypetin in their *Primula* flowers as major yellow pigment (Harborne, 1969). Furthermore, gossypetin was found at least in *P. velis* L., *P. vulgaris* Hudson and *P. elatior* (L.) Hill of the two sections Sikkimensis and Primula (Harborne, 1969). Pigments of cyanic flower species in the section Sikkimensis were isolated from the flowers of *P. secundiflora* Franch and *P. ioessa* W.W.Sm., and

anthocyanins, malvidin and delphinidin, were identified, respectively (Harborne, 1968).

In this study, we isolated and identified the flavonols from two *Primula* taxa, *P. alpicola* and *P. sikkimensis* var. *hopeana* (Balfour f. & Cooper) W.W.Smith & Fletcher having whitish flowers in the section Sikkimensis. *Primula alpicola* is growing in stream sides of 3600–4100 m elev. in southeastern Tibet, Bhutan and northeastern India, and has campanulate white, yellow or purple flower (Aitken *et al.*, 1999). On the other hand, *P. sikkimensis* var. *hopeana* having campanulate ivory white flowers is mainly growing in wet meadows or scrub of 3600–4700 m elev. in southeastern Tibet, Bhutan and eastern Nepal (Aitken *et al.*, 1999). Moreover, we performed aglycone analysis using crude extracts for the elucidation of the flower color expression and discussed the white color expression of *Primula* species of the section Sikkimensis.

Materials and Methods

Plant materials

Fresh flowers of *Primula alpicola* (Fig. 1A) were collected between Zizingoma–Omta Tsho and tent site of Omta Tsho, Thampe La zone, Bhutan on 30 June 2017. On the other hand, *P. sikkimensis* var. *hopeana* (Fig. 1B) were collected between Yakuchu–Wangzhi, Black Mountains, Bhutan on 31 July 2018. Species were identified by one of the authors (R. Yangzom) and the voucher specimens were deposited in the Herbarium of National Biodiversity Centre (THIM), Bhutan. Flowers were immediately dried in collection sites.

Extraction and isolation

Dry flowers of *P. alpicola* (15.1 g) and *P. sikkimensis* var. *hopeana* (10.0 g) were extracted with MeOH for an overnight at room temperature. After filtration and concentration, the extracts were applied to column chromatography on Diaion HP-20SS (Mitsubishi Chemical Co., Japan) eluted with 200 mL 0.5% TFA, 200 mL 10% MeCN containing 0.5% TFA and 200 mL 25% MeCN containing 0.5% TFA. Separated fractions were subjected to Sephadex LH-20 column chromatography eluted with 70% MeOH. The fractions were purified by preparative HPLC with Shimadzu HPLC systems equipped with a SPD-20A UV–Vis detector using Inertsil ODS–4 (I.D. 10 × 250 mm, GL Science Inc., Japan), at a flow-rate of 1.5–3.0 mL min^{-1}; injection of 350–400 µL; eluent of 15 or 25% MeCN containing 5% HCOOH.

Identification of the flavonoids

Isolated flavonols were identified by UV-Vis spectral survey (500–220 nm) according to Mabry *et al.* (1970), LC-MS, acid hydrolysis, and direct TLC (BAW, 15% HOAc and BEW) and HPLC comparisons with authentic samples. In addition, **1** was performed sugar analysis by HPLC, and NMR measurement.

HPLC analysis was performed with Shimadzu HPLC systems equipped with a SPD–20A UV–Vis detector using InertSustain C18 (I.D. 4.6 × 250 mm, GL Science Inc.), at a flow-rate of 1.0 mL min^{-1}. Water containing 5% HCOOH (solvent A) and 90% MeCN containing 5% HCOOH (solvent B) were used as the mobile phase under gradient elution condition. The gradient solution program was as follows: a linear gradient from 10 to 50% solvent B for 35 min, 50% solvent B for 10 min.

Liquid chromatograph-mass spectra (LC-MS) was performed with Shimadzu LC-MS systems equipped with a SPD-20A UV–Vis detector using Inertsil ODS–4 (I.D. 2.1 × 100 mm, GL Science Inc.), at a flow-rate of 0.2 mL min^{-1}, and the eluent with 12% MeCN containing 5% HCOOH. ESI$^+$ 4.5 kV and ESI$^-$ 3.5 kV, 250°C.

NMR spectra (^{1}H NMR,^{13}C NMR,^{1}H-^{1}H COSY, TOCSY, HMQC and HMBC) were recorded on a Brucker AV-600 in pyridine-d_5 at 600 MHz (^{1}H NMR) and 150 MHz (^{13}C NMR). Chemical shifts (δ) were given in ppm.

Sugar identification was performed to hydrolysate of **1** according to Tanaka *et al.* (2007) and Mizuno *et al.* (2015). After acid hydrolysis by 0.5 mM HCl, sugars were separated from the

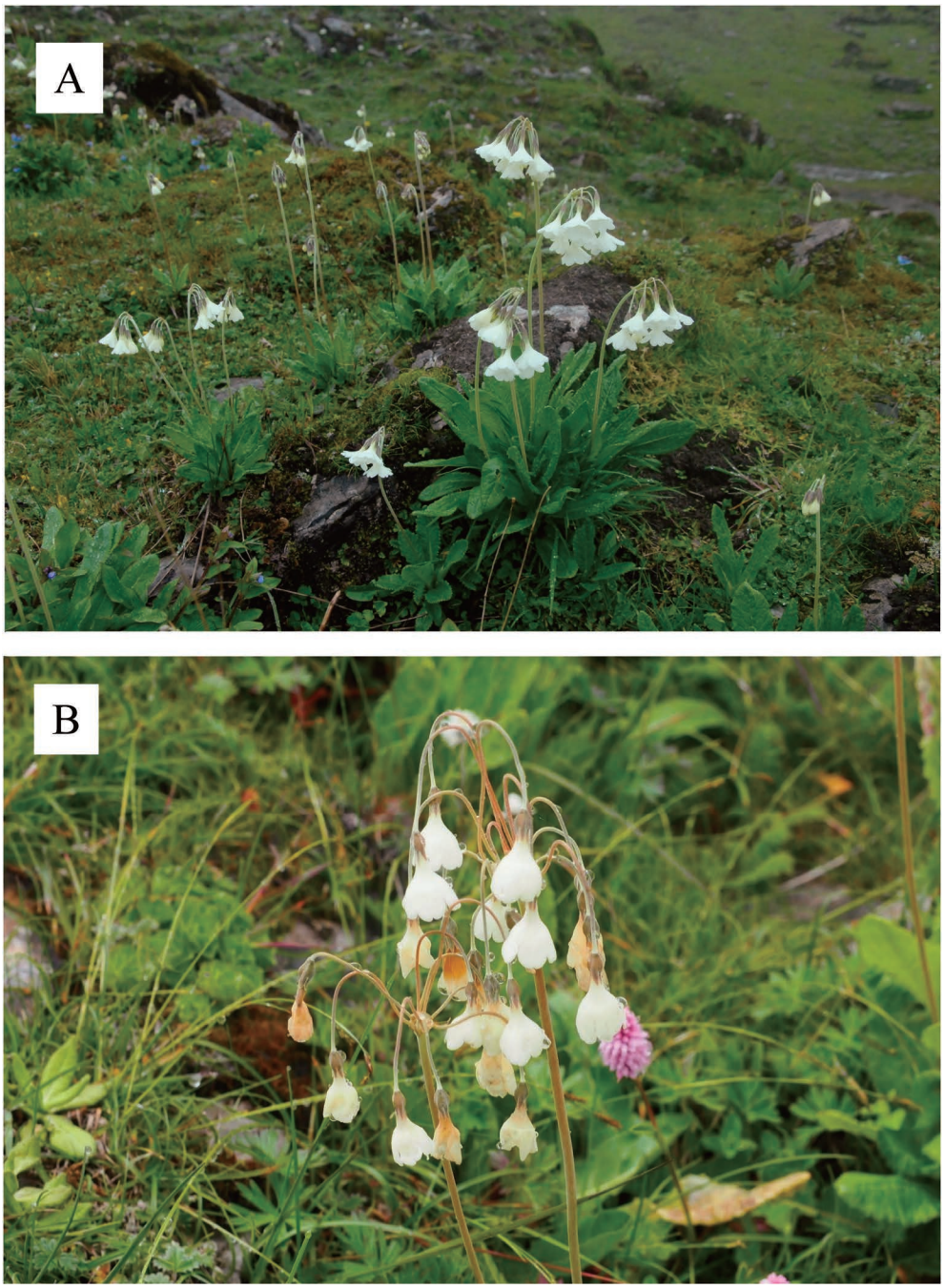

Fig. 1. Habitat of *Primula alpicola* (**A**, Tent site of Omta Tsho, Thampe La zone, Bhutan, 30 June 2017) and *P. sikkimensis* var. *hopeana* (**B**, Between Yakuchu and Wangzhi, Black Mountains, Bhutan, 31 July 2018).

aglycone with Sep-pack C18 and the neutralized with WAX column (Waters Corp., Milford, MA). The residues were dissolved in pyridine (50 µL) containing L-cysteine methyl ester hydrochloride (10 mg mL^{-1}) and heated at 60°C for 60 min. The solution was added to 50 µL pyridine containing *o*-tolyl isothiocyanate (10 mg mL^{-1}), and then heated at 60°C for 60 min. The reaction solution

and authentic sugars were analyzed using column, Cosmosil 5C18-AR-II (4.6 × 250 mm, Nacalai Tesque Inc., Japan). Elution was performed with 25% MeCN containing 0.2% H_3PO_4 at a flow rate of 0.8 mL min^{-1}, and detection at 250 nm.

Aglycone analysis

Crude extracts (0.1 mL) were added into 12% aq. HCl and treated for 30 min at 100℃ for acid hydrolysis. After cooling, the solutions were diluted with water and were applied to Sep-pack C18 cartridge eluted with 2 mL H_2O, 2 mL 10% MeCN containing 0.5% TFA and 2 mL MeOH. The organic fractions were evaporated and applied to HPLC analysis with Shimadzu HPLC system equipped with PDA detector using Inertsil Sustain C18 (I.D. 4.6 × 250 mm), at a flow-rate of 1.0 mL min^{-1}. The gradient solution program of the mobile phase was as follows: a linear gradient from 20 to 100% solvent B for 16 min, 100% solvent B for 6 min.

Authentic samples

Origins of the authentic samples were as follows; quercetin 3-*O*-glucoside, quercetin 3-*O*-rutinoside and kaempferol 3-*O*-rutinoside from the aerial parts and fruits of *Osyris alba* L. (Santalaceae) (Iwashina *et al.*, 2008), isorhamnetin 3-*O*-rutinoside from the leaves of *Asarum caulescens* Maxim. (Aristolochiaceae) (Iwashina *et al.*, 2005) (TNS collection number **87-1**), quercetin from the flowers of *Astrophytum ornatum* (DC.) Web. (Cactaceae) (Iwashina *et al.*, 1988) (TNS collection number **81-3**), kaempferol from hydrolysate of kaempferol 3-*O*-glucoside from *Camptotheca acuminata* Decne. leaves (Cornaceae) (Iwashina and Hatta, 1998) (TNS collection number **77-14**), and isorhamnetin from hydrorysate of isorhamnetin 3-*O*-rhamnosylglucoside from *Epiphyllum hybrida* flowers (Cactaceae) (Iwashina, unpublished data).

Quercetin 3-*O*-xylosyl-(1→2)-[rhamnosyl-(1→6)-glucoside] (**1**, Fig. 2). TLC: Rf 0.50 (BAW), 0.71 (15%HOAc), 0.58 (BEW); Color UV (365 nm): dark purple, UV/NH_3: yellow. HPLC: *t*R (min) 15.5. UV: λmax (nm) MeOH 258, 357; +NaOMe 278, 331, 414 (inc.); +$AlCl_3$ 273, 412; +$AlCl_3$/HCl 270, 297, 361, 398; +NaOAc 271, 389; +NaOAc/H_3BO_3 262, 379; LC-MS: *m/z* 743 $[M+H]^+$, 741 $[M-H]^-$; Sugar analysis: *tR* (min): 16.1, 18.6 and 27.2 min (authentic samples: D-glucose, 16.1 min, D-xylose, 18.6, L-rhamnose, 27.2 min).

Quercetin 3-*O*-rutinoside (**2**, rutin, Fig. 3). TLC: Rf 0.59 (BAW), 0.48 (15%HOAc), 0.66 (BEW); Color UV (365 nm): dark purple, UV/NH_3: yellow. HPLC: *t*R (min) 18.1. UV: λmax (nm) MeOH 257, 357; +NaOMe 275, 414 (inc.); +$AlCl_3$

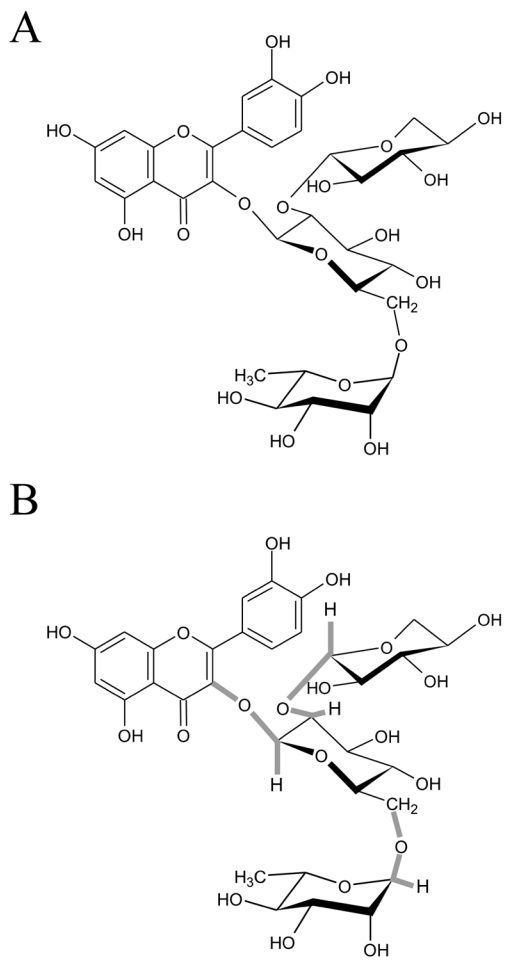

Fig. 2. Quercetin 3-*O*-xylosyl-(1→2)-[rhamnosyl-(1→6)-glucoside] (**1**). A = Chemical structure. B = Significant HMBC correlations were shown as the greyed lines.

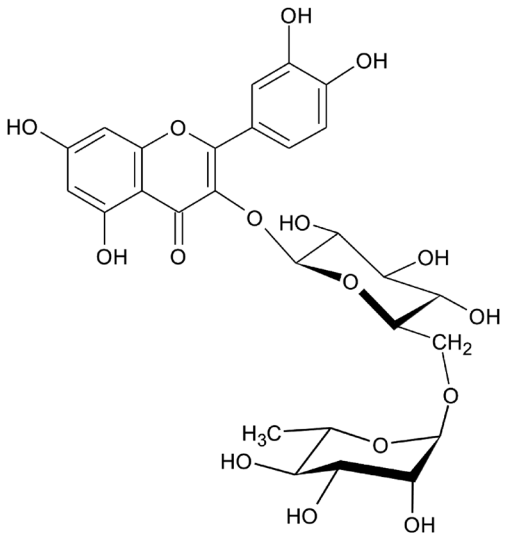

Fig. 3. Quercetin 3-*O*-rutinoside (**2**, rutin).

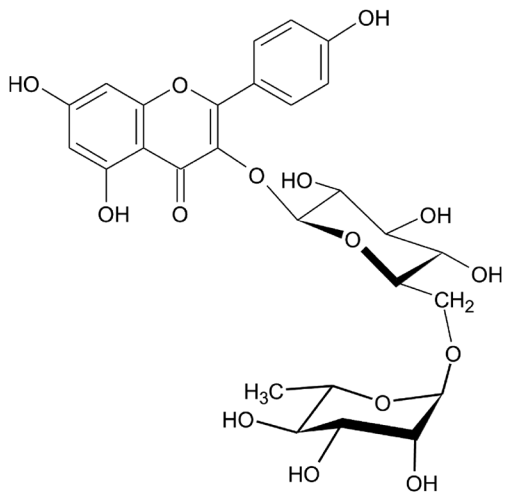

Fig. 5. Kaempferol 3-*O*-rutinoside (**4**, nicotiflorin).

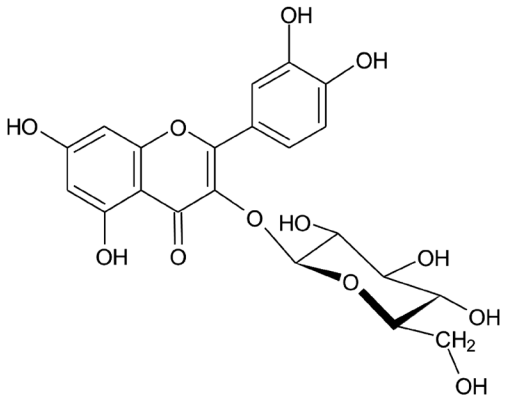

Fig. 4. Quercetin 3-*O*-glucoside (**3**, isoquercitrin).

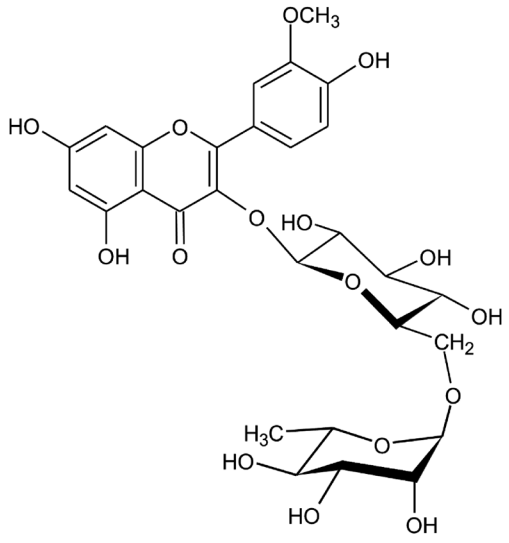

Fig. 6. Isorhamnetin 3-*O*-rutinoside (**5**, narcissin).

273, 420; + $AlCl_3$/HCl 268, 297, 362, 398; + NaOAc 262, 409; + NaOAc/H_3BO_3 271, 306, 399; LC-MS: *m/z* 611 $[M + H]^+$, 609 $[M - H]^-$.

Quercetin 3-*O*-glucoside (**3**, isoquercitrin, Fig. 4). TLC: Rf 0.67 (BAW), 0.28 (15%HOAc), 0.73 (BEW); Color UV (365 nm): dark purple, UV/NH_3: yellow. HPLC: *t*R (min) 19.1. UV: λmax (nm) MeOH 257, 358; + NaOMe 275, 325, 408 (inc.); + $AlCl_3$ 267, 369, 402; + $AlCl_3$/HCl 267, 297, 360, 403; + NaOAc 269, 372; + NaOAc/H_3BO_3 262, 379; LC-MS: *m/z* 465 $[M + H]^+$, 463 $[M - H]^-$.

Kaempferol 3-*O*-rutinoside (**4**, nicotiflorin, Fig. 5). TLC: Rf 0.70 (BAW), 0.54 (15%HOAc), 0.76 (BEW); Color UV (365 nm): dark purple, UV/NH_3: dark greenish yellow. HPLC: *t*R (min) 20.5. UV: λmax (nm) MeOH 265, 351; + NaOMe 277, 322, 397 (inc.); + $AlCl_3$ 268, 303, 351, 406sh.; + $AlCl_3$/HCl 271, 301, 348, 387; + NaOAc 270, 306, 364; + NaOAc/H_3BO_3 265, 354; LC-MS: *m/z* 595 $[M + H]^+$, 593 $[M - H]^-$.

Isorhamnetin 3-*O*-rutinoside (**5**, narcissin, Fig. 6). TLC: Rf 0.68 (BAW), 0.54 (15%HOAc), 0.73 (BEW); Color UV (365 nm): dark purple, UV/

NH_3: yellow. HPLC: *t*R (min) 21.1. UV: *λ*max (nm) MeOH 255, 356; +NaOMe 276, 324, 405 (inc.); +$AlCl_3$ 266, 301, 358, 411sh.; +$AlCl_3$/HCl 266, 302, 358, 402sh.; +NaOAc 270, 371; +NaOAc/H_3BO_3 255, 360; LC-MS: *m/z* 623 $[M-H]^-$.

Results and Discussion

HPLC chromatograms of flower extracts of *Primula alpicola* and *P. sikkimensis* var. *hopeana* were shown in Fig. 7. Flavonoid composition was qualitatively the same to each other. Of five flavonoids isolated from two *Primula* species, **1** was mainly identified by NMR, together with LC-MS, acid hydrolysis and UV-Vis spectra. On the other hand, **2**–**5** were characterized by LC-MS, UV-Vis spectra, and direct HPLC comparisons with authentic samples.

LC-MS of **1** showed the molecular ion peaks at *m/z* 743 $[M+H]^+$ and 741 $[M-H]^-$ and fragment ion peaks, *m/z* 611 $[M-132+H]^+$, 465 $[M-132-146+H]^+$ and 303 $[M-132-146-162+H]^+$, showing the attachment of each 1 mol of hexose, rhamnose and pentose to pentahydroxyflavone. Quercetin, D-glucose, L-rhamnose and D-xylose were liberated by acid hydrolysis of **1**. In NMR analysis of **1**, four aromatic proton signals, δ_H 8.33 (1H, *d*, H-2′), 8.23 (1H, *dd*, H-6′), 7.39 (1H, *d*, H-5′), 6.67 (1H, *d*, H-6) and 6.60 (1H, *d*, H-8), appeared (Table 1). The *β*-pyranose structure of glucose was shown by the coupling constant ($J=7.7$ Hz) of anomeric proton signal (Markham and Geiger, 1994). HMBC correlation between Glc-1 proton and δ_C 134.7 corresponding to C-3 of quercetin showed that glucose is attached to 3-positon of quercetin. The *α*-pyranose structure of rhamnose was shown by the $^3J_{H,C}$ correlation of δ_H 5.24 (1H, *brs*, Rham-1) with δ_C 69.6 (Rham-5) and occurrence as broad-singlet of anomeric proton signal. HMBC correlation between Rham-1 proton and

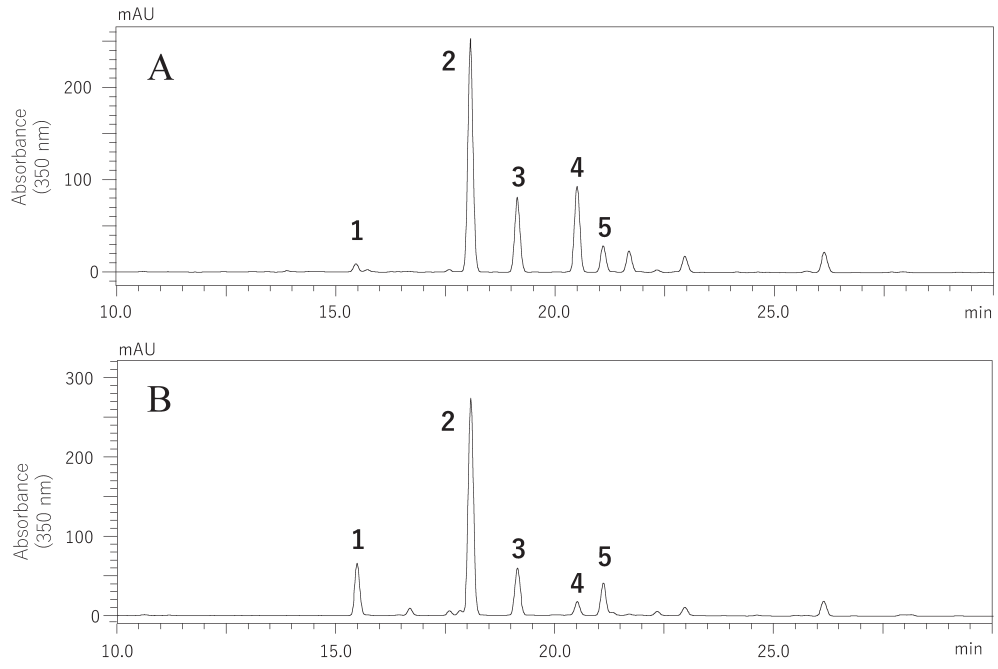

Fig. 7. HPLC analysis of the flowers of *Primura alpicola* (A) *and P. sikkimensis* var. *hopeana* (B). **1** = quercetin 3-*O*-xylosyl-(1→2)-[rhamnosyl-(1→6)-*O*-glucoside] (15.5 min), **2** = quercetin 3-*O*-rutinoside (18.1 min), **3** = quercetin 3-*O*-glucoside (19.1 min), **4** = kaempferol 3-*O*-rutinoside (20.5 min), **5** = isorhamnetin 3-*O*-rutinoside (21.1 min).

Table 1. ^{1}H and ^{13}C NMR data of **1** from *P. sikkimensis* var. *hopeana*

Position	Pyridine-d_5		
	δH		δC
Quercetin			
2			157.6
3			134.7
4			178.7
5			162.9
6	6.67	*d*, 1.8	99.6
7			165.5
8	6.60	*d*, 1.8	94.0
9			157.5
10			105.4
1′			123.1
2′	8.33	*d*, 2.0	117.8
3′			146.8
4′			150.4
5′	7.39	*d*, 8.3	116.3
6′	8.23	*dd*, 8.3, 2.1	122.8
3-O-Glucose			
1	6.42	*d*, 7.7	100.5
2	4.44	*dd*, 7.6, 8.8	82.4
3	4.34	*m*	78.6
4	4.34	*m*	72.5
5	4.03	*m*	77.3
6a	4.43	*dd*, 13.2, 2.9	68.0
6b	4.93	*m*	
6″-O-Rhamnose			
1	5.24	*brs*	102.3
2	4.34	*m*	72.1
3	3.95	*t*, 8.7	71.3
4	4.11	*m*	73.9
5	4.14	*m*	69.6
Me	1.47	*d*, 5.9	
2″-O-Xylose			
1	5.67	*d*, 6.2	105.7
2	4.24	*m*	74.9
3	4.24	*m*	77.1
4	4.21	*m*	71.0
5a	4.54	*dd*, 11.2, 4.1	66.6
5b	3.84	*dd*, 11.2, 8.5	

C-6 of glucose at δ_C 68.0 showed that rhamnose is attached to 6-position of glucose. Furthermore, β-xylopyranose was shown by the $^{3}J_{H,C}$ correlation of δ_H 4.54 (1H, *dd*, Xyl-5) with δ_C 105.7 (Xyl-1) and coupling constant ($J = 6.2$ Hz) of anomeric proton signal. It was proved by HMBC (correlation of Xyl-1 proton and C-2 carbon signal of glucose at δ_C 82.4) that xylose is attached to 2-positon of glucose. Significant HMBC correlations are shown in Fig. 2B. Thus, **1** was identified as quercetin 3-*O*-β-D-xylopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside]. This flavonol glycoside has already been reported from the leaves of *Orixa japonica* Thunb. (Rutaceae) (Nishida *et al.*, 1990) and *Actinidia arguta* (Sieb. & Zucc.) var. *giraldii* (Diels) Vorosch. (Actinidiaceae) (Webby, 1991).

UV spectral properties showed that **2** and **3** are flavonols having free 5-, 7-, 3′- and 4′-hydroxyl and a substituted 3-hydroxyl groups. LC-MS of **2** showed that the molecular ion peaks, *m/z* 611 $[M+H]^+$ and 609 $[M-H]^-$, and fragment ion peaks, *m/z* 465 $[M-146+H]^+$ and 303 $[M-146-162+H]^+$. suggesting that **2** is quercetin which attached each 1 mol of hexose and rhamnose. LC-MS of **3** showed molecular ion peaks at *m/z* 465 $[M+H]^+$ and 463 $[M-H]^-$ and fragment ion peak at *m/z* 303 $[M-162+H]^+$, showing that the compound is quercetin which attached 1 mol hexose. Finally, they are identified as quercetin 3-*O*-rutinoside (**2**) and 3-*O*-glucoside (**3**) by direct HPLC and TLC comparisons with authentic samples.

Compound **4** was characterized as flavonol with free 5-, 7- and 4′-hydroxyl and a substituted 3-hydroxyl groups by UV spectral survey. LC-MS of **4** showed the molecular ion peaks at *m/z* 595 $[M+H]^+$ and 593 $[M-H]^-$, and fragment ion peaks at *m/z* 449 $[M-162+H]^+$ and *m/z* 287 $[M-162-146+H]^+$, suggesting that **4** is kaempferol which attached each 1 mol of hexose and rhamnose. From the results of HPLC and TLC comparison with authentic sample, **4** was identified as kaempferol 3-*O*-rutinoside.

Compound **5** was characterized as 5-, 7- and 4′-hydroxylated, and 3- and 3′-substituted flavonol by UV spectral survey. Since the molecular ion peak, *m/z* 623 $[M-H]^-$, and fragment ion peak, *m/z* 317 $[M-162-146+H]^+$ was shown by LC-MS, **5** is tetrahydroxy-monomethoxyflavone which attached each 1 mol of hexose and rhamnose. Finally, **5** was identified as isorhamnetin 3-*O*-rutinoside by HPLC and TLC comparison with authentic sample.

Some flavonols which were isolated from the

Primula species have been shown to be flavonols which were lineally attached sugars to 3-position, e.g. quercetin and isorhamnetin 3-*O*-glucosyl-(1→2)-gentiobiosides from the leaves of *Primula auricula* L. (Fico *et al.*, 2007.), quercetin 3-*O*-arabinosyl-(1→2)-arabinosyl-(1→4)-glucoside from the leaves of *Primula turkestanica* (Regel) E.A.White (Zakharov *et al.*, 1970), and quercetin and kaempferol 3-*O*-xylosyl-(1→2)-glucosyl-(1→6)-glucosides and kaempferol 3-*O*-glucosyl-(1→2)-glucosyl-(1→6)-glucoside from the leaves and flowers of *Primula sieboldii* (Hashimoto *et al.*, 2015). In this study, triglycosylated flavonol, quercetin 3-*O*-xylosyl-(1→2)-[rhamnosyl-(1→6)-glucoside] (**1**), was identified as branched flavonol glycoside. Although many *Primula* species are not surveyed for flavonoids, we presumed that **1** is the characteristic flavonol of *P. sikkimensis* var. *hopeana*. Flavonoid **1** from *Orixa japonica* was reported as oviposition deterrent of a Rutaceae-feeding swallowtail butterfly, *Papilio xuthus* (Nishida *et al.*, 1990). However, the function in *Primula* flowers is unknown.

Aglycone analyses of two *Primula* species by HPLC were shown in Fig. 8. These peaks were compared with authentic flavonol aglycones, i.e. quercetin, kaempferol and isorhamnetin. The proportion of each flavonol was followed, quercetion (Qu) 63.8%, kaempferol (Km) 17.7% and isorhamnetin (Is) 10.5% in *P. alpicola*, and Qu 82.8%, Km 4.0% and Is 9.2% in *P. sikkimensis* var. *hopeana*. Quercetin was highest amount than those of kaempferol and isorhamnetin in both *Primula* species. Thus, it was shown that major flavonoid in their species is quercetin (each 63.8% and 82.85).

Yellow color due to 6- or 8-hydroxylated flavonols such as gossypetin and quercetagetin has been reported from *Centaurea* (Asteraceae) (Mishio *et al.*, 2006, 2015), *Rudbeckia* (Asteraceae) (Thompson *et al.*, 1972), *Eriophyllum* (Asteraceae) (Harborne and Smith, 1978), *Gossypium* (Malvaceae) (Harborne, 1966) and *Tagetes* flowers (Asteraceae) (Morita, 1957; Bhardwaj *et al.*, 1980). Harborne (1969) reported that yellowish *Primula* flowers in section Sikkimensis, are due to gossypetin. In this study, we isolated five flavonols, i.e. quercetin 3-*O*-xylosyl-(1→2)-[rhamnosyl-(1→6)-glucoside], 3-*O*-rutinoside and 3-*O*-glucoside, and kaempferol and isorhamnetin 3-*O*-rutinosides from two whitish flowers of *Primula* species.

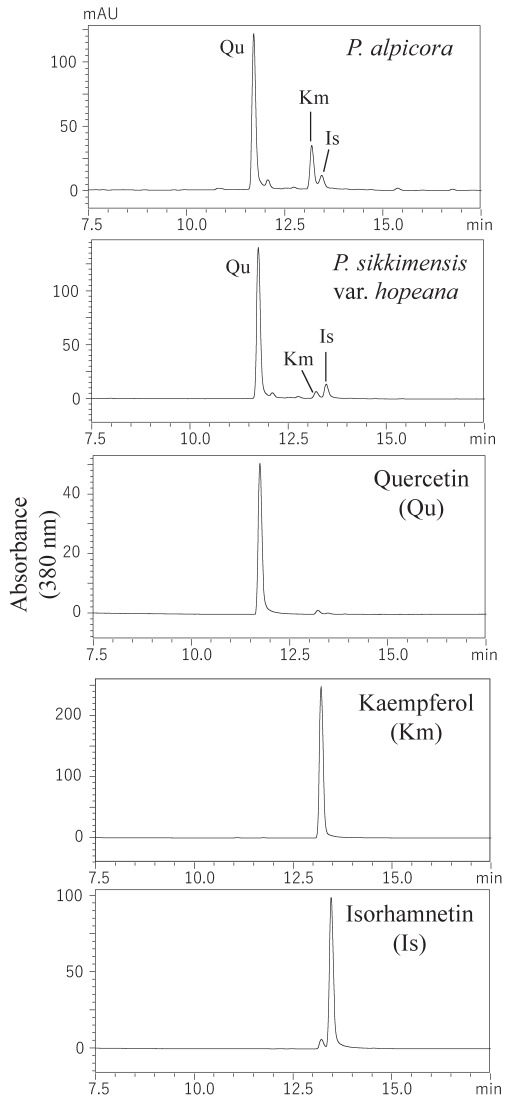

Fig. 8. Aglycone analysis of the flowers of *Primura alpicola* and *P. sikkimensis* var. *hopeana*. These aglycones were compared with authentic flavonols, quercetin (Qu,11.7 min), kaempferol (Km,13.2 min) and isorhamnetin (Is,13.5 min) by HPLC.

Quercetin as yellow pigment has been reported from the yellow flowers of *Astrophytum* species (Cactaceae) (Iwashina *et al.*, 1988) and *Clematis* cultivars and species (Ranunculaceae) (Hashimoto *et al.*, 2008), *Chimonanthus praecox* (Calycanthaceae) (Iwashina *et al.*, 2001) and *Hibiscus rosa-sinensis* (Malvaceae) (Subramanian and Nair, 1972). In this study, the high quercetin concentration in the flowers of two *Primula* species was revealed by aglycone analysis. However, their *Primula* flowers are white to ivory white. As the results, we presumed that quercetin amount is quantitatively insufficient for expression of yellow.

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