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Qualitative and Quantitative Variation of Anthocyanins and Flavonols among the Different Organs of *Cercidiphyllum japonicum*

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Abstract Anthocyanins and flavonols of the reddish sprouting leaves, male flowers and female flowers of *Cercidiphyllum japonicum* were qualitatively and quantitatively analyzed. Five anthocyanins and 13 flavonols were isolated from their organs, together with chlorogenic acid and ellagic acid, with various combination. Their anthocyanins were identified as cyanidin 3-*O*-glucoside, 3-*O*-galactoside, 3-*O*-arabinoside, 3,5-di-*O*-glucoside and 3-*O*-arabinoside-5-*O*-glucoside by UV-Vis, LC-MS, acid hydrolysis, and TLC and HPLC comparisons with authentic samples. On the other hand, flavonols were characterized as kaempferol and its 3-*O*-glucoside, 3-*O*-rutinoside and 3-*O*-sophoroside, quercetin and its 3-*O*-glucoside, 3-*O*-rhamnoside, 3-*O*-rutinoside, 3-*O*-sophoroside and 3-*O*-arabinofuranoside, sexangularetin 3-*O*-glucoside, corniculatusin and its 3-*O*-diglucoside by the same manners described above. Although flavonoid composition of the sprouting leaves and female flowers was comparatively similar, that of male flowers was qualitatively and quantitatively different.

Key words: anthocyanins, *Cercidiphyllum japonicum*, female flowers, flavonols, inter-organic chemical variation, male flowers, sprouting leaves.

Introduction

The genus *Cercidiphyllum* belongs to the family Cercidiphyllaceae and consists of only two species, *C. japonicum* Siebold et Zucc. and *C. magnificum* (Nakai) Nakai. Of their species, *C. japonicum* is deciduous tree and distributed in Japan and China (Akiyama, 2006). In Japan, the species is widely cultivated in gardens and parks, or as street trees. Autumn leaves are yellow but rarely reddish yellow. However, sprouting leaves, male and female flowers are red in spring. Flavonoids of this species have been reported from the sprouting and mature leaves, heartwoods and bark by a few researches.

Two flavonol glycosides were isolated from the leaves and identified as quercetin 3-*O*-rhamnoside and rhamnocitrin 3-*O*-rhamninoside (Egger and Reznik, 1961; Harborne and Baxter, 1999). Two flavonol aglycones, myricetin and quercetin, dihydroflavonol, ampelopsin, and flavan and proanthocyanidins, (+)-taxifolin and procyanidin B_2, were found in the heartwoods (Towatari *et al.*, 2002). Moreover, other four flavonol glycosides, kaempferol 3-*O*-glucoside, kaempferol 7-*O*-glucoside, quercetin 3-*O*-glucoside and sexangularetin 3-*O*-glucoside, were isolated from the bark (Kasuga *et al.*, 2008). An anthocyanin, cyanidin 3,5-di-*O*-glucoside, was found in the reddish sprouting leaves (Yoshitama *et al.*, 1972). Two anthocyanins were isolated from the reddish mature leaves and partially characterized as cyanidin diglucoside and 3-*O*-monohexoside (Hayashi and Abe, 1955). In

this survey, anthocyanins and flavonols were isolated from the reddish sprouting leaves, male flowers and female flowers by various chromatography and characterized by UV-Vis, LC-MS, acid hydrolysis, and TLC and HPLC comparisons with authentic samples. Moreover, anthocyanin and flavonol composition was qualitatively and quantitatively compared among their organs.

Materials and Methods

Plant materials

The sprouting leaves, male flowers and female flowers of *Cercidiphyllum japonicum* Siebold et Zucc. were collected in Mt. Sugana-dake, Niigata Pref., Japan in spring, 2002.

General

Analytical high performance liquid chromatography (HPLC) was performed with Shimadzu HPLC systems using Inertsil ODS-4 (I.D. 6.0 × 150 mm, Chemicals Evaluation and Research Institute, Tokyo) at a flowe-rate of 1.0 ml min^{-1}. Detection wave-length was 350 nm (flavonols and organic acids) and 530 nm (anthocyanins). Eluents were MeCN/H_2O/H_3PO_4 (20 : 80 : 0.2) (solv. I) or (40 : 60 : 0.2) (solv. II) (flavonols and organic acids) and MeCN/HOAc/H_2O/H_3PO_4 (8 : 8 : 81 : 3) (solv. III) (anthocyanins). Liquid chromatograph-mass spectra (LC-MS) was performed with Shimadzu LC-MS systems using Inertsil ODS-4 (I.D. 2.1 × 100 mm) at flow-rate of 0.2 ml min^{-1}, ESI^+ 4.5 kV and ESI^- 3.5 kV, 250℃. Eluents were MeCN/H_2O/HCOOH (20 : 75 : 5 or 40 : 55 : 5) (flavonols and organic acids) and MeCN/H_2O/HCOOH (10 : 85 : 5) (anthocyanins). Acid hydrolysis was performed in 12% HCl, 100℃, 30 min. After shaking with diethy ether (flavonols) and isoamyl alcohol (anthocyanins), aglycones and anthocyanidins were migrated to the organic layer, and sugars were left in aqueous layer. Anthocyanidins and flavonol aglycones were identified by HPLC comparisons with authentic samples. On the other hand, sugars were identified by paper chromatographic comparisons with authentic sugars using solvent systems, BBPW (*n*-BuOH/benzene/pyridine/H_2O = 5 : 1 : 3 : 3) and BTPW (*n*-BuOH/toluene/pyridine/H_2O = 5 : 1 : 3 : 3). Sugar spots were visualized by spraying 1% methanolic aniline hydrochloride on the chromatograms and heating. Partial acid hydrolysis of **A1** was performed in 1%MeOH–HCl/20%HCl (1 : 1), 70℃, 5–90 min. Retention times of the intermediates were compared with those of cyanidin 3-*O*-arabinoside and cyanidin 3-*O*-glucoside by HPLC. Preparative PC was performed using solvent systems, BAW (*n*-BuOH/HOAc/H_2O = 4 : 1 : 5, upper phase) and 15% HOAc. Analytical thin layer chromatography (TLC) was performed with Cellulose F plastic plate (Merck, Germany) using solvent systems, BAW, BEW (*n*-BuOH/EtOH/H_2O = 4 : 1 : 2.2), 15% HOAc and Forestal (HOAc/HCl/H_2O = 30 : 3 : 10).

Extraction and isolation

Dry sprouting leaves (2.84 g), male flowers (1.60 g) and female flowers (1.90 g) of *C. japonicum* were extracted with MeOH/HCOOH (92 : 8). The concentrated extracts were applied to prep. PC using solvent system BAW and then 15% HOAc. Isolated anthocyanins, and flavonols and organic acids were purified by Sephadex LH-20 column chromatography using solvent systems, 70% MeOH and MeOH/H_2O/HCOOH (70 : 25 : 5), respectively.

Identification of anthocyanins, flavonols and organic acids

The compounds were identified by UV-Vis spectral survey according to Mabry *et al.* (1970) for flavonols, and Harborne (1958) for anthocyanins, LC-MS, characterization of acid hydrolysates, and TLC and HPLC comparisons with authentic samples. TLC, HPLC, UV-Vis spectral and LC-MS data of the isolated compounds were as follows.

Cyanidin 3-*O*-arabinoside-5-*O*-glucoside (**A1**). HPLC: Retention time (Rt) (min) 10.26 (solv. III). UV-Vis: λmax (nm) 0.1%MeOH-HCl 273, 527; E_{440}/E_{max} (%) 14.8. LC-MS: *m/z* 581 $[M]^+$ (molecular ion peak, cyanidin + each 1 mol of

arabinose and glucose), *m/z* 449 $[M-132]^+$ (fragment ion peak, cyanidin + 1 mol glucose), *m/z* 419 $[M-162]^+$ (fragment ion peak, cyanidin + 1 mol arabinose) and *m/z* 287 $[M-294]^+$ (fragment ion peak, cyanidin).

Cyanidin 3,5-di-*O*-glucoside (cyanin, **A2**). HPLC: Rt (min) 5.55 (solv. III).

Cyanidin 3-*O*-galactoside (idaein, **A3**). HPLC: Rt (min) 7.93 (solv. III).

Cyanidin 3-*O*-glucoside (chrysanthemin, **A4**). HPLC: Rt (min) 8.50 (solv. III). UV-Vis: λmax (nm) 0.1%MeOH-HCl 281, 529; E_{440}/E_{max} (%) 24.8. LC-MS: *m/z* 449 $[M]^+$ (molecular ion peak, cyanidin + 1 mol glucose).

Cyanidin 3-*O*-arabinoside (**A5**). HPLC: Rt (min) 10.26 (solv. III). LC-MS: *m/z* 419 $[M]^+$ (molecular ion peak, cyanidin + 1 mol arabinose).

Quercetin 3-*O*-glucoside (isoquercitrin, **F1**). TLC: Rf 0.65 (BAW), 0.75 (BEW), 0.26 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 yellow. HPLC: Rt (min) 12.99 (solv. I). UV-Vis: λmax (nm) MeOH 256, 265sh, 357; + NaOMe 273, 331, 408 (inc.); + $AlCl_3$ 274, 429; + $AlCl_3$/HCl 269, 297, 359, 398; + NaOAc 273, 325, 395; + NaOAc/H_3BO_3 261, 297, 378. LC-MS: *m/z* 465 $[M+H]^+$, 463 $[M-H]^-$ (molecular ion peaks, quercetin + 1 mol glucose), *m/z* 303 $[M-162+H]^+$ (fragment ion peak, quercetin).

Quercetin 3-*O*-rutinoside (rutin, **F2**). TLC: Rf 0.41 (BAW), 0.65 (BEW), 0.45 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 yellow. HPLC: Rt (min) 10.66 (solv. I). UV-Vis: λmax (nm) MeOH 257, 264sh, 358; + NaOMe 274, 326, 412 (inc.); + $AlCl_3$ 273, 428; + $AlCl_3$/HCl 263, 298sh, 360, 396; + NaOAc 273, 324, 401; + NaOAc/H_3BO_3 260, 295sh, 378. LC-MS: *m/z* 609 $[M-H]^-$ (molecular ion peak, quercetin + each 1 mol of rhamnose and glucose), *m/z* 303 $[M-308+H]^+$ (fragment ion peak, quercetin).

Kaempferol 3-*O*-sophoroside (**F3**). TLC: Rf 0.62 (BAW), 0.75 (BEW), 0.51 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 dark greenish yellow. HPLC: Rt (min) 10.21 (solv. I). UV-Vis: λmax (nm) MeOH 265, 349; + NaOMe 275, 324, 396 (inc.); + $AlCl_3$ 274, 302, 348, 402; + $AlCl_3$/HCl 274, 299sh, 349, 392; + NaOAc 273, 320, 389; + NaOAc/H_3BO_3 265, 302, 357. LC-MS: *m/z* 633 $[M+H+Na]^+$, 609 $[M-H]^-$ (molecular ion peaks, kaempferol + 2 mol glucose), *m/z* 287 $[M-324+H]^+$ (fragment ion peak, kaempferol).

Kaempferol 3-*O*-glucoside (astragalin, **F4**). TLC: Rf 0.76 (BAW), 0.79 (BEW), 0.35 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 dark greenish yellow. HPLC: Rt (min) 21.51 (solv. I). UV-Vis: λmax (nm) MeOH 265, 350; + NaOMe 274, 326, 399 (inc.); + $AlCl_3$ 275, 302, 351, 405; + $AlCl_3$/HCl 273, 299sh, 349, 393; + NaOAc 274, 320, 390; + NaOAc/H_3BO_3 264, 302, 360. LC-MS: *m/z* 449 $[M+H]^+$, 447 $[M-H]^-$ (molecular ion peaks, kaempferol + 1 mol glucose), *m/z* 287 $[M-162+H]^+$ (fragment ion peak, kaempferol).

Kaempferol 3-*O*-rutinoside (nicotiflorin, **F5**). TLC: Rf 0.62 (BAW), 0.75 (BEW), 0.51 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 dark greenish yellow. HPLC: Rt (min) 17.65 (solv. I). UV-Vis: λmax (nm) MeOH 268, 352; + NaOMe 279, 327, 404 (inc.); + $AlCl_3$ 277, 305, 353, 396; + $AlCl_3$/HCl 277, 303, 347, 395; + NaOAc 277, 311, 398; + NaOAc/H_3BO_3 268, 299, 357. LC-MS: *m/z* 617 $[M+H+Na]^+$, 593 $[M-H]^-$ (molecular ion peaks, kaempferol + each 1 mol of rhamnose and glucose), *m/z* 449 $[M-146+H]^+$ (fragment ion peak, kaempferol + 1 mol glucose), *m/z* 287 $[M-308+H]^+$ (fragment ion peak, kaempferol).

Quercetin 3-*O*-rhamnoside (quercitrin, **F6**). TLC: Rf 0.80 (BAW), 0.83 (BEW), 0.39 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 yellow. HPLC: Rt (min) 19.78 (solv. I). UV-Vis: λmax (nm) MeOH 257, 264sh, 356; + NaOMe 272, 330, 407 (inc.); + $AlCl_3$ 274, 430; + $AlCl_3$/HCl 270, 297sh, 359, 397; + NaOAc 273, 330, 393; + NaOAc/H_3BO_3 262, 301, 377.

Quercetin 3-*O*-arabinofuranoside (avicularin, **F7**). TLC: Rf 0.80 (BAW), 0.83 (BEW), 0.24 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 yellow. HPLC: Rt (min) 20.06 (solv. I). UV-Vis: λmax (nm) MeOH 257, 264sh, 358; + NaOMe 276, 327, 407 (inc.); + $AlCl_3$ 274, 428; + $AlCl_3$/HCl 266, 301, 358, 396sh; + NaOAc 272,

327, 391; +NaOAc/H_3BO_3 262, 376. LC-MS: *m/z* 435 $[M+H]^+$, 433 $[M-H]^-$ (molecular ion peaks, quercetin + 1 mol arabinose), *m/z* 303 $[M-132+H]^+$ (fragment ion peak, quercetin).

Sexangularetin 3-*O*-glucoside (**F8**). HPLC: Rt (min) 20.40 (solv. I). UV-Vis: λmax (nm) MeOH 272, 357; +NaOMe 283, 329, 406 (inc.); +$AlCl_3$ 281, 310, 352, 405sh; +$AlCl_3$/HCl 280, 308, 349, 405sh; +NaOAc 282, 309, 402; +NaOAc/H_3BO_3 273, 325, 358sh. LC-MS: *m/z* 479 $[M+H]^+$, 477 $[M-H]^-$ [molecular ion peaks, sexangularetin (3,5,7,4′-tetrahydroxy-8-methoxyflavone) + 1 mol glucose], *m/z* 317 $[M-162+H]^+$ (fragment ion peak, sexangularetin).

Quercetin 3-*O*-sophoroside (**F9**). TLC: Rf 0.35 (BAW), 0.60 (BEW), 0.59 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 yellow. HPLC: Rt (min) 7.20 (solv. I). UV-Vis: λmax (nm) MeOH 256, 264sh, 355; +NaOMe 273, 330, 407 (inc.); +$AlCl_3$ 274, 427; +$AlCl_3$/HCl 269, 298sh, 359, 396; +NaOAc 273, 325, 394; +NaOAc/H_3BO_3 262, 296, 376. LC-MS: *m/z* 625 $[M-H]^-$ (molecular ion peak, quercetin + 2 mol glucose), *m/z* 465 $[M-162+H]^+$ (fragment ion peak, quercetin + 1 mol glucose), *m/z* 303 $[M-324+H]^+$, 301 $[M-324-H]^-$ (fragment ion peaks, quercetin).

Corniculatusin 3-*O*-diglucoside (**F10**). HPLC: Rt (min) 6.60 (solv. I). UV-Vis: λmax (nm) MeOH 260, 268sh, 358; +NaOMe 279, 332, 414 (inc.); +$AlCl_3$ 279, 434; +$AlCl_3$/HCl 276, 304sh, 357, 402; +NaOAc 278, 328, 408; +NaOAc/H_3BO_3 266, 378. LC-MS: *m/z* 679 $[M+H+Na]^+$, 655 $[M-H]^-$ [molecular ion peaks, corniculatusin (3,5,7,3′,4′-pentahydroxy-8-methoxyflavone) + 2 mol glucose], *m/z* 495 $[M-162+H]^+$ (fragment ion peak, corniculatusin + 1 mol glucose), *m/z* 333 $[M-324+H]^+$ (fragment ion peak, corniculatusin).

Corniculatusin (**F11**). HPLC: Rt (min) 7.29 (solv. II). UV-Vis: λmax (nm) MeOH 259, 271sh, 379; +NaOMe decomposition; +$AlCl_3$ 259sh, 274, 362, 461; +$AlCl_3$/HCl 266, 307sh, 361, 437; +NaOAc 281, 332, 414; +NaOAc/H_3BO_3 265, 310sh, 395. LC-MS: *m/z* 333 $[M+H]^+$, 331 $[M-H]^-$ (molecular ion peaks, corniculatusin).

Quercetin (**F12**). TLC: Rf 0.69 (BAW), 0.03 (15%HOAc), 0.31 (Forestal); color UV (365 nm) and UV/NH_3 yellow. HPLC: Rt (min) 7.36 (solv. II). UV-Vis: λmax (nm) MeOH 256, 272sh, 374; +NaOMe decomposition; +$AlCl_3$ 269, 450; +$AlCl_3$/HCl 257, 308, 358, 424; +NaOAc 271, 324, 402; +NaOAc/H_3BO_3 258, 388. LC-MS: *m/z* 301 $[M-H]^-$ (molecular ion peaks, quercetin).

Kaempferol (**F13**). TLC: Rf 0.90 (BAW), 0.00 (15%HOAc), 0.56 (Forestal); color UV (365 nm) and UV/NH_3 yellow. HPLC: Rt (min) 11.17 (solv. II). LC-MS: *m/z* 285 $[M-H]^-$ (molecular ion peak, kaempferol).

Chlorogenic acid. TLC: Rf 0.59 (BAW), 0.54 (15%HOAc), 0.77 (Forestal); color UV (365 nm) blue, UV/NH_3 blue-green. HPLC: Rt (min) 5.39 (solv. I). UV-Vis: λmax (nm) MeOH 297sh, 327; +NaOMe 264, 378 (inc.); +$AlCl_3$ 259, 307sh, 357; +$AlCl_3$/HCl 243sh, 298sh, 327; +NaOAc 309sh, 341, 368sh; +NaOAc/H_3BO_3 254sh, 303sh, 348. LC-MS: *m/z* 355 $[M+H]^+$, 353 $[M-H]^-$ (molecular ion peaks, each 1 mol of caffeic acid and quinic acid), *m/z* 191 $[M-162-H]^-$ (fragment ion peak, quinic acid).

Ellagic acid. TLC: Rf 0.29 (BAW), 0.03 (15%HOAc), 0.23 (Forestal); color UV (365 nm) purple, UV/NH_3 dark red. HPLC: Rt (min) 12.67 (solv. I). UV-Vis: λmax (nm) MeOH 254, 353sh, 366; +NaOMe 251, 290, 402; +$AlCl_3$ 247, 271, 316sh, 380; +$AlCl_3$/HCl 254, 354sh, 366; +NaOAc 253, 278, 355, 373sh; +NaOAc/H_3BO_3 263, 313sh, 364sh, 380. LC-MS: *m/z* 303 $[M+H]^+$, 301 $[M-H]^-$ (molecular ion peaks, ellagic acid).

Results and Discussion

Anthocyanins from Cercidiphyllum japonicum

Five anthocyanins **A1**–**A5** were found by HPLC survey and isolated from the sprouting leaves, male flowers and/or female flowers (Table 1). Of their anthocyanins, **A2** and **A3** were identified as cyanidin 3,5-di-*O*-glucoside (cyanin) and cyanidin 3-*O*-galactoside (idaein) by HPLC comparisons with authentic samples from the flowers of *Dahlia variabilis* L. (Astera-

Table 1. Distribution of anthocyanins among the sprouting leaves, male flowers and female flowers of *Cercidiphyllum japonicum*

	A1	A2	A3	A4	A5
Sprouting leaves	70.4	13.0		6.7	9.9
Female flowers	50.3	15.8		13.3	20.6
Male flowers	15.7		6.9	30.6	46.8

A1 = Cyanidin 3-*O*-arabinoside-5-*O*-glucoside, **A2** = Cyanidin 3,5-di-*O*-glucoside, **A3** = Cyanidin 3-*O*-galactoside, **A4** = Cyanidin 3-*O*-glucoside and **A5** = Cyanidin 3-*O*-arabinoside.

*Their relative amounts (%) were calculated by peak area (530 nm) of HPLC chromatograms.

ceae) (Hayashi, 1933) and pericarp of *Fatsia japonica* (Thunb.) Decne. et Planch. (Araliaceae) (Hayashi, 1939). Glucose and arabinose were liberated by acid hydrolysis of **A4** and **A5**, together with cyanidin, respectively. The attachment of 1 mol of hexose or pentose to cyanidin was shown by LC-MS survey. Finally, **A4** was identified as cyanidin 3-*O*-glucoside (chrysanthemin) by HPLC comparison with authentic sample from the autumn leaves of *Acer* spp. (Sapindaceae) (Hattori and Hayashi, 1937). On the other hand, **A5** was characterized as cyanidin 3-*O*-arabinoside. Anthocyanin **A1** produced cyanidin, glucose and arabinose by acid hydrolysis. E_{440}/E_{max} value of **A1** was 14.8%, showing that this pigment is cyanidin 3,5-*O*-glycoside but not 3-*O*-glycoside (Harborne, 1958). The attachment of each 1 mol of glucose and arabinose to cyanidin was shown by LC-MS, i.e. the occurrence of molecular ion peak, *m/z* 581 $[M]^+$, and fragment ion peaks, *m/z* 449 and 419. Thus, **A1** was presumed to be cyanidin 3-*O*-arabinoside-5-*O*-glucoside or 3-*O*-glucoside-5-*O*-arabinoside. Finally, **A1** was identified as cyanidin 3-*O*-arabinoside-5-*O*-glucoside, since retention time of an intermediate which was produced by partial acid hydrolysis, agreed with that of **A5** (cyanidin 3-*O*-arabinoside) but not **A4** (cyanidin 3-*O*-glucoside). Of five anthocyanins which were isolated in this survey, cyanidin 3,5-di-*O*-glucoside (**A2**) has already been reported from the sprouting leaves of this species (Yoshitama *et al.*, 1972). In this survey, **A2** was also found in the

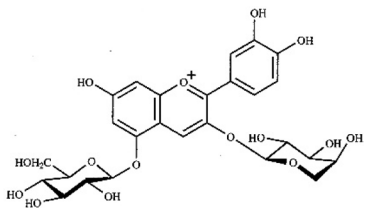

Fig. 1. Cyanidin 3-*O*-arabinoside-5-glucoside (**A1**).

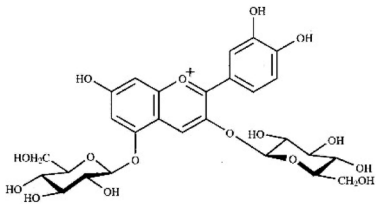

Fig. 2. Cyanidin 3,5-di-O-glucoside (**A2**).

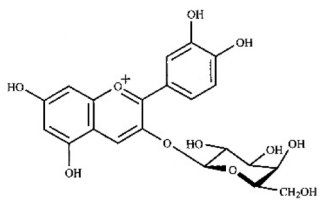

Fig. 3. Cyanidin 3-*O*-galactoside (**A3**).

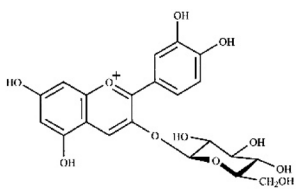

Fig. 4. Cyanidin 3-*O*-glucoside (**A4**).

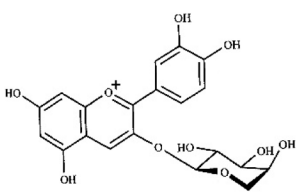

Fig. 5. Cyanidin 3-*O*-arabinoside (**A5**).

female flowers. Other four anthocyanins, **A1**, **A3**–**A5**, were reported from this species for the first time. Cyanidin 3-*O*-arabinoside-5-*O*-glucoside (**A1**) has been reported from other two plant

species, the flowers of *Rhododendron simsii* Planchon (Ericaceae) (Asen and Budin, 1966) and the sepals of *Polygonum* spp. (Polygonaceae) (Yoshitama *et al.*, 1984).

Flavonols from Cercidiphyllum japonicum

Thirteen flavonols (**F1**–**F13**) were isolated from the sprouting leaves, male flowers and female flowers (Table 2). Major flavonoids **F1** and **F4** from the sprouting leaves were liberated quercetin and kaempferol by acid hydrolysis, together with glucose, respectively. It was shown by UV-Vis spectral properties that glucose is attached to 3-position of aglycones (Mabry *et al.*, 1970). Finally, **F1** and **F4** were identified as quercetin 3-*O*-glucoside (isoquercitrin) and kaempferol 3-*O*-glucoside (astragalin) by TLC and HPLC comparisons with authentic samples from the leaves of *Barringtonia asiatica* (L.) Kurz. (Lecythidaceae) (Iwashina and Kokubugata, 2016) and *Phytolacca americana* L. (Phytolaccaceae) (Iwashina and Kitajima, 2009). UV spectral properties of **F2** and **F5** were those of 3-substituted 5,7,3′,4′-tetrahydroxyflavone and 5,7,4′-trihydroxyflavone (Mabry *et al.*, 1970). Quercetin and kaempferol were liberated by acid hydrolysis of **F2** and **F5**, together with both rhamnose and glucose. Since molecular ion peaks, *m/z* 609 $[M-H]^-$ and *m/z* 593 $[M-H]^-$, appeared on LC-MS, it was shown that each 1 mol of rhamnose and glucose is attached to 3-position of quercetin and kaempferol. Finally, **F2** and **F5** were identified as quercetin 3-*O*-rutinoside and kaempferol 3-*O*-rutinoside by TLC and HPLC comparisons with authentic rutin from the aerial parts of *Osyris alba* L. (Santalaceae) (Iwashina *et al.*, 2008a) and the fronds of *Cyrto-*

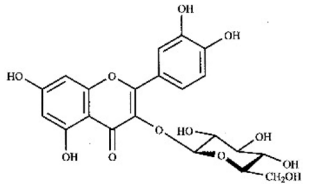

Fig. 6. Quercetin 3-*O*-glucoside (**F1**).

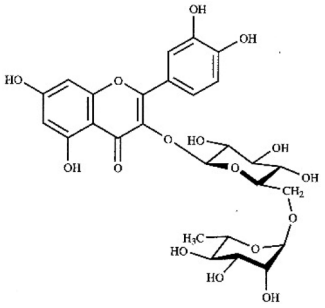

Fig. 7. Quercetin 3-*O*-rutinoside (**F2**).

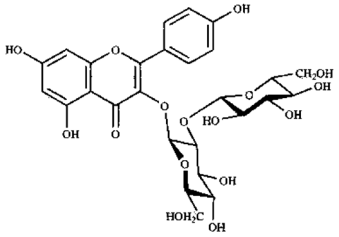

Fig. 8. Kaempferol 3-*O*-sophoroside (**F3**).

Table 2. Distribution of flavonols among the sprouting leaves, male flowers and female flowers of *Cercidiphyllum japonicum*

	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13
Sprouting leaves	25.1	5.2	19.7	25.9	4.0	13.1	7.0					tr	tr
Female flowers	21.6	31.3	24.5	5.5	5.1	3.3	4.0	4.7					
Male flowers	12.7	3.0	14.8	5.6	1.7			2.5	33.8	25.5	tr	tr	tr

F1 = Quercetin 3-*O*-glucoside, **F2** = Quercetin 3-*O*-rutinoside, **F3** = Kaempferol 3-*O*-sophoroside, **F4** = Kaempferol 3-*O*-glucoside, **F5** = Kaempferol 3-*O*-rutinoside, **F6** = Quercetin 3-*O*-rhamnoside, **F7** = Quercetin 3-*O*-arabino furanoside, **F8** = Sexangularetin 3-*O*-glucoside, **F9** = Quercetin 3-*O*-sophoroside, **F10** = Corniculatusin 3-*O*-diglucoside, **F11** = Corniculatusin, **F12** = Quercetin, and **F13** = Kaempferol. tr = trace amounts.

* Their relative amounts (%) were calculated by peak area (350 nm) of HPLC chromatograms.

** Chlorogenic acid and ellagic acid were found in all three organs as major compounds.

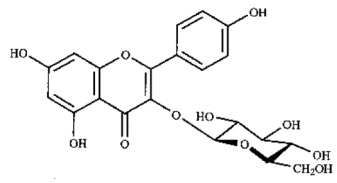

Fig. 9. Kaempferol 3-*O*-glucoside (**F4**).

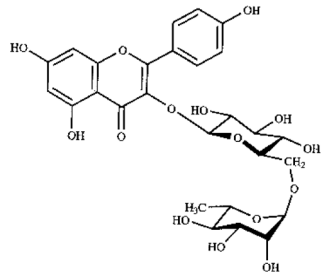

Fig. 10. Kaempferol 3-*O*-rutinoside (**F5**).

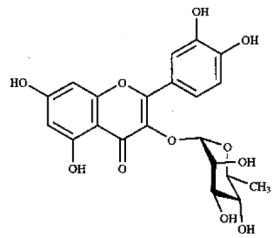

Fig. 11. Quercetin 3-*O*-rhamnoside (**F6**).

mium spp. (Dryopteridaceae) (Iwashina *et al.*, 2006b), respectively.

Flavonoids **F3** and **F9** were obtained as major compounds from the female and male flowers, respectively. Kaempferol and quercetin were liberated by acid hydrolysis of their flavonoids, together with glucose. Molecular ion peaks, *m/z* 609 $[M\text{-}H]^-$ and 625 $[M\text{-}H]^-$, and fragment ion peaks, *m/z* 287 $[M\text{-}324+H]^+$ and 303 $[M\text{-}324+H]^+$, appeared on LC-MS, showing the attachment of each 2 mol of glucose to kaempferol and quercetin. Since UV-Vis spectral properties of **F3** and **F9** showed those of 3-substituted kaempferol and quercetin, they were indicated to be kaempferol 3-*O*-diglucoside and quercetin 3-*O*-diglucoside. Finally, **F3** and **F9** were identified as kaempferol 3-*O*-glucosyl-(1→2)-glucoside (= kaempferol 3-*O*-sophoroside) and quercetin 3-*O*-sophoroside, by TLC and HPLC comparisons with authentic samples from the flowers of *Dianthus caryophyllus* L. (Caryophyllaceae) (Iwashina *et al.*, 2010) and the leaves of *Asarum yakusimense* Masam. (Aristolochiaceae) (Iwashina *et al.*, 2005). Flavonoid **F6** was isolated from the sprouting leaves and female flowers. UV-Vis spectral properties of **F6** were typical those of 3,5,7,3′,4′-pentahydroxyflavone 3-*O*-glycoside (Mabry *et al.*, 1970). Quercetin and rhamnose were liberated by acid hydrolysis. Thus, **F6** was identified as quercetin 3-*O*-rhamnoside by TLC and HPLC comparison with authentic quercitrin (Extrasynthèse, France).

Quercetin and arabinose were produced by acid hydrolysis of **F7**. Molecular ion peaks, *m/z* 435 $[M+H]^+$ and 433 $[M\text{-}H]^-$, and fragment ion peak, *m/z* 303 $[M\text{-}132+H]^+$ appeared, showing the attachment of 1 mol arabinose to quercetin. The attachment of the sugar to 3-position of quercetin was shown by UV-Vis spectral survey according to Mabry *et al.* (1970). Thus, **F7** was identified as quercetin 3-*O*-arabinofuranoside (avicularin) but not 3-*O*-arabinopyranoside (guaijaverin) by TLC and HPLC comparison with authentic sample from the leaves of *Fallopia japonica* (Houtt.) Ronse Decr. (Polygonaceae) (Murai *et al.*, 2015). Flavonoid **F8** was obtained as minor compound of the male and female flowers. Its UV-Vis spectral properties were similar to those of kaempferol 3-*O*-glycoside. An aglycone and glucose were liberated by acid hydrolysis. However, retention time of the aglycone was not agreed with that of kaempferol. Molecular ion peaks, *m/z* 479 $[M+H]^+$ and 477 $[M\text{-}H]^-$, and fragment ion peak, *m/z* 317 $[M\text{-}162+H]^+$, appeared on LC-MS, showing the attachment of 1 mol glucose to tetrahydroxy-monomethoxyflavone. Thus, it was shown that an additional methoxyl group was attached to kaempferol. Since sexangularetin (3,5,7,4′-tetrahydroxy-8-methoxyflavone) 3-*O*-glucoside has already been found in the bark of *Cercidiphyllum japonicum* (Kasuga *et al.*, 2008), **F8** was presumed as this flavonoids. Flavonoid **F10** was obtained as major compound of male flowers. The presence of free 5-, 7-, 3′-

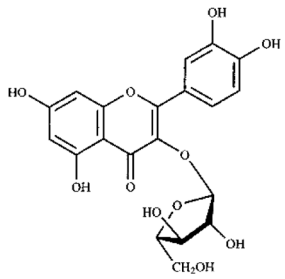

Fig. 12. Quercetin 3-*O*-arabinofuranoside (**F7**).

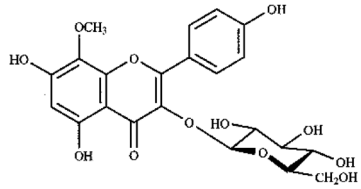

Fig. 13. Sexangularetin 3-*O*-glucoside (**F8**).

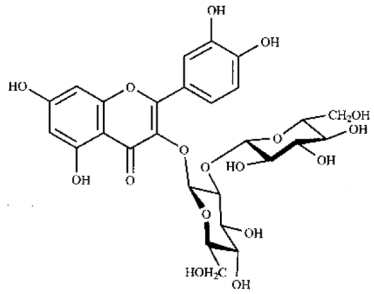

Fig. 14. Quercetin 3-*O*-sophoroside (**F9**).

and 4′-hydroxyl and substituted 3-hydroxyl groups was shown by UV-Vis spectral survey. An aglycone and glucose were produced by acid hydrolysis. The attachment of 2 mol glucose to aglycone was proved by the occurrence of the molecular ion peak, *m/z* 655 $[M\text{-}H]^-$, and fragment ion peaks, *m/z* 495 $[M\text{-}162+H]^+$ and 333 $[M\text{-}324+H]^+$ on LC-MS. The occurrence of the latter fragment ion peak showed the attachment of an additional methoxyl group to quercetin. Thus, **F10** was presumed as coruniculatusin (3,5,7,3′,4′-pentahydroxy-8-methoxyflavone) 3-*O*-diglucoside, since 3,5,7,4′-tetrahydroxy-8-methoxyflavone (sexangularetin) 3-*O*-glucoside has been found in this species (Kasuga *et al.*, 2008). Flavonoid aglycone **F11** was isolated from

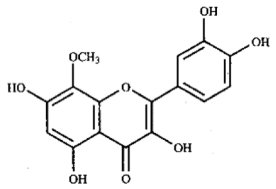

Fig. 15. Corniculatusin (**F11**).

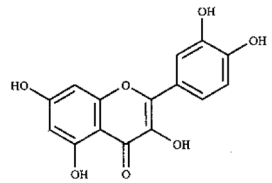

Fig. 16. Quercetin (**F12**).

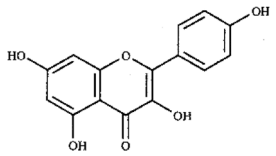

Fig. 17. Kaempferol (**F13**).

the male flowers as very minor compound. Since UV-Vis spectral properties, and LC-MS and HPLC data of this flavonoid agreed with those of aglycone of **F11**, it was presumed as corniculatusin. Minor flavonoids **F12** and **F13** were also aglycones but not glycosides. Their UV-Vis spectral properties were those of 3,5,7,3′,4′-pentahydroxyflavone and 3,5,7,4′-tetrahydroxyflavone, respectively (Mabry *et al.*, 1970). Finally, they were identified as quercetin and kaempferol themselves by TLC and HPLC comparisons with authentic samples from the flowers of *Astrophytum* spp. (Cactaceae) (Iwashina *et al.*, 1988). Two organic acids were isolated from all organs, i.e. sprouting leaves, male flowers and female flowers as major compounds. Their UV-Vis spectral, LC-MS, TLC and HPLC data were agreed with those of authentic chlorogenic acid from the seeds of *Coffea arabica* L. (Rubiaceae) (Hayashi, unpublished data) and ellagic acid (Yazaki, private communication).

Of 13 flavonols and two organic acids isolated from this species, eight flavonols, i.e. quercetin

3-*O*-rutinoside (**F2**), 3-*O*-arabinofuranoside (**F7**) and 3-*O*-sophoroside (**F9**), kaempferol (**F13**) and its 3-*O*-rutinoside (**F5**) and 3-*O*-sophoroside (**F3**) and corniculatusin (**F11**) and its 3-*O*-diglucoside (**F10**), and chlorogenic acid and ellagic acid were found in *Cercidiphyllum japonicum* for the first time. Although their organic acids were found as major compounds, they are common compounds in plants.

Qualitative and quantitative variation of anthocyanins and flavonols among the sprouting leaves, female flowers and male flowers

Distribution patterns of anthocyanins and flavonols among the sprouting leaves, female flowers and male flowers were shown in Tables 1 and 2. Anthocyanin and flavonol composition of the sprouting leaves and female flowers is comparatively similar to each other. Although three anthocyanins **A1**, **A4** and **A5** were present in all organs, major anthocyanins of the sprouting leaves and female flowers were cyanidin 3-*O*-arabinoside-5-*O*-glucoside (**A1**). On the other hand, those of the male flowers were cyanidin 3-*O*-arabinoside (**A5**) and cyanidin 3-*O*-glucoside (**A4**). Moreover, cyanidin 3,5-di-*O*-glucoside (**A2**) was present in the sprouting leaves and female flowers, but absent in male flowers. In contrast, minor anthocyanin, cyanidin 3-*O*-galactoside (**A3**) occurred in the male flowers only.

Thirteen flavonol glycosides and aglycones were isolated from their organs. Of their flavonols, quercetin 3-*O*-glucoside (**F1**) and 3-*O*-rutinoside (**F2**), and kaempferol 3-*O*-glucoside (**F4**), 3-*O*-sophoroside (**F3**) and 3-*O*-rutinoside (**F5**) were found in all organs, i.e. sprouting leaves, male flowers and female flowers. Flavonoids **F1** (25.1%) and **F4** (25.9%) were major flavonoids in the sprouting leaves. On the other hand, **F2** (31.3%) and **F3** (24.5%) were major ones in female flowers. In male flowers of *C. japonicum*, although **F1**–**F5** were also present, major compounds were quercetin 3-*O*-sophoroside (**F9**, 33.8%) and corniculatusin 3-*O*-diglucoside (**F10**, 25.5%). Quercetin 3-*O*-rhamnoside (**F6**) and quercetin 3-*O*-arabinofuranoside (**F7**) were detected from the sprouting leaves and female flowers. Sexangularerin 3-*O*-glucoside (**F8**) was found in the female and male flowers as minor compound (4.7% and 2.5%, respectively). Thus, it was shown that qualitative and quantitative flavonoid variation occur among three organs, sprouting leaves, female flowers and male flowers.

In *Glycine max* (L.) Merr. (Leguminosae), flavonoids of the flowers, leaves, pubescence on leaves, and roots and seeds were isolated. They were reported as anthocyanins such as malvidin 3,5-di-*O*-glucoside and flavonols such as kaempferol 3-*O*-gentiobioside from the flowers (Iwashina *et al.*, 2007, 2008b), various kaempferol, quercetin and isorhamnetin 3-*O*-glycosides from the leaves (Murai *et al.*, 2013), apigenin and luteolin from the pubescence on leaves (Iwashina *et al.*, 2006a), and isoflavonoids such as daidzein 7-*O*-glucoside, and glyceollins I, II and III from the roots and seeds (e.g., Burden and Bailey, 1975; Ohta *et al.*, 1979). They are known or presumed as pollinator attractant (flower), UV shields and anti-stress compounds (leaves), defensive agents against fungi (pubescence), and phytoalexins and rhizobia attractant (roots and seeds), respectively (Bohm, 1998). Their flavonoid variation among the sprouting leaves, female flowers and male flowers of *Cercidiphyllum japonicum* may be produced due to the difference of the function, which is performed by their flavonoids, in each organ.

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