

Flavonoid Properties in the Leaves of *Barringtonia asiatica* (Lecythidaceae)

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Abstract Six C-glycosylflavones and four flavonol O-glycosides were isolated from the leaves of *Barringtonia asiatica*. Of their flavonoids, four flavones and four flavonols were identified as isovitexin, vitexin, isoorientin and orientin, and kaempferol 3-O-glucoside, kaempferol 3-O-rutinoside, quercetin 3-O-glucoside and quercetin 3-O-rutinoside, respectively. Other two were unidentified C-glycosylflavones. Though ellagitannins were also present in the leaves, they were not characterized. The flavonoids of *B. asiatica* were reported for the first time. In this survey, it was presumed that major flavonoids of the Lecythidaceae were not only flavonols such as kaempferol and quercetin but also C-glycosylflavones such as isovitexin, vitexin, isoorientin and orientin.

Key words : *Barringtonia asiatica*, flavonoids, flavonols, C-glycosylflavones, Lecythidaceae.

Introduction

As a series of flavonoid survey of endemic and/or endangered plants in Japan, we have isolated and identified the flavonoids from the leaves and/or flowers of *Japonolirion osense* Nakai (Petrosaviaceae) (Iwashina *et al.*, 2005a), *Asarum* spp. (Aristolochiaceae) (Iwashina *et al.*, 2005b), *Cassytha* spp. (Lauraceae) (Murai *et al.*, 2008), *Cladopus japonicus* Imamura and *Hydrobryum japonicum* Imamura (Podostemaceae) (Murai *et al.*, 2009), *Ranzania japonica* (T.Ito) T.Ito (Berberidaceae) (Iwashina and Kitajima, 2009a), *Pothos chinensis* (Raf.) Merr. (Araceae) (Iwashina *et al.*, 2010), *Callianthemum hon-doense* Nakai & Hara (Ranunculaceae) (Asakawa *et al.*, 2010), *Myoporum bontiodese* (Sieb. et Zucc.) A.Gray (Myoporaceae) (Iwashina and Kokubugata, 2010), *Petrosavia sakuraii* (Makino) J.J.Sm. (Petrosaviaceae) (Iwashina *et al.*, 2011a), *Nipponanthemum nipponicum* (Franch ex Maxim.) Kitam. (Asteraceae) (Uehara and Iwashina, 2012), *Iris rossii* Baker (Iridaceae)

(Mizuno *et al.*, 2012), *Crossostephium chinense* (L.) Makino (Asteraceae) (Uehara *et al.*, 2014), *Torenia concolor* Lindl. var. *formosana* Yamazaki (Scrophulariaceae) (Iwashina and Kokubugata, 2014), *Primula sieboldii* E.Morr. (Primulaceae) (Hashimoto *et al.*, 2015), *Asplenium ruta-muraria* L. (Aspleniaceae) (Iwashina *et al.*, 2015a), *Iris gracilipes* A.Gray (Iridaceae) (Mizuno *et al.*, 2015) and so on.

The genus *Barringtonia* belongs to the family Lecythidaceae and consists of ca. 40 species (Hatusima, 1989). In Japan, *B. asiatica* (L.) Kurz. and *B. racemosa* (L.) Spreng. are growing. *B. asiatica* is evergreen trees to 15 m tall, and growing in Ishigaki and Iriomote Islands in Ryukyus and Ogasawara Islands, and also Taiwan, Southern China, the Philippines, Indochina, Malaysia, New Guinea, northern Australia, Melanesia and Micronesia (Setoguchi, 1999). The species is designated to endangered plant by Red-data book in Japan.

The flavonoid compounds of *Barringtonia* species have been reported by a few authors. A

flavanone, one flavone and two flavonols were isolated from the leaves of *B. racemosa* and identified as naringenin, luteolin, and kaempferol and quercetin 3-*O*-rutinoside, together with two organic acids, gallic acid and ferulic acid, as antifungal activity substances (Hussin *et al.*, 2009). Three flavonols, kaempferol, quercetin and its 3-*O*-rutinoside, were also found in the leaves and stems of the same species with three organic acids, gallic acid, ellagic acid and protocatechuic acid (Kong *et al.*, 2014). Two anthocyanins, cyanidin and delphinidin 3-*O*-sambubiosides were isolated from the flowers of *B. racemosa* and *B. macrostachya* Kurz. (Lowry, 1976). Three flavan 3-ols, (+)-epigallocatechin, (+)-gallocatechin 4'-methyl ether and (+)-gallocatechin 4'-methyl ether 5-*O*-glucoside, were isolated from the barks of *B. acutangula* (L.) Gaertn. (Nguyen *et al.*, 2014).

In *B. asiatica*, four saponins were isolated from the seeds (Errington and White, 1967; Itô *et al.*, 1967; Herlt *et al.*, 2002). Eleven triterpenes were also found in the leaves (Ragasa *et al.*, 2011). However, flavonoids were not reported.

In this paper, we describe the isolation and identification of the flavonoids from the leaves of *Barringtonia asiatica* for the first time.

Materials and Methods

Plant materials

Barringtonia asiatica (L.) Kurz. was collected in Nakama-zaki, Iriomote Island, Okinawa Pref., Japan, 4 July 2012. Voucher specimen was deposited in the Herbarium of National Museum of Nature and Science, Japan (TNS).

General

High performance liquid chromatography (HPLC) was performed with Shimadzu HPLC systems using L-column2 ODS (I.D. 6.0×150 mm, Chemicals Evaluation and Research Institute, Japan) at a flow-rate of 1.0 ml min^{-1} . Detection was 350 nm. Eluents were MeCN/H₂O/H₃PO₄ (20:80:0.2 for flavonoid glycosides, 40:60:0.2 for flavonoid aglycones and 18:82:0.2 for ella-

gitannins). Liquid chromatograph-mass spectra (LC-MS) was performed with Shimadzu LC-MS systems using L-column2 ODS (I.D. 2.1×100 mm, Chemicals Evaluation and Research Institute) at a flow-rate of 0.2 ml min^{-1} . ESI⁺ 4.5 kV and ESI⁻ 3.5 kV, 250°C. Eluent was MeCN/H₂O/HCOOH (15:85:5). Acid hydrolysis was performed in 12% HCl, 100°C, 30 min. After shaking with diethyl ether, aglycones migrated to the organic layer, and sugars and C-glycosylflavones were left in aqueous layer. Thin layer chromatography (TLC) was performed with Cellulose F plastic plate (Merck, Germany) using solvent systems, BAW (*n*-BuOH/HOAc/H₂O = 4:1:5, upper phase), BEW (*n*-BuOH/EtOH/H₂O = 4:1:2.2) and 15% HOAc. Paper chromatography (PC) for sugar identification was performed using solvent systems, BBPW (*n*-BuOH/benzene/pyridine/H₂O = 5:1:3:3) and BTPW (*n*-BuOH/toluene/pyridine/H₂O = 5:1:3:3). The sugar spots were visualized by spraying 1% methanolic aniline hydrochloride on the paper chromatograms and heating.

Extraction and separation

Fresh leaves (154.8 g) were extracted with MeOH. The concentrated extracts were applied to preparative PC using solvent systems, BAW, 15%HOAc, re-BAW and then re-15% HOAc. The isolated compounds were purified by Sephadex LH-20 column chromatography using solvent system, 70% MeOH.

Identification of the flavonoids

Flavonoids were identified by UV spectral survey according to Mabry *et al.* (1970), LC-MS, characterization of acid hydrolysates, and TLC and HPLC comparisons with authentic samples. Origins of the authentic samples which were used in this survey were as follows: isovitexin from the inflorescence of *Amorphophallus titanum* (Becc.) Becc. ex Arcangeli (Araceae) (Iwashina *et al.*, 2015b), vitexin from the fronds of *Adiantum venustum* Don (Parkeriaceae) (Iwashina *et al.*, 1995), isoorientin from the leaves of *Vitex rotundifolia* L.fil. (Verbenaceae)

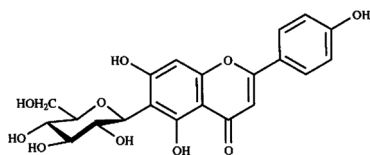


Fig. 1. Isovitexin (1).

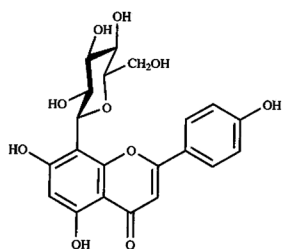


Fig. 2. Vitexin (2).

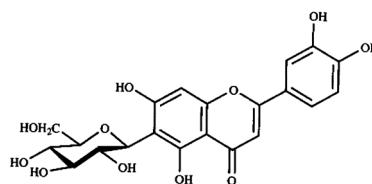


Fig. 3. Isoorientin (3).

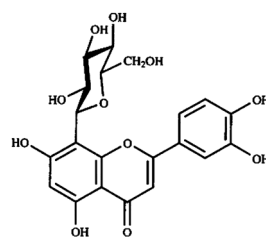


Fig. 4. Orientin (4).

(Iwashina *et al.*, 2011b), orientin from the leaves of *Acer palmatum* Thunb. (Sapindaceae) (Iwashina and Murai, 2008), kaempferol 3-*O*-glucoside from the leaves of *Calystegia japonica* Choisy (Convolvulaceae) (Murai *et al.*, 2015), kaempferol 3-*O*-rutinoside from the leaves of *Glycine max* (L.) Merr. (Leguminosae) (Murai *et al.*, 2013), quercetin 3-*O*-glucoside from the leaves of *Phytolacca americana* L. (Phytolaccaceae) (Iwashina and Kitajima, 2009b) and quercetin 3-*O*-rutinoside from the leaves of *Saruma henryi* Oliver (Aristolochiaceae) (Iwashina *et al.*, 2002). TLC, HPLC, UV and LC-MS data of the isolated flavonoids were as follows.

Isovitexin (1). TLC: *R_f* 0.69 (BAW), 0.63 (BEW), 0.40 (15%HOAc); color UV (365 nm) dark purple, UV/NH₃ dark greenish yellow. HPLC: retention time (*t_R*) (min) 14.93. UV: λ_{max} (nm) MeOH 271, 333; + NaOMe 278, 330, 399 (inc.); + AlCl₃ 278, 303, 348, 378sh; + AlCl₃/HCl 279, 301, 344, 377sh; + NaOAc 278, 394; + NaOAc/H₃BO₃ 272, 347. LC-MS: *m/z* 433 [M + H]⁺, 431 [M - H]⁻ (molecular ion peaks, apigenin + 1 mol glucose).

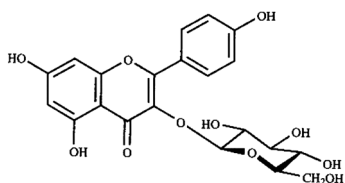
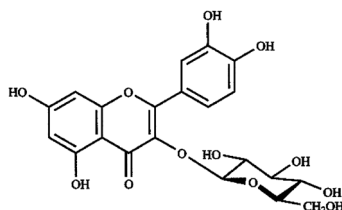
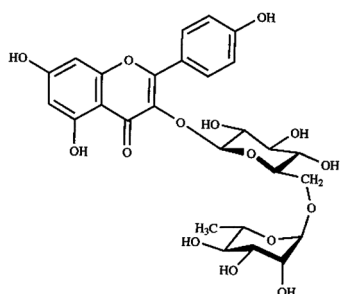
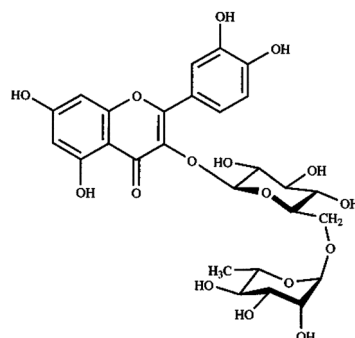
Vitexin (2). TLC: *R_f* 0.46 (BAW), 0.44 (BEW), 0.17 (15%HOAc); color UV (365 nm) dark purple, UV/NH₃ dark greenish yellow. HPLC: *t_R* (min) 14.38. UV: λ_{max} (nm) MeOH

270, 332; + NaOMe 279, 330, 394 (inc.); + AlCl₃ 276, 303, 346, 382; + AlCl₃/HCl 277, 302, 340, 379sh; + NaOAc 279, 389; + NaOAc/H₃BO₃ 271, 345. LC-MS: *m/z* 433 [M + H]⁺, 431 [M - H]⁻ (molecular ion peaks, apigenin + 1 mol glucose).

Isoorientin (3). TLC: *R_f* 0.45 (BAW), 0.43 (BEW), 0.27 (15%HOAc); color UV (365 nm) dark purple, UV/NH₃ dark yellow. HPLC: *t_R* (min) 14.01. UV: λ_{max} (nm) MeOH 257, 268sh, 351; + NaOMe 275, 412 (inc.); + AlCl₃ 274, 422; + AlCl₃/HCl 261, 276sh, 360, 377sh; + NaOAc 276, 333, 400; + NaOAc/H₃BO₃ 265, 380, 427sh. LC-MS: *m/z* 449 [M + H]⁺, 447 [M - H]⁻ (molecular ion peaks, luteolin + 1 mol glucose).

Orientin (4). TLC: *R_f* 0.30 (BAW), 0.30 (BEW), 0.09 (15%HOAc); color UV (365 nm) dark purple, UV/NH₃ dark yellow. HPLC: *t_R* (min) 10.47. UV: λ_{max} (nm) MeOH 256, 268sh, 348; + NaOMe 276, 332, 407 (inc.); + AlCl₃ 273, 422; + AlCl₃/HCl 261, 275sh, 297sh, 357, 386sh; + NaOAc 278, 330, 398; + NaOAc/H₃BO₃ 264, 375, 427sh. LC-MS: *m/z* 447 [M - H]⁻ (molecular ion peak, luteolin + 1 mol glucose).

Kaempferol 3-*O*-glucoside (astragalin, 5). TLC: *R_f* 0.79 (BAW), 0.87 (BEW), 0.39

Fig. 5. Kaempferol 3-*O*-glucoside (astragalin, **5**).Fig. 7. Quercetin 3-*O*-glucoside (isoquercitrin, **7**).Fig. 6. Kaempferol 3-*O*-rutinoside (nicotiflorin, **6**).Fig. 8. Quercetin 3-*O*-rutinoside (rutin, **8**).

(15%HOAc); color UV (365nm) dark purple, UV/NH₃ dark greenish yellow. HPLC: *t*R (min) 25.45. UV: λ_{max} (nm) MeOH 266, 346; + NaOMe 275, 324, 398 (inc.); + AlCl₃ 273, 303, 351, 392; + AlCl₃/HCl 275, 302, 345, 391; + NaOAc 275, 313, 392; + NaOAc/H₃BO₃ 266, 353. LC-MS: *m/z* 447 [M – H][–] (molecular ion peak, kaempferol + 1 mol glucose).

Kaempferol 3-*O*-rutinoside (nicotiflorin, **6**). TLC: R_f 0.64 (BAW), 0.73 (BEW), 0.59 (15%HOAc); color UV (365nm) dark purple, UV/NH₃ dark greenish yellow. HPLC: *t*R (min) 20.51. UV: λ_{max} (nm) MeOH 266, 347; + NaOMe 275, 324, 400 (inc.); + AlCl₃ 274, 304, 350, 393; + AlCl₃/HCl 275, 302, 347, 392; + NaOAc 275, 316, 395; + NaOAc/H₃BO₃ 267, 354. LC-MS: *m/z* 593 [M – H][–] (molecular ion peak, kaempferol + each 1 mol glucose and rhamnose), *m/z* 287 [M – 308 + H]⁺ (fragment ion peak, kaempferol).

Quercetin 3-*O*-glucoside (isoquercitrin, **7**). TLC: R_f 0.65 (BAW), 0.73 (BEW), 0.31 (15%HOAc); color UV (365nm) dark purple, UV/NH₃ dark yellow. HPLC: *t*R (min) 16.97.

UV: λ_{max} (nm) MeOH 258, 265sh, 352; + NaOMe 274, 331, 403 (inc.); + AlCl₃ 275, 417; + AlCl₃/HCl 270, 299sh, 354, 393sh; + NaOAc 274, 323, 395; + NaOAc/H₃BO₃ 263, 297, 376. LC-MS: *m/z* 463 [M – H][–] (molecular ion peak, quercetin + 1 mol glucose), *m/z* 303 [M – 162 + H]⁺ (fragment ion peak, quercetin).

Quercetin 3-*O*-rutinoside (rutin, **8**). TLC: R_f 0.54 (BAW), 0.55 (BEW), 0.55 (15%HOAc); color UV (365nm) dark purple, UV/NH₃ dark yellow. HPLC: *t*R (min) 13.69. UV: λ_{max} (nm) MeOH 257, 264sh, 358; + NaOMe 273, 328, 412 (inc.); + AlCl₃ 274, 428; + AlCl₃/HCl 269, 298sh, 362, 396; + NaOAc 273, 326, 402; + NaOAc/H₃BO₃ 262, 294, 379. LC-MS: *m/z* 610 [M – H][–] (molecular ion peak, quercetin + each 1 mol glucose and rhamnose), *m/z* 303 [M – 308 + H]⁺ (fragment ion peak, quercetin).

Unknown C-glycosylflavone (**9**). TLC: R_f 0.76 (BAW), 0.76 (BEW), 0.50 (15%HOAc); color UV (365nm) dark purple, UV/NH₃ dark greenish yellow. HPLC: *t*R (min) 24.08. UV: λ_{max} (nm) MeOH 270, 332; + NaOMe 280, 329, 399 (inc.); + AlCl₃ 277, 303, 335, 377sh; + AlCl₃/HCl 277,

302sh, 345, 376sh; + NaOAc 279, 316, 395; + NaOAc/H₃BO₃ 274, 284sh, 302, 344sh, 406sh. LC-MS: *m/z* 585 [M + H]⁺, 583 [M – H][–] (molecular ion peaks).

Unknown *C*-glycosylflavone (**10**). TLC: Rf 0.70 (BAW), 0.76 (BEW), 0.45 (15%HOAc); color UV (365 nm) dark purple, UV/NH₃ dark greenish yellow. HPLC: *t*R (min) 21.82. UV: λ_{max} (nm) MeOH 271, 334; + NaOMe 279, 330, 396 (inc.); + AlCl₃ 278, 303, 341, 379; + AlCl₃/HCl 277, 299sh, 345, 377sh; + NaOAc 279, 320, 393; + NaOAc/H₃BO₃ 273, 285sh, 303, 348sh, 400sh. LC-MS: *m/z* 585 [M + H]⁺, 583 [M – H][–] (molecular ion peaks).

Results and Discussion

In this survey, ten flavonoids (**1–10**) were isolated from the leaves of *Barringtonia asiatica*. It was shown by UV spectral survey that **1** and **2** were flavones having free 5-, 7- and 4'-hydroxyl groups (Mabry *et al.*, 1970). They were unhydrolyzable by hot acid treatment, showing that they are *C*-glycosylflavones. Their LC-MS survey showed the attachment of 1 mol hexose to trihydroxyflavone. Finally, they were identified as isovitexin (5,7,4'-trihydroxyflavone 6-*C*-glucoside, **1**, Fig. 1) and vitexin (5,7,4'-trihydroxyflavone 8-*C*-glucoside, **2**, Fig. 2) by TLC and HPLC comparisons with authentic samples.

Similarly, **3** and **4** were shown to be flavones with free 5-, 7-, 3'- and 4'-hydroxyl groups by UV spectra, and *C*-glycosylflavones by hot acid treatment. LC-MS survey showed that both flavones are luteolin which attached each 1 mol hexose. Thus, **3** and **4** were identified as isoorientin (5,7,3',4'-tetrahydroxyflavone 6-*C*-glucoside, **3**, Fig. 3) and orientin (5,7,3',4'-tetrahydroxyflavone 8-*C*-glucoside, **4**, Fig. 4).

Kaempferol was liberated by acid hydrolysis of **5** and **6**. As sugar portions, glucose, and glucose and rhamnose were found in the aqueous layer. It was shown by UV spectral properties that they are flavonols having free 5-, 7-, 4'-hydroxyl and a substituted 3-hydroxyl groups. Finally, **5** and **6** were identified as kaempferol

3-*O*-glucoside (Fig. 5) and kaempferol 3-*O*-rutinoside (Fig. 6) by direct TLC and HPLC comparisons with authentic astragalin and nicotiflorin.

Flavonoids **7** and **8** were shown to be 5,7,3',4'-tetrahydroxy-3-substituted flavones by UV spectral survey (Mabry *et al.*, 1970). By acid hydrolysis, quercetin and glucose, and quercetin, glucose and rhamnose were liberated from them, respectively. LC-MS survey of **7** and **8** showed the attachment of 1 mol glucose, and each 1 mol glucose and rhamnose to quercetin. Thus, they were identified as quercetin 3-*O*-glucoside (isquercitrin, **7**, Fig. 7) and quercetin 3-*O*-rutinoside (rutin, **8**, Fig. 8) by TLC and HPLC comparisons with authentic specimens.

UV spectral survey of **9** and **10** showed that they are flavones having free 5-, 7- and 4'-hydroxyl groups. Since **9** and **10** were unhydrolyzable by hot acid treatment, they are *C*-glycosylflavones. Their molecular weights were 584 (see Experimental), presuming that they are new *C*-glycosylflavones. However, their further characterization could not perform for a small amounts of the compounds. As other compounds, some ellagitannins, which were shown by UV spectral properties and characterization of acid hydrolysates (ellagic acid), were found.

In this survey, four *C*-glycosylflavones (**1–4**) and four flavonol *O*-glycosides (**5–8**) were identified. Of their flavonoids, though quercetin 3-*O*-rutinoside (**8**) has been reported from the leaves of *B. racemosa* (Hussin *et al.*, 2009; Kong *et al.*, 2014), other compounds were found in the genus *Barringtonia* for the first time. The flavonoids of the family Lecythidaceae were hardly surveyed. However, it has been reported that major flavonoids of the family are quercetin and/or kaempferol, together with another compound, ellagic acid (Giannasi, 1988). Practically, quercetin and its 3-*O*-rutinoside and kaempferol were found in another Lecythidaceous species, *Napoleona imperialis* P.Beav. (Ukachukwu *et al.*, 2013). Quercetin was also isolated from *Bertholletia excelsa* Humb. & Borpl., together with gallic acid, gallocatechin, protocatechuic acid, myricetin, ellagic acid etc. (John and Shahidi, 2010).

In another Lecythidaceous species, *Planchonia grandis* Ridley and *P. careya* (F.Muell.) R. Knuth, three kaempferol *p*-coumaroylglycosides, and kaempferol 3-*O*-gentiobioside, 3-*O*-glucoside and two acylated 3-*O*-glycosides were reported from the leaves, respectively (Crublet *et al.*, 2003; McRae *et al.*, 2008). Kaempferol and quercetin glycosides were isolated from *Barringtonia asiatica* in this survey. However, C-glycosylflavones, isovitexin (1), vitexin (2), isoorientin (3) and orientin (4), were also found as major flavonoids.

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