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Flavonoids from *Theligonum japonicum* Endemic to Japan and their Chemical Character

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Abstract Flavonoids from the aerial parts of *Theligonum japonicum* endemic to Japan were surveyed. Eleven flavonoids were found and isolated by various chromatography including paper, column and HPLC. Of their compounds, four were identified as quercetin 3-*O*-glucoside, quercetin 3-*O*-galactoside, isorhamnetin 3-*O*-glucoside and isorhamnetin 3-*O*-galactoside by UV, LC-MS, acid hydrolysis and TLC and HPLC comparisons with authentic samples. Other seven were characterized as 3-*O*-hexosides and 3-*O*-rhamnosylhexosides of quercetin and isorhamnetin which attached 1 mol unknown substance of molecular weight 162. Although the genus belonged to the family Theligonaceae, it was recently incorporated to the family Rubiaceae by APG III. In this paper, flavonoid character of *T. japonicum* was compared with *Rubia* and *Hedyotis* species which phylogenetically or morphologically related to *Theligonum*.

Key words: chemosystematics, flavonoids, flavonols, Rubiaceae, Theligonaceae, *Theligonum japonicum*.

Introduction

The genus *Theligonum* consists of four species, i.e. *T. cynocrambe* L. in the Mediterranean and Macronesia, *T. macranthum* Franch. in China, *T. formosanum* Ohwi et T.S.Liu in Taiwan and *T. japonicum* Okubo et Makino in Japan. *Theligonum japonicum* was endemic to Japan, found in Kochi Pref. and recorded by Japanese taxonomist, Tomitaro Makino, for the first time (Makino, 1889). Although Makino was first belonged *Theligonum* to the family Urticaceae, the species was afterward contained to the independent family Theligonaceae. However, the genus *Theligonum* was recently incorporated to the family Rubiaceae by APG III.

The present of anthocyanin in the seeds of *Theligonum cynocrambe* is shown without its identification (Mabry *et al.*, 1975). However, chemical compound including flavonoids in *Theligonum* species was never reported. As a series of flavonoid survey of Japanese endemic and endangered plants, in this paper, the isolation and characterization of flavonoid and related compounds from *Theligonum japonicum* are reported for the first time.

Materials and Methods

Plant materials

Theligonum japonicum Okubo et Makino was collected in Mt. Tsukuba, Ibaraki Pref., Japan, 27 May 2013 and Daiyu-zan, Hakone, Kanagawa Pref., Japan, 25, April 2015. Voucher specimens were deposited to the Herbarium of National Museum of Nature and Science, Japan (TNS).

General

Analytical 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 250 and 350 nm. Eluents were MeCN/H_2O/H_3PO_4 (20 : 80 : 0.2). 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℃. Eluent was MeCN/H_2O/HCOOH (20 : 75 : 5, 15 : 5 : 80 or 10 : 85 : 5). Acid hydrolysis was performed in 12% HCl, 100℃, 30 min. After shaking with diethyl ether, aglycones migrated to the organic layer, and sugars were left in aqueous layer. Sugars were applied to paper chromatography (PC) 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. Thin layer chromatography (TLC) was performed with Cellulose F plastic plate (Merck, Germany) using solvent systems: BAW (*n*-BuOH/HOAc/H_2O = 4 : 1 : 5, upper phase), BEW (*n*-BuOH/EtOH/H_2O = 4 : 1 : 2.2) and 15%HOAc.

Extraction and isolation

Fresh aerial parts (33.1 g for Mt. Tsukuba and 38.7 g for Daiyu-zan) were extracted with MeOH. The filtrated and concentrated extracts were applied to preparative PC using solvent systems, BAW, 15%HOAc, re-15%HOAc and then re-BAW. The isolated compounds were purified by Sephadex LH-20 column chromatography using solvent system, 70% MeOH.

Identification

The compounds were characterized by UV spectral survey according to Mabry *et al.* (1970), LC-MS, acid hydrolysis, and TLC and HPLC comparisons with authentic samples. TLC, HPLC, UV and LC-MS data of the isolated compounds were as follows.

Quercetin 3-*O*-glucoside (isoquercitrin, **1**)

TLC: Rf 0.64 (BAW), 0.74 (BEW), 0.27 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 yellow. HPLC: *t*R (min) 8.79. UV: *λ*max (nm) MeOH 257, 265sh, 360; + NaOMe 273, 330, 410 (inc.); + $AlCl_3$ 274, 422; + $AlCl_3$/HCl 268, 299, 364, 399; + NaOAc 273, 325, 397; + NaOAc/H_3BO_3 261, 298, 378. LC-MS: *m/z* 465 $[M+H]^+$, 463 $[M-H]^-$ (molecular ion peaks, quercetin + 1 mol hexose), *m/z* 303 $[M-162+H]^+$, 301 $[M-162-H]^-$ (fragment ion peaks, quercetin).

Quercetin 3-*O*-galactoside (hyperine, **2**)

TLC: Rf 0.64 (BAW), 0.74 (BEW), 0.27 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 yellow. HPLC: *t*R (min) 8.44. UV: *λ*max (nm) MeOH 257, 265sh, 357; + NaOMe 273, 329, 406 (inc.); + $AlCl_3$ 275, 430; + $AlCl_3$/HCl 269, 299, 359, 395; + NaOAc 273, 326, 396; + NaOAc/H_3BO_3 262, 297, 376. LC-MS: *m/z* 465 $[M+H]^+$, 463 $[M-H]^-$ (molecular ion peaks, quercetin + 1 mol hexose), *m/z* 303 $[M-162+H]^+$, 301 $[M-162-H]^-$ (fragment ion peaks, quercetin).

Isorhamnetin 3-*O*-glucoside (**3**)

TLC: Rf 0.71 (BAW), 0.79 (BEW), 0.27 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 yellow. HPLC: *t*R (min) 13.77. UV: *λ*max (nm) MeOH 255, 266sh, 356; + NaOMe 275, 330, 408 (inc.); + $AlCl_3$ 270, 301sh, 364sh, 405; + $AlCl_3$/HCl 267, 300, 360, 396; + NaOAc 273, 324, 403; + NaOAc/H_3BO_3 258, 297sh, 365. LC-MS: *m/z* 501 $[M+H+Na]^+$, *m/z* 477 $[M-H]^-$ (molecular ion peaks, isorhamnetin + 1 mol hexose), *m/z* 317 $[M-162+H]^+$ (fragment ion peak, isorhamnetin).

Isorhamnetin 3-*O*-galactoside (cacticin, **4**)

TLC: Rf 0.71 (BAW), 0.79 (BEW), 0.27 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 yellow. HPLC: *t*R (min) 12.77. UV: *λ*max (nm) MeOH 255, 266sh, 356; + NaOMe 275, 330, 408 (inc.); + $AlCl_3$ 270, 301sh, 365sh, 405; + $AlCl_3$/HCl 267, 300, 360, 396; + NaOAc 273, 324, 403; + NaOAc/H_3BO_3 256, 297sh, 365. LC-MS: *m/z* 501 $[M+H+Na]^+$, *m/z* 477 $[M-H]^-$ (molecular ion peaks, isorhamnetin + 1 mol hexose), *m/z* 317 $[M-162+H]^+$

(fragment ion peak, isorhamnetin).

Quercetin 3-*O*-glycoside (**5a** and **5b**)

TLC: Rf 0.73 (BAW), 0.83 (BEW), 0.44 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 yellow. HPLC: *t*R (min) 10.70 (**5a**) and 10.47 (**5b**). UV: λmax (nm) MeOH 257, 265sh, 360; + NaOMe 273, 330, 410 (inc.); + $AlCl_3$ 274, 422; + $AlCl_3$/HCl 268, 299, 364, 399; + NaOAc 273, 325, 397; + NaOAc/H_3BO_3 261, 298, 378. LC-MS: *m/z* 609 $[M+H]^+$, 607 $[M-H]^-$ (molecular ion peaks, quercetin + 1 mol hexose + 1 mol unknown compound of molecular weight 162), *m/z* 303 $[M-306+H]^+$ (fragment ion peak, quercetin).

Quercetin 3-*O*-glycoside (**6a** and **6b**)

TLC: Rf 0.53 (BAW), 0.47 (BEW), 0.80 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 yellow. HPLC: *t*R (min) 16. 93 (**6a**) and 16. 21 (**6b**). UV: λmax (nm) MeOH 257, 266sh, 356; + NaOMe 273, 328, 412 (inc.); + $AlCl_3$ 274, 428; + $AlCl_3$/HCl 269, 298sh, 362, 396; + NaOAc 273, 326, 402; + NaOAc/H_3BO_3 262, 294, 379. LC-MS: *m/z* 777 $[M+H+Na]^+$, 753 $[M-H]^-$ (molecular ion peaks, quercetin + each 1 mol hexose and rhamnose + 1 mol unknown compound of molecular weight 162), *m/z* 303 $[M-452+H]^+$ (fragment ion peak, quercetin).

Isorhamnetin 3-*O*-glycoside (**7a** and **7b**)

TLC: Rf 0.80 (BAW), 0.89 (BEW), 0.51 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 yellow. HPLC: *t*R (min) 20.29 (**7a**) and 17.48 (**7b**). UV: λmax (nm) MeOH 255, 266sh, 357; + NaOMe 274, 330, 410 (inc.); + $AlCl_3$ 270, 301sh, 367, 403; + $AlCl_3$/HCl 268, 300, 362, 395; + NaOAc 274, 323, 402; + NaOAc/H_3BO_3 256, 304sh, 365. LC-MS: *m/z* 645 $[M+H+Na]^+$, 621 $[M-H]^-$ (molecular ion peaks, isorhamnetin + 1 mol hexose + 1 mol unknown compound of molecular weight 162), *m/z* 317 $[M-306+H]^+$ (fragment ion peak, isorhamnetin).

Isorhamnetin 3-*O*-glycoside (**8**)

TLC: Rf 0.64 (BAW), 0.61 (BEW), 0.64 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 yellow. HPLC: *t*R (min) 25.22. UV: λmax (nm) MeOH 254, 266sh, 351; + NaOMe 271, 320, 402 (inc.); + $AlCl_3$ 268, 300sh, 365, 405sh; + $AlCl_3$/HCl 265, 300sh, 358, 400sh; + NaOAc 273, 315, 375; + NaOAc/H_3BO_3 257, 364. LC-MS: *m/z* 791 $[M+H+Na]^+$, 767 $[M-H]^-$ (molecular ion peaks, isorhamnetin + each 1 mol hexose and rhamnose + 1 mol unknown compound of molecular weight 162), *m/z* 317 $[M-452+H]^+$ (fragment ion peak, isorhamnetin).

Chlorogenic acid

TLC: Rf 0.80 (BAW), 0.64 (BEW), 0.63 (15%HOAc); color UV (365 nm) blue, UV/NH_3 blue-green. HPLC: *t*R (min) 4.07. UV: λmax (nm) MeOH 243sh, 298sh, 327; + NaOMe 267, 379; + $AlCl_3$ 257sh, 351; + $AlCl_3$/HCl 234sh, 326; + NaOAc 298sh, 339, 374sh; + NaOAc/H_3BO_3 253sh, 303sh, 347. LC-MS: *m/z* 355 $[M+H]^+$, 353 $[M-H]^-$ (molecular ion peaks, each 1 mol caffeic acid and quinic acid), *m/z* 181 $[M-174+H]^+$ (fragment ion peak, caffeic acid).

Results and Discussion

Eleven peaks of the flavonoids appeared on the chromatogram of the aerial part extracts of *Theligonum japonicum* and isolated by various chromatography. Although two populations of the species were used as plant materials, their flavonoid composition was essentially the same. Of their compounds, four were completely identified by UV spectral survey, characterization of acid hydrolysates, measurement of molecular weight by LC-MS, and TLC and HPLC comparisons with authentic samples. Molecular weight of flavonoids **1** and **2** were both 464, showing the attachment of 1 mol hexose to pentahydroxyflavone. Quercetin was liberated by acid hydrolysis of **1** and **2**. Moreover, it was shown by UV spectral properties that the attachment of a hexose to 3-position of quercetin (Mabry *et al.*, 1970). Thus, both **1** and **2** were determined as quercetin 3-*O*-hexosides. Finally, they were identified as quercetin 3-*O*-glucoside (isoquercitrin, **1**) (Fig. 1) and quercetin 3-*O*-galactoside (hyperine, **2**) (Fig. 2) by TLC and HPLC comparisons with

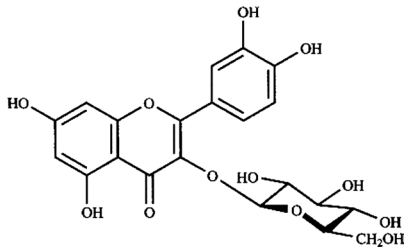

Fig. 1. Quercetin 3-*O*-glucoside (isoquercitrin, **1**).

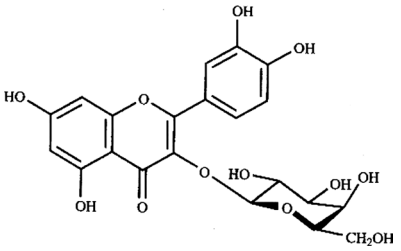

Fig. 2. Quercetin 3-*O*-galactoside (hyperine, **2**).

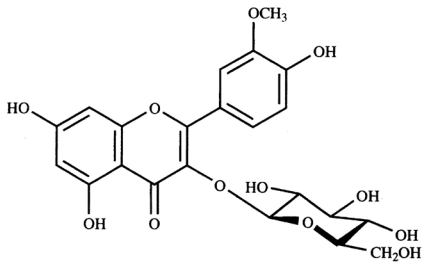

Fig. 3. Isorhamnetin 3-*O*-glucoside (**3**).

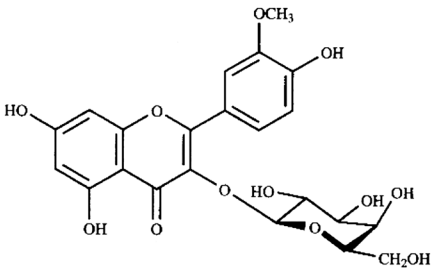

Fig. 4. Isorhamnetin 3-*O*-galactoside (cacticin, **4**).

authentic samples from *Rheum nobile* (Polygonaceae) (Iwashina *et al.*, 2004). Similarly, flavonoids **3** and **4** were shown to be tetrahydroxymonomethoxyflavone 3-*O*-hexosides by UV spectral and LC-MS analysis. Since isorhamnetin (3,5,7,4′-tetrahydroxy-3′-methoxyflavone) was liberated by acid hydrolyses, they were determined to be isorhamnetin 3-*O*-hexosides. Finally, they were identified as isorhamnetin 3-*O*-glucoside (**3**) (Fig. 3) and isorhamnetin 3-*O*-galactoside (**4**) (Fig. 4) by TLC and HPLC comparisons with authentic samples (Extrasynthèse, France).

It was shown by HPLC analyses that flavonoids **5** and **6** are mixtures of two flavonoids, respectively. They were liberated quercetin by acid hydrolysis. Glucose and galactose, and glucose, galactose and rhamnose produced by acid hydrolysis of **5** and **6** as sugars, respectively. Moreover, since their UV spectral properties showed those of 3-substituted quercetin, **5** and **6** were presumed as quercetin 3-*O*-glucoside (**5a**) and 3-*O*-galactoside (**5b**), and quercetin 3-*O*-rhamnosylglucoside (**6a**) and 3-*O*-rhamnosylgalactoside (**6b**). However, their molecular weights were proved to be 608 (**5a** and **5b**) and 754 (**6a** and **6b**), showing the additional attachment of 1 mol unknown compound of molecular weight 144 (162 as free state) to quercetin 3-*O*-glucoside, 3-*O*-galactoside, 3-*O*-rhamnosylglucoside and 3-*O*-rhamnosylgalactoside. Unfortunately, their flavonoids are partially characterized for small amount of the plant materials. Similarly, UV spectral properties and acid hydrolysis of flavonoids **7** and **8** showed that they are isorhamnetin 3-*O*-glucoside (**7a**), isorhamnetin 3-*O*-galactoside (**7b**) and isorhamnetin 3-*O*-rhamnosylglucoside (**8**). However, the attachment of unknown compound of molecular weight 162 to isorhamnetin 3-*O*-glucoside, 3-*O*-galactoside and 3-*O*-rhamnosylglucoside was proved by LC-MS data. They could not also be identified for small amount of plant materials.

Thus, 11 flavonoids were found in the aerial parts of *Theligonum japonicum*, and four were identified as quercetin 3-*O*-glucoside (**1**), quercetin 3-*O*-galactoside (**2**), isorhamnetin 3-*O*-glucoside (**3**) and isorhamnetin 3-*O*-galactoside (**4**). Other seven flavonoids were characterized as quercetin 3-*O*-glucoside, quercetin 3-*O*-galactoside, isorhamnetin 3-*O*-glucoside, isorhamnetin 3-*O*-galactoside, quercetin 3-*O*-rhamnosylgluco-

side, quercetin 3-*O*-rhamnosylgalactoside and isorhamnetin 3-*O*-rhamnosylglucoside which attached 1 mol unknown compound of molecular weight 162, respectively. Moreover, as other phenolic compounds, the presence of some cinnamic acid derivatives were shown by HPLC. Of their compounds, chlorogenic acid was isolated as a major compound, together with the flavonoids, and identified by UV, LC-MS, and TLC and HPLC comparison with authentic sample which was obtained from the beans of *Coffea arabica* L. (Hayashi, unpublished data). Flavonoids of the genus *Theligonum* species (formerly the family Theligonaceae) were reported for the first time. Thus, flavonoid character of the genus *Theligonum* is the presence of flavonols such as quercetin and isorhamnetin, and some flavonoids attached on unknown compound of molecular weight 162.

Although the genus *Theligonum* has been monotypic family Theligonaceae, it was recently incorporated to the family Rubiaceae by APG III. In the Rubiaceae, it was cleared that the genus is related with the genera *Didymaea* and *Rubia* by analysis of chloroplast DNA (Bremer and Manen, 2000). Flavonoid of *Didymaea* species are not reported until now. *Rubia* species was hardly analyzed for flavonoid except for anthocyanins, cyanidin 3-*O*-rutinoside and 3-*O*-glucoside from the fruits of *Rubia argyi* (Lév.) Hara (= *R. akane* Nakai) (Hayashi, 1944; Ishikura and Sugahara, 1979). In Rubiaceae, morphologically related *Hedyotis* species were surveyed for flavonoids. Thus, a flavanone, liquiritigenin 4′-*O*-glucoside, was isolated from *H. lindleyana* Hook. which is most morphologically similar to *Theligonum japonicum* (Kim *et al.*, 2015). Common 3-*O*-glycosides such as kaempferol, quercetin and isorhamnetin were found in some *Hedyotis* species, e.g. kaempferol, quercetin and isorhamnetin 3-*O*-rutinosides from *H. chrysotricha* (Peng *et al.*, 1999), kaempferol 3,7-di-*O*-rhamnoside from *H. verticillata* Lam. (Hamzah *et al.*, 1994), kaempferol 3-*O*-rutinoside and 3-*O*-arabinoside from *H. herbacea* L. (Hamzah *et al.*, 1996; Hamzah and Lajis, 1998), quercetin, quercetin 3-*O*-sambubioside, 3-*O*-sophoroside, 3-*O*-glucoside and 3-*O*-glucosyl-(1→2)-galactoside, kaempferol, and some kaempferol and quercetin 3-*O*-synapoyl and feruloyl glycosides from *H. diffusa* Willd. (Lu *et al.*, 2000; Tan *et al.*, 2002; Si *et al.*, 2006; Ye *et al.*, 2015). As described above, flavonoids of *Hedyotis* species were also flavonol glycosides such as kaempferol, quercetin and isorhamnetin. However, flavonol glycosides which attached the compound of molecular weight 162 were not found. Although flavonoids of *Theligonum japonicum* were partially characterized, the synthesis of quercetin and isorhamnetin 3-*O*-glycosides, and the attachment of unknown compound of molecular weight 162 to flavonols may be chemical characters of the genus *Theligonum* alone in Rubiaceae.

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