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Flavonoids from the Leaves of Betalain-containing Species, *Phytolacca americana* (Phytolaccaceae)

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Abstract Flavonoids in the leaves of *Phytolacca americana* (Phytolaccaceae), which is native to North America and growing in Japan as a cosmopolitan weed, were surveyed. They were isolated by paper, column and high performance liquid chromatography, and identified as kaempferol 3-*O*-glucoside, quercetin 3-*O*-glucoside and kaempferol 3-*O*-apiofuranosyl-(1→2)-glucoside by UV spectral survey, acid hydrolysis, LC-MS, ^{1}H and ^{13}C NMR, and direct TLC and HPLC comparisons with authentic samples. The species exclusively synthesizes the betalain pigments and can not make anthocyanins, which are major pigments in almost vascular plants. However, it was reconfirmed by this survey that *P. americana* can make flavonol glycosides which biosynthetically related to anthocyanins. Moreover, it was presumed by comparisons with the previous reports that the geographic chemical variations are produced in the species.
Key words: Betalain-containing species, Flavonols, Kaempferol, *Phytolacca americana*, Phytolaccaceae, Quercetin.

Introduction

Phytolacca americana L. (=*P. decandra* L.) is originally native to North America, but now growing in Japan as a cosmopolitan weed. Four betacyanin pigments, betanin, isobetanin, prebetanin, isoprebetanin, and four acylated betacyanins, betanidin and isobetanidin 5-*O*-[(5″-*O*-*E*-feruloyl)-2′-*O*-apiofuranosyl]-glucosides, lampranthin II and isolampranthin II, have been reported from the stems, ripening fruits and cell cultures (Wyler and Dreiding, 1961; Piattelli and Minale, 1964; Schliemann *et al*., 1996). However, anthocyanin pigments, which occur in almost vascular plants, never been reported. On the other hand, some flavonols, *i.e.*, kaempferol 3-*O*-diglycoside and quercetin 3,7-di-*O*-glycoside (Saunders and McClure, 1976), quercetin and kaempferol glycosides (Richardson, 1978; Burret *et al*., 1981), rhamnocitrin and rhamnetin 3-*O*-glucosides and 3-*O*-rhamnosylglucosides, and quercetin 3-*O*-rhamnosylglucoside (Caulkins and Wyatt, 1990), kaempferol 3-*O*-glucoside, 3-*O*-xylosyl-(1→2)-glucoside and 3-*O*-neohesperidoside (Bylka and Matlawska, 2001) have been reported from the leaves. In also other *Phytolacca* species, flavonol glycosides, *e.g*., rhamnocitrin, rhamnetin and quercetin 3-*O*-rhamnosylglucosides, and rhamnocitrin and rhamnetin 3-*O*-glucosides from *P. rigida* Small. (Caulkins and Wyatt, 1990), kaempferol and isokaempferide 3-*O*-glucosyl-(1→2)-galactoside and 3-*O*-xylosyl-(1→2)-galactoside from *P. thyrsiflora* Fenzl. ex Schmidt (Haraguchi *et al*., 1988), and ombuin 3-*O*-rutinoside from *P. dioica* L. (Marini-Bettólo *et al*., 1950; Hörhammer *et al*., 1968), together with betacyanin pigments. Moreover, the presence of proanthocyanidins, which closely biosynthetically related to the anthocyanins, has been shown in the seeds of *P. americana* (Bittrich and Amaral, 1991). It has been shown that betalain pigments, betacyanins and betaxanthins, coexist with almost flavonoid classes except for anthocyanins in the betalain-producing nine families, Aizoaceae, Amaranthaceae, Basellaceae, Cactaceae, Chenopodiaceae, Didiereaceae, Nyctaginaceae, Phytolaccaceae and Portulacaceae belonging to the order Caryophyllales (Iwashina, 2001). However, it was recently proved that dihydroflavonol 4-reductase (DFR) and anthocyanin synthases (ANSs), which directly participate anthocyanin synthesis, are present in *P. americana* and also *Spinacia oleracea* L. (Shimada *et al*., 2004, 2005). Thus, the accumulation of flavonols in *P. americana* suggests that the steps of anthocyanin biosynthesis from dihydroflavonols to anthocyanins are blocked (Fig. 1) (Shimada *et al*., 2007).

In this paper, characterization of the flavonols from the leaves of *P. americana* in Japan is described and qualitatively compared with those of foreign populations previously reported.

Materials and Methods

Extraction and isolation of flavonoids from the plant materials

Fresh leaves (306 g) of *Phytolacca americana* L. which are naturally growing in Tsukuba Botanical Garden, National Museum of Nature and Science, Tsukuba, Japan, were extracted with MeOH. After concentration, petroleum ether was added in aqueous residue and lipophilic components were removed. The concentrated extracts were applied to preparative high performance liquid chromatography (prep. HPLC). Five fractions (fr. 1–5) obtained by prep. HPLC were purified by Sephadex LH-20 column chromatography using solvent system: 70% MeOH, respectively. Flavonoid **1** was obtained as pale yellow powder (ca. 30 mg) from fr. 5. Fractions 1–4 were applied to preparative paper chromatography using solvent system: BAW (*n*-BuOH/HOAc/H_2O=4 : 1 : 5, upper phase) and the isolated flavonoids were purified by Sephadex LH-20 column chromatography (70% MeOH). Flavonoids **2** (ca. 20 mg) and **3** (ca. 30 mg) were obtained as pale yellow powders, respectively.

High performance liquid chromatography (HPLC)

Analytical HPLC was performed with Shimadzu HPLC systems using a Senshu Pak PEGASIL ODS column (I.D. 6.0×150 mm: Senshu Scientific Co., Ltd.), at a flow-rate of 1.0 ml min^{-1}. Detection was 350 nm and eluent was $MeCN/H_2O/H_3PO_4$ (22 : 78 : 0.2). Prep. HPLC was performed with Tosoh HPLC systems using Senshu Pak PEGASIL ODS column (I.D. 10.0×250 mm: Senshu Scientific Co., Ltd.), a flow-rate of 2.0 ml min^{-1} and eluent was $MeCN/H_2O$ (22 : 78).

Liquid chromatograph-mass spectra (LC-MS)

LC-MS was performed with Shimadzu LC-MS systems using Senshu Pak PEGASIL ODS column (I.D. 2.0×150 mm: Senshu Scientific Co., Ltd.), at a flow-rate of 0.1 ml min^{-1}, eluent: $MeCN/H_2O/HCOOH$ (15 : 80 : 5), and ESI^+ 4.5 kV and ESI^- 3.5 kV, 250°C.

Identification of flavonoids

The isolated flavonoids were identified by UV spectral survey according to Mabry *et al*. (1970), LC-MS, acid hydrolysis (in 12% aq. HCl, 100°C, 30 min) and characterization of its products, ^1H and ^{13}C NMR, and direct TLC [solvent systems: BAW, BEW (*n*-BuOH/EtOH/H_2O=4 : 1 : 2.2) and 15% HOAc] and HPLC comparisons with authentic samples. TLC, HPLC, UV, acid hydrolysis, ^1H and ^{13}C NMR and LC-MS data of the isolated flavonoids are as follows.

Kaempferol 3-*O*-glucoside (astragalin, **1**). TLC: Rf 0.80 (BAW), 0.81 (BEW), 0.40 (15%HOAc); UV—dark purple, UV/NH_3—dark greenish yellow. HPLC: Rt (min) 9.41. UV: λmax (nm) MeOH 266, 296sh, 347; +NaOMe 275, 325, 396 (inc.); +$AlCl_3$ 275, 304, 348, 393; +$AlCl_3$/HCl 275, 303, 347, 394; +NaOAc 274, 310, 383; +NaOAc/H_3BO_3 267, 296sh, 348. Acid hydrolysis: kaempferol and glucose. LC-MS: *m/z* 449 $[M+H]^+$, 447 $[M–H]^-$ (kaempferol+1 mol glucose); *m/z* 287 $[M–162+H]^+$ (kaempferol).

Quercetin 3-*O*-glucoside (isoquercitrin, **2**).

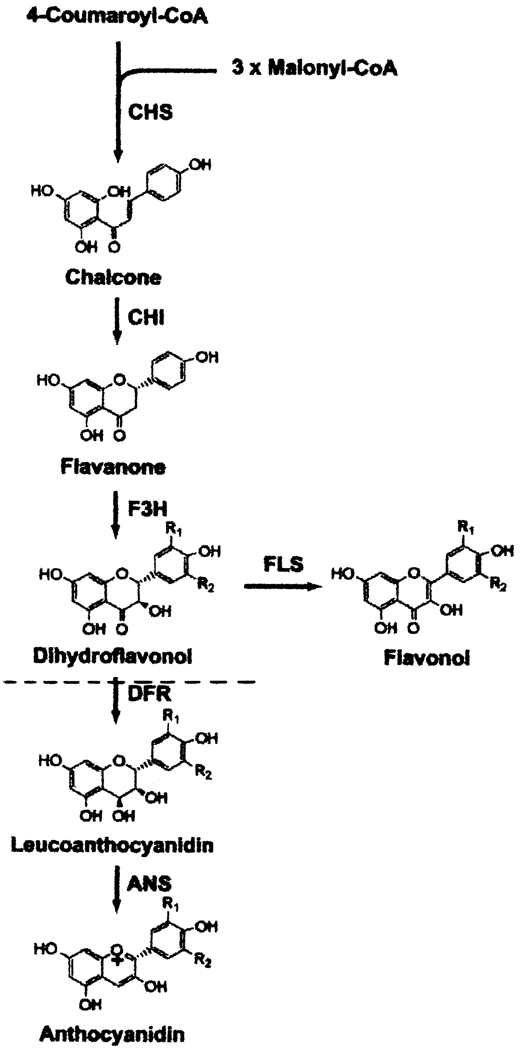

Fig. 1. Biosynthetic pathway of anthocyanidins and flavonols (partially altered from Shimada *et al*., 2005). Anthocyanin biosynthetic pathway is blocked between dihydroflavonol and leucoanthocyanidin in *Phytolacca americana*. CHS: chalcone synthase, chalcone isomerase, F3H: flavanone 3-hydrogenase, FLS: flavonol synthase, DFR: dihydroflavonol 4-reductase and ANS: anthocyanidin synthase.

TLC: Rf 0.67 (BAW), 0.67 (BEW), 0.29 (15%HOAc); UV–dark purple, UV/NH_3–yellow. HPLC: Rt (min) 6.31. UV: λmax (nm) MeOH 257, 266sh, 296sh, 358; +NaOMe 273, 329, 409 (inc.); +$AlCl_3$ 275, 433; +$AlCl_3$/HCl 269, 298, 362, 399; +NaOAc 273, 326, 394; +NaOAc/H_3BO_3 261, 296, 378. Acid hydrolysis: quercetin and glucose. LC-MS: *m*/*z* 465 $[M+H]^+$, 463 $[M–H]^-$ (quercetin+1 mol glucose); *m*/*z* 303 $[M–162+H]^+$ (quercetin).

Kaempferol 3-*O*-apiofuranosyl-(1→2)-glucoside (**3**). TLC: Rf 0.74 (BAW), 0.80 (BEW), 0.63 (15%HOAc); UV–dark purple, UV/NH_3–dark greenish yellow. HPLC: Rt (min) 6.86. UV: λmax (nm) MeOH 266, 296sh, 349; +NaOMe 275, 324, 396 (inc.); +$AlCl_3$ 275, 303, 350, 392; +$AlCl_3$/HCl 275, 302, 347, 393; +NaOAc 274, 309, 384; +NaOAc/H_3BO_3 267, 296sh, 351. Acid hydrolysis: kaempferol, apiose and glucose. ^{1}H NMR (600 MHz, pyridine-d_5): δ 13.46 (1H, *s*, 5-OH), 8.54 (2H, *dd*, *J*=2.0 and 8.9 Hz, H-2′,6′), 7.31 (2H, *dd*, *J*=2.0 and 8.9 Hz, H-3′,5′), 6.71 (1H, *d*, *J*=2.0 Hz, H-6), 6.70 (1H, *d*, *J*=2.1 Hz, H-8), 6.62 (1H, *d*, *J*=0.9 Hz, apiosyl H-1), 6.61 (1H, *d*, *J*=7.9 Hz, glucosyl H-1), 4.83 (1H, *d*, *J*=9.3 Hz, apiosyl H-4), 4.58 (1H, *dd*, J=7.9 and 15.1 Hz, glucosyl H-2), 3.9–4.5 (*m*, sugar protons). ^{13}C NMR (150 MHz, pyridine-d_5): δ (kaempferol) 156.7 (C-2), 134.5 (C-3), 178.7 (C-4), 162.9 (C-5), 99.7 (C-6), 165.7 (C-7), 94.4 (C-8), 157.4 (C-9), 105.3 (C-10), 122.2 (C-1′), 131.8 (C-2′, 6′), 116.2 (C-3′,5′), 161.6 (C-4′); δ (apiose) 110.7 (C-1), 78.1 (C-2), 81.0 (C-3), 75.9 (C-4), 66.4 (C-5); δ (glucose) 100.5 (C-1), 78.6 (C-2), 79.0 (C-3), 71.6 (C-4), 78.9 (C-5), 62.3 (C-6). LC-MS: *m*/*z* 579 $[M–H]^-$ (kaempferol+each 1 mol apiose and glucose); *m*/*z* 449 $[M–132+H]^+$ (kaempferol+1 mol glucose).

Results and Discussion

Four flavonol peaks appeared on the chromatograms in this survey. Of their flavonoids, three ones (**1**–**3**) could be isolated as pale yellow powders. Flavonoid **1** was kaempferol glucoside which was shown by acid hydrolysis and characterization of its products. The attachment of 1 mol glucose to 3-position of kaempferol was shown by LC-MS and UV spectral survey according to Mabry *et al.* (1970). Finally, compound **1** was identified as kaempferol 3-*O*-glucoside (astragalin, Fig. 2) by direct TLC and HPLC

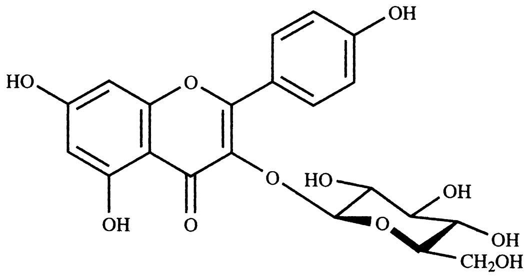

Fig. 2. Chemical structure of kaempferol 3-*O*-glucoside (astragalin, **1**).

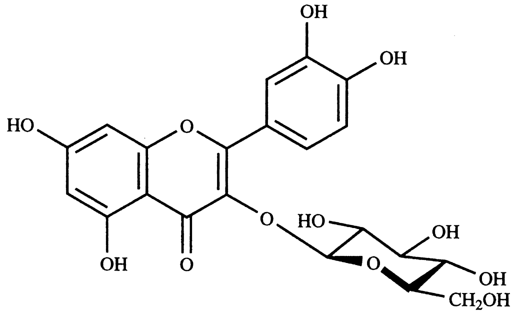

Fig. 3. Chemical structure of quercetin 3-*O*-glucoside (isoquercitrin, **2**).

comparison with authentic sample obtained from the fronds of *Cyrtomium falcatum* (L. f) C. Presl (Dryopteridaceae) (Iwashina *et al*., 2006).

Astragalin has been widespread distributed in vascular plants including the betalain-producing species, *e.g*., *Conophytum* spp. (Aizoaceae) (Reznik, 1957), *Echinocereus* spp. (Cactaceae) (Leuck and Miller, 1982; Miller, 1988), *Chenopodium fremontii* S. Watson (Chenopodiaceae) (Crawford and Mabry, 1978) and *Claytonia virginica* L. (Portulacaceae) (Doyle, 1983). This glycoside has already been found in *P. americana* from Poland (Bylka and Matlawska, 2001).

Flavonoid **2** liberated quercetin and glucose by acid hydrolysis and was shown to be 3-substituted quercetin by UV spectral properties. Since the molecular ion peak, *m*/*z* 465 $[M+H]^+$ appeared by LC-MS, the attachment of 1 mol glucose to quercetin nucleus was suggested. By direct TLC and HPLC comparison with authentic isoquercitrin from the fronds of *Cyrtomium falcatum* (Iwashina *et al*., 2006), compound **2** was identified as quercetin 3-*O*-glucoside (Fig. 3).

Isoquercitrin is also common flavonol glycoside in plants and has already been reported from *Claytonia* spp. (Miller, 1981), *Chenopodium* spp. (Crawford, 1975; Crawford and Mabry, 1978), *Opuntia* spp. (Cactaceae) (Clark and Parfitt, 1980; Clark *et al*., 1980), *Echinocereus* spp. (Leuck and Miller, 1982; Miller, 1982) and *Neria meyeri* Schwantes (Aizoaceae) (Kolodziej, 1982) etc. in the betalain-producing species. However, quercetin 3-*O*-glucoside was found in *P. americana* for the first time.

It was shown by UV spectral survey that flavonoid **3** has free 5-, 7- and 4′-hydroxyl and substituted 3-hydroxyl groups. Since kaempferol as an aglycone, and apiose and glucose as glycosidic sugars were liberated by acid hydrolysis, original glycoside is kaempferol 3-*O*-apiosylglucoside or 3-*O*-glucosylapioside. A fragment ion peak, *m*/*z* 449 $[M-132+H]^+$ (calculated for kaempferol+1 mol glucose) appeared by LC-MS survey, showing that flavonoid **3** is kaempferol 3-*O*-apiosylglucoside.

Four aromatic proton signals corresponding to H-2′,6′ (δ 8.54), H-3′,5′ (δ 7.31), H-6 (δ 6.71) and H-8 (δ 6.70), and two sugar anomeric protons, apiosyl H-1 (δ 6.62, *d*, $J=0.9$ Hz) and glucosyl H-1 (δ 6.61, *d*, $J=7.9$ Hz) appeared on ^{1}H NMR spectrum. Sugar-sugar linkage between apiose and glucose was determined as apiosyl-(1→2)-glucoside by ^{13}C NMR spectrum, *i.e*. significant shift to lower field of C-2 carbon signal (δ 78.6) of glucose (Fossen and Andersen, 2006). Thus, original glycoside **3** was completely identified as kaempferol 3-*O*-β-D-apiofuranosyl-(1→2)-*O*-β-D-glucopyranoside (Fig. 4). Kaempferol 3-*O*-apiosylglucoside is very rare compound and has been isolated from only one plant species, *Securidaca diversifolia* S. F. Blake (Polygalaceae) until now (Hamburger *et al*., 1985).

Twelve flavonoids have been isolated from *P. americana* leaves and completely or partially characterized (Table 1). All of them were flavonol glycosides based on kaempferol and quercetin, and their 7-methyl ethers. It was shown that the betalain-producing families, *i.e.* Aizoaceae, Amaranthaceae, Basellaceae, Cactaceae, Chenopodiaceae, Didiereaceae, Nyctaginaceae, Phytolaccaceae and Portulacaceae, can synthesize the almost classes of flavonoids, flavones, flavonols, isoflavonoids, chalcones, dihydroflavonols, aurones, flavanones, and also favans and proanthocyanidins which closely biosynthetically related to anthocyanins, except for dihydrochalcones, anthocyanins and biflavonoids (Iwashina, 2001). Of their flavonoid classes, major ones are flavone and flavonol. It was re-confirmed by the present survey that the flavonoid class of *P. americana* is flavonol. However, their flavonoid composition was geographically different with collection sites, *i.e.*, methoxylated flavonols, rhamnocitrin and rhamnetin 3-*O*-glucosides and 3-*O*-rhamnosylglucosides, and quercetin 3-*O*-rhamnosylglucoside from USA (Caulkins and Wyatt, 1990), kaempferol 3-*O*-glucoside, 3-*O*-neohesperidoside and 3-*O*-xylosyl-(1→2)-glucoside from Europe, Poland (Bylka and Matlawska, 2001), and kaempferol 3-*O*-glucoside and 3-*O*-apiosyl-(1→2)-glucoside, and quercetin 3-*O*-glucoside from Asia, Japan in this survey. Though *Phytolacca americana* is originally native to North America, but now growing in the world as a cosmopolitan weed, and geographic variations or chemical races may be occur in the species.

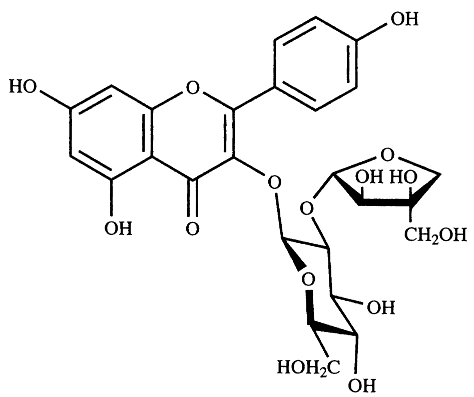

Fig. 4. Chemical structure of kaempferol 3-*O*-apiofuranosyl-(1→2)-glucoside (**3**).

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Table 1. Reports of flavonoids from *Phytolacca americana*

Flavonoids		References
Kaempferol	3-*O*-diglycoside	Saunders and McClure (1976)
	3-*O*-glucoside	Bylka and Matlawska (2001) present paper
	3-*O*-xylosyl-(1→2)-glucoside	Bylka and Matlawska (2001)
	3-*O*-apiosyl-(1→2)-glucoside	present paper
	3-*O*-neohesperidoside	Bylka and Matlawska (2001)
Rhamnocitrin	3-*O*-glucoside	Caulkins and Wyatt (1990)
	3-*O*-rhamnosylglucoside	Caulkins and Wyatt (1990)
Quercetin	3,7-di-O-glycoside	Saunders and McClure (1976)
	3-*O*-glucoside	present paper
	3-*O*-rhamnosylglucoside	Caulkins and Wyatt (1990)
Rhamnetin	3-*O*-glucoside	Caulkins and Wyatt (1990)
	3-*O*-rhamnosylglucoside	Caulkins and Wyatt (1990)

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