Flavonoids from the Leaves of *Vitex rotundifolia* (Verbenaceae), and their Qualitative and Quantitative Comparison between Coastal and Inland Populations

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Abstracts  The flavonoid compounds in the leaves of *Vitex rotundifolia* growing in coast and Lake Biwa were surveyed. Six flavonoids were isolated and five of them were identified as isoorientin (1), luteolin 7-O-glucuronide (2), luteolin 7-O-glucoside (3), luteolin 3’-O-glucuronide (5) and isovitexin (6). Another one (4) was characterized as luteolin diglucoside. Nine populations of *V. rotundifolia* from coastal populations, i.e. each three Ise Bay in Pacific Ocean side and Wakasa Bay in Sea of Japan side, and inland populations, i.e. Lake Biwa, were qualitatively and quantitatively compared by HPLC for flavonoids. It is known that the flavonoids in plants act as anti-stress products against UV radiation, salinity and so on. The seashore is a harsh environment for plants to inhabit due to their various stresses. Salinity and UV radiation are two of the major stresses in the coastal region, where limited plant species tolerable to those stresses become dominant. However, the flavonoids in the leaves of inland and coastal *V. rotundifolia* were qualitatively and quantitatively the almost same from each other. In general, it is shown that anti-stress activities of catechol type flavonoids such as luteolin and quercetin are stronger than those of B-ring monohydroxylated flavonoids such as apigenin and kaempferol. Since 96.4–97.1% of total flavonoids of *V. rotundifolia* is luteolin type, *V. rotundifolia* can originally synthesize a much amount of luteolin glycosides, so that we presumed that the species could adapt in coastal environment.

Key words: anti-stress activities, coastal populations, flavonoids, inland populations, luteolin, *Vitex rotundifolia*.

Introduction

*Vitex rotundifolia* L. fil. (Verbenaceae) commonly grows on sandy seashores in China, Southeastern Asia, Polynesia, Australia and Japan. However, it exceptionally inhibits the sandy lakeshore in inland fresh water lake, Lake Biwa, Central Japan. This lake is an ancient lake which was formed about four million years ago (Kawabe, 1989). In addition to *V. rotundifolia*, common seashore plant species, e.g. *Arabis kawasakiana* Makino (Brassicaceae), *Calystegia soldanella* (L.) Roem. et Schult. (Convolvulaceae), *Dianthus japonicus* Thunb. (Caryophyllaceae), *Lathyrus japonicus* Willd. (Leguminosae) and *Raphanus sativus* L. var. *rphanistroides* Makino (Brassicaceae), inhibit in the lake side. It is presumed that their species have migrated to the inland lake from coastal populations during the period when Lake Biwa had been adjacent to the seashore, and lake populations might have later become isolated from the
coastal populations (Takaya, 1963; Kitamura, 1968). Commonly, the seashore plants may be exposed to various stresses, e.g. UV radiation, salinity etc. than inland plants of the same species. Coastal plants protect themselves from these stresses by various manners. In their plants, chemical compounds such as flavonoids act as stress scavengers. In this paper, we describe the isolation and identification of the flavonoids in the leaves of *Vitex rotundifolia*, and qualitatively and quantitatively compare the flavonoids in coastal and inland populations.

**Materials and Methods**

**Plant materials**

Each ten individuals were collected from nine populations, i.e. 1) Sakajiri-kaigan, 2) Takanoshu-hama and 3) Sanri-hama in Sea of Japan side; 4) Maiami-hama, 5) Sanami-hama and 6) Shinkai-hama in Lake Biwa; and 7) Utsue, 8) Nishino-hama and 9) Kojjiga-hama in Pacific Ocean side. Voucher specimens were deposited in the herbarium of National Museum of Nature and Science, Japan (TNS).

**Extraction and isolation of flavonoids**

Fresh leaves (1272 g) were extracted with MeOH for isolation. After concentration, crude extracts were applied to preparative paper chromatography using solvent systems: BAW (n-BuOH/HOAc/H2O = 4:1:5, upper phase), 15% HOAc and then BEW (n-BuOH/EtOH/H2O = 4:1:2.2). The isolated flavonoids were finally purified by Sephadex LH-20 column chromatography using solvent system: 70% MeOH. Of six flavonoids (1–6) detected in this experiment, 1 (ca. 270 mg), 2 (ca. 10 mg), 3 (ca. 20 mg) and 5 (ca. 70 mg) were obtained as pale yellow powder.

Fresh leaves (5 g) of each sample were extracted with MeOH (40 ml) for quantitative HPLC survey.

**High performance liquid chromatography (HPLC)**

HPLC was performed with Shimadzu HPLC systems using 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/H2O/H3PO4 (20:80:0.2).

**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, ESI⁺ 4.5 kV and ESI⁻ 3.5 kV, 250°C. The eluent was MeCN/H2O/HCOOH = 18:77:5).

**Acid hydrolysis**

Acid hydrolysis was performed in 12% HCl, 100°C, 30 min. After shaking with diethyl ether, aglycones migrated to organic layers, and glycosidic sugars and C-glycosyl flavones were left in aqueous layers.

**Identification of flavonoids**

Flavonoids were identified by UV spectral survey according to Mabry *et al.* (1970), acid hydrolysis and characterization of its products, LC-MS, ¹H and ¹³C NMR, and direct TLC and HPLC comparisons with authentic samples. TLC, HPLC, LC-MS, and ¹H and ¹³C NMR data of the isolated flavonoids are as follows.

Isoorientin (Luteolin 6-C-glucoside, 1). TLC: Rf 0.49 (BAW), 0.57 (BEW), 0.28 (15%HOAc); Color UV – dark purple, UV/NH3 – yellow. HPLC: tR (min) 5.67. UV: λmax (nm) MeOH 257, 271, 350; +NaOMe 272, 327sh, 412 (inc.); +AlCl₃ 276, 425; +AlCl₃/HCl 257, 271, 350; +AlCl₃/HCl/H₂O 266, 379; +NaOAc/H₂O/H₂BO₃ 266, 379. LC-MS: *m/z* 449 [M+H]+, 447 [M−H]− (luteolin +1 mol glucose).

Luteolin 7-O-glucuronide (2). TLC: Rf 0.36 (BAW), 0.38 (BEW), 0.10 (15%HOAc); Color UV – dark purple, UV/NH₃ – dark yellow. HPLC: tR (min) 9.40. UV: *λ*max (nm) MeOH 255, 265sh, 349; +NaOMe 266, 390 (inc.); +AlCl₃ 274, 426; +AlCl₃/HCl 264sh, 273, 294sh, 360, 385; +NaOAc 260, 403; +NaOAc/H₂BO₃ 260,
372. LC-MS: m/z 463 [M+H]+ (luteolin +1 mol glucuronic acid). 1H NMR (600 MHz, pyridine-d$_5$): δ 7.82 (1H, d, J=2.3 Hz, H-2'), 7.51 (1H, dd, J=1.5 and 8.3 Hz, H-6'), 7.26 (1H, d, J=8.3 Hz, H-5'), 7.04 (1H, d, J=1.7 Hz, H-8), 6.82 (1H, s, H-3), 6.77 (1H, d, J=2.1 Hz, H-6), 5.73 (1H, d, J=7.2 Hz, glucuronyl H-1), 4.54 (1H, t, J=8.4 Hz, glucuronyl H-5), 4.32 (2H, t, J=8.8 Hz, glucuronyl H-3, H-4), 4.24 (1H, m, glucuronyl H-2). 13C NMR (150 MHz, pyridine-d$_5$): δ 165.6 (C-2), 104.0 (C-3), 183.1 (C-4), 162.1 (C-5), 100.9 (C-6), 164.2 (C-7), 95.6 (C-8), 158.1 (C-9), 104.0 (C-10), 122.8 (C-1'), 114.5 (C-2'), 147.5 (C-3'), 151.6 (C-4'), 116.9 (C-5'), 120.0 (C-6'); (glucuronic acid) δ 101.8 (C-1), 74.5 (C-2), 77.8 (C-3), 73.5 (C-4), 76.5 (C-5), 174.7 (C-6).

Luteolin 7-O-glucoside (3). TLC: Rf 0.44 (BAW), 0.51 (BEW), 0.08 (15%H$_2$OAc); Color UV – dark purple, UV/NH$_3$ – dark yellow. HPLC: tR (min) 8.96. UV: λ$_{max}$ (nm) MeOH 255, 266sh, 348; +NaOMe 267, 391 (inc.); +AlCl$_3$ 273, 426; +AlCl$_3$/HCl 263sh, 273, 295sh, 358, 381; +NaOAc 260, 402; +NaOAc/H$_2$BO$_3$ 260, 372. LC-MS: m/z 449 [M+H]+ (luteolin +1 mol glucose). 1H NMR (600 MHz, pyridine-d$_5$): δ 7.76 (1H, d, J=2.2 Hz, H-2'), 7.54 (1H, dd, J=2.2 and 8.3 Hz, H-6'), 7.24 (1H, d, J=8.3 Hz, H-5'), 6.94 (1H, d, J=2.1 Hz, H-8), 6.80 (1H, s, H-3), 6.73 (1H, d, J=2.2 Hz, H-6), 5.60 (1H, d, J=7.2 Hz, glucosyl H-1), 4.40 (1H, d, J=12.8 Hz, glucosyl H-6a), 4.19 (2H, m, glucosyl H-3, H-6b), 4.12 (1H, t, J=16.6 Hz, glucosyl H-2), 4.06 (2H, m, glucosyl H-4, H-5). 13C NMR (150
Results and Discussion

Identification of flavonoids

Six flavonoid peaks appeared on HPLC, and five compounds were completely identified except for 4. UV spectral properties of major flavonoid 1 were those of typical luteolin (5,7,3′,4′-tetrahydroxyflavone). However, it could not be hydrolyzed by hot acid treatment, showing that the compound is C-glycosylflavone. It was indicated by LC-MS that 1 mol hexose is attached to luteolin. Finally, flavonoid 1 was identified as isoorientin by direct TLC and HPLC comparison with authentic sample from the leaves of Japanolirion osense Nakai (Petrosaviaceae) (Iwashina et al., 2005). Though isoorientin has been isolated from a few Vitex species, e.g. V. megapotamica (Spreng.) Moldenke and V. agnus-castus L. (Hänsel et al., 1965), it was found from V. rotundifolia for the first time.

Flavonoid 2 liberated luteolin and glucuronic acid by acid hydrolysis. The attachment of the sugar to 7-position of luteolin was proved by UV spectral survey, i.e. no shift of Band II in addition to NaOAc. Molecular ion peak, m/z 463 [M+H]+ appeared on LC-MS, showing the attachment of 1 mol glucuronic acid to luteolin. Attachment of glucuronic acid to 7-position of luteolin was confirmed from the HMBC correlation between glucuronyl anomeric proton at δ 5.73 and C-7 carbon signal at δ 164.2. Finally, flavonoid 2 was identified as luteolin 7-O-β-glucuronopyranoside by TLC and HPLC comparison with authentic sample from the leaves of Myoporum bontiioides (Sieb. et Zucc.) A. Gray (Myoporaceae) (Iwashina and Kokubugata, 2010). Luteolin 7-O-glucuronicid was newly reported from Vitex species.

UV spectral properties of flavonoid 3 were essentially the same with those of 2, showing that the compound is 7-O-glycosylated luteolin. Practically, luteolin was liberated by acid hydrolysis, together with glucose. The attachment of 1 mol glucose to luteolin was proved by LC-MS survey, i.e. appearance of a molecular ion peak, m/z 449 [M+H]+. Moreover, its chemical structure was...
also determined by $^1$H and $^{13}$C NMR. Finally, flavonoid 3 was estimated as luteolin 7-0-$\beta$-glucopyranoside by TLC and HPLC comparison with authentic sample from the leaves of Schmalhausenia nidulans Petrak (Asteraceae) (Iwashina and Kadota, 1999). Though luteolin 7-0-glucoside is widely distributed in plant kingdom and has been reported from a few Vitex species (Hänsel et al., 1965), it was isolated from V. rotundifolia for the first time.

Luteolin and glucuronic acid were produced by acid hydrolysis of 5. However, its UV spectral properties, especially in addition to AlCl$_3$, were similar to those of apigenin, showing the presence of monohydroxyl or substituted dihydroxyl group in B-ring. Attachment of 1 mol glucuronic acid to luteolin was shown by the occurrence of a molecular ion peaks, $m/z$ 463 [M+H]$^+$ and 461 [M−H]$^-$ on LC-MS. In $^1$H NMR, six aromatic protons corresponding to H-3, H-6, H-8, H-2', H-5' and H-6', and an anemic proton ($\delta$ 5.42, $d$, $J$=7.3 Hz) appeared. The attachment of glucuronic acid to 3'-position of luteolin was determined from the HMBC correlation between glucuronyl anemic proton at $\delta$ 5.42 and C-3' carbon signal at $\delta$ 147.3. Thus, flavonoid 5 was identified as luteolin 3'-0-$\beta$-glucuronopyranoside. Luteolin 3'-0-glucuronide was comparatively rare glycoside in nature and has been reported from a few plant species, e.g. Lunularia cruciata (L.) Dumort. ex Lindb. (Lunulariaceae, liverwort) (Markham and Porter, 1974) and Melissa officinalis L. (Lamiaceae) (Heitz et al., 2000). However, it has not been found from Vitex species.

It was shown by UV spectral properties and hot acid treatment that flavonoid 6 is C-glycosylflavone of apigenin type. Its TLC and HPLC behaviors completely agreed with those of authentic isovitexin from the flowers of Iris ensata Thumb. (Iwashina et al., 1996). Thus, flavonoid 6 was identified as isovitexin (apigenin 6-C-gluco-side). Isovitexin is common in plants and has been reported from Vitex species such as V. lucens T. Kirk. (Horowitz and Gentili, 1964).

Flavonoid 4 was presumed as luteolin diglucoside by HPLC and LC-MS.
Qualitative and quantitative comparisons of flavonoids between coastal and inland Vitex rotundifolia

The genus Vitex consists of ca. 250–1,000 species and some ones have been surveyed for flavonoids (e.g. Hänsel et al., 1965; Horowitz and Gentili, 1966; Banerji et al., 1969; Misra and Subramanian, 1980; Thuy et al., 1998; Cheng et al., 2007; Chen et al., 2008). Various flavones and flavonols and their O-glycosides, flavanone and chalcones have been reported from their species, together with C-glycosylflavones. Flavonoids have already been isolated from V. rotundifolia (Kondo et al., 1986; Yoshioka et al., 2004). Two flavones, luteolin and 5,5′-dihydroxy-6,7,4′-trimethoxyflavone, and two flavonols, artemetin (5-hydroxy-3,6,7,3′,4′-pentamethoxyflavone) and casticin (5,3′-dihydroxy-3,6,7,4′-tetramethoxyflavone) have been found in the fruits and leaves. However, they are all free flavonoids and may be present as external flavonoids. In this survey, six flavone glycosides were isolated from the leaves of V. rotundifolia for the first time. Major flavonoid is isoorientin, i.e. luteolin 6-C-glucoside and 36.8–45.9% of total flavonoids. Though total flavonoid content (0.97–0.98) of Lake Biwa populations slightly decrease than those of Sea of Japan (1.00–1.22) and Pacific Ocean (1.05–1.10), they did not significantly vary. In general, it is shown that anti-

Table 1. Quantitative HPLC analysis of foliar flavonoids from Vitex rotundifolia growing in Lake Biwa, Sea of Japan side and Pacific Ocean side

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Each fresh leaves (5 g) was extracted with MeOH (40 ml).

aPeak area at 350 nm. bEach flavonoid percentage. cRelative amounts of the flavonoids as peak area of the samples collected in Sakajiri-kaigan is 1.00. Lu=Total luteolin percentage.

1=Isoorientin, 2=Luteolin 7-O-glucuronide, 3=Luteolin 7-O-glucoside, 4=Luteolin diglucoside, 5=Luteolin 3′-O-glucuronide and 6=Isovitexin.

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aPeak area at 350 nm. bEach flavonoid percentage. cRelative amounts of the flavonoids as peak area of the samples collected in Sakajiri-kaigan is 1.00. Lu=Total luteolin percentage.

1=Isoorientin, 2=Luteolin 7-O-glucuronide, 3=Luteolin 7-O-glucoside, 4=Luteolin diglucoside, 5=Luteolin 3′-O-glucuronide and 6=Isovitexin.
stress activities of catechol type flavonoids such as luteolin and quercetin are stronger than those of B-ring monohydroxylated flavonoids such as apigenin and kaempferol. Practically, though wild-type Arabidopsis leaves exposed to low UV-B conditions contained predominantly kaempferol glycosides, with low levels of quercetin glycosides, the flavonoid level doubled on treatment with UV-B and an increase in the ratio of quercetin: kaempferol was observed (Ryan et al., 2001). Moreover, it has been reported that B-ring ortho-dihydroxylated flavonoids notably increased than mono-hydroxylated ones with increasing altitude in common weed, Plantago asiatica (Murai et al., 2009). Of the flavonoids in V. rotundifolia, five ones are luteolin glycosides which have stronger anti-oxidative activities, and total luteolin percentage is 96.0–97.1% of total flavonoids in all populations. Vitex rotundifolia can originally synthesize a much amount of luteolin glycosides, so that the species could grow and need not has more powerful activities in coastal environment. Vitex rotundifolia is a deciduous creeping trees, but some herbaceous species are growing in both coast and Lake Biwa. We know that some herbaceous species such as Calystgia soldanella and Lathyrus japonicus are occurred the quantitatively or qualitatively different flavonoids between coastal and Lake Biwa populations (Iwashina, Setoguchi and Murai, unpublished data), and are now studying about them.

References


Takaya, Y. 1963. Stratigraphy of the Paleo-Biwa group.
