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Citation	Natural Product Communications, 13(12): 1641-1644
Issue Date	2018-12-01
URL	http://hdl.handle.net/20.500.12000/45105
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NPC Natural Product Communications

2018
Vol. 13
No. 12
1641 - 1644

Flavonoids from three Wild *Glycine* Species in Japan and Taiwan

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Received: September 12th, 2018; Accepted: October 23rd, 2018

Fourteen flavonols, four flavones and six isoflavones were isolated from the aerial parts of two Japanese *Glycine* species, *G. tabacina* and *G. koidzumii*, and the leaves of Taiwanese *G. max* subsp. *formosana*. Of their flavonoids, twelve flavonols were identified as kaempferol 3-*O*-sophoroside (**1**), 3-*O*-rutinoside (**2**), 3-*O*-robinobioside (**3**) and 3-*O*-rhamnosyl-(1→4)-[rhamnosyl-(1→6)-galactoside] (**4**), quercetin 3-*O*-gentiobioside (**5**), 3-*O*-glucoside (**6**), 3-*O*-galactoside (**7**), 3-*O*-rutinoside (**8**), 3-*O*-robinobioside (**9**) and 3-*O*-rhamnosyl-(1→4)-[rhamnosyl-(1→6)-galactoside] (**10**), and isorhamnetin 3-*O*-rutinoside (**11**) and 3-*O*-robinobioside (**12**). Other two flavonols were characterized as isorhamnetin 3-*O*-rhamnosylrhamnosylglucoside (**13**) and 3-*O*-rhamnosylrhamnosylgalactoside (**14**). Four flavones and six isoflavones were estimated as schaftoside (**15**), apigenin 6,8-di-*C*-arabinoside (**16**), luteolin 7-*O*-glucoside (**17**) and chrysoeriol 7-*O*-glucoside (**18**), and daidzein 7-*O*-glucoside (**19**), 4′-*O*-glucoside (**20**) and 7-*O*-xylosylglucoside (**21**), genistein 7-*O*-glucoside (**22**) and 4′-*O*-glucoside (**23**), and 3′-*O*-methylorobol 7-*O*-glucoside (**24**). Although flavonoid composition of *G. tabacina* and *G. koidzumii* was similar to each other, that of *G. max* subsp. *formosana* was different with those of two Japanese *Glycine* species described above. Flavonoids of their *Glycine* species were reported for the first time except for those of *G. tabacina*.

Keywords: *Glycine tabacina*, *Glycine koidzumii*, *Glycine max* subsp. *formosana*, Flavonoids, Flavonols, Flavones, Isoflavones.

The genus *Glycine* consists of ca. 20 species and is distributed in Asia and Australia [1]. Three and four *Glycine* taxa are growing in Japan and Taiwan, i.e. *G. max* (L.) Merr. subsp. *soja* (Siebold ex Zucc.) H. Ohashi, *G. tabacina* (Labill.) Benth. and *G. koidzumii* Ohwi in Japan [2], and *G. delichocarpa* Tateishi & H. Ohashi, *G. max* subsp. *formosana* (Hosokawa) Tateishi & H. Ohashi, *G. tabacina* and *G. tomentella* Hayata in Taiwan [3]. Of their species, *G. koidzumii* is endemic to Japan (Ryukyu Islands) [2]. *Glycine max* subsp. *max* is cultivated in the world as an important crop, and many flavonoids including isoflavonoids, flavonols, *C*-glycosylflavones, flavones, dihydroflavonols, aurone, chalcone and anthocyanins have been reported from the leaves, seeds, cotyledons, roots, seedlings and flowers [e.g. 4]. However, flavonoids of wild species of the genus *Glycine* are hardly reported except for phytoalexins. A wild species of soybean, *G. max* subsp. *soja* has been analyzed for flavonoids, and some anthocyanins, flavonols and isoflavonoids were found in the flowers, leaves and hypocotyls [5].

Of three *Glycine* taxa which were used as plant materials in this survey, pterocarpans, glyceollins I, II and III, and clandestacarpin were isolated as phytoalexins from the leaves of *G. tabacina* which was inoculated with a suspension of *Pseudomonas syringae* pv. *pisi* [6]. Two flavonols, kaempferol and quercetin, and three isoflavonoids, genistein, daidzein and coumestrol, were found in the leaf tissue of *G. tabacina* after and before acid hydrolysis, respectively, together with unknown *C*-glycosylflavone [7]. However, flavonoids are not reported from *G. koidzumii* and *G. max* subsp. *formosana*. In this survey, isolation and identification of flavonoids in Japanese and Taiwanese *Glycine* taxa, *G. tabacina*, *G. koidzumii* and *G. max* subsp. *formosana* were performed and their flavonoid properties are described.

Two flavonols, one *C*-glycosylflavone and six isoflavones were isolated from the aerial parts of *G. tabacina* (Table 1, Figure 1). Of their flavonoids, flavonols and *C*-glycosylflavone were identified as kaempferol 3-*O*-sophoroside (**1**), quercetin 3-*O*-gentiobioside (**5**) and schaftoside (apigenin 6-*C*-glucoside-8-*C*-arabinoside, **15**) by UV spectral properties, LC-MS, acid hydrolysis, and TLC and HPLC comparisons with authentic samples. On the other hand, six isoflavones were identified as daidzein 7-*O*-glucoside (**19**), genistein 7-*O*-glucoside (**22**), daidzein 4′-*O*-glucoside (**20**), daidzein 7-*O*-xylosylglucoside (**21**), genistein 4′-*O*-glucoside (**23**) and 3′-*O*-methylorobol 7-*O*-glucoside (**24**). Of their isoflavones, **19** and **22**, and **20**, **21** and **23** were characterized by LC-MS, acid hydrolysis, and HPLC comparisons with authentic samples, and UV spectral properties, LC-MS and acid hydrolysis, respectively. 3′-*O*-Methylorobol (5,7,4′-trihydroxy-3′-methoxyisoflavone) 7-*O*-glucoside (**24**) was identified by LC-MS, acid hydrolysis and ^{1}H and ^{13}C NMR spectra. Of two flavonols, although **2** has been reported from the leaves of *G. max* subsp. *max* and subsp. *soja* [4a, 5a], **1** was found in the genus *Glycine* for the first time. The presence of *C*-glycosylflavone in *G. tabacina* has been pointed out by Vaughan and Hymowitz [7]. In this survey, that of *G. tabacina* was shown to be schaftoside. As *C*-glycosylflavones, carlinoside, isocarlinoside, gen kwanin 6,8-di-*C*-hexoside, isoschaftoside, vitexin and vitexin 2′′-*O*-rhamnoside, were reported from soybean

Table 1: Distribution of flavonoids among *Glycine tabacina*, *G. koidzumii* and *G. max* subsp. *formosana*.

Spp.	Flavonols														Flavones				Isoflavones					
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
Gt	+				+										+				+	+	+	+	+	+
Gk	+				+										+	+			+			+		+
Gm		+	+	+		+	+	+	+	+	+	+	+	+			+	+	+			+		+

Gt = *Glycine tabacina*, Gk = *Glycine koidzumii*, Gm = *Glycine max* subsp. *formosana*, **1** = Kaempferol 3-*O*-sophoroside, **2** = Kaempferol 3-*O*-rutinoside, **3** = Kaempferol 3-*O*-robinobioside, **4** = Kaempferol 3-*O*-rhamnosyl-(1→4)-[rhamnosyl-(1→6)-galactoside], **5** = Quercetin 3-*O*-gentiobioside, **6** = Quercetin 3-*O*-glucoside, **7** = Quercetin 3-*O*-galactoside, **8** = Quercetin 3-*O*-rutinoside, **9** = Quercetin 3-*O*-robinobioside, **10** = Quercetin 3-*O*-rhamnosyl-(1→4)-[rhamnosyl-(1→6)-galactoside], **11** = Isorhamnetin 3-*O*-rutinoside, **12** = Isorhamnetin 3-*O*-robinobioside, **13** = Isorhamnetin 3-*O*-rhamnosylrhamnosylglucoside, **14** = Isorhamnetin 3-*O*-rhamnosylrhamnosylgalactoside, **15** = Schaftoside, **16** = Apigenin 6,8-di-*C*-arabinoside, **17** = Luteolin 7-*O*-glucoside, **18** = Chrysoeriol 7-*O*-glucoside, **19** = Daidzein 7-*O*-glucoside, **20** = Daidzein 4′-*O*-glucoside, **21** = Daidzein 7-*O*-xylosylglucoside, **22** = Genistein 7-*O*-glucoside, **23** = Genistein 4′-*O*-glucoside and **24** = 3′-*O*-Methylorobol 7-*O*-glucoside.

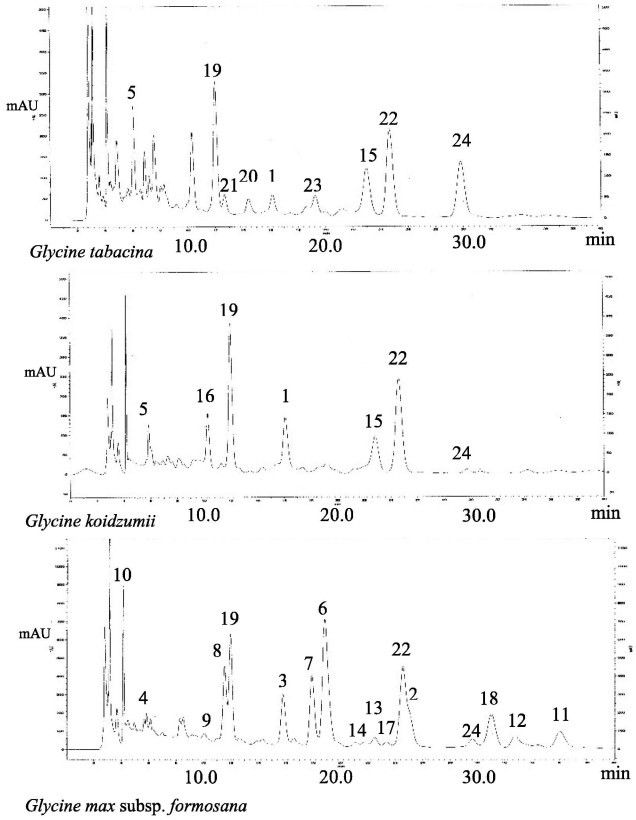
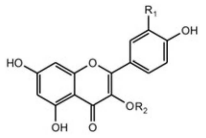
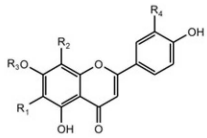


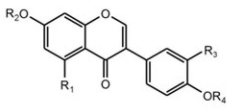
Figure 1: HPLC chromatograms of crude extracts from the leaves or aerial parts of three *Glycine* species. Numbers, see Table 1. Other peaks were organic acids and unknown isoflavones.



(1) R_1 = H, R_2 = glucosyl-(1→2)-glucosyl
(2) R_1 = H, R_2 = rhamnosyl-(1→6)-glucosyl
(3) R_1 = H, R_2 = rhamnosyl-(1→6)-galactosyl
(4) R_1 = H, R_2 = rhamnosyl-(1→4)-[rhamnosyl-(1→6)-galactosyl]
(5) R_1 = OH, R_2 = glucosyl-(1→6)-glucosyl
(6) R_1 = OH, R_2 = glucosyl
(7) R_1 = OH, R_2 = galactosyl
(8) R_1 = OH, R_2 = rhamnosyl-(1→6)-glucosyl
(9) R_1 = OH, R_2 = rhamnosyl-(1→6)-galactosyl
(10) R_1 = OH, R_2 = rhamnosyl-(1→4)-[rhamnosyl-(1→6)-galactosyl]
(11) R_1 = OMe, R_2 = rhamnosyl-(1→6)-glucosyl
(12) R_1 = OMe, R_2 = rhamnosyl-(1→6)-galactosyl
(13) R_1 = OMe, R_2 = rhamnosyl-rhamnosyl-glucosyl
(14) R_1 = OMe, R_2 = rhamnosyl-rhamnosyl-galactosyl



(15) R_1 = glucosyl, R_2 = arabinosyl, R_3 = R_4 = H
(16) R_1 = R_2 = arabinosyl, R_3 = R_4 = H
(17) R_1 = R_2 = H, R_3 = glucosyl, R_4 = OH
(18) R_1 = R_2 = H, R_3 = glucosyl, R_4 = OMe



(19) R_1 = R_3 = R_4 = H, R_2 = glucosyl
(20) R_1 = R_2 = R_3 = H, R_4 = glucosyl
(21) R_1 = R_2 = R_3 = H, R_4 = xylosylglucosyl
(22) R_1 = OH, R_2 = glucosyl, R_3 = R_4 = H
(23) R_1 = OH, R_2 = R_3 = H, R_4 = glucosyl
(24) R_1 = OH, R_2 = glucosyl, R_3 = OMe, R_4 = H

Figure 2: Chemical structures of flavonoids isolated from *Glycine tabacina*, *G. koidzumii* and *G. max* subsp. *formosana*.

[4g]. Many isoflavonoids were reported from the genus *Glycine* [8]. In *G. tabacina*, four pterocarpans, clandestacarpin and glyceollins I-III, were found as phytoalexins, together with coumestrol, daidzein and genistein [6, 7]. Of six isoflavone glycosides which were isolated from *G. tabacina*, **19** and **22** have already been reported from *G. max* subsp. *max* [e.g. 9]. However, other four isoflavone glycosides **20**, **21**, **23** and **24** were not reported from the genus *Glycine*. Although rare 3′-*O*-methylorobol 7-*O*-glucoside (**24**) was isolated from other Leguminosae species, *Thermopsis montana* Nutt. and *T. angustata* F. Greene [10], it was found in the genus *Glycine* for the first time. Other a few isoflavonoids were detected in *G. tabacina* by HPLC survey in this survey. However, they could not be characterized for small amounts of the compounds.

Each two of flavonols and flavones, and three isoflavones were isolated from *G. koidzumii*. They were identified as kaempferol 3-*O*-sophoroside (**1**), quercetin 3-*O*-gentiobioside (**5**), schaftoside (**15**), apigenin 6,8-di-*C*-arabinoside (**16**), daidzein 7-*O*-glucoside (**19**), genistein 7-*O*-glucoside (**22**) and 3′-*O*-methylorobol 7-*O*-glucoside (**24**). Flavonoids of *G. koidzumii* were reported for the first time. Flavonoid composition of this species was similar to that of *G. tabacina* except for the presence of **16** and the absence of **20**, **21** and **23**. *C*-Glycosylflavone **16** was characterized by UV spectra, LC-MS, and TLC and HPLC comparison with authentic sample and newly found in the genus *Glycine*.

Eleven flavonols, two flavones and three isoflavones, were isolated from the leaves of *Glycine max* subsp. *formosana* in Taiwan

(Table 1, Figure 1). Flavonols were 3-*O*-glycosides based on kaempferol, quercetin and isorhamnetin and identified as kaempferol 3-*O*-rutinoside (**2**) and 3-*O*-robinobioside (**3**), quercetin 3-*O*-glucoside (**6**), 3-*O*-galactoside (**7**), 3-*O*-rutinoside (**8**) and 3-*O*-robinobioside (**9**), and isorhamnetin 3-*O*-rutinoside (**11**) and 3-*O*-robinobioside (**12**) by UV spectra, LC-MS, acid hydrolysis and TLC and HPLC comparisons with authentic samples. Although **2**, **6** and **8** were reported from *G. max* subsp. *max* [4a, 4e], other glycosides were not found in soybean. Flavonoids **4** and **10** were identified as 3-*O*-rhamnosyl-(1→4)-[rhamnosyl-(1→6)-galacto-sides] of kaempferol and quercetin by UV spectra, LC-MS, acid hydrolysis, and ^{1}H and ^{13}C NMR. They were recently isolated from soybean leaves as new compounds [4a, Murai et al., unpublished data]. Other two flavonol glycosides were partially characterized as 3-*O*-rhamnosyl-rhamnosylglucoside and 3-*O*-rhamnosylrhamnosyl-galactoside of isorhamnetin by UV spectra, LC-MS and acid hydrolysis. Two flavone glycosides **17** and **18** were flavone *O*-glycosides, and identified as luteolin 7-*O*-glucoside and chrysoeriol 7-*O*-glucoside by UV spectra, LC-MS, acid hydrolysis, and TLC and HPLC comparisons with authentic samples. Although **17** was reported from soybean [4d], **18** was newly found in the genus *Glycine*. Three isoflavone glycosides were isolated from *G. max* subsp. *formosana* and identified as diadzein 7-*O*-glucoside (**19**), genistein 7-*O*-glucoside (**22**) and 3′-*O*-methylorobol 7-*O*-glucoside (**24**). A few isoflavone glycosides were accompanied with their compounds in *G. max* subsp. *formosana* leaves and characterized as dihydroxy-monomethoxyisoflavone, monohydroxy-monomethoxy-isoflavone and monohydroxy-dimethoxyisoflavone monohexosides by LC-MS. However, their complete identification was not performed for small amounts of the compounds.

Many isoflavonoids and flavonols have been reported, together with some anthocyanins, aurone, chalcone, dihydroflavonol, flavones and *C*-glycosylflavones from the flowers, leaves, seeds, roots, hypocotyls and cotyledons of soybean cultivars. However, major flavonoids of the leaves are flavonol glycosides based on kaempferol and quercetin [4]. Those of *G. max* subsp. *formosana* were also flavonol glycosides. Its flavonoid composition was comparatively similar to those of many soybean cultivars. *G. max* subsp. *formosana* is a subspecies of *G. max*. The similarity of flavonoid composition between both subspecies supported their taxonomic relationship. On the other hand, flavonols of *G. tabacina* and *G. koidzumii* were different with those of soybean, i.e. presence or absence of **1** and **5** (Table 1, Figure 1). Insofar as I know, major flavonoids of the leaves in soybean cultivars are flavonol glycosides including kaempferol, quercetin and sometimes isorhamnetin [8; Iwashina et al., unpublished data]. However, flavonol composition is different among soybean cultivars. Of flavone glycosides which were isolated in this survey, **15** and **16** were *C*-glycosylflavones and present in both *G. tabacina* and *G. koidzumii*. On the other hand, **17** and **18** were flavone *O*-glycosides and detected in *G. max* subsp. *formosana* (Table 1, Figure 1). Flavonoid surveys were performed in some *Glycine* species for phytoalexins [6]. However, flavonoids in the healthy leaves were fragmentarily surveyed except for some soybean cultivars. Although we investigated the flavonoids of *G. tabacina*, *G. koidzumii* and *G. max* subsp. *formosana* in Japan and Taiwan, much wild *Glycine* species and cultivars must be surveyed for flavonoids, because soybean is one of the important crop in the world, and flavonoids including isoflavonoids may be act as the functional compounds in the leaves, seeds and roots.

Experimental

General: UV spectra were measured with a Shimadzu MPS-2000 multipurpose recording spectrophotometer (Shimadzu, Kyoto). Analytical high performance liquid chromatography (HPLC) of the isolated compounds and crude extracts were performed with Shimadzu HPLC systems using *L*-column2 ODS column (Chemicals Evaluation and Research Institute, Tokyo) or Inertsil ODS-4 column (GL Sciences Inc., Tokyo) (I.D. 6.0 ×150 mm) at flow-rate of 1.0 ml min^{-1}, detection wave-length of 250 nm, and eluent was MeCN/H_2O/H_3PO_4 (17:83:0.2). Preparative HPLC were performed with Shimadzu HPLC systems using Inertsil ODS-4 column (I.D. 10 × 250 mm) at flow-rate of 3.0 mL min^{-1}, detection wave-length of 250 nm, and eluents were MeCN/H_2O/HCOOH (20:79:1). Liquid-chromatograph-mass spectra (LC-MS) were measured with Shimadzu LCMS-2010 EV systems using Inertsil ODS-4 column (I.D. 2.1 × 100 mm) at flow-rate of 0.2 mL min^{-1}, detection wave-length of 350 (flavonols and flavones) and 250 nm (isoflavones), ionizing voltage, 4.5 kV for ESI^+ and 3.5 kV for ESI^-, and eluents were MeCN/H_2O/HCOOH (20:79:1 or 20:75:5). Nuclear magnetic resonance (NMR) spectra (^{1}H and ^{13}C NMR, $m^{1}H$-^{1}H COSY, ^{1}H-^{1}H NOESY, HMQC and HMSC) were recorded on a Bruker AV-600 in pyridine-d_5 at 600 MHz (^{1}H NMR) and 150 MHz (^{13}C NMR). Thin layer chromatography (TLC) was performed with Cellulose 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. Preparative paper chromatography (PC) was performed with solvent systems, BAW and then 15% HOAc.

Plant materials: *Glycine* species used as plant materials were collected in the following sites and dates: *G. tabacina* (Labill.) Benth., Ie Island, Okinawa Pref., Japan, 20 Sept. 2014; *G. koidzumii* Ohwi, Miyako Island and Irabu Island, Okinawa Pref., Japan, 8 April 2015; and *G. max* (L.) Merr. subsp. *formosana* (Hosokawa) Tateishi & H. Ohashi, Hualien, Taiwan, 31 Oct. 2011 and 2 Nov. 2011. Voucher specimens were deposited in the Herbarium of National Museum of Nature and Science (TNS), Japan.

Extraction and isolation: Dry aerial parts (25.9 g) except for the flowers of *G. tabacina*, fresh aerial parts except for flowers (62.8 g) of *G. koidzumii* and dry leaves (165.2 g) of *G. max* subsp. *formosana* were extracted with MeOH, respectively. After concentration, extracts were applied to preparative PC using solvent systems, BAW, 15% HOAc, re-BAW and then re-15% HOAc. Roughly separated compounds were applied to preparative HPLC. The isolated flavonoids were finally purified by Sephadex LH-20 column chromatography using solvent system, 70% MeOH. Flavonoids **1** (ca 5 mg), **15** (ca. 10 mg), **22** (ca. 5 mg) and **24** (ca 5 mg) were obtained from *G. tabacina* and *G. koidzumii* as yellow or white powders. On the other hand, **2** (ca 15 mg), **3** (ca. 5 mg), **4** (ca. 5 mg), **8** (ca. 50 mg), **10** (ca 10 mg), **19** (ca 5 mg) and **22** (ca 3 mg) were obtained from *G. max* subsp. *formosana* as pale yellow or white powders. Other flavonoids were obtained as pure MeOH solution.

Identification: Of isolated flavonoids, **3**, **4**, **10** and **24** were identified as UV, LC-MS, acid hydrolysis, and ^{1}H and ^{13}C NMR. Other flavonoids were characterized by UV, LC-MS, acid hydrolysis and/or TLC and HPLC comparisons with authentic samples. NMR and MS data of **3**, **4**, **10** and **24** were as follows.

Authentic samples: Origins of authentic flavonoids which were used in this survey were as follows: kaempferol 3-*O*-sophoroside from the flowers of *Dianthus caryophyllus* L. [11]; kaempferol 3-*O*-rutinoside from the leaves of *Calystegia japonica* Choisy [12]; kaempferol 3-*O*-robinobioside and quercetin 3-*O*-galactoside from the aerial parts of *Cassytha filimormis* L. [13]; quercetin 3-*O*-gentiobioside from the leaves of *Hydrastis canadense* L. [14]; quercetin 3-*O*-glucoside, quercetin 3-*O*-rutinoside and schaftoside

from the aerial parts of *Osyris alba* L. [15]; quercetin 3-*O*-robinobioside from the leaves of *Asarum canadense* L. [16]; isorhamnetin 3-*O*-rutinoside and isorhamnetin 3-*O*-robinobioside from the leaves of *Asarum* spp. [17]; apigenin 6,8-di-*C*-arabinoside from the leaves of *Ajuga* spp. [18]; luteolin 7-*O*-glucoside from the leaves of *Schmalhausenia nidulans* Petrak [19]; daidzein 7-*O*-glucoside and genistein 7-*O*-glucoside from Extrasynthèse, France.

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