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Flavonoids from the cocoon of *Rondotia menciana*

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ABSTRACT

Two flavonol glycosides along with four known flavonoids were isolated from the cocoon of the mulberry white caterpillar, *Rondotia menciana* (Lepidoptera: Bombycidae: Bombycinae), a closely related species of the domesticated silkworm *Bombyx mori*, both of which feed on leaves of mulberry (*Morus alba*). The two glycosides were characterized as quercetin 3-*O*-β-D-galactopyranosyl-(1 → 3)-β-D-galactopyranoside and kaempferol 3-*O*-β-D-galactopyranosyl-(1 → 3)-β-D-galactopyranoside, based on spectroscopic data and chemical evidence. The flavonol galactosides found in the cocoon were not present in the host plant, nor in the cocoon of the silkworm, *B. mori*. Notably, flavonol glucosides, which are the main constituents of cocoon flavonoids in *B. mori mori*, were not found in the *R. menciana* cocoon. The present result strongly suggests that *R. menciana* is quite unique in that they predominantly use an UDP-galactosyltransferase for conjugation of dietary flavonoids, whereas UDP-glucosyltransferases are generally used for conjugation of plant phenolics and xenobiotics in other insects.

1. Introduction

It has been known that cocoon shells of some strains of the domesticated silkworm, *Bombyx mori* (L.), contain flavonoids, which are derived from the leaves of their host plant, the mulberry tree (*Morus alba*) (Fujimoto and Hayashiya, 1972; Tamura et al., 2002; Kurioka and Yamazaki, 2002; Hirayama et al., 2006). Orally administrated quercetin (**5**) was preferably glucosylated at the 5-*O*-position in the midgut, which is the first step for the biosynthesis of cocoon flavonoids (Hirayama et al., 2008). It was also found that cocoon flavonoids increase the UV-shielding activity of cocoons, and protect individuals from harmful effects of sunlight during metamorphosis (Daimon et al., 2010).

Uptake and utilization of dietary flavonoids is relatively widespread in the Lepidoptera (Ferreres et al., 2007). Flavonoids sequestered by Lepidoptera larvae are subsequently metabolized, stored, and transferred into the target organs such as the wings (Geuder et al., 1997). However, as far as is known, there is no report on cocoon flavonoids of insects other than *B. mori*. The aim of this study was to increase the knowledge of cocoon flavonoids of insects. The mulberry white caterpillar, *Rondotia menciana* has a close relationship to *B. mori*, both belonging to the subfamily Bombycinae and feeding on mulberry leaves. It is considered herein that comparative studies on ecological aspects of *R. menciana* and *B. mori* (e.g. metabolism of plant secondary metabolites, such as flavonoids and alkaloids) would facilitate an understanding of molecular mechanisms underlying interactions of insects and plants.

In the present study, cocoon flavonoids of *R. menciana* were isolated and identified based on spectroscopic methods and chemical evidence. These results indicated that the main constituents of the insect cocoon flavonoids were flavonol galactosides. Flavonol glycosides, which have been found in the cocoon of *B. mori* and their host plant, were not detected in the cocoon of *R. menciana*. This is the first report suggesting that flavonoids are conjugated with galactose in animal species, highlighting the complex metabolism of flavonoids in insects and its great diversity. This study further suggests that insect metabolites are a source of potential bioactive compounds that are not found in plants.

2. Results and discussion

2.1. Structural determination of cocoon shell flavonoids of R. menciana

The HPLC-DAD profile of the aqueous methanolic extract of a *R. menciana* cocoon shows six peaks with UV spectra characteristic of flavonols (Fig. 1A, see also Table S1). These flavonols were isolated and identified as follows. Acid hydrolysis of peaks a and b gave quercetin (**5**) (Fig. 2) as the aglycone and D-galactose (**7**) as the sugar residue. The UV spectra of both peaks a and b in the

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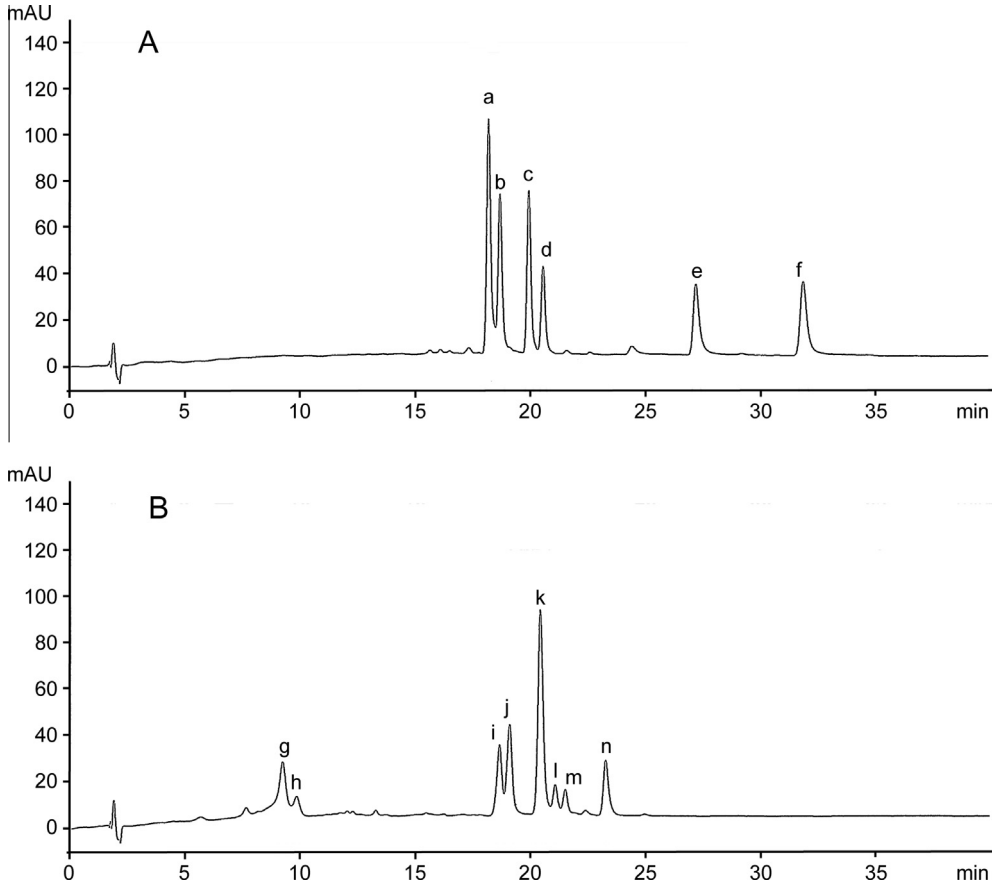

Fig. 1. Representative HPLC-DAD profiles of aqueous methanolic extracts of *R. menciana* cocoon (A) and *M. alba* leaves (B). Detection was performed at 365 nm. a, quercetin 3-*O*-galactosyl-galactoside (**1**); b, quercetin 3-*O*-galactoside (**3**); c, kaempferol 3-*O*-galactosyl-galactoside (**2**); d, kaempferol 3-*O*-galactoside (**4**); e, quercetin (**5**); f, kaempferol (**6**); g, 5-caffeoylquinic acid; h, caffeoylquinic acid; i, quercetin 3-*O*-rutinoside; j, quercetin 3-*O*-glucoside; k, quercetin 3-*O*-(6-malonylglucoside); l, quercetin derivative; m, kaempferol 3-*O*-glucoside; n, kaempferol derivative.

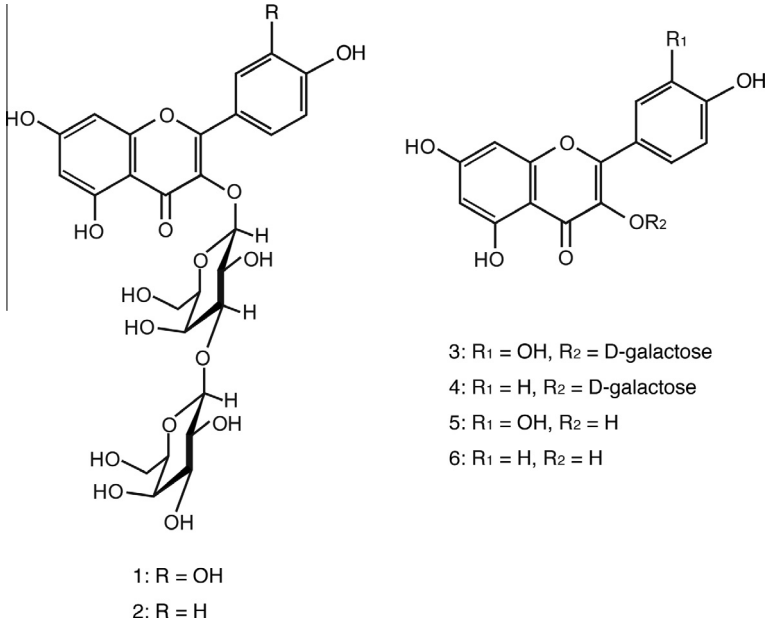

Fig. 2. Structures of flavonoids isolated from the cocoon of *Rondotia menciana*.

presence of shift reagents suggested free hydroxyl groups at C-5, C-7, C-3′ and C-4′, and substitution at the 3-*O* position of quercetin (**5**). LC–MS analysis of peaks a and b showed deprotonated ions $[M-H]^-$ with peaks at *m*/*z* 625, 463, respectively. Based on these data, peaks a and b were identified as quercetin 3-*O*-galactosyl-galactoside (**1**) and quercetin-3-*O*-galactoside (**3**), respectively.

After acid hydrolysis of peaks c and d, kaempferol (**6**) and D-galactose (**7**) were also obtained. The UV spectra of peaks c and d in the presence of the shift reagents suggested free hydroxyls at C-5, C-7, and C-4′, and substitution at the 3-*O* position of kaempferol (**6**). LC–MS analysis of peaks c and d showed deprotonated ions $[M-H]^-$ with peaks at *m*/*z* 609, 447, respectively. Based on these data, peaks c and d were identified as kaempferol 3-*O*-galactosyl-galactoside (**2**) and kaempferol 3-*O*-galactoside (**4**), respectively. In addition, peaks e and f were confirmed to be quercetin (**5**) and kaempferol (**6**), by comparing their HPLC-DAD and LC–MS profiles with those of authentic standards. To determine the detailed chemical structures of the novel compounds (**1** and **2**), both compounds were characterized by ^{1}H NMR, ^{13}C NMR, and HR-ESI-MS analysis. The molecular formula of **1** was determined to be $C_{27}H_{30}O_{17}$ by analysis of its HR-ESI-MS spectrum (*m*/*z* calcd. for $C_{27}H_{30}O_{17}Na$ ($[M+Na]^+$), 649.13752, found: 649.1375); this corresponds to a digalactosyl derivative of quercetin (**5**). The ^{1}H NMR and ^{13}C NMR spectra of **1** (Table 1) were also similar to those of quercetin 3-*O*-galactoside (**3**) (Barberá et al., 1986; Yasukawa and Takido, 1987; Bennini et al., 1992; Foo et al., 2000). Substitution at the 3-*O* position of quercetin (**5**) was confirmed by the HMBC correlation between the anomeric proton H-1″ (δ 5.30) of galactose and the carbon (δ 135.7) of C-3 (Fig. 3). In the ^{13}C NMR spectrum, a large downfield shift of the galactose C-3″ carbon (Δd11.1∼11.5) was observed, compared to the corresponding signal of quercetin 3-*O*-galactoside (**3**) (Barberá et al., 1986; Yasukawa and Takido; 1987; Bennini et al., 1992; Foo et al., 2000), suggesting an interglycosidic linkage at this position. This was corroborated by the HMBC correlation between the anomeric proton H-1‴ (δ 4.54) of the terminal galactose residue and a carbon (δ 84.7) assigned to C″-3 of the initial galactose residue. It was further confirmed by the HMBC correlation between the initial galactose H-3″ proton (δ 3.71) and the C‴-3 carbon of the terminal galactose (δ 106.5) (Fig. 3). Further, the β-configuration of each galactosyl residue was deduced from the large coupling constants (7.6–7.9 Hz) at the anomeric position in the ^{1}H NMR spectra (Table 1). Therefore, compound **1** was identified as quercetin 3-*O*-β-D-galactopyranosyl-(1 → 3)-β-D-galactopyranoside.

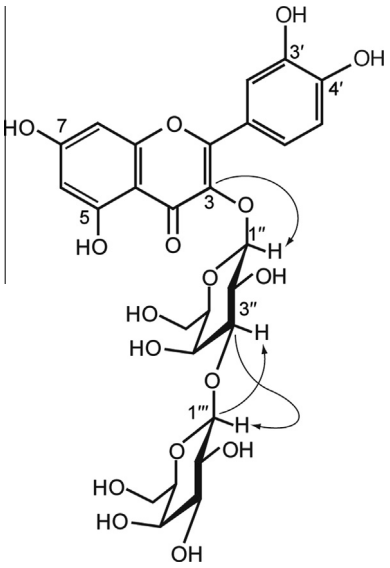

Fig. 3. Key HMBC correlation of **1**.

The structure of **2** was also characterized by the same methods used in the analysis of **1**. Its molecular formula was determined to be $C_{27}H_{30}O_{16}$ by HR-ESI-MS spectrum (*m*/*z* calcd. for $C_{27}H_{30}O_{16}Na$ ($[M+Na]^+$), 633.14261, found: 633.1431), corresponding to a digalactosyl derivative of kaempferol (**6**). The ^{1}H NMR and ^{13}C NMR spectra of **2** were very similar to those of **1**, except for signals assigned to the aglycone moiety. An HMBC experiment indicated

Table 1
^{1}H and ^{13}C NMR spectroscopic data for compounds **1** and **2**.

Position		Compound **1**		Compound **2**	
		^{1}H δ [ppm] multiplicity (*J* [Hz])	^{13}C δ [ppm]	^{1}H δ [ppm] multiplicity (*J* [Hz])	^{13}C δ [ppm]
Aglycone	2		158.7		159.0
	3		135.7		135.5
	4		179.4		179.5
	4a		105.6		105.7
	5		163.0		163.1
	6	6.20 *d* (2.0)	99.9	6.20 *d* (2.1)	100.0
	7		166.2		165.7
	8	6.40 *d* (2.0)	94.7	6.41 *d* (2.1)	99.7
	8a		158.5		158.5
	1′		122.9		122.7
	2′	7.84 *d* (2.1)	117.7	8.09 *d* (8.9)	132.4
	3′		145.8	6.88 *d* (8.9)	116.1
	4′		150.0		161.6
	5′	6.86 *d* (8.5)	116.1	6.88 *d* (8.9)	116.1
	6′	7.58 *dd* (8.5, 2.1)	122.9	6.88 *d* (8.9)	132.4
3-*O*-β-Gal*p*	1″	5.30 *d* (7.9)	104.7	5.29 *d* (7.9)	104.3
	2″	3.98 *dd* (9.6, 7.9)	72.3	3.94 *dd* (9.6, 7.9)	72.2
	3″	3.71 *dd* (9.6, 3.3)	84.7	3.70 *dd* (9.6, 3.3)	84.6
	4″	4.16 *brd* (3.3)	69.6	4.11 *dd* (3.3, 0.9)	69.5
	5″	3.53 *brdd* (6.2, 5.8)	76.9	3.50 *ddd* (6.4, 5.8, 0.9)	76.8
	6″	3.67 *dd* (11.3, 5.8)	62.0	3.64 *dd* (11.3, 5.8)	62.1
		3.58 *dd* (11.4, 6.2)		3.54 *dd* (11.4, 6.4)	
3″-*O*-β-Gal*p*	1‴	4.54 *d* (7.6)	106.5	4.52 *d* (7.6)	106.4
	2‴	3.64 *dd* (9.7, 7.6)	73.0	3.63 *dd* (9.7, 7.6)	73.0
	3‴	3.51 *dd* (9.7, 3.3)	74.6	3.50 *dd* (9.7, 3.3)	74.6
	4‴	3.83 *brd* (3.3)	70.2	3.82 *dd* (3.3, 0.8)	70.2
	5‴	3.55 *brdd* (7.1, 5.0)	76.8	3.54 *ddd* (7.1, 5.2, 0.8)	76.8
	6‴	3.74 *dd* (11.4, 7.1)	62.6	3.74 *dd* (11.4, 7.1)	62.6
		3.70 *dd* (11.4, 5.0)		3.69 *dd* (11.4, 5.2)	

that the residual galactose was attached at the 3-*O* position of the kaempferol (**6**), and that the other galactose residue was attached to the 3″-*O* position of the initial galactose. These data identified compound **2** as kaempferol 3-*O*-β-D-galactopyranosyl-(1 → 3)-β-D-galactopyranoside.

2.2. Host plant flavonoids uptake and metabolism by R. menciana

The flavonoids of mulberry (*M. alba*) leaves have been isolated and characterized by many researchers. These study described that mulberry leaves contained quercetin 3-*O*-glucoside, quercetin 3-*O*-rutinoside, quercetin 3-*O*-(6-malonylglucoside), quercetin 3-*O*-(6-acetylglucoside), kaempferol 3-*O*-glucoside, kaempferol 3-*O*-(6-acetylglucoside), kaempferol 3-*O*-(6-malonylglucoside) (Naito, 1968, 1979; Onogi et al., 1994; Doi et al., 2001; Katsube et al., 2006, 2009). The present study confirmed that the main constituent of the mulberry leaves was quercetin 3-*O*-(6-malonylglucoside), followed by quercetin 3-*O*-rutinoside and quercetin 3-*O*-glucoside (Fig. 2B). Comparison of the flavonoid profile of *R. menciana* with that of mulberry established that there were no compounds in common for the insect and its host plant (Fig. 1, see also Table S1). Interestingly, even though no flavonol galactosides have been found from the food source plant, flavonol galactosides were the main constitutes of the cocoon extracts (Fig. 1A, see also Table S2). This can be ascribed to metabolism of the flavonols in the insect. It is thus considered that flavonol glycosides derived from the mulberry are hydrolyzed to aglycones (quercetin (**5**) and kaempferol (**6**)) during uptake by the midgut tissue, then conjugated to galactose for further transport to the silk gland, where the raw materials for cocoon shells are produced. Schittko et al. (1999) showed that quercetin 3-*O*-galactoside (**3**) was the main component of flavonoids accumulated in the common blue butterfly, *Polyommatus icarus*. However, *P. icarus* larvae probably uptake and store quercetin 3-*O*-galactoside (**3**) without modification since inflorescences of *Trifolium repens*, the food plant source of *P. icarus*, is also rich in quercetin 3-*O*-galactoside (**3**) (Schittko et al., 1999).

Thus, so far, there have been no reports on galactosyl-conjugation reaction of plant phenolics in animal tissues. In general, insects have been known to use UDP-glucosyltransferase to modify plant phenolics and xenobiotics (Ahmad and Hopkins, 1993; Ahn et al., 2011; Rausell et al., 1997; Real et al., 1991; Sasai et al.,2009). It was found, however, that *B. mori* also uses UDP-glucosyltransferase in the metabolism of dietary flavonoids (Hirayama et al., 2008; Daimon et al., 2010). Although it is necessary to show the actual enzyme activity in the near future, the present study suggests that *R. menciana* uses UDP-galactosyltransferase instead of UDP-glucosyltransferase. It is very interesting to now consider why *R. menciana* uses an UDP-galactosyltransferase, even though *R. menciana* and *B. mori* are closely related and reared on the same food plant. Further investigation of the biological functions of flavonol galactosides found in *R. menciana* may help to understand its evolution, as well as the ecological significance of the galactosyl conjugation reaction of flavonoids in insects, i.e. thereby potentially constituting a source of potential bioactive compounds not found so far. In addition, molecular and biochemical analysis of the UDP-galactosyltransferase of *R. menciana* could be useful for understanding the molecular basis of substrate specificity of UDP-glycosyltransferase (UGT) family enzymes in animals.

3. Conclusion

Cocoon flavonoids of *R. menciana* were isolated and identified based on spectroscopic methods and chemical evidence, indicating that the main constituents of the cocoon flavonoids are flavonol galactosides. This is the first report suggesting that flavonoids are conjugated with galactose in animal species. The present results contribute the understanding the complex metabolism of flavonoids in insects.

4. Experimental

4.1. Chemicals and reagents

Quercetin 3-*O*-glucoside, kaempferol 3-*O*-glucoside, rutin, quercetin 3-*O*-galactoside (**3**), quercetin (**5**), and kaempferol (**6**) were from Extrasynthese (Genay, France). Qurcetin 3-*O*-(6-malonylglucoside) was a gift from Dr. Takuya Katsube (Shimane institute for industrial technology, Shimane, Japan). A galactose assay kit was purchased from Biovision (Milpitas, CA), whereas a, glucose assay kit was from Wako pure chemical (Osaka, Japan). All other chemicals and solvents used were of either analytical or HPLC grade. Water (H_2O) was treated with a Milli-Q water purification system (Millipore, Bedford, MA).

4.2. Biological materials

R. menciana were maintained in the Anhui Agricultural University, the National Institute of Agrobiological Sciences, and the University of Tokyo. The larvae of *R. menciana* were reared on fresh leaves of mulberry (*M. alba* L). Cocoon shells produced by the larvae were harvested after adult emergence, then cut into small pieces and stored at −80 °C until needed. Collected mulberry leaves were frozen, and then lyophilized. Powdered mulberry samples were kept at −80 °C until analysis.

4.3. LC–MS analysis

LC–ESI-MS analysis was performed using a HP 1100 series HPLC (Agilent Technologies, Santa Clara, CA) equipped with an HP 1100 MSD mass spectrometer. Flavonoids were extracted from either cocoon shells or lyophilized mulberry powder by MeOH–H_2O (7:3, V/V) at 60 °C for 2 h. After centrifugation at 20,000*g* for 10 min, an aliquot (10 μl) of the supernatants was injected into the LC–MS system and separated by a Nova-Pak C18 reversed phase column, 150 mm × 2.0 mm i.d. (Waters, Milford, MA) at a flow rate of 0.3 ml/min. Column temperature was maintained at 40 °C. The mobile phase consisted of solvents A (0.2% aq. HCO_2H formic acid) and B (0.2% HCO_2H in CH_3CN). Flavonoids were separated with a linear gradient from 7% B to 40% B over 40 min and then to 100% B for 5 min. UV detection was carried out using a HP 1100 photodiode array detector (DAD) to facilitate peak assignment. The eluent was ionized by negative electrospray ionization. The electrospray mass spectrometer conditions were as follows: negative ion mode; fragmentor voltage, 70 V; capillary voltage, 3500 V; drying gas (N_2) flow, 10.0 L/min; nebulizer pressure, 25 psig; drying gas temperature, 350 °C. Some flavonoids were identified based on their co-elution with commercially available standards and their MS profile.

4.4. Isolation of flavonoids from the cocoon shell of R. menciana

Flavonoids were extracted from cocoon shells (10 g) using MeOH–H_2O (7:3, V/V) at 60 °C for 2 h. Crude extracts were filtered and concentrated by evaporation and diluted with H_2O. Each diluted solution was applied to a solid phase extraction cartridge (Oasis HLB, 35 ml, Waters, Milford, MA). After washing with H_2O, the column was eluted with MeOH. Eluted flavonoids were concentrated by evaporation and loaded to a preparative reversed-phase column (Sunfire C18, 19 × 150 mm, Waters) connected to a Shimadzu HPLC system equipped with an LC-7A pump, a

CTO-10A column oven, and an SPD-7AV UV–Vis detector monitoring at 365 nm. Flavonoids were eluted by a linear gradient of 14–25% CH_3CN in 0.2% HCO_2H over 90 min, at a flow rate of 10 ml/min at 40 °C.

4.5. Sugar and aglycone analysis

Acid hydrolysis of flavonoids was carried out in 2 M HCl, at 100 °C for 30 min. After cooling, HCl was removed by evaporation and the hydolysate was dried under a stream of N_2. Aglycones formed by acid hydrolysis were analyzed by LC–MS as described above using commercially available standards. Sugars released by hydrolysis were analyzed by a glucose assay kit and a galactose assay kit according to the manufactures procedures to determine their absolute configuration.

4.6. Identification of conjugation position by UV spectra

Conjugation positions of flavonoids were determined by UV spectra according to Markham (1982). Each isolated flavonoid was dissolved in MeOH and diluted until the absorbance of the maxima was between 0.6 and 0.7. Shift reagents were added sequentially to the freshly prepared solution, and the UV spectra were recoded on a UV-2500PC spectrometer (Shimadzu, Tokyo, Japan).

4.7. Structural analysis for novel flavonol galactosides

Flavonoid **1** and **2** were analyzed by ^1H NMR and ^{13}C NMR spectroscopic analyses using a Bruker Avance 800 spectrometer (Karlsruhe, Germany, 800 MHz for ^1H; 201 MHz for ^{13}C) in CD_3OD with tetramethylsilane (TMS) as an internal standard. Chemical shifts were expressed in ppm downfield from TMS. High-resolution ESI mass analysis for them was carried out using an Apex II 70e Fourier-transform ion cyclotron resonance (FTICR) mass spectrometer (Bruker Daltonics, Billerica, MA).

4.8. Quercetin 3-O-β-D-galactopyranosyl-(1→3)-β-D-galactopyranoside (*1*)

Yellow powder; UV λ_{max} nm; 257, 359 (MeOH); 273, 332, 413 (+NaOMe); 273, 381 (+NaOAc); 263, 379 (+NaOAc + H_3BO_3); 272, 450 (+$AlCl_3$); 270, 404 (+$AlCl_3$ + HCl). HR-ESI-MS; *m/z* 649.1375 $[M+Na]^+$ (calcd. for $C_{27}H_{30}O_{17}Na$, 649.13752). For ^1H NMR and ^{13}C NMR, see Table 1.

4.9. Kaempferol 3-O-β-D-galactopyranosyl-(1→3)-β-D-galactopyranoside (*2*)

Yellow powder; UV λ_{max} nm; 267, 351 (MeOH); 275, 326, 403 (+NaOMe); 275, 376 (+NaOAc); 267, 353 (+NaOAc + H_3BO_3); 274, 304, 351, 398 (+$AlCl_3$); 274, 305, 351, 398 (+$AlCl_3$ + HCl). HR-ESI-MS; *m/z* 633.1431 $[M+Na]^+$ (calcd. for $C_{27}H_{30}O_{16}Na$, 633.14261). For ^1H NMR and ^{13}C NMR, see Table 1.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2013.05.023.

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