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Article

Flavonoids from the Stems of Eastern Picklypear *Opuntia humifusa*, Cactaceae

Si Hyung Park*, Hui Kim and Dong Young Rhyu

Division of Bioscience, Mokpo National University, Muan 534-729, Korea

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Five flavonoids, isorhamnetin 3-*O*-β-D-galactosyl-4'-*O*-β-D-glucoside (1), isorhamnetin 3,4'-di-*O*-β-D-glucoside (2), isorhamnetin 3-*O*-β-D-(6-*O*-α-L-rhamnosyl)glucosyl-4'-*O*-β-D-glucoside (3), isorhamnetin 3-*O*-β-D-(6-*O*-α-L-rhamnosyl)glucoside (4), and isorhamnetin 3-*O*-β-D-(6-*O*-α-L-rhamnosyl) galactoside (5) were isolated from the stems of *Opuntia humifusa* (Raf.) Raf. and their structures were identified based on LC-MS and NMR data.

Key words: *isorhamnetin glycoside,* Opuntia humifusa

Some species of the genus *Opuntia*, (Family Cactaceae) are widely distributed in the southern regions of the Korean peninsula. Although their origins can be traced back to the Northern and Central Americas, it is unknown when these species were introduced into Korea. *Opuntia ficus-indica* (L.) Mill., an Indian-fig picklypear, is intensively cultivated in the Jeju Island for industrial food production. Its fruits and stems have been used in folk medicines to treat burns, edema, diabetes, and indigestion [Lopez, 1995]. It has also been reported that the extracts of *O. ficus-indica* show anti-inflammatory [Park *et al.*, 1998], anti-ulcer [Galati *et al.*, 2001], and hypoglycemic activities [Ibanez and Roman, 1979]. Isolation of two alkaloids from the fruit and several flavonoids from the stems of *O. ficus-indica* were reported [Jeong *et al.*, 1999; Strack *et al.*, 1987; Lee *et al.*, 2003; Saleem *et al.*, 2006]. Another cactus, the eastern picklypear, *O. humifusa* (Raf.) Raf. is also found in the southern coastal area of Korea, and its cultivation is increasing: the characteristics of its stem and the spine are different from those of *O. ficus-indica*. *O. ficus-indica* is 2-3 m tall with a definite trunk and spineless or 1-4 spines in each areole. *O. humifusa* is a prostrate cactus having oblong pads with tiny hair-like bristles that occur in little tufts. Although *O. humifusa* shrinks in winter, it is frost-resistant under the snow at −10°C. It has also been reported that the extracts of *O. humifusa* show several bioactivities [Lee *et al.*, 2004; Lee *et al.*, 2005]. However, there are no reports yet on the secondary metabolites of *O. humifusa* except taxifolin [Lee *et al.*, 2007]. This paper describes the structure elucidation of five flavonoids isolated from the stems of *O. humifusa* by means of LC-MS/MS and NMR.

*Corresponding author
Phone: +82-61-450-2662; Fax +82-61-450-6443
E-mail: shp@mokpo.ac.kr

Materials and Methods

Plant material. Stems of *O. humifusa* were harvested from Jido, Sinan-gun in September 2006 and a voucher specimen has been deposited in our laboratory.

General experiments. A reverse phase (RP-18) silica gel (70-230 mesh, YMC GEL ODS-A) was purchased from YMC Co. (Japan). Compounds **1-5** were isolated through RP open column chromatography, MPLC (Eyela VSP3050 system, Japan) with an Ultrapack ODS-S-50C column (37 × 300 mm, Yamazen Co.), and Sepadex LH-20 column chromatography. The NMR spectra were recorded using a Bruker Avanace 400 spectrometer. The LC-MS were obtained using Agilent 1100 series and Bruker HCT 3000 model equipped with ESI.

Extraction and isolation. Fresh stems of *O. humifusa* (6 kg) were chopped and extracted with MeOH (10 L, three times). The extract was concentrated under reduced pressure and the residue (308 g) was suspended in 2 L of water and successively extracted three times with 2 L of hexane, EtOAc and BuOH. The BuOH extract was concentrated *in vacuo* to yield a residue (16.5 g), which was subjected to Sephadex LH-20 column (3.5 × 60 cm) eluting with MeOH to give three fractions. Fraction 2 (10 g) was subjected to RP-18 chromatography eluting with MeOH-H_2O (4 : 6) to yield six sub-fractions. Fraction 2-2 was subjected to Ultrapack ODS-S-50C column (37 × 300 mm) and purified by Sephadex LH-20 column (1.5 × 140 cm) eluting with 80% MeOH solution to afford

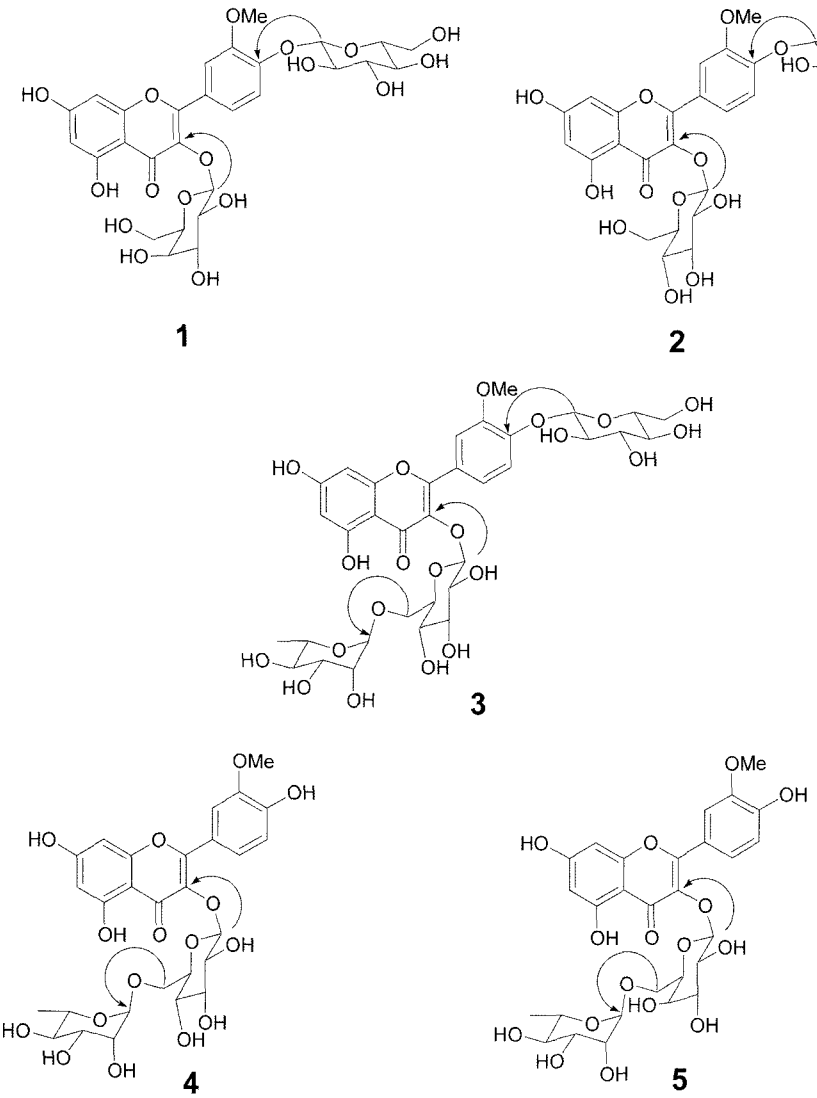

Fig. 1. Structures and important HMBC interactions of compounds 1-5.

compound **1** (30 mg). Fraction 2-3 was subjected to Ultrapack ODS-S-50C column (37 × 300 mm) and purified by Sephadex LH-20 column (1.5 × 140 cm) eluting with 80% MeOH solution to afford compounds **2** (42 mg) and **3** (40 mg). Fraction 2-5 was subjected to Ultrapack ODS-S-50C column (37 × 300 mm) and purified by Sephadex LH-20 column (1.5 × 140 cm) eluting with 80% MeOH solution to afford compounds **4** (33 mg) and **5** (54 mg).

LC-MS analysis. Agilent 1100 series LC system equipped with a gradient controller, an automatic sample injector, and a PDA detector was used. The separation was performed on 150 × 3 mm, 3.5 μm, Xterra MSC-18 column (Waters, US). A mobile phase consisting of 1% acetic acid in water (B), and 1% acetic acid in acetonitrile (A) was used for the separation. The flow rate was kept at 0.5 mL/min. The system was run with the following gradient program: 10% A for 5 min, from 10% A to 25% A for the next 35 min, from 25% A to 10% A for 1 min, then kept at 10% A for 9 min. The sample injection volume was 5 μL. Column temperature was kept at 25°C. A Bruker HCT 3000 ion trap mass spectrometer was coupled to the HPLC column. Dry temperature was 350°C, nebulizer gas was 40 psi, and dry gas was 9 L/min. The scan mass range was *m/z* 100-1000, target mass was *m/z* 700 and other parameters were set using the Smart parameter setting in negative auto MS/MS mode.

Isorhamnetin 3-*O*-β-D-galactosyl-4'-*O*-β-D-glucoside (1): Yellow solid; ESI-MS *m/z* 639 $[M-H]^-$; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.13 (1H, d, J = 2.0 Hz, H-2'), 7.60 (1H, dd, J = 2.0, 8.9 Hz, H-5'), 7.30 (1H, d, J = 8.9 Hz, H-6'), 6.52 (1H, d, J = 2.0 Hz, H-8), 6.28 (1H, d, J = 2.0 Hz, H-6), 5.59 (1H, d, J = 7.7 Hz, H-1''), 5.13 (1H, d, J = 7.4 Hz, H-1''''), 3.91 (3H, s, 3'-OMe); ^{13}C-NMR (100 MHz) in Table 2.

Isorhamnetin 3,4'-di-*O*-β-D-glucoside (2): Yellow solid; ESI-MS *m/z* 639 $[M-H]^-$; ^{1}H-NMR (400 MHz,

Table 1. LC-MS data of compounds 1-5

Flavonoid	Rt (min)	λ_{max} (nm)	$[M-H]^-$ *m/z*	Fragment ions[a] *m/z*
1	19.5	251, 346	639	477 [M-Hex-H], 315 [M-2Hex-H]
2	20.3	251, 346	639	477 [M-Hex-H], 315 [M-2Hex-H]
3	21.2	251, 350	785	623 [M-Hex-H], 477 [M-Hex-dHex-H], 315 [M-2Hex-dHex-H]
4	29.5	254, 356	623	315 [M-Hex-dHex-H]
5	30.4	254, 356	623	315 [M-Hex-dHex-H]

[a]Hex; hexose, dHex; deoxyhexose

DMSO-d_6) δ 8.03 (1H, d, J = 2.0 Hz, H-2'), 7.61 (1H, dd, J = 2.0, 8.9 Hz, H-5'), 7.30 (1H, d, J = 8.9 Hz, H-6'), 6.52 (1H, d, J = 2.0 Hz, H-8), 6.28 (1H, d, J = 2.0 Hz, H-6), 5.65 (1H, d, J = 7.4 Hz, H-1''), 5.12 (1H, d, J = 7.5 Hz, H-1''''), 3.90 (3H, s, 3'-OMe); ^{13}C-NMR (100 MHz) in Table 2.

Isorhamnetin 3-*O*-β-D-(6-*O*-α-L-rhamnosyl)glucosyl-4'-*O*-β-D-glucoside (3): Yellow solid; ESI-MS *m/z* 785 $[M-H]^-$; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.90 (1H, d, J = 2.0 Hz, H-2'), 7.56 (1H, dd, J = 2.0, 8.9 Hz, H-5'), 7.24 (1H, d, J = 8.9 Hz, H-6'), 6.45 (1H, d, J = 2.0 Hz, H-8), 6.22 (1H, d, J = 2.0 Hz, H-6), 5.46 (1H, d, J = 7.3 Hz, H-1''), 5.10 (1H, d, J = 7.4 Hz, H-1''''), 4.43 (1H, J = 0.9 Hz, H-1'''), 3.84 (3H, s, 3'-OMe); ^{13}C-NMR (100 MHz) in Table 2.

Isorhamnetin 3-*O*-β-D-(6-*O*-α-L-rhamnosyl)glucoside (4): Yellow solid; ESI-MS *m/z* 623 $[M-H]^-$; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.87 (1H, d, J = 2.0 Hz, H-2'), 7.52 (1H, dd, J = 2.0, 8.4 Hz, H-5'), 6.92 (1H, d, J = 8.4 Hz, H-6'), 6.44 (1H, d, J = 2.0 Hz, H-8), 6.21 (1H, d, J = 2.0 Hz, H-6), 5.45 (1H, d, J = 7.4 Hz, H-1''), 4.42 (1H, d, J = 0.9 Hz, H-1'''), 3.91 (3H, s, 3'-OMe); ^{13}C-NMR (100 MHz) in Table 2.

Isorhamnetin 3-*O*-β-D-(6-*O*-α-L-rhamnosyl)galactoside (5): Yellow solid; ESI-MS *m/z* 623 $[M-H]^-$; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.07 (1H, d, J = 2.0 Hz, H-2'), 7.57 (1H, dd, J = 2.0, 8.4 Hz, H-5'), 6.97 (1H, d, J = 8.4 Hz, H-6'), 6.50 (1H, d, J = 2.0 Hz, H-8), 6.26 (1H, d, J = 2.0 Hz, H-6), 5.52 (1H, d, J = 7.7 Hz, H-1''), 4.49 (1H, d, J = 1.1 Hz, H-1'''), 3.84 (3H, s, 3'-OMe); ^{13}C-NMR (100 MHz) in Table 2.

Results and Discussion

The ESI-MS (negative mode) of **1** gave an $[M-H]^-$ peak at *m/z* 639, fragment ion peaks for [M-hexose-H]$^-$ at *m/z* 477, and [M-2hexose-H]$^-$ at *m/z* 315. This MS fragmentation pattern indicated the compound **1** had two hexose moieties. The ^{1}H NMR spectrum of **1** also indicated a flavonol glycoside moiety, as it showed two aromatic proton signals at δ 6.52 (d, J = 2.0 Hz) and δ 6.28 (d, J = 2.0 Hz) for ring A, and three resonances at δ 8.13 (d, J = 2.0 Hz), δ 7.60 (dd, J = 2.0 and 8.9 Hz) and δ 7.30 (d, J = 8.9 Hz) corresponding to the ABX spin system of ring B. The presence of a methoxy signal at δ 3.91 revealed that the flavonol aglycone could be isorhamnetin, which was confirmed by ^{13}C NMR and other 2D NMR analyses. Two anomeric proton signals at δ 5.59 (d, J = 7.7 Hz) and δ 5.13 (d, J = 7.4 Hz) were observed in the ^{1}H NMR spectrum and two sets of carbon signals corresponding to the galactose and the glucose moieties were observed in the ^{13}C NMR spectrum. The glycosyl linkages to aglycone, as determined by an HMBC experiment to be the galactose anomeric proton at δ 5.59 (H-1''), showed a long range interaction with the carbon at δ 134.2 (C-3), and the glucose anomeric proton at δ 5.13 (H-1'''') showed an interaction with the carbon at δ 149.3 (C-4'). On the basis of these data and comparison with the reported NMR data [Kim *et al.*, 2006], the structure of the compound **1** was identified as isorhamnetin 3-*O*-β-D-galactosyl-4'-*O*-β-D-glucoside.

The ESI-MS (negative mode) of **2** gave an $[M-H]^-$ peak at *m/z* 639, and the same fragmentation pattern as that of the compound **1**, which indicated the compound **2** was a structure isomer of the compound **1**. The ^{1}H NMR spectrum of **2** also indicated a flavonol glycoside moiety very similar to that of the compound **1**. Two anomeric proton signals at δ 5.65 (d, J = 7.4 Hz) and δ 5.12 (d, J = 7.5 Hz) were observed in the ^{1}H NMR spectrum, and two sets of the carbon signals corresponding to two glucose moieties instead of a galactosyl moiety in the compound **1** were observed in the ^{13}C NMR spectrum. The glucosyl linkages to aglycone were determined by an HMBC experiment, because the one glucose anomeric proton at δ 5.65 (H-1'') showed a long range interaction with the carbon at δ 134.2 (C-3) and the other glucose anomeric proton at δ 5.12 (H-1'''') showed an interaction of the carbon at δ 149.3 (C-4'). On the basis of these data and comparison with the reported NMR data [Norbak *et al.*, 1999], the structure of the compound **2** was identified as isorhamnetin 3,4'-di-*O*-β-D-glucoside (dactylin).

The ESI-MS (negative mode) of **3** gave an $[M-H]^-$ peak

Table 2. ^{13}C NMR data of compounds 1-5 (100 MHz, DMSO-d_6)

	C	1	2	3	4	5
Flavonol	2	156.8	157.3	156.9	157.3	157.3
	3	133.9	134.2	133.8	133.8	133.9
	4	177.8	178.3	177.7	178.2	178.2
	5	161.6	162.1	161.5	162.0	162.0
	6	99.2	99.7	99.2	99.6	99.6
	7	165.0	165.5	164.9	165.1	165.2
	8	94.2	94.7	94.3	94.7	94.6
	9	156.0	156.6	156.3	157.3	157.2
	10	104.4	104.9	104.4	104.8	104.8
	1'	121.5	122.2	121.9	121.9	121.9
	2'	113.8	114.3	113.7	114.1	114.3
	3'	148.6	148.9	148.4	147.7	147.8
	4'	148.8	149.3	148.8	150.2	150.3
	5'	114.8	115.4	114.8	116.1	116.0
	6'	124.0	124.5	124.0	123.1	122.8
	-OMe	56.3	56.5	56.0	56.5	56.8
Sugar at C-3	1"	101.9	101.5	101.5	102.0	102.6
	2"	71.6	75.2	74.6	75.1	72.0
	3"	73.4	77.7	76.3	76.8	72.7
	4"	68.3	70.6	70.4	70.9	68.8
	5"	76.4	78.4	76.7	77.2	74.4
	6"	60.9	61.4	67.1	67.7	66.0
	1'''			101.2	101.8	100.9
	2'''			70.7	71.2	71.4
	3'''			70.9	71.4	71.3
	4'''			72.1	72.6	72.7
	5'''			68.6	69.2	69.1
	6'''			18.1	18.6	18.7
Sugar at C-4'	1''''	100.3	100.3	99.7		
	2''''	74.0	74.0	73.5		
	3''''	77.2	77.2	77.2		
	4''''	70.4	70.4	69.9		
	5''''	77.9	77.9	77.4		
	6''''	61.4	61.4	60.9		

at *m/z* 785, fragment ion peaks for [M-hexose-H]$^-$ at *m/z* 623, [M-hexose-deoxyhexose-H]$^-$ at *m/z* 477, and [M-2hexose-deoxyhexose-H]$^-$ at *m/z* 315. This MS fragmentation pattern indicated the compound **3** had two hexose and one deoxyhexose moieties. The ^1H and ^{13}C NMR spectra of aglycone part of **3** were the same as those of **1** and **2**, which indicated the aglycone of **3** was isorhamnetin. Three anomeric proton signals at δ 5.46 (d, J = 7.3 Hz), δ 5.10 (d, J = 7.4 Hz) and δ 4.43 (d, J = 0.9 Hz) were observed in the ^1H NMR spectrum. These anomeric protons showed interactions of the carbon signals at δ 101.05 (C-1" of glucose), δ 99.7 (C-1"" of glucose) and δ 101.2 (C-1''' of rhamnose). The ^{13}C NMR signals were assigned by HMBC experiments and the reference data. Upon HMBC experiments on the glycosyl linkages between the aglycone and glycosyl moieties, the glucose anomeric protons at δ 5.46 (H-1") and δ 5.10 (H-1"") showed long range interactions with the carbons at δ 133.8 (C-3) and δ 148.8 (C-4'), respectively. The down- and up-field shifts of about 5.7 ppm for C-6" and 1.7 ppm for C-5" of glucose pointed to the inter-glycosidic linkage at C-6" of glucose. The interaction between the C-6" of glucose and H-1''' of rhamnose in the HMBC spectrum indicated the internal and the external sugars were glucose and rhamnose, respectively. On the basis of these data and comparison with the reported NMR data [Aquino *et al.*, 1987], the structure of the compound **3** was identified as isorhamnetin 3-*O*-β-D-(6-*O*-α-L-rhamnosyl)glucosyl-4'-*O*-β-D-glucoside (isorhamnetin 3-*O*-rutinosyl-4'-*O*-β-D-glucoside).

The ESI-MS (negative mode) of **4** and **5** gave an [M-H]$^-$ peak at *m/z* 623, and a fragment ion peak for [M-hexose-deoxyhexose-H]$^-$ at *m/z* 315. This MS fragmentation pattern indicated the compounds **4** and **5** had a hexose and a deoxyhexose moiety. The ^1H and ^{13}C NMR spectra of the aglycone parts of **4** and **5** were the same as those of **1-3**, which indicated the aglycones of **4** and **5** were also isorhamnetin. Glucose and rhamnose signals were observed in the ^{13}C NMR spectrum of **4**, and these signals showed the same pattern as those of the compound **3**. The glycosidic linkages of **4** were determined by HMBC experiment to be from C-6" of glucose to C-1''' of rhamnose. On the basis of these data and comparison with the reported NMR data [Lee *et al.*, 2003; Yeskaliyeva *et al.*, 2006], the structure of the compound **4** was identified as isorhamnetin 3-*O*-β-D-(6-*O*-α-L-rhamnosyl)glucoside (narcissin).

The compound **5** was a structure isomer of **4**. Galactose and rhamnose signals were observed in the ^{13}C NMR spectrum of **5**. Two anomeric proton signals at δ 5.52 (d, J = 7.7 Hz) and δ 4.49 (d, J = 1.1 Hz) observed in the ^1H NMR spectrum showed interactions of the carbon signals at δ 133.9 (C-3) and δ 66.0 (C-6" of galactose) in the HMBC spectrum, respectively. The downfield shift of about 5.1 ppm for C-6" and an upfield shift of about 2.0 ppm for C-5" of galactose further indicated the inter-glycosidic linkage at C-6" of galactose. These results revealed the internal sugar was galactose and external sugar was rhamnose. On the basis of these data and comparison with the reported NMR data [Buschi and Pomilo, 1982; Halim *et al.*, 1995], the structure of the compound **5** was identified as isorhamnetin 3-*O*-β-D-(6-*O*-α-L-rhamnosyl)galactoside (isorhamnetin 3-robinobioside).

The five isorhamnetin glycosides were isolated and identified as isorhamnetin 3-*O*-β-D-galactosyl-4'-*O*-β-D-glucoside (**1**), isorhamnetin 3,4'-di-*O*-β-D-glucoside (**2**), isorhamnetin 3-*O*-β-D-(6-*O*-α-L-rhamnosyl)glucosyl-4'-*O*-β-D-glucoside (**3**), isorhamnetin 3-*O*-β-D-(6-*O*-α-L-rhamnosyl)glucoside (**4**), and isorhamnetin 3-*O*-β-D-(6-*O*-α-L-rhamnosyl)galactoside (**5**) from *O. humifusa*. Although these compounds had been isolated from other plants, this is the first report from *O. humifusa*. In a recent paper, the isolation and antimicrobial effects of taxifolin from *O. humifusa* were reported [Lee *et al.*, 2007], but other secondary metabolites from *O. humifusa* have not yet been reported. On other hand, 14 flavonoids had been isolated and reported as eridictyol, dihydrokaempferol, kaempferol, kaempferol 3-*O*-methyl ether, kaempferol 7-*O*-glucoside, dihydroquercetin (taxifolin), quercetin, quercetin 3-*O*-methyl ether, isorhamnetin 3-*O*-glucoside, 3-*O*-β-D-(6-*O*-α-L-rhamnosyl)glucoside (narcissin), isorhamnetin 3-*O*-neohesperidoside, isorhamnetin 3-*O*-(2,6-dirhamnosyl)glucoside, isorhamnetin 3-*O*-rutinosyl 4'-*O*-glucoside, and isorhamnetin 3-*O*-(6"-*O*-feruloyl)neohesperidoside from *O. ficus-indica* [Lee *et al.*, 2003; Saleem *et al.*, 2006]. Two compounds, isorhamnetin 3-*O*-β-D-(6-*O*-α-L-rhamnosyl)glucosyl- 4'-*O*-β-D-glucoside (**3**), and isorhamnetin 3-*O*-β-D-(6-*O*-α-L-rhamnosyl)glucoside (**4**) were isolated from both *O. ficus-indica* and *O. humifusa*. Although it is possible to find other metabolites from them, the metabolic profiles of the two cactus species are quite different (data not shown). The metabolic profiling of these two cactuses will be another interesting subject.

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