

Three New Di-*O*-glycosyl-*C*-glucosyl Flavones from the Leaves of *Caesalpinia ferrea* Mart.

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Three hitherto unknown di-*O*-xylosyl-*C*-glycosyl flavones were isolated from the leaves of *Caesalpinia ferrea*. The structures of all isolated compounds were elucidated by conventional methods and spectroscopic analysis, including 1D and 2D NMR, as well as by HRESIMS.

Key words: *Caesalpinia ferrea*, Fabaceae, Di-*O*-glycosyl-*C*-glucosyl Flavones

Introduction

The little studied genus *Caesalpinia* (subfamily, Caesalpinioideae; family, Fabaceae), which is comprised of tropical or subtropical trees or shrubs, contains more than 500 species of worldwide distribution (Joly, 1998).

Previous studies of species of this genus reported remarkable biological activities such as antimicrobial (*C. bonducella*; Saeed and Sabir, 2001), antidiabetic (*C. bonducella*; Sharma *et al.*, 1997), antimalarial (*C. volkensii* and *C. pluviosa*; Kuria *et al.*, 2001; Deharo *et al.*, 2001), and anti-inflammatory (*C. sappan*; Hikino *et al.*, 1977; Carvalho *et al.*, 1996). Some species, such as *C. sappan*, *C. ferrea*, and *C. bonducella*, exhibit antinociceptive activity (Baldim Zanin *et al.*, 2012).

To date, less than 30 species of this genus have been studied for their phytoconstituents. The metabolites described include predominantly flavonoid derivatives, steroids, triterpenoids, and cassane diterpenes.

C. ferrea Mart., known in English as leopard tree, is a leguminous tree of worldwide distribution. The tree grows up to 15 m, forming a broad, flat-topped crown.

The trunk is typically short, branching often close to the base. The leaves are bi-pinnately compound, 15–19 cm long. The individual leaflets range between 8–24 per pinnae. The flowers are bright yellow, typical of the Caesalpinioideae in form, and appear in terminal or axillary inflorescences. The seeds are dark brown, hard, and contained in a flat pod (Lorenzi, 2002).

The aqueous extract from fruits of this species is used in the treatment of diabetes and coughs, and exhibits antifungal, antiulcerogenic, anti-inflammatory, and analgesic properties. The antitumour effects of the fruits of *C. ferrea* were tested *in vitro* by assessing the activation of the Epstein-Barr virus-early antigen (EBV-EA), which causes mononucleosis and other diseases, such as malignancy. The active constituents were identified as gallic acid and methyl galate (Baldim Zanin *et al.*, 2012).

Recently, two chalcone dimers, pauferrol B and pauferrol C, were isolated from the stems of *C. ferrea*. These chalcone dimers exhibited potent inhibitory activities against human topoisomerase II and cell proliferation via the induction of apoptosis in human leukemia HL 60 cells (Ohira *et al.*, 2013). Pauferrol A, a unique chalcone trimer, was also isolated from the

stems of the plant. This chalcone trimer is a potent inhibitor of human topoisomerase II (Nozaki *et al.*, 2007).

In the present study, the leaves of *C. ferrea* Mart. were extracted with aqueous ethanol (3:1). Column chromatography of the extract gave twelve polyphenols, **1**–**12**, including three hitherto unknown, *i. e.* isovitexin 2''-O- β -[xylopyranosyl-(1''' \rightarrow 2''')-O- β -xylopyranosyl] (**3**) and the vitexin and orientin analogues **4** and **5**. The structures of all isolates were elucidated by conventional methods and spectroscopic analysis, including 1D and 2D NMR spectroscopy.

Results and Discussion

Repeated column and preparative paper chromatography of an aqueous EtOH extract of *Caesalpinia ferrea* leaves gave compounds **3**–**5**, each as a faintly yellow amorphous powder. The UV spectra of **3** and **4** were almost identical [λ_{\max} (MeOH) = 268, 330 nm] and reminiscent to that of an apigenin derivative in which all phenolic OH groups are unsubstituted [a shift with NaOAc, stable shift with NaOMe, small changes (2 nm) between the shifts with AlCl₃ only or with AlCl₃/HCl] (Mabry *et al.*, 1970; Harborne and Williams, 1975). This finding, together with the chromatographic behaviour (*R_f* values, colour on chromatograms under UV light), suggests that **3** and **4** are C-glycosyl apigenins. The UV spectrum of **5** was typical for a mono-C-glycosyl luteolin, a view which was supported by its chromatographic properties. Both **3** and **4** exhibited closely similar ESI mass spectra, whereby an [M + Na]⁺ ion at *m/z* 719 showed up in the positive ion mode ESI spectra, corresponding to a molecular mass of 696. A fragment at *m/z* 455 ([fragment + Na]⁺), corresponding to a mono-C-hexosyl apigenin, was also observed in both spectra. The molecular formula of **3** and **4** was concluded to be C₃₁H₃₆O₁₈ from its positive HRESI mass spectrum which showed an [M + Na]⁺ ion at *m/z* = 719.5948 (calcd. for C₃₁H₃₆O₁₈Na, 719.5965). The above described analytical data suggested that **3** and **4** could be the C-6 and C-8 hexosyl apigenin isomers, and furthermore, that both **3** and **4** contain either a single di-O-pentosyl moiety or two mono-pentosyl moieties linked to the C-hexosyl apigenin moiety through one (or two) of the hydroxy groups of the hexosyl moiety. Complete acid hydrolysis in 2 M methanolic (H₂O/MeOH, 1:1, v/v) HCl, for 7 h, of either **3** or **4** gave isovitexin and vitexin in each case (Co-PC, UV, and ¹H and ¹³C NMR spectral analyses) and xylose (Co-PC). To find

out the site(s) of attachment of the two xylosyl units to the hexosyl moiety in **3** and **4**, ¹H and ¹³C NMR analyses were employed. The ¹H NMR spectrum of **3** revealed the distinct pattern of proton resonances belonging to di-O-xylosyl isovitexin which was found to contain well separated signals of isovitexin [δ_{H} 7.92 (d, *J* = 8 Hz, H-2' and H-6'), 6.93 (d, *J* = 8 Hz, H-3' and H-5'), 6.77 (s, H-3), 6.53 ppm (s, H-8)]. The spectrum also revealed three anomeric resonances at δ_{H} 4.91 (d, *J* = 7 Hz, anomeric xylosyl proton), 4.80 (d, *J* = 7 Hz, anomeric xylosyl proton), 4.60 ppm (d, *J* = 8.5 Hz, anomeric glucosyl proton), and a multiplet between δ_{H} 3.3 and 3.9 ppm corresponding to other sugar protons overlapping with the water signal. The ¹H NMR spectrum of **4** was quite similar to that of **3**, but differed from it by lacking the H-8 singlet resonance and having instead a singlet at δ_{H} 6.25 ppm, assignable to the apigenin H-6 proton, thus proving that **3** is isovitexin di-O-xylosyl and **4** is the respective vitexin isomer. All sugar moieties had β -glycosyl linkages from the magnitude of the vicinal proton couplings of the anomeric protons (Nawwar *et al.*, 2012).

Confirmation of the structures of **3** and **4** was achieved by 2D ¹³C NMR spectroscopy, including HMQC and HMBC, which allowed the full assignment of all carbon and proton resonances. The 1D ¹H and ¹³C NMR spectra unambiguously identified **3** as an isovitexin derivative with a 2''-O-dixylosyl substituent and **4** as the vitexin analogue. The number and characteristic shifts of the ¹³C NMR glycosyl signals in the spectra of **3** and **4** indicated the presence of an identical system consisting of one hexosyl and two xylosyl moieties, respectively, all in the pyranose form. The assignment of the ¹³C NMR signals followed directly from the HMQC and HMBC spectra. In all cases the ¹³C NMR shifts of the glucosyl signal C-2'' (at δ_{C} 83.33 ppm for **3**, and 83.26 ppm for **4**) and xylosyl signal C-2''' (at δ_{C} 83.53 ppm for **3**, and 83.51 ppm for **4**) indicated that these positions were substituted. This followed from the downfield location of these signals. That the hexose is C-C linked to the apigenin moiety at its C-6 atom in **3** and at its C-8 atom in **4**, was readily deduced from the lowfield shift of its anomeric proton H-1'' (at δ_{H} 4.60 ppm in **3**, and at 4.62 ppm in **4**) and unambiguously confirmed by a long-range correlation between this proton and the flavone C-6 atom of **3** (δ_{C} 109.51 ppm) and the flavone C-8 of **4** (δ_{C} 103.8 ppm) in the HMBC spectrum. This latter spectrum also allowed the unambiguous determination of the interconnectivity of the two xylosyl units in both compounds. For **3**, the xylosyl anomeric

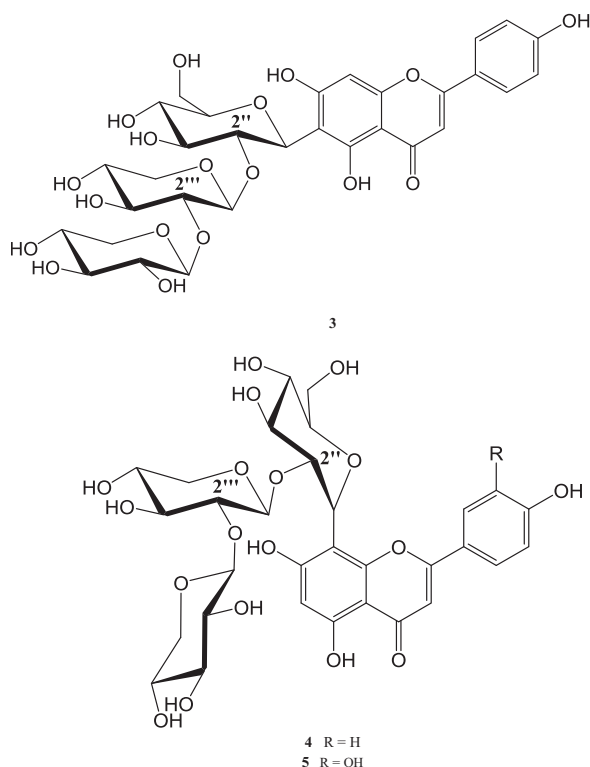


Fig. 1. Chemical structures of isovitexin 2''-O-β-[xylopyranosyl-(1''' → 2'')]-O-β-xylopyranosyl (3), vitexin 2''-O-β-[xylopyranosyl-(1''' → 2'')]-O-β-xylopyranosyl (4), and orientin 2''-O-β-[xylopyranosyl-(1''' → 2'')]-O-β-xylopyranosyl (5).

protons at δ_H 4.91 ppm showed a correlation with the C-2'' signal (δ_C 83.33 ppm) of the glucosyl moiety, while the anomeric proton of the second (terminal) xylose moiety (δ_H 4.80 ppm) was shown to possess a long-range correlation with C-2''' (δ_C 83.53 ppm) of the first (intermediary) xylosyl unit. The shift of C-2''' was compatible with the terminal sugar unit being xylose. This was indirectly confirmed, as the ^{13}C NMR shifts of the terminal sugar carbon atoms were only compatible with those of a β -xylopyranose moiety. Typical sets of correlations were also recognized in case of 4. Hence, the structure of 3 was determined to be isovitexin 2''-O-β-[xylopyranosyl-(1''' → 2'')]-O-β-xylopyranosyl, and that of 4 was determined to be vitexin 2''-O-β-[xylopyranosyl-(1''' → 2'')]-O-β-xylopyranosyl (Fig. 1).

UV spectral analysis of 5 indicated that it is an orientin derivative. The positive ESI mass spectrum

showed a molecular ion at m/z 713, corresponding to a molecular mass of 712, and a fragment ion at m/z 449 ([orientin + H] $^+$). The molecular formula of 5 was concluded to be $C_{31}H_{36}O_{19}$ from the positive HRESI mass spectrum which showed an $[M + H]^+$ ion at m/z 712.6058 (calcd. for $C_{31}H_{36}O_{19}$, 712.6061). Acid hydrolysis indicated xylose (Co-PC), and orientin and isoorientin as the aglycones (Co-PC, UV, 1H NMR for both aglycones and ^{13}C NMR for orientin). The 1H and ^{13}C NMR spectra confirmed the presence of a 2''-O-β-[xylopyranosyl-(1''' → 2'')]-O-β-xylopyranosyl system, linked at C-8 of the flavone moiety in 5. The full assignment of all proton resonances by HMBC and HMQC analysis was guided by the assignments accomplished in the case of 3 and 4. All ^{13}C NMR shifts from the sugar system agreed with those of the corresponding system in 3 and 4, while those from the luteolin moiety were like those reported for that moiety in orientin (Kato and Morita, 1990), therefore 5 was identified as orientin 2''-O-β-[xylopyranosyl-(1''' → 2'')]-O-β-xylopyranosyl (Fig. 1).

Experimental

General

NMR spectra were acquired in DMSO- d_6 on a Bruker 400-MHz NMR spectrometer (El-Menya, Egypt) at 400 MHz. Standard pulse sequence and parameters were used to obtain one-dimensional 1H and ^{13}C NMR, and two-dimensional COSY, HSQC, and HMBC spectra, respectively. Chemical shifts (δ) were measured in ppm, 1H NMR chemical shifts relative to tetramethylsilane (TMS) and ^{13}C NMR chemical shifts to acetone- d_6 and were converted to the TMS scale by adding 29.8. High-resolution ESI mass spectra were measured using a Finnigan LTQ FT Ultra mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Nanomate ESI interface (Advion, Berlin, Germany). An electrospray voltage of 1.7 kV (+/−) and a transfer capillary temperature of 200 °C were applied. Collision-induced dissociation (CID) was performed in the ion trap using a normalized collision energy of 35 kV, activation time of 30 ms, 0.25 activation Q, and a precursor ion isolation width of 2 amu. High-resolution product ions were detected in the Fourier transform ion cyclotron resonance (FTICR) cell of the mass spectrometer. UV spectra were recorded on a Shimadzu UV-Visible-1601 spectrophotometer (Kyoto, Japan). Flame atomic absorption analysis was performed on

a Varian Spectra-AA220 instrument (Cairo, Egypt) (lamp current, 5 mA; fuel, acetylene; oxidant, air). Paper chromatography (PC) was carried out on Whatman No. 1 paper, using the following solvent systems: H₂O; 15 vol.-% HOAc; 6 vol.-% HOAc; BAW [*n*-BuOH/HOAc/H₂O (4:1:5, v/v/v), upper layer].

Plant material

Leaves of *C. ferrea* were collected from a tree cultivated in the Zoological Garden, Cairo, Egypt, in May 2012. The plant was identified by Prof. Salwa Quashti, National Research Centre (NRC), Cairo, Egypt. A voucher specimen (C 253) has been deposited at the herbarium of the NRC.

Extraction and isolation

Leaves (2.5 kg), dried in the shadow in the air, were crushed and exhaustively extracted with 70% (v/v) aqueous EtOH under reflux (three times, each extraction for 8 h with 2 l). Two hundred g of the dried extract were dissolved in 150 ml aqueous EtOH and applied to a polyamide column (2 kg; Macherey-Nagel, Düren, Germany). Separation was initiated with H₂O followed by H₂O/MeOH (9:1, v/v), and the MeOH content was gradually increased in 10% steps. The flavonoid-containing fraction IV was eluted with H₂O/MeOH (3:7), to yield a faintly brown amorphous powder (14 g). Further fractionation of fraction IV was performed on an MCI gel column (CHP-20P, 75–150 µm; Mitsubishi Chemical Co., Düsseldorf, Germany) and gradient elution with H₂O/MeOH mixtures to afford eight individual sub-fractions. Two-dimensional paper chromatography (TDPC) revealed the presence of flavonoids **3**–**5** in sub-fraction 6 (1 g, eluted with 80% MeOH). Polyamide column fractionation of the material of sub-fraction 6, using gradient elution with H₂O/MeOH mixtures, followed by preparative PC, using BAW as solvent, of the material desorbed by 60% MeOH (284 mg), afforded individual chromatographically pure samples of **3** (61 mg), **4** (39 mg), and **5** (54 mg).

Isovitexin 2''-O-β-[xylopyranosyl-(1''' → 2''')-O-β-xylopyranosyl] (**3**): *R*_f = 0.42 (H₂O), 0.74 (15% HOAc), 0.50 (BAW). – UV: λ_{max} (MeOH) = 268, 330; (+ NaOAc) 278, 305, 381; (+ NaOAc + H₃BO₃) 272, 282, 309, 324, 342; (+ AlCl₃) 279, 350, 380; (+ AlCl₃ + HCl) 280, 343, 381 nm. – ESI (positive mode): *m/z* = 719 [M + Na]⁺. – Positive HRESIMS:

m/z = 719.5948 [M + Na]⁺ (calcd. for C₃₁H₃₆O₁₈Na, 719.5965).

Compound **3** was hydrolyzed with 2 M methanolic (H₂O/MeOH, 1:1, v/v) HCl, for 7 h at 100 °C, following removal of MeOH *in vacuo* and extraction with EtOAc. Preparative PC gave vitexin and isovitexin. Washing the remaining aqueous hydrolysate with 10% *N*-methyl dioctyl amine, followed by drying and Co-PC, proved the presence of xylose.

Vitexin: ¹H NMR: δ = 8.05 (d, *J* = 8.5 Hz, H-2' and H-6'), 6.89 (d, *J* = 8.5 Hz, H-3'' and H-5'), 6.67 (s, H-3), 6.1 (s, H-6), 4.66 (1H, d, *J* = 9.5 Hz, H-1''). – ¹³C NMR: see Table I.

Isovitexin: ¹H NMR: δ = 7.95 (d, *J* = 8.5 Hz, H-2' and H-6'), 6.92 (d, *J* = 8.5 Hz, H-3' and H-5'), 6.60 (s, H-3), 6.76 (s, H-8), 4.62 (d, *J* = 9 Hz, H-1''). – ¹³C NMR: see Table I.

3: ¹H NMR: δ = 7.92 (d, *J* = 8 Hz, H-2' and H-6'), 6.93 (d, *J* = 8 Hz, H-3' and H-5'), 6.77 (s, H-3), 6.53 (s, H-8), 4.91 (d, *J* = 7 Hz, anomeric xylosyl proton), 4.80 (d, *J* = 7 Hz, anomeric xylosyl proton), 4.60 (d, *J* = 8.5 Hz, anomeric glucosyl proton), 3.3 and 3.9 (m, glycosyl protons). – ¹³C NMR: see Table I.

Vitexin 2''-O-β-[xylopyranosyl-(1''' → 2''')-O-β-xylopyranosyl] (**4**): *R*_f = 0.32 (H₂O), 0.65 (15% HOAc), 0.48 (BAW). – UV: λ_{max} (MeOH) = 268, 330; (+ NaOAc) 280, 303, 380; (+ NaOAc + H₃BO₃) 272, 282, 309, 324, 344; (+ AlCl₃) 279, 353, 384; (+ AlCl₃ + HCl) 279, 343, 382 nm. – ESI (positive mode): *m/z* = 696, 719 [M + Na]⁺. – Positive HRESIMS: *m/z* = 719.5948 [M + Na]⁺ (calcd. for C₃₁H₃₆O₁₈Na, 719.5965).

Compound **4** was hydrolyzed with 2 M methanolic (H₂O/MeOH, 1:1, v/v) HCl, for 7 h at 100 °C, following removal of MeOH *in vacuo* and extraction with EtOAc. Preparative PC gave vitexin and isovitexin. Washing the remaining aqueous hydrolysate with 10% *N*-methyl dioctyl amine, followed by drying and Co-PC, proved the presence of xylose.

Vitexin and isovitexin: Analytical data as given above.

4: ¹H NMR: δ = 7.95 (d, *J* = 8 Hz, H-2' and H-6'), 6.98 (d, *J* = 8 Hz, H-3' and H-5'), 6.76 (s, H-3), 6.25 (s, H-6), 4.87 (d, *J* = 7 Hz, anomeric xylosyl proton), 4.80 (d, *J* = 7 Hz, anomeric xylosyl proton), 4.62 (d, *J* = 8.5 Hz, anomeric glucosyl proton), 3.3–3.9 (m, glycosyl protons). – ¹³C NMR: see Table I.

Orientin 2''-O-β-[xylopyranosyl-(1''' → 2''')-O-β-xylopyranosyl] (**5**): *R*_f = 0.12 (H₂O), 0.17 (15%

Table I. ^{13}C NMR chemical shifts (in ppm) for compounds **3**–**5** and their aglycones.

Aglycone	3	4	5	Isovitexin	Vitexin	Orientin
2	163.99	163.72	163.08	163.20	164.22	164.21
3	102.38	102.44	102.35	102.39	102.43	102.48
4	182.3	182.00	182.48	182.00	182.24	182.00
5	161.90	161.2	160.83	160.14	160.98	160.07
6	109.51	98.3	97.76	108.96	98.10	97.09
7	163.99	161.69	164.57	163.48	162.33	162.20
8	94.18	103.8	104.46	93.66	104.61	104.50
9	156.73	156.50	156.45	156.27	156.00	156.63
10	103.22	104.53	104.99	103.53	104.46	103.81
1'	121.56	121.5	122.43	121.11	121.60	122.82
2'	128.90	128.78	114.47	128.38	129.12	114.01
3'	116.49	115.98	146.27	116.00	115.78	145.84
4'	161.10	160.70	150.11	160.6	161.14	149.66
5'	116.49	115.98	116.16	116.00	11.78	115.63
6'	128.90	128.78	119.83	128.38	129.12	119.39
Glucosyl						
1''	71.07	71.22	71.25	73.344	73.42	73.72
2''	83.33	83.26	83.31	70.60	70.85	70.81
3''	79.42	79.30	79.21	78.98	78.87	78.85
4''	69.81	69.80	69.79	69.83	70.05	70.32
5''	81.97	82.44	82.42	82.00	82.36	81.80
6''	61.92	61.95	62.00	61.33	61.30	61.44
Xylosyl intermediary						
1'''	97.76	97.73	97.76			
2'''	83.53	83.51	83.49			
3'''	73.25	73.25	73.24			
4'''	68.12	68.13	68.14			
5'''	65.41	65.45	65.46			
Xylosyl terminal						
1''''	103.77	103.86	104.46			
2''''	73.66	73.80	73.83			
3''''	77.29	77.31	77.25			
4''''	68.12	68.12	68.14			
5''''	65.45	68.46	65.46			

HOAc), 0.31 (BAW). – UV: λ_{max} (MeOH) = 260, 270, 350; (+NaOAc) 272, 280, 325, 382; (+NaOAc + H_3BO_3) 264, 380; (+ AlCl_3) 278, 304, 423 nm. – ESI (positive mode): m/z = 713 $[\text{M} + \text{H}]^+$. – Positive HRESIMS: m/z = 712.6058 $[\text{M} + \text{H}]^+$ (calcd. for $\text{C}_{31}\text{H}_{36}\text{O}_{19}$, 712.6061).

Compound **5** was hydrolyzed with 2 M methanolic ($\text{H}_2\text{O}/\text{MeOH}$, 1:1, v/v) HCl, for 7 h at 100 °C, following removal of MeOH *in vacuo* and extraction with EtOAc. Preparative PC gave orientin and isoorientin. Proceeding as described above, Co-PC proved the presence of xylose.

Orientin: ^1H NMR: δ = 7.58 (dd, J = 8 Hz and 2 Hz, H-6'), 7.5 (d, J = 2 Hz, H-2'), 6.7 (s, H-3), 6.25 (s, H-6), 4.70 (1H, d, J = 9.5 Hz, H-1''), 3.2–3.9 (m, sugar protons). – ^{13}C NMR: see Table I.

Isoorientin: ^1H NMR: δ = 7.44 (dd, J = 8.5 Hz and 1.5 Hz, H-6'), 7.45 (d, J = 1.5 Hz, H-2'), 6.92 (d, J = 8 Hz, H-5'), 6.68 (s, H-3), 6.55 (s, H-8), 4.65 (d, J = 9 Hz, H-1''), 3.2–3.9 (m, sugar protons).

5: ^1H NMR: δ = 7.53 (dd, J = 8 Hz and 1.5 Hz, H-6'), 7.45 (d, J = 1.5 Hz, H-2'), 6.65 (s, H-3), 6.29 (s, H-6), 4.8 (d, J = 7 Hz, anomeric xylosyl proton), 4.87 (d, J = 7 Hz, anomeric xylosyl proton), 4.67 (d, J = 8.5 Hz, anomeric glucosyl proton), 3.2–3.9 (m, sugar protons). – ^{13}C NMR: see Table I.

Conclusion

The present study has revealed that *Caesalpinia ferrea* is capable of synthesizing and accumulating different types of flavonoids. It adds to the previ-

ously characterized unique chalcone-type compounds pauferrol B, pauferrol C, and pauferrol A (Nozaki *et al.*, 2007; Ohira *et al.*, 2013) another flavonoid type represented by the hitherto unknown C-glycosyl flavone isovitexin 2''-O-β-[xylopyranosyl]-(1''' → 2''')-O-β-xylopyranosyl] and the respective vitexin and orientin analogues.

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