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Biflavonoids from *Lonicera japonica* ☆

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Abstract

Two biflavonoids, 3′-*O*-methyl loniflavone [5,5″,7,7″-tetrahydroxy 3′-methoxy 4′,4‴-biflavonyl ether (**1**)] and loniflavone [5,5″,7,7″,3′-pentahydroxy 4′,4‴-biflavonyl ether (**2**)] along with luteolin (**3**) and chrysin (**4**) were isolated from the leaves of *Lonicera japonica*. The structures were established on the basis of UV/vis, 1D, 2D NMR (HMQC and HMBC) and ESI-QTOF-MS/MS spectroscopic methods and chemical evidences.

Keywords: *Lonicera japonica*; Caprifoliaceae; Biflavone; 3′-*O*-methyl loniflavone; Loniflavone

1. Introduction

Lonicera (Caprifoliaceae) is a genus of more than 150 species of erect climbing or scrambling shrub distributed chiefly in the sub-tropical and temperate regions of the Northern Hemisphere. Approximately 45 species are known to grow in India and few species are used in indigenous system of medicine as an antipyretic, stomachic and diuretic and in dysentery (Wealth of India, 1962; Kirtikar and Basu, 1935). *Lonicera japonica* Thunb., Japanese honeysuckle, has been used to treat urinary disorders, fever and headache. It has been known as an anti-inflammatory agent in Korea from ancient times and is used widely for upper respiratory tract infections, diabetes mellitus and rheumatoid arthritis (Lee et al., 1998). A number of compounds such as iridoid glycosides, saponins, flavonoids and tannins have been reported from this species (Mehrotra et al., 1988; Kawai et al., 1988a,b; Machida et al., 2002; Kwak et al., 2003; Son et al., 1992, 1994a,b). Although the chemical composition of *L. japonica* from few countries is well documented, there has been no report on the chemical composition from India. We, therefore, report here the isolation and structure elucidation of two new biflavonoids (**1** and **2**) (Fig. 1) together with luteolin (**3**) and chrysin (**4**) from the leaves.

2. Results and discussion

Dried powdered leaves of *L. japonica* were extracted with MeOH–H_2O (80:20; v/v) and fractionated successively with hexane, $CHCl_3$, EtOAc, and *n*-BuOH. EtOAc extract was subjected to repeated chromatographic purification to yield two new biflavones (**1** and **2**) along with luteolin (**3**) and chrysin (**4**).

Compound **1** was isolated as a yellowish amorphous powder. The presence of paired signals in ^{13}C NMR spectrum (Table 1) suggested either a dimer or a mixture of two closely related compounds. However, negative HR-ESI-QTOF-MS showed a molecular ion peak at *m*/*z* 551.4558 $[M-H]^-$ and its positive HR-ESI-MS gave a molecular ion peak at *m*/*z* 553.4949 $[M+H]^+$, consistent with a dimer and corresponding to molecular formula $C_{31}H_{20}O_{10}$. The UV spectrum showed absorption maxima at 271 and 330 nm, characteristic of flavone derivatives (Mabry et al., 1970), addition of aluminium chloride produced a large bathochromic shift of band I (60 nm)

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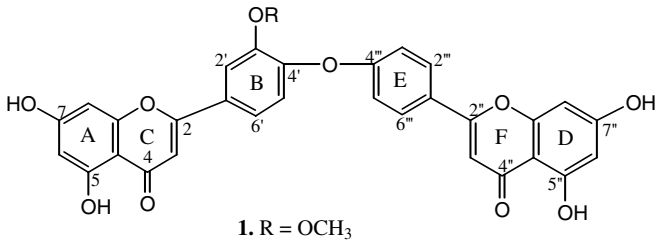

Fig. 1. Structure of compounds **1** and **2**.

characteristic of 5,7 dihydroxy flavone which did not show any change on addition of dil. HCl (Castro and Blanco, 2004). The IR spectrum exhibited a broad band hydroxyl absorption band at 3418 cm^{-1}, a carbonyl band at 1618 cm^{-1} and methoxyl band at 2928 cm^{-1}.

Acetylation of **1** gave a tetraacetate (**1a**), $[M + Na]^+$ (*m/z* 743.6181). The ^1H NMR (Table 1) and HR-ESI-QTOF mass spectra of **1** and **1a** proved the presence of four hydroxyl, one methoxyl group, 11 aromatic and two methine protons in **1** indicating it to be a biflavone with an interflavonoid ether linkage. The ^1H NMR spectrum revealed the signal of one methoxyl group (δ 3.66, 3H, *s*). Two of the four hydroxyl protons resonated at downfield δ 13.62 indicating the presence of chelated hydroxyl groups at the 5 and 5″ positions. The presence of two sets of *meta*-coupled doublets at δ 6.65 (2H, *d*, $J = 2.4$ Hz) and 6.69 (2H, *d*, $J = 2.4$ Hz) each one integrating for two protons were described to 6, 6″ and 8, 8″ protons, respectively. The presence of a set of A_2B_2 doublets at δ 7.88 (2H, *d*, $J = 8.8$ Hz), 7.09 (2H, *d*, $J = 8.8$ Hz) were assigned to the 2‴,6‴ and 3‴,5‴ protons of ring E, respectively. Further, three protons signals at δ 7.16 (1H, *d*, $J = 8.8$ Hz), 7.95 (1H, *s*) and 7.86 (1H, *dd*, $J = 8.8$, 2.3 Hz) corresponding to 5′,2′ and 6′, respectively. The presence of two singlets at δ 6.84 and 6.92 corresponding to protons at 3 and 3″ positions of two flavone moieties, indicate that compound **1** is a biflavone derivative. The mass spectra generated for **1** and **1a** by ion spray in the negative mode (ESI-QTOF-MS) gave the deprotonated molecule $[M - H]^-$ (*m/z* 551 and 743, respectively). The presence of two fragment peak at 283 and 269 in MS^2 spectrum of *m/z* 551 showed the cleavage of both flavone moieties through their interflavonoid ether linkage. Cleavage of ring C and ring F by an *retro*-Diels-Alder mechanism led to $^{1,3}A^-$ (*m/z* 151) and $^{1,3}B^-$(*m/z* 133) and $^{1'',3''}D^-$ (*m/z* 151), $^{1'',3''}E^-$ (*m/z* 117) ions, which proved the

Table 1
^1H and ^{13}C NMR (300.13 and 75.46 MHz) spectral data in pyridine-d_5 for **1**, **2**, chrysoeriol and chrysin

Compounds					
1			**2**		
Position	δ C (ppm)	δ H (ppm) *m* (*J* Hz)	δ C (ppm)	δ H (ppm) *m* (*J* Hz)	Chrysoeriol δ C (ppm)
2	162.9		163.1		163.7
3	104.9	6.84 *s*	104.8	6.82 *s*	103.8
4	182.4		182.5		181.8
4a	104.1		105.0		103.3
5	162.7		161.1		157.4
6	99.5	6.65 *d* (2.4)	99.9	6.67 *d* (2.4)	99.8
7	165.8	8.67 (OH)	166.1	8.76 (OH)	164.2
8	94.7	6.69 *d* (2.4)	94.7	6.70 *d* (2.4)	94.0
8a	158.2		157.4		161.6
1′	124.7		125.1		120.4
2′	113.6	7.95 *d* (2.3)	118.8	7.97 *d* (2.4)	110.2
3′	154.9		154.7		150.8
OCH_3	55.1	3.66 *s*			56.0
4′	143.3		144.4		148.0
5′	121.2	7.16 *d* (8.8)	121.4	7.32 *d* (9.0)	115.8
6′	125.3	7.86 *dd* (8.8, 2.3)	124.8	7.75 *dd* (8.8, 2.4)	121.7
					Chrysin
2″	163.3		163.4		163.0
3″	105.1	6.92 *s*	105.0	6.93 *s*	105.0
4″	182.4		182.5		181.6
4a″	104.1		104.7		103.9
5″	162.9	13.23 *s* (OH)	161.1	12.83 *s* (OH)	161.5
6″	99.5	6.65 *d* (2.4)	99.9	6.67 *d* (2.5)	98.9
7″	165.9	8.67 (OH)	166.1	8.76 (OH)	164.3
8″	94.7	6.69 *d* (2.4)	94.7	6.70 *d* (2.5)	94.0
8a″	158.2		157.4		157.3
1‴	125.3		125.8		131.7
2‴	128.5	7.88 *d* (8.8)	128.4	7.80 *d* (9.0)	126.1
3‴	116.2	7.09 *d* (8.8)	116.5	7.10 *d* (9.0)	128.9
4‴	161.3		161.0		130.6
5‴	116.2	7.09 *d* (8.8)	116.5	7.10 *d* (9.0)	128.9
6‴	128.5	7.88 *d* (8.8)	128.4	7.80 *d* (9.0)	126.1

5,7 dihydroxyl substituted A and D ring and one methoxyl in B ring along with one ether linkage to other flavone. The presence of $^{1'',3''}E^{-}$ (*m*/*z* 117) suggested only one oxygenation in ring E. The nomenclature adopted for the RDA cleavage was taken from the one proposed by Ma et al. (1997) and Fabre et al. (2001).

The decoupled ^{13}C NMR spectra of **1** displayed superimposable signals, including two flavone carbonyls and nine oxygen bearing quaternary carbons. Four carbons were attached to hydroxyl groups, two to pyranone oxygen and one to a methoxyl group. This suggested that remaining two should be involved in interflavonoid ether linkage. Thus, **1** must be the biflavonoid with either 4′-4‴ or 3′-4‴ ether linkage. Okigawa et al. (1976) had reported the characterization of later biflavonoid from *Ochna squarrosa* which was proved synthetically to have C-3′-4‴ ether linkage. Okigawa et al. did not report the NMR data of their compound, however, ^{1}H NMR data for their tetraacetate was reported and was found to be quite different from tetracetate of **1**. Comparison of ^{13}C NMR spectral data of **1** with chrysoeriol and chrysin (Aggarwal, 1989; Wagner et al., 1976) depicted that C-4′ of ring B and C-4‴ of ring E in **1** are involved in inerflavonoid ether linkage as the resonance of hydroxylated carbon shifted upfield by 4.7 and the resonance of C-4‴ shifted downfield by 30.7 ppm. C-4′-4‴ ether linkage was further confirmed by analyzing HMBC spectral data (Fig. 2). The protons of methoxyl methyl correlated with C-3′ (δ 154.9) suggesting the attachment of methoxyl group to C-3′. Further, the correlation of H-6′ with the carbon involved in the ether linkage C-4′ (δ 143.3) and H-2′ and H-5′ with C-3′ and C-4′ suggested the C-4′-4‴ ether linkage.

The negative ion HR-ESI-QTOF-MS of compound **2** showed a molecular ion $[M-H]^{-}$ at *m*/*z* 537.4363 and was consistent with the molecular formula $C_{30}H_{18}O_{10}$, again indicating a dimeric flavonoid structure. ^{1}H and ^{13}C NMR (Table 1), UV, IR and MS/MS spectra of compound **2** closely resemble that of **1**, except for the appearance of a hydroxyl group at C-3′ instead of a methoxyl group, which clearly showed that compound **2** could be a demethyl derivative of **1**. The structure of **2** was further confirmed by mass and ^{1}H NMR spectral data of its acetate derivative **2a**. The ^{1}H NMR chemical shift values of its pentaacetate **2a** were also quite different from those quoted for the 3′-4‴ ether linked structure.

Compound **3** and **4** were identified as luteolin and chrysin, respectively by comparison of their ^{1}H, ^{13}C NMR and negative ion ESI-QTOF-MS/MS spectra with reported values (Aggarwal, 1989; Fabre et al., 2001; Careri et al., 2002) and TLC with authentic markers.

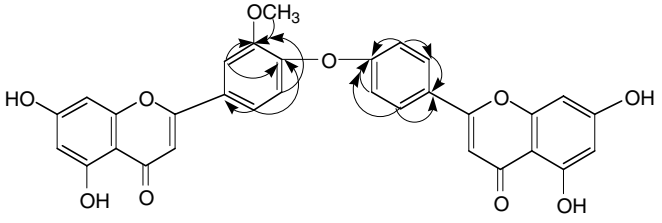

Fig. 2. Key HMBC (→) correlations.

3. Experimental

3.1. General experimental procedures

MPs were determined on a Mettler FP 800 (Central Processor) and were uncorrected. IR spectra were recorded in KBr disks on a Jasco FT-IR-5300, and UV spectra with Specord 200, Analytikjena spectrophotometer. Mass spectra were recorded on QTOF-Micro of Waters Micromass. NMR experiments were performed on Bruker Avance-300 spectrometer. Silica gel (60–120 mesh, MERCK) for Column chromatography, TLC silica gel 60 F_{254} plates and all other chemicals were produced by Merck India Ltd.

3.2. Plant material

Leaves of *L. japonica* were collected from IHBT campus, Palampur, Himachal Pradesh, India. A voucher specimen (No. 5909) has been deposited in the Herbarium of IHBT, Palampur, India.

3.3. Extraction and isolation

Shade dried and powdered leaves (2.2 kg) were extracted at room temperature with MeOH:H_2O (80:20 v/v; 3L × 4). After filteration and removal of solvent by evaporation in vacuo, a residue (613.60 g) was obtained. This was suspended in H_2O (500 ml) and fractionated with *n*-hexane (800 ml × 3), $CHCl_3$ (600 ml × 4), EtOAc (500 ml × 4) and *n*-BuOH (500 ml × 4), successively. Evaporation of each solvent *in vacuo*, yielded *n*-hexane extract (27 g), $CHCl_3$ extract (19 g), EtOAc extract (4.6) and *n*-BuOH extract (134.6 g). The EtOAc extract (4.6 g) was subjected to column chromatography (1.3 cm × 130 cm) over silica gel 60–120 mesh (115 g) and eluted with $CHCl_3$, $CHCl_3$–MeOH (9:1), (8.5:1.5), (7.5:2.5), (6.5:3.5), (5:5), (2.5:7.5), (1:9) and MeOH to give a total of 162 fractions (100 ml each).

Fractions 24–27, eluted with $CHCl_3$–MeOH (8.5:1.5), were evaporated, and the resulting pale yellow residue (78 mg) was crystallized from MeOH to give compound **1** (26 mg). Fractions 40–42 eluted with $CHCl_3$–MeOH (8:2), were evaporated, and the yellow residue (70 mg) obtained was crystallized from MeOH to give compound **2** (21 mg). Fractions 50–56, eluted with $CHCl_3$–MeOH (7:3), were separated by preparative thin layer chromatography (1.5 mm layer thickness), eluted with $CHCl_3$-MeOH (97:3, v/v), to yield compound **3** (16 mg) and **4** (14 mg).

3.4. 3′-O-Methyl loniflavone (*1*)

Yellowish powder, m.p. 242–244 °C; $C_{31}H_{20}O_{10}$; UV (MeOH) λ_{max} nm (log ε): 271 (3.5), 330 (3.4); +$AlCl_3$: 283,

389; $+AlCl_3 + HCl$: 282, 390; IR (KBr) ν_{max} 3418, 2928, 1618, 1508, 1460, 1330, 1157 cm^{-1}; ^1H NMR (300.13 MHz, pyridine-d_5) and ^{13}C NMR (75.46 MHz, pyridine-d_5), see Table 1; HR-ESI-MS m/z 551.4558 $[M - H]^-$ (calcd. for $C_{31}H_{21}O_{10}$, 551.4851) and m/z 553.4949 $[M + H]^+$ (calcd. for $C_{31}H_{21}O_{10}$, 553.5009); MS^2 m/z (rel. Int.): 536 $[M - H - CH_3]^-$ (100), 283 [M − H − Ist unit]$^-$ (37), 269 [M − H − IInd unit]$^-$ (9), 255 [IInd unit-CO]$^-$ (51), 509 $[M - H - C_2H_2O]^-$ (10), 507 $[M - H - CO_2]^-$ (8), 483 $[M - H - C_3O_2]^-$ (28), 483 $[M - H - C_2H_2O - C_2H_2]^-$ (28), 467 $[M - H - 2 \times C_2H_2O]^-$ (13), 442 $[M - H - C_3O_2 - C_2H_2O]^-$ (10), 151 [$^{1,3}A^-$ and $^{1'',3''}D^-$] (8), 133 [$^{1,3}B^-$] (6), 117 [$^{1'',3''}E^-$] (20), 107 [$^{1,3}A^-$ and/or $^{1'',3''}$D-CO_2]$^-$ (20).

3.5. Tetraacetate of 1 (1a)

Acetylation of **1** with acetic anhydride and pyridine at room temperature for 48 h resulted in a colourless powder. **1a** was isolated from preparative TLC of reaction product (EtOAc:hexane, 2:8, v/v). IR (KBr) ν_{max} 1772, 1685 cm^{-1}; ^1H NMR (300.13 MHz, $CDCl_3$): δ 7.75 (2H, *d*, $J = 8.7$ Hz, H-2‴, 6‴), 7.67 (2H, *m*, H-2′, 6′), 7.25 (1H, *d*, $J = 8.5$ Hz, H-5′), 7.07 (2H, *d*, $J = 8.7$ Hz, H-3‴, 5‴), 6.95 (1H, *d*, $J = 2.2$ Hz, H-8″), 6.76 (1H, *d*, $J = 2.2$ Hz, H-6″), 6.64 (2H, *s*, H-3, 3″), 6.54 (1H, *d*, $J = 2.2$ Hz, H-8), 6.51 (1H, *d*, $J = 2.2$ Hz, H-6), 3.84 (3H, *s*, OMe-3′), 2.41 (3H, *s*, OAc-5″), 2.36 (6H, *s*, OAc-7, 7″), 2.34 (3H, *s*, OAc-5); HR-ESI-MS m/z $[M + Na]^+$ 743.6181 (calcd. for $C_{39}H_{28}O_{14}Na$, 743.6315).

3.6. Loniflavone (2)

Yellowish powder, m.p. 212–214 °C; $C_{30}H_{18}O_{10}$; UV (MeOH) λ_{max} nm (log ε) 270 (3.9), 337 (3.4), $+AlCl_3$: 281, 386; $+AlCl_3 + HCl$: 280, 390; IR (KBr) ν_{max} 3420, 1630, 1510, 1460, 1340, 1167 cm^{-1}; ^1H NMR (300.13 MHz, pyridine-d_5) and ^{13}C NMR (75.46 MHz, pyridine-d_5), see Table 1; HR-ESI-MS m/z 537.4322 $[M - H]^-$ (calcd. for $C_{31}H_{21}O_{10}$, 537.4583) and m/z 539.4399 $[M + H]^+$ (calcd. for $C_{30}H_{19}O_{10}$, 539.4741); MS^2 m/z (rel. int.): 285 [M − H − Ist unit]$^-$ (88), 269 [M − H − IInd unit]$^-$ (63), 257 [IInd unit − CO]$^-$ (100), 199 [Ist unit − C_2H_2O − CO_2]$^-$ (9), 227 [IInd unit-C_2H_2O]$^-$ (12), 495 $[M - H - C_2H_2O]^-$ (3), 451 $[M - H - CO_2 - C_2H_2O]^-$ (4), 469 $[M - H - C_3O_2]^-$ (3), 469 $[M - H - C_2H_2O - C_2H_2]^-$ (3), 427 $[M - H - C_3O_2 - C_2H_2O]^-$ (4), 151 [$^{1,3}A^-$ and $^{1'',3''}D^-$] (53), 133 [$^{1,3}B^-$] (22), 117 [$^{1'',3''}E^-$] (20), 107 [$^{1,3}A^-$ and/or $^{1'',3''}D - CO_2$]$^-$ (15), 385 [M − H − $^{1,3}A^-$]$^-$(54).

3.7. Pentaacetate of 2 (2a)

Acetylation of **2** with acetic anhydride and pyridine at room temperature for 48 h resulted in a colourless powder. **2a** was isolated from preparative TLC of reaction product (EtOAc:hexane, 2:8, v/v). IR (KBr) ν_{max}: 1770, 1688 cm^{-1}; ^1H NMR (300.13 MHz, $CDCl_3$) δ 7.71 (2H, *d*, $J = 8.7$ Hz, H-2‴, 6‴), 7.66 (2H, *m*, H-2′, 6′), 7.26 (1H, *d*, $J = 8.5$ Hz, H-5′), 7.09 (2H, *d*, $J = 8.7$ Hz, H-3‴, 5‴), 6.88 (1H, *d*, $J = 2.2$ Hz, H-8″), 6.85 (1H, *d*, $J = 2.2$ Hz, H-6″), 6.66 (2H, *s*, H-3, 3″), 6.43 (*d*, 1H, $J = 2.2$ Hz, H-8), 6.40 (*d*, 1H, $J = 2.2$ Hz, H-6), 2.17 (3H, *s*, OAc-3′), 2.40 (3H, *s*, OAc-5″), 2.36 (6H, *s*, OAc-7, 7″), 2.30 (3H, *s*, OAc-5); HR-ESI-MS m/z $[M + Na]^+$ 771.6122 (calcd. for $C_{40}H_{28}O_{15}Na$, 771.6419).

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