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Chemical constituents of *Limnophila indica*

G Brahmachari*, S K Jash, A Gangopadhyay, S Sarkar, S Laskar & D Gorai

Natural Products Laboratory, Department of Chemistry, Visva-Bharati University, Santiniketan 731 235, India

E-mail: brahmg2001@yahoo.co.in

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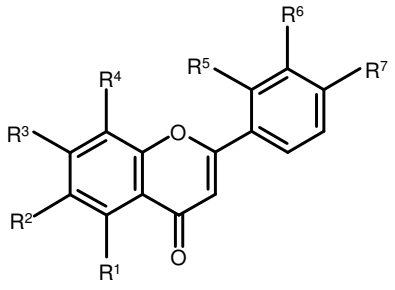
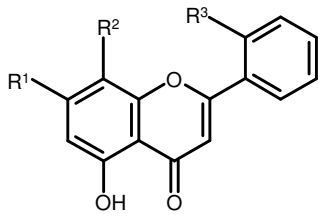
Two flavonoids, 5,6-dihydroxy-7,8,4'-trimethoxy flavone **1** and 5,2'-dihydroxy-8,3',4'-trimethoxyflavone **2** together with three known compounds, 5-hydroxy-7,2'-dimethoxyflavone **3**, 5,2'-dihydroxy-7,8-dimethoxyflavone **4** and β-sitosterol **5**, have been isolated from the aerial parts and roots of *Limnophila indica* (Scrophulariaceae). The structures of compounds **1**-**5** have been elucidated on the basis of spectral and chemical studies.

Keywords: Chelated, unsaturated, *Limnophila indica,* flavone, flavonoids

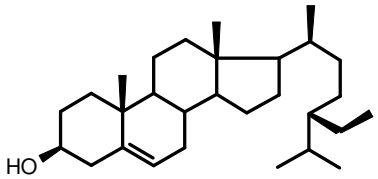
Limnophila indica (Linn.) Druce (Scrophulariaceae)[1,2] is a small aquatic herb, used widely in traditional Indian medicine in the treatment of various diseases like pestilent fever, dysentery, and elephantiasis[3-5]; the plant has also been reported to possess immense anti-microbial activity[6]. In continuation to our previous works on this plant[7-9], we herein report the isolation and structural elucidation of two novel flavonoids, 5,6-dihydroxy-7,8,4'-trimethoxyflavone **1** and 5,2'-dihydroxy-8,3',4'-trimethoxyflavone **2**, together with three known compounds, 5-hydroxy-7,2'-dimethoxyflavone **3**, 5,2'-dihydroxy-7,8-dimethoxyflavone **4** and β-sitosterol **5**, from the aerial parts and roots of *L. indica*. The structures of compounds **1**-**5** were elucidated on the basis of spectral and chemical studies.



1: $R^1 = R^2 = OH$; $R^3 = R^4 = R^7 = OCH_3$; $R^5 = R^6 = H$
1a: $R^1 = R^2 = R^3 = R^4 = R^7 = OCH_3$; $R^5 = R^6 = H$
2: $R^1 = R^5 = OH$; $R^4 = R^6 = R^7 = OCH_3$; $R^2 = R^3 = H$



3: $R^1 = R^3 = OCH_3$; $R^2 = H$
4: $R^1 = R^2 = OCH_3$; $R^3 = OH$



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Results and Discussion

The compound **1**, $C_{18}H_{16}O_7$ ($[M]^+$ at *m/z* 344), responded positively towards Shinoda test[10] and an alcoholic solution of the compound imparted an intense green colour with ferric chloride solution, indicating thereby that **1** is a flavonoid derivative with a free hydroxyl at C_5-position[11]. Its UV spectrum gave the absorption bands at λ_{max} 282 and 329 nm, suggestive of a flavone derivative unsubstituted at 3-position[12,13]. IR spectrum of **1** showed characteristic absorption bands for the presence of bonded hydroxyl function (3411 cm^{-1}), chelated α,β-unsaturated carbonyl attached with aromatic nucleus (1661, 1590, 1507.5, 1388 cm^{-1}), and methoxy group(s) (2939, 2842, 1266, 1025 cm^{-1}) into the molecule; presence of three methoxyl functions in the molecule was also supported from the results of functional group analysis (Zeisel estimation) as well as from the results of other spectroscopic experiments.

The ^1H NMR spectrum of **1** displayed signals at (i) δ 12.78 (1H, s) due to a strongly hydrogen-bonded phenolic hydroxyl function (D_2O exchangeable); (ii) δ 6.58 (1H, s) attributed to C_3-H; (iii) δ 6.44 (1H, s) due to a free phenolic hydroxyl group (D_2O exchangeable) (iv) δ 4.04 (3H, s), 4.02 (3H,s), 3.90 (3H,s) for three methoxyl functions; (v) δ 7.89 (2H, d, *J* = 9Hz, H-2' and H-6') and (vii) δ 7.04 (2H, d, *J* = 9Hz, H-3' & H-5'). The characteristic mass spectral

(EIMS) fragmentations are strongly indicative of the presence of flavone skeleton containing hydroxyl and methoxyl functions in the parent molecule[14]; the mass values of fragmented ions at *m/z* 212 and 132, arising out of retro-Diels-Alder fragmentation around ring C, along with the significant mass-ion peaks at *m/z* 184, 169, 135 and 107 clearly suggest that two methoxyl and two hydroxyl functions are attached to ring-A, while the remaining methoxyl group is linked with the ring-B in the parent compound and unambiguously it must be placed at C-4' as evidenced from the ^{1}H NMR spectral analysis. The above contention receives further support from the experimental results that the compound **1** on methylation with methyl iodide and potassium carbonate furnished a dimethyl flavonoid derivative **1a**, which was found to be completely identical with 5,6,7,8,4'-pentamethoxyflavone (tangeretin) from comparison of its physical and spectral data with the literature values[15-17]. The appearance of intense green colour with ferric chloride imparted by the parent compound **1** locates one of the hydroxyls at C_5-position[6] as also revealed from its IR and ^{1}H NMR spectra. Again, bathochromic shift of band I by 27 nm (329→356) in the UV spectrum of **1** in the presence of $AlCl_3$/HCl confirmed the presence of a hydroxyl function at C_5 and the other at C_6-position[18]. Hence, C_7 and C_8-positions are blocked by the remaining two methoxyls — this contention is supported by the facts that the parent compound did not response to gossypetone test[19], remained insoluble in sodium bicarbonate solution, and also did not exhibit any bathochromic shift of Band II in presence of sodium acetate[20,21]. Thus, the aforediscussed evidences led us to designate the compound **1** as 5,6-dihydroxy-7,8,4'-trimethoxyflavone. This structural formulation was confirmed by its ^{13}C NMR and HMQC (heteronuclear multiple-quantum coherence) spectral studies (**Table I**).

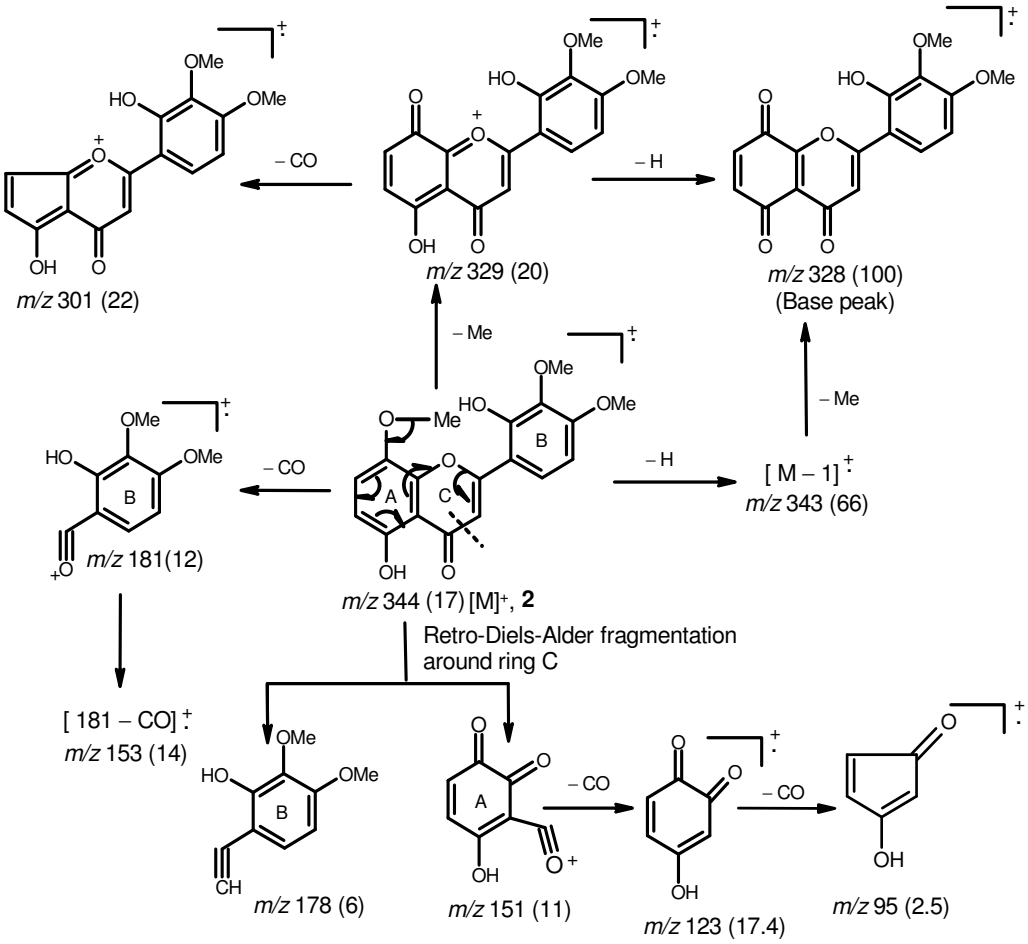
Table I — ^{13}C NMR data and HMQC results for compound **1**

C-atom	δ_C- value	HMQC
2	164.2	-
3	104.1	δ_{H-3} 6.58
4	183.4	-
5	146.2	-
6	131.1	-
7	149.2	-
8	127.8	-
9	148.8	-
10	104.9	-
1′	123.9	-
2′,6′	128.4	$\delta_{H-2',6'}$ 7.89
3′,5′	115.0	$\delta_{H-3',5'}$ 7.04
4′	163.1	-
7-O*C*H$_3$	62.2	δ_H 4.04
8-O*C*H$_3$	61.4	δ_H 4.02
4′-O*C*H$_3$	55.9	δ_H 3.90

Compound **2** responded positively towards flavonoid colour reactions; it exhibited UV absorption bands at λ_{max} 285 and 329 nm, suggesting that **2** may be a flavone derivative unsubstituted at C-3 position[12,13]. The IR spectrum of **2** showed characteristic absorption bands due to the presence of bonded hydroxyl function (3400.7 cm^{-1}), chelated α,β-unsaturated carbonyl attached with aromatic nucleus (1666.3, 1588.7, 1511.3 cm^{-1}), and methoxy group(s) (2917.6, 1059.7 cm^{-1}). Functional group analysis (Zeisel estimation) revealed the presence of three methoxyl functions in the molecule and this contention has received further support from spectral evidences.

The high resolution ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **2** displayed signals at (i) δ12.71 (s, 1H) due to a strongly H-bonded phenolic hydroxyl function (D_2O exchangeable); (ii) δ 6.52 (s, 1H) attributed to C-3 proton; (iii) δ 3.97 (s, 3H), 3.95 (s, 3H), 3.83 (s, 3H) for three methoxy groups; (iv) δ 6.97 (d, *J* = 9Hz, 2H) for two *ortho*-coupled protons of ring-A, and (v) δ 7.82 (d, *J* = 9Hz, 2H) for two *ortho*-coupled protons of ring-B — suggesting thereby that only two positions in each ring of **2** remain unsubstituted. This contention was substantiated by the appearance of significant fragmented mass ion peaks in its EIMS spectrum at *m/z* 329, 328, 181, 178, 153 and 151 (**Scheme I**), which clearly indicates that one hydroxyl and one methoxyl function are attached to ring-A, while the remaining two methoxyl and one hydroxyl functions are linked with the ring-B in the present flavonoid molecule.

The appearance of intense green colour with ferric chloride imparted by the parent compound **2** locates one of the hydroxyls at C-5 position[11] as also revealed from its IR and ^{1}H NMR spectra. Again, the bathochromic shift of Band I by 55 nm (329→384 nm) in the UV spectrum of **2** in presence of $AlCl_3$/HCl, confirmed the presence of a hydroxyl function at C-5 and one of the methoxyls at C-8 position[18]. That the C-8 position is blocked by a methoxyl function was also evidenced from the negative response towards gossepetone test[19] by the parent compound as well as the characteristic mass spectral fragmentation pattern[22]



Scheme I — Partial EIMS fragmentation of compound 2 (% intensity in parentheses)

(**Scheme I**). Thus, ring-A is unsubstituted at C-6 and C-7 positions as revealed from the appearance of ^{1}H NMR signals at δ 6.97 (2H, d, $J_{6,7}$ =9Hz, C_6-H & C_7-H). Further, ^{1}H NMR signal for ring-B protons at δ 7.82 (d, J =9Hz, 2H) is indicative of either 2',3',4' or 2',5',6' or 2',3',6'-trisubstitued B-ring in **2**; the exact constitution was established with the help of hydrolysis experiment — isolation and identification of 2-hydroxy-3,4-dimethoxybenzoic acid[23] as one of the hydrolysis-degradated products during alkaline hydrolysis of the parent flavone **2** along with all the afore-discussed observations ultimately led the present investigators to formulate the compound as 5,2'-dihydroxy-8,3',4'-trimethoxyflavone **2**.

The known compounds isolated from the whole plants of *L. indica* were identified as 5-hydroxy-7,2'-dimethoxyflavone **3** (ref. 24, 25) and 5,2'-dihydroxy-7,8-dimethoxyflavone **4** (ref. 26, 27), and β-sitosterol **5** (ref. 28, 29) by comparison of their physical and spectral data with literature values.

Experimental Section

All m.p.s are uncorrected. TMS has been used as internal standard in recording NMR spectra on a Bruker DRX-300 NMR spectrometer. UV (MeOH) and IR spectra (KBr discs) were recorded respectively on a Shimadzu UV-Vis and Shimadzu 8201 PC – IR spectrophotometer. Mass spectra were recorded on a Jeol SX-102 mass spectrometer. Shinoda test, Gibbs test, gossypetone test and Zeisel experiment were performed with the samples in respective usual procedure as reported. Whole plants of *Limnophila indica* were collected at and around Santiniketan during October-November 2007, and their identity were verified by Dr H R Chowdhury and Dr S Mondal (Visva-Bharati University). A voucher specimen (NPL-0023) is deposited in the Natural Products Laboratory of this University.

Extraction and isolation of flavone 1

The shade air-dried whole plants (aerial parts and roots; 5 kg) of *L. indica* were extracted successively

with petrol ether, benzene and chloroform in a Soxhlet apparatus; the respective extracts were then concentrated under reduced pressure. The concentrated benzene extract was shaken successively with aqueous sodium-bicarbonate, sodium hydroxide and dilute HCl. The sodium hydroxide soluble portion was neutralized by dropwise addition of dilute HCl; neutralized liquor was vigorously shaken with ethyl acetate — the organic layer was separated out with the help of a separating funnel. On removal of ethyl acetate, a dark green mass (45 g) was obtained, which was subjected to column chromatography over silica gel (60-120 mesh); petrol ether – ethyl acetate (5:1) eluent afforded 5,6-dihydroxy-7,8,4'-trimethoxyflavone as yellow crystalline solid (acetone; yield 0.375g), R_f 0.83 (petrol ether: ethylacetate = 1:2), m.p. 184-86°C ($C_{18}H_{16}O_7$ requires C, 62.79; H, 4.65. Found: C, 62.95; H, 4.64%). UV (MeOH), IR (KBr), ^{1}H NMR (300 MHz, $CDCl_3$), ^{13}C NMR (75 MHz, $CDCl_3$), HMQC (75 MHz) results and spectral data are described in the text; EIMS (70 eV): *m/z* (% rel. int.) at 344 ($[M]^+$, 100, base peak), 329 $[M–Me]^+$ (22.51), 316 $[M–CO]^+$ (4.32), 315 $[M–CO–H]^+$ (8.45), 301 $[M–CO–Me]^+$ (5.24), 212 (15.23) and 132 (9.8) (retro-Diels-Alder fragmented ion peaks of **1**), 184 $[212–CO]^+$(4.16), 183$[184–H]^+$(4.98), 169 $[184–Me]^+$ (3.58), 135 (fragmented ion peak) (13.21), 107$[135–Me]^+$(6.24).

Methylation of compound 1. A mixture of 60 mg of compound **1**, 80 mL of dry acetone, 3 g of anhydrous potassium carbonate and 2 mL of methyl iodide was refluxed for 36 hr. Fresh amounts of potassium carbonate (0.5 g) and methyl iodide (0.5 mL) were added and refluxing was continued for another 24 hr. The mixture was filtered, the residual solid was washed with acetone and the combined filtrate and washings were evaporated. The residue was taken up in water and extracted with ether; the dried ether extract was evaporated. The residue was crystallized from acetone-hexane to give the methylated derivative (**1a**, yield 22 mg), found to be identical with 5,6,7,8,4'-pentamethoxyflavone (tangeretin) (referred in the text); m.p. 153-54°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.90 (2H, d, *J* = 9Hz), 7.04 (2H, d, *J* = 9Hz) [A_2B_2 quartet, H-2', H-3', H-5', H-6'], 6.61(1H,s,H-3), 4.12 (3H,s), 4.04 (3H,s), 3.96 (6H, s), 3.90 (3H,s), for five methoxyl functions; other physical and IR spectral data are very much close to those as reported for tangeretin.

Extraction and isolation of flavone 2. On chromatographic resolution over silica gel (60-120 mesh), the chloroform fraction (concentrated under reduced pressure) of ethanolic extract of air-dried whole plants of *L. indica* (5 kg) afforded the 5,2'-dihydroxy-8,3',4'-trimethoxyflavone when eluted with benzene; the compound was crystallized from ethanol as bright yellow needles (yield 0.275 g), R_f 0.72 (C_6H_6:$CHCl_3$ = 2:3), m.p. 182°C; $C_{18}H_{16}O_7$ requires C, 62.79; H, 4.65. Found: C, 62.95; H, 4.64%; UV (MeOH), IR (KBr), ^{1}H NMR (300 MHz, $CDCl_3$) spectral data are described in the text; EIMS (70 eV): *m/z* (% rel. int.) 344 ($[M]^+$,17), 343 $[M–H]^+$ (66), 329 (20), 328 [329–H]+ (base peak, 100), 315 $[M–CO–H]^+$ (6.8), 314 $[M–2Me-H]^+$ (6.1), 299 $[329–2Me]^+$ (5.2), 181 (12), 151 (11) & 178 (6), 153 $[181-CO]^+$ (14), 123$[151-CO]^+$ (4), 95 $[123-CO]^+$ (21).

Alkali degradation of compound 2. Compound **2** (200 mg) was treated with 40% aqueous KOH solution and heated at reflux temperature for 20 hr in nitrogen atmosphere; the reaction-mixture was then cooled, acidified with 10% aqueous HCl and filtered. A solid mass of about 0.03 g was separated out and was identified as 2-hydroxy-3,4-dimethoxybenzoic acid (m.p. 168-71°C) by comparison of its physical and spectral (IR and ^{1}H NMR) data with those reported in the literature[23] along with performing m.m.p. and CO-TLC experiments with authentic sample.

Extraction and isolation of known compounds 3-5. Silica gel (60-120 mesh) column packed with the petrol extract as obtained from the plant materials, when eluted with petroleum ether-benzene (4:1), yielded β-sitosterol **5** (ref. 28,29) as white amorphous powder (15 mg). Compounds **3** and **4** were obtained from the same column from where compound **1** was eluted with petroleum ether-ethyl acetate (10:1 v/v) to afford 5-hydroxy-7,2'-dimethoxyflavone (**3**, yield 25 mg): $C_{17}H_{15}O_5$ ($[M]^+$ at *m/z* 299), pale yellow solid; m.p. 222-25°C; UV, IR, ^{1}H NMR and EIMS spectral data are closely related to those as reported in literature[24,25]; while petroleum ether-ethyl acetate (8:1 v/v) eluent afforded 5,2'-dihydroxy-7,8-dimethoxyflavone (**4**, yield 28 mg): $C_{17}H_{15}O_6$ ($[M]^+$ at *m/z* 315), pale yellow solid; m.p. 253-54°C; UV, IR, ^{1}H NMR and EIMS spectral data are closely related to those as reported in literatures[26,27].

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