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ISOFLAVONOIDS FROM *FLEMINGIA STROBILIFERA* (L) R. BR. ROOTS

SWATI MADAN[1], GYANENDRA NATH SINGH[1], KANCHAN KOHLI[3], MOHAMMED ALI[*3], YATENDRA KUMAR[2], RAMAN MOHAN SINGH[1] and OM PRAKASH[1]

[1]Central Indian Pharmacopoeia Laboratory, Ministry of Health and Family Welfare, Sector-23, Raj Nagar, Ghaziabad, U.P.-201002, India.
[2]I.T.S. Paramedical College (Pharmacy), Delhi-Meerut Road, Murad Nagar, Ghaziabad, U.P. -2012006, India.
[3]Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Jamia Hamdard Nagar (Hamdard University), New Delhi-110062, India.

Abstract: A new isoflavone (**1**) isolated from the roots of *Flemingia strobilifera* (L) R. Br. was identified as 5,7,4'-trihydroxy 8,2',5'-tri(3-methylbut-2-enyl)isoflavone along with the known phytoconstituents: 5,7,2',4'-tetrahydroxyisoflavone (**2**), 5,7,4'-trihydroxyisoflavone (**3**) and β-sitosterol (**4**). Structure assignments were performed on the basis of spectroscopic data including homo- and heteronuclear 1D and 2D NMR (COSY, HMBC and DEPT) and MS studies. The compounds were tested *in vitro* for antimicrobial activity and antioxidant activity and compounds (**1-3**) proved to be moderately active.

Keywords: *Flemingia strobilifera*, Leguminosae, roots, isoflavones, antimicrobial, antioxidant

The genus *Flemingia* (family Leguminosae) comprises over fourty species in the world. In India, this genus is represented by fifteen species, including *Flemingia strobilifera* (1, 2). *F. strobilifera* (R.Br.), an important medicinal plant, is commonly known as Kusrunt and is found in Sind, Rajputana, Bengal, South India and Andamans (3). Several plants of this genus have been used in folk medicine to treat fever, diarrhoea, indigestion and as vermifuge (4, 5). The plants are reported to possess antimicrobial, antifungal, anthelmintic, anticancer, anti-rheumatic, anti-inflammatory, antioxidant and anti-histamine activities (6-11). Previous chemical studies showed that flavonoids, flavonoid glycosides, chalcones, epoxychromenes and pterocarpans were the main constituents found in this genus (12, 13). Reports on chemical composition of *F. stobilifera* are limited. The presence of flavonoids and flavonoid glycosides was confirmed in *F. strobilifera* herb (14, 15) and a chalcone from the roots was identified as 3',6'-dihydroxy-2',4',5',4'-tetramethoxychalcone (16).

This paper reports the isolation and structural elucidation of a new isoflavone (**1**) along with three known compounds (**2-4**) isolated for the first time from the roots of *F. strobilifera*.

The compounds were tested *in vitro* for antimicrobial and antioxidant activity.

EXPERIMENTAL

General procedures

Melting points were measured on a Büchi Melting Point B-540 apparatus (Switzerland) and are uncorrected. Precoated silica gel 60 F_{254} plates of 0.2 mm thickness (Merck, Germany) were used for TLC. The spots were visualized by spraying with anisaldehyde-sulfuric acid (AS) reagent, followed by heating at 110°C for 5 min and also by using 10% MeOH/$FeCl_3$ reagent. UV spectra were taken on UV-Perkin Elmer double beam UV spectrophotometer (Germany). IR spectra were recorded on a Jasco FT/IR 410 (USA) instrument with KBr pellets. 1D and 2D NMR experiments were obtained in

* Corresponding author: phone: +91-11-26059692, e-mail: mali_hamdard@yahoo.co.in; maliphyto@gmail.com

DMSO-d_6 using Bruker 400 MHz Ultrashield, Advance 400 (Germany) spectrometer, using TMS as an internal standard. 2D NMR experiment included the HSQC, HMBC, and COSY pulse sequences. Coupling constants (*J* values) were given in Hz. Mass spectra were obtained using a WATERS 2496 separations module-LC system with a MS-MSD Quattro micro in ESI mode (multimode ionization). 2,2-Diphenyl-1-picrylhydrazyl (molecular formula $C_{18}H_{12}N_5O_6$, DPPH) was obtained from Merck (Darmstadt, Germany).

Plant material

The roots of *F. strobilifera* were collected from forests of Shann Power House, Joginder Nagar, (Dist. Mandi) Himachal Pradesh in October 2006; voucher specimen number NISCAIR/RHMD/ Consult/06/757/74 was deposited at the Herbarium of National Institute of Science Communication and Information Resources, New Delhi.

Extraction and isolation

The air-dried roots (2.94 kg) of *F. strobilifera* were extracted with methanol for 24 h by cold-maceration. The methanolic extract was evaporated in rota-vapor to yield a residue (1000 g), 900 g of which was suspended in water (5 L) and partitioned with dichloromethane (DCM) (20 L) to obtain DCM fraction (18 g).

The DCM fraction (16 g) was column chromatographed over silica gel using petroleum ether (PE) with increasing amount of ethyl acetate (EtOAc), step gradient to afford fractions A-D. The combined fractions A (eluted with PE-EtOAc, 9:1, v/v) were separated on TLC (PE: EtOAc, 3:1, v/v) to give compound **1** (30 mg) and **4** (66 mg)**.** The combined fractions D (48 mg), was column chromatographed over silica gel eluting with chloroform : methanol ($CHCl_3$: MeOH, 19:1, v/v) to give compound **2** (6.1 mg). Fraction C was combined and eluted with PE : EtOAc, (4:1, v/v). It was separated on TLC with PE : EtOAc (1:1, v/v) to give compound **3** (83 mg).

5,7,4'-Trihydroxy-8,2',5'-tri(3-methylbut-2-enyl)isoflavone (1)

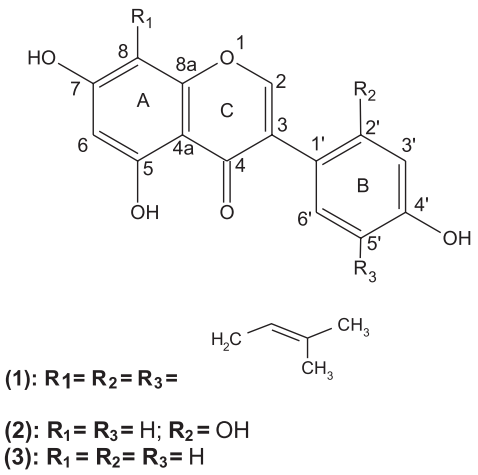
Creamish crystals, m.p. 170-172°C (17), $C_{30}H_{34}O_5$, MS: m/z 474 $[M]^+$, UV: λ max^{MeOH}, 268 nm. IR (KBr) cm^{-1}: 1625 (C=O), 3300 (OH). ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (400 MHz, $CDCl_3$) data are given in Table 1.

5,7,2',4'-Tetrahydroxyisoflavone (2)

Creamish amorphous, m.p. 270-272°C (18, 19), $C_{15}H_{10}O_6$, MS: m/z 286 $[M]^+$, UV: λ max^{MeOH}: 258 and 315 (sh) nm; + $AlCl_3$: 268 and 315 (sh); + $AlCl_3$-HCl: 268 and 315 (sh); + NaOAc: 270 + NaOAc-H_3BO_3: 258 nm. IR (KBr) cm^{-1}: 1625 cm^{-1} (C=O), 3300 cm^{-1} (OH). ^{1}H NMR (400 MHz, MeOD-d_4) and ^{13}C NMR (400 MHz, MeOD-d_4) data are given in Table 1.

Genistein (3)

Creamish amorphous, m.p. 290-292°C (19, 20), $C_{15}H_{10}O_5$, MS: m/z $[270]^+$. UV: λ_{max} [MeOH, nm]: 261, 315 (sh); + NaOAc: 270; + $AlCl_3$: 273, 332; + NaOAc-H_3BO_3: 262. IR (KBr) cm^{-1}: 3340



(1): R_1= R_2= R_3=

(2): R_1= R_3= H; **R_2=** OH

(3): R_1 = R_2= R_3= H

Figure 1. Structure of isoflavones 1, 2 and 3

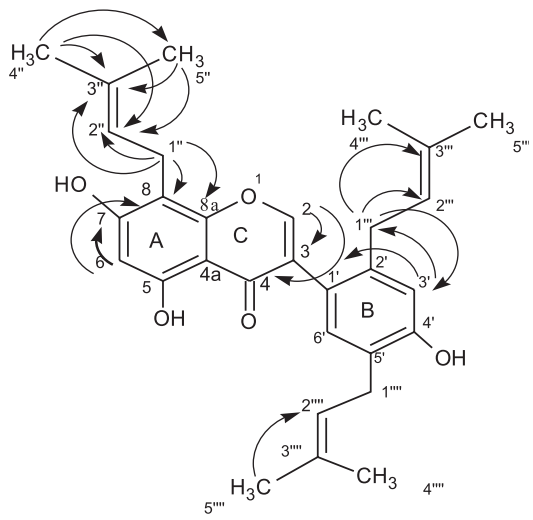


Figure 2. ^{1}H-^{13}C HMBC correlations of compound **1**

Table 1. ^{1}H NMR and ^{13}C NMR spectral data ™ (ppm) of isoflavones **1**, **2** and **3.**

Compounds	**1**			**2**			**3**		
Position	^{1}H NMR (*J*, Hz)	^{13}C NMR	DEPT moiety	^{1}H NMR (*J*, Hz)	^{13}C NMR	DEPT moiety	^{1}H NMR (*J*, Hz)	^{13}C NMR	DEPT moiety
2	7.80 *s*	152.6	CH	7.9 *s*	155.6	CH	8.32 *s*	153.9	CH
3		122.6	C		126.9	C		122.2	C
4		181.0	C		180.4	C		180.1	C
5-OH	13.29 *s*	159.6	C		161.4	C	12.96 *s*	161.9	C
6	6.36 *s*	93.9	CH	6.13 *d* (2.03)	103.4	CH	6.22 *d* (2.0)	98.9	CH
7-OH	5.5 *s*	161.3	C		163.8	C	10.89 *s*	164.2	C
8		109.8	C	6.29 *d* (2.03)	108.6	CH	6.38 *d* (2.0)	93.6	CH
4a		106.4	C		105.9	C		104.4	C
8a		159.6	C		154.2	C		158.1	C
1’		123.8	C		120.3	C		121.1	C
2’		127.5	C		157.5	C	7.37 *dd* (1.1, 8.52)	130.1	CH
3’	7.11 *s*	128.5	CH	6.29 *d* (2.24)	92.6	CH	6.82 *dd* (1.0, 8.6)	115.0	CH
4’-OH	5.5 *s*	153.1	C		158.0	C	9.60 *s*	157.4	C
5’		127.5	C	6.26 *dd* (8.24, 2.24)	97.9	CH	6.82 *dd* (1.0, 8.6)	115.0	CH
6’	7.11 *s*	128.5	CH	6.94 *d* (8.24)	130.9	CH	7.37 *dd* (1.1, 8.52)	130.1	CH
1’’	3.46 *d* (7.0)	21.5	CH_2						
2’’	5.29 *t* (7.0)	121.8	CH						
3’’		136.1	C						
4’’	1.77 *s*	17.9	CH_3						
5’’	1.84 *s*	25.8	CH_3						
1’’’/1’’’’	3.37 *t* (7.0)	29.7	CH_2						
2’’’/2’’’’	5.34 *t* (7.0)	121.0	CH						
3’’’/3’’’’		134.6	C						
4’’’/4’’’’	1.77 *s*	17.9	CH_3						
5’’’/5’’’’	1.84 *s*	25.8	CH_3						

(OH) and 1655 cm^{-1} (C=O). ^{1}H NMR (400 MHz, DMSO-d_6) and ^{13}C NMR (400 MHz, DMSO-d_6) data are given in Table 1.

β-Sitosterol (4)

Colorless crystals, m.p. 139-140^{o}C; $[\alpha]_D^{25}$ -36^{o} (c = 1.5 in $CHCl_3$), $C_{29}H_{50}O$, MS: m/z [414]$^{+}$, IR (KBr) cm^{-1}: 3465, 2955, 2845, 1640, 1475, 1365, 1210, 1103 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H [ppm]: 5.30 (d, *J* = 5.5 Hz, H-6), 3.51 (1H, br, $w_{1/2}$ 16.5 Hz, H-3α), 1.01 (3H, brs, Me-19), 0.97 (3H, d, *J* = 6.5 Hz, Me-21), 0.86 (3H, d, *J* = 6.0 Hz, Me-26), 0.83 (3H, d, *J* = 6.0 Hz, Me-27), 0.82 (3H, t, *J* = 6.2 Hz, Me-29), 0.67 (3H, brs, Me-18).

^{13}C NMR (400 MHz, $CDCl_3$): 37.33 (C-1), 31.63 (C-2), 71.73 (C-3), 41.98 (C-4), 141.17 (C-5), 121.63 (C-6), 31.15 (C-7), 31.81 (C-8), 49.57 (C-9), 36.74 (C-10), 21.66 (C-11), 39.80 (C-12), 41.98 (C-13), 56.04 (C-14), 24.19 (C-15), 28.60 (C-16), 55.41 (C-17), 11.36 (C-18), 19.30 (C-19), 36.74 (C-20), 18.75 (C-21), 33.30 (C-22), 25.73 (C-23), 45.14 (C-24), 29.15 (C-25), 20.37 (C-26), 19.30 (C-27), 23.56 (C-28), 11.03 (C-29). Properties and spectra were identical to those reported earlier (21).

Antimicrobial activity method

The minimal inhibitory concentration (MIC) of extract and isolated compounds were determined by

Table 2. Selected data from COSY experiments of isoflavones **1, 2 and 3.**

proton-proton connectivities between:						
Compounds	**1**		**2**		**3**	
	3.46 (H_2-1'')	5.29 (H-2'')	6.94 (H-6')	6.26 (H-5')	7.37 (H-2'/ 6')	6.82 (H-3'/5')
	5.29 (H-2'')	1.84 (H_3-5'')				

Table 3. Selected long-range couplings (δ, ppm) observed in HMBC experiments of isoflavones **1, 2** and **3.**

Compounds	**1**		**2**		**3**	
	Proton	Carbon	Proton	Carbon	Proton	Carbon
	7.80 (H-2)	122.6 (C-3), 181.0 (C-4)	7.9 (H-2)	180.4 (C-4), 120.3 (C-1'),	8.32 (H-2)	122.2 (C-3), 180.1 (C-4)
	6.36 (H-6)	161.3 (C-7), 109.8 (C-8)	6.29 (H-8)	103.4 (C-6)	6.22 (H-6)	164.2 (C-7), 93.6 (C-8), 104.4 (C-4a)
	7.11 (H-3')	29.7 (C-1'''), 123.8 (C-1')	6.94 (H-6')	120.3 (C-1'), 157.5 (C-2')	6.38 (H-8)	104.4 (C-4a), 164.2 (C-7)
	3.46 (H_2-1'')	109.8 (C-8), 121.8 (C-2''), 136.1 (C-3''), 159.6 (C-8a)			7.37 (H-2'/6')	122.2 (C-3), 115.0 (C-3'/C-5')
	1.77 (H_3-4'')	25.8 (C-5''), 121.8 (C-2''), 136.1 (C-3'')			6.82 (H-3'/5')	121.1(C-1')
	1.84 (H_3-5'')	121.8 (C-2''), 136.1 (C-3'')				
	3.37 (H_2-1'''/1'''')	121.0 (C-2'''/2''''), 134.6 (C-3'''/C-3''''), 28.5 (C-3')				
	1.84 (H_3-5'''/5'''')	121.0 (C-2'''/ 2'''')				

the broth microdilution method according to National Committee for Clinical Laboratory Standards guidelines (NCCLS, 2000) (22) as well as for non-filamentous fungi in 96-well microtitre plates with MHB (Muller Hinton broth) made in-house. Polypropylene 96-well microtitre plates contained the antimicrobial agents in serial twofold dilutions from 136 to 0.53 µg/mL, depending on the antimicrobial agent being tested. The samples were inoculated into broth again. Inocula were prepared in MHB from cultures grown on tryptic soya agar. The final concentration was 1×10^5 CFU/mL. All microtitre plates were prepared in duplicate and incubated at 35°C for 24 h. The susceptibility of the standard drugs: vancomycin, linezolid, fluconazole and itraconazole, were defined as the lowest concentration of drug that resulted in total inhibition of microbial growth.

DPPH radical-scavenging assay

The antioxidant activities (23) of compounds **1-3** were assessed on the basis of radical scavenging effect of the stable DPPH free radical. To 6 mL of DPPH (20µg/mL) methanolic solution, 20 µL of DMSO solution of each compound was added separately, at room temperature. The mixture was shaken vigorously and kept aside for 5 min and absorbance was measured at about 517 nm with Beckman (DU 640B) spectrophotometer against corresponding test blanks. All tests were run in triplicate and mean values were taken for calculation.

RESULTS AND DISCUSSION

The methanolic extract of the dried roots of *F. strobilifera* was evaporated in a rotavapor, and the residue was suspended in water and partitioned with

Table 4. Antimicrobial activity of isoflavones **1**, **2**, **3** and dichloromethane (DCM) extract on selected test organisms.

Test organism	MIC µg/mL							
	Test compounds and extract				Standards*			
	Cpd. (**1**)	Cpd. (**2**)	Cpd. (**3**)	DCM Ext.	V	L	F	I
Gram- positive								
Staphylococcus aureus ATCC 25923	28	136	34	2.1	1	2		
Staphylococcus epidermidis ATCC 12228	28	136	34	2.1		2.1	2	1
Methicillin resistant Staphylococcus aureus 562	36	–	136	2.1		2.1	1	2
Gram-negative								
Pseudomonas aeruginosa ATCC 7853	133	136	136	34		34	16	16
Escherichia coli ATCC 25922	130	136	136	17		17	2	2
Non-Filamentous fungi								
Candida albicans ATCC 1122	36	68	136	17			> 64	> 16

* V: Vancomycin, L: Linezolid, F: Fluconazole, I: Itraconazole.

Table 5. *In-vitro* antioxidant activity of isoflavones **1**, **2** and **3**.

IC_{50} values ± SE (µg/mL)*	
Compound	DPPH radical scavenging
1	52.4 ± 1.28
2	32.7 ± 0.61]
3	170.0 ± 1.06

* Average of three determinations, SE: standard error

dichloromethane (DCM). Compounds **1-4** were isolated from the DCM-soluble fraction of *F. strobilifera* by column chromatography. The compounds **2-4** have been reported from the *Flemingia* species (18-21) but their presence in the roots of *F. strobilifera* has been detected for the first time. Compound **1** was isolated for the first time from the genus *Flemingia*.

Compound **1** named strobiliferyllin, was obtained as creamish crystals from petroleum ether-ethyl acetate (3:1) eluants. It yielded green color with alcoholic ferric chloride solution indicating the phenolic nature of the compound. The presence of UV absorption maxima at 268 nm (24) and negative Shinoda test (25) suggested that the compound was genistein like isoflavone. The bathochromic shifts of band II from 268 nm to 276 nm on addition of sodium acetate and to 281 nm on addition of aluminium chloride-hydrochloric acid supported existence of 7-hydroxyl and 5-hydroxyl groups, respectively, in the isoflavone nucleus (24). The IR spectrum of **1** exhibited characteristic absorption bands for hydroxyl groups at 3300 cm^{-1} and carbonyl group at 1625 cm^{-1}. Its mass spectrum displayed a molecular ion peak m/z 474 corresponding to a triprenylated isoflavone, $C_{30}H_{34}O_5$.

The ^{1}H NMR spectrum of **1** showed two one-proton deshielded signals at δ 7.80 ppm and 6.36 ppm assigned to H-2 methine and H-6 aromatic protons, respectively, indicating the existence of one of the prenyl group at C-8 atom. A two-proton signal at δ 7.11 ppm was ascribed to *p*-coupled H-3' and H-6' aromatic protons suggesting the attachment of the prenyl groups at C-2' and C-5' atoms. A set of signals as a two-proton doublet at δ 3.46 ppm (J = 7.0 Hz) and a four proton triplet at δ 3.37 ppm (J = 7.0 Hz) was associated with H_2-1'', H_2-1''' and H_2-1'''' methylene protons attached between aromatic and vinylic carbons. A one-proton triplet at δ 5.29 ppm (J = 7.0 Hz) and a two-proton triplet at δ 5.34 ppm (J = 7.0 Hz) were accounted to vinylic H-2'' and H-2''', H-2'''', respectively. Two broad signals at δ 1.77 ppm and 1.84 ppm, integrated for nine protons each, were accommodated to six methyl protons located on the vinylic carbons. A two-proton broad signal at δ 5.50 ppm and a one-proton signal at δ 13.29 ppm, exchangeable with D_2O, were due to C-7, C-4' and C-5 hydroxyl groups.

The ^{13}C NMR spectrum of **1** showed a set of carbon signals at δ 152.6 ppm (C-2), 122.6 ppm (C-3) and 181.0 ppm (C-4) characteristic of isoflavone type molecules. The carbon signals at δ 159.6, 161.3 and 153.1 ppm were assigned to phenolic carbons C-5, C-7 and C-4', respectively. The vinylic carbons appeared at δ 121.8 ppm (C-2''), 121.0 ppm (C-2''', C-2''''), 136.1 ppm (C-3'') and 134.6 ppm (C-3''', C-3''''). The methyl carbon signals resonated at 17.9 and 25.8 ppm. The carbon signals of **1** were compared with the related isoflavones (26, 27). DEPT spectrum of **1** exhibited the presence of six methyl, three methylene, seven methine and fourteen quaternary carbons. The HSQC experiments displayed seven methine carbons at δ 152.6 (C-2), 93.9 (C-6), 128.5 (C-3'/6'), 121.8 (C-2'') and 121.0 ppm (C-2'''/C-2'''') (Table 1).

In ^{1}H-^{1}H COSY spectrum of **1**, H_2-1'' proton interacted with H-2'' proton and H-2'' proton of ring-A showed correlation with H_3-5'' proton (Table 2). The ^{1}H-^{13}C HMBC spectrum of **1** exhibited correlation of H-2 with C-3 and C-4; H-6 with C-7 and C-8; H-3' with C-1''' and C-1'; H_2-1'' with C-8, C-2'', C-3'' and C-8a; H-4'' with C-5'', C-2'' and C-3''; H-5'' with C-2'' and C-3''; H_2-1'''/H_2-1'''' with C-2'''/C-2'''', C-3'''/C-3'''', C-3'; H-4'''/H-4'''' with C-2'''/C-2'''' (Table 3). On the basis of the above discussion the structure of **1** has been elucidated as 5,7,4'- trihydroxy-8,2',5'-tri(3-methylbut-2-enyl)isoflavone.

The isomeric compound (Flemiphyllin) identified as 5,7,4'-trihydroxy-8,3',5'-tri(3-methylbut-2-enyl)isoflavone has been reported from the stems of *F. macrophylla* (17).

Data on antimicrobial properties of flavonoids prompted us to test the activity of compounds **1-3** against some bacterial and fungal strains. The minimum inhibitory concentration (MIC) (22) values of compounds are shown in Table 4. Compound **1** and **3** showed the moderate activity against Gram positive bacteria: *Staphylococcus aureus*, *Staphylococcus epidermidis* with MIC of 28 and 34 µg/mL, while choloroform extract showed potent activity with MIC of 2.1 µg/mL against Gram positive bacteria: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Methicillin resistant Staphylococcus aureus* and MIC of 34 and 17 µg/mL against Gram negative bacteria: *Pseudomonas aeruginosa*, *Escherichia coli* and 17 µg/mL of MIC against fungi *Candida albicans*.

In addition, compounds **1-3** were also studied for *in-vitro* antioxidant activity by DPPH radical scavenging assay (23). Compound **2** had showed the IC_{50} value of 32.7 µg/mL (Table 5).

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