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FLAVONOIDS FROM THE LEAVES OF *NICOTIANA TABACUM* AND THEIR ANTI-TOBACCO MOSAIC VIRUS ACTIVITY

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Abstract –Three new flavonoids, 8-formyl-4'-hydroxy-7-methoxy-6-methyl-flavone (**1**), 8-formyl-4',7-dimethoxy-6-methylflavone (**2**), 4',6-dihydroxy-7-methoxy-8-methoxycarbonylflavone (**3**) together with two known flavonoids (**4** and **5**) were isolated from the leaves of *Nicotiana tabacum.* Their structures were determined by means of HRESIMS and extensive 1D and 2D NMR spectroscopic studies. Compounds **1-3** were tested for their anti-tobacco mosaic virus (anti-TMV) activities. The results showed that compounds **1-3** exhibited inhibition rates (%) of 18.4, 22.1 and 16.4, respectively.

Nicotiana tabacum L. is the most commonly grown of all plants in the *Nicotiana* genus, and its leaves are commercially grown in many countries to be processed into tobacco.[1,2] In addition, *N. tabacum* is also used as insecticide, anesthetic, diaphoretic, sedative, and emetic agents in Chinese folklore medicine because of it containing many useful chemical compounds.[1,3-5] In previous work, a number of bioactive compounds, such as terpenoids,[6-8] alkaloids,[9,10] lignans,[11,12] flavonoids,[13] phenylpropanoids,[14] and the homologous, were isolated from this plant. Therefore, the multipurpose utilization of flue-cured tobacco is an interesting topical and attracts more and more attentions.[15,16] Motivated by search for more bioactive metabolites from this plant, an investigation on the chemical constituents of the leaves of Yunyan-200 (a variant of *N. tabacum*) was carried out. As a result, three new (**1-3**) and two known flavonoids (**4** and **5**) were isolated from this plant. In addition, the anti-tobacco mosaic virus (anti-TMV) activities of compounds **1**–**3** were evaluated. This article deals with the isolation, structural elucidation and biological activities of the new flavonoids.

A 70% aq. methanol extract prepared from the leaves of tobacco was subjected repeatedly to column

chromatography on silica gel and preparative HPLC to afford three new flavonoids, 8-formyl-4'-hydroxy-7-methoxy-6-methylflavone (**1**), 8-formyl-4',7-dimethoxy-6-methylflavone (**2**), 4',6-dihydroxy-7-methoxy-8-methoxycarbonylflavone (**3**), and two known flavonoids (**4** and **5**). The structures of the compounds **1**-**5** were as shown in Figure 1, and the ^{1}H and ^{13}C NMR data of **1**-**3** were listed in Table 1. The known compounds, compared with literature, were identified as kaempferol (**4**)[17] and quercetin (**5**).[17]

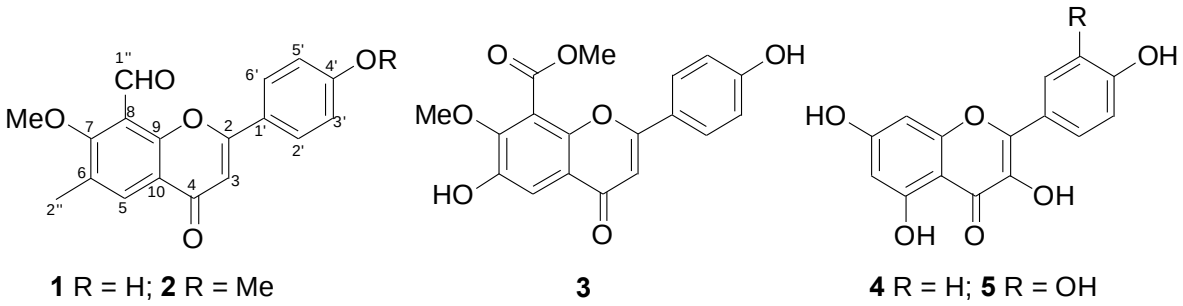

Figure 1. The structures of Compounds **1** - **5**

Compound **1** was obtained as an orange-yellow gum. It has the molecular formula $C_{18}H_{14}O_5$ from HRESIMS (*m/z*: 333.0732 $[M+Na]^+$, calcd 333.0739). The ^{1}H and ^{13}C NMR spectra of **1** (Table 1) along with analysis of the DEPT spectra displayed 18 carbon signals and 8 hydrogen signals for 14 protons, respectively, corresponding to a pentasubstituted benzene ring (δ_C 137.0 d, 120.2 s, 166.1 s, 113.8 s, 154.0 s, and 119.0 s; δ_H 7.69 s), a 1,4-disubstitued benzene ring [δ_C 122.8 s, 131.3 d (2C), 116.4 d (2C), and 158.2 s; δ_H 7.87 (d) and 6.88 (d)], a pair of double bond (δ_C 163.5 s and 107.0 d; δ_H 6.58 s), a carbonyl group (δ_C 178.8), one aldehyde group (δ_C 191.1; δ_H 10.23), one methyl group (δ_C 16.2; δ_H 2.32), one methoxy group (δ_C 61.0; δ_H 3.83), and one low-field hydroxy proton (δ_H 11.09). These NMR data together with the HMBC correlations (Figure 2) of H-3 (δ_H 6.58 s) with C-4 (δ_C 178.8), C-10 (δ_C 119.0), and C-1′ (δ_C 122.8) suggested that **1** should be a flavone.[17] The HMBC correlations of the hydroxy proton signal (δ_H 11.09) with C-3′,5′ (δ_C 116.4) and C-4′ (δ_C 158.2), suggested the attachment position of the hydroxy group at C-4′. Since the substituents on ring C were evident, the surplus substituents (one aldehyde group, one methyl group, and one methoxy group) should be located at ring B. The HMBC correlations of aldehyde proton signal (δ_H 11.09) with C-7 (δ_C 166.1), C-8 (δ_C 113.8), and C-9 (δ_C 154.0) suggested the placement of the aldehyde group at C-8. The HMBC correlations of methyl proton signal (δ_H 2.32) with C-5 (137.0), C-6 (120.2), and C-7 (166.1) suggested that the methyl group located at C-6. The methoxy group located at C-7 was also supported by the HMBC

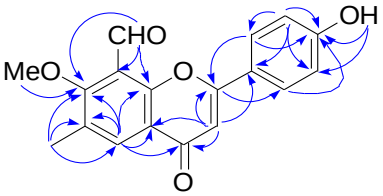

Figure 2. The HMBC (↷) correlations of **1**

correlation of the methoxy proton signal (δ_H 3.83) with C-7 (δ_C 166.1). Thus, the structure of **1** was established as 8-formyl-4'-hydroxy- 7-methoxy- 6-methylflavone.

Table 1. ^{1}H NMR and ^{13}C NMR Data (in C_5D_5N) of compounds **1-3**

No.	Compound **1**		Compound **2**		Compound **3**	
	δ_C (mult.)	δ_H (mult., *J*, Hz)	δ_C (mult.)	δ_H (mult., *J*, Hz)	δ_C (mult.)	δ_H (mult., *J*, Hz)
2	163.5 s		163.9 s		163.0 s	
3	107.0 d	6.58 s	106.7 d	6.65 s	105.3 d	6.67 s
4	178.8 s		179.7 s		182.0 s	
5	137.0 d	7.69 s	136.7 d	7.68 s	121.4 d	
6	120.2 s		120.2 s		142.3 s	
7	166.1 s		166.4 s		159.7 s	
8	113.8 s		113.2 s		113.2 s	
9	154.0 s		154.1 s		149.0 s	
10	119.0 s		118.3 s		118.1 s	
1′	122.8 s		122.9 s		123.0 s	
2′,6′	131.3 d	7.87 (d) 8.6	130.2 d	7.80 (d) 8.6	131.1 d	7.88 (d) 8.8
3′,5′	116.4 d	6.88 (d) 8.6	115.5 d	6.81 (d) 8.6	116.6 d	6.88 (d) 8.8
4′	158.2 s		161.0 s		157.8 s	
1″	191.1 s	10.23 s	192.7 s	10.12 s	168.7 s	
2″	16.2 s	2.32 s	16.9 s	2.30 s		
-OMe-7	61.0 q	3.83 s	61.0 q	3.83 s	61.1 q	3.80 s
-OMe-4′			56.0 q	3.81 s		
-OMe-1″					52.8 s	3.97 s
Ar-OH-6						11.29 s
Ar-OH-4′		11.09 brs				11.07 s

Compounds **2** was obtained as orange-yellow gum, and showed a quasi-molecular ion peak at *m/z* 347.0888 $[M+Na]^+$ in the HRESIMS (calcd *m/z* 347.0895), corresponding to the molecular formula of $C_{19}H_{16}O_5$. The ^{1}H and ^{13}C NMR spectra of **2** were very similar to these of **1**. The only difference was due to a hydroxy group in **1** was substituted by a methoxy group in **2** on the aromatic rings. This was supported by the disappearance of a phenolic proton signal and appearance of methoxy group signals in NMR spectrum of **2**. The HMBC correlations of two methoxy proton signals (3.83 s and 3.81 s) with C-4′ (δ_C 161.0) and C-7 (δ_C 166.4) indicated that two methoxy groups were located at C-4′ and C-7 respectively. The down-shift of ^{13}C NMR signal of C-4′ from δ_C 158.2 ppm to δ_C 161.0 ppm also

suggested that the substituent group should be varied at C-4′. Therefore, the structure of 8-formyl-4',7-dimethoxy-6-methylflavone (**2**) was assigned as shown.

Compound **3** was also obtained as an orange-yellow gum. It was assigned the molecular formula $C_{18}H_{14}O_7$ by its HRESIMS at *m/z* 365.0637 $[M+Na]^+$. The ^1H and ^{13}C NMR spectra of **3** are also similar to those of compound **1**. Analysis of the ^1H and ^{13}C NMR spectral data of **3** with those of **1** suggested that the difference was due to the disappearance of aldehyde group signals (δ_C 191.1; δ_H 10.23), methyl group signal (δ_C 16.2 s; δ_H 2.32 s) and appearance of methoxycarbonyl group signals (δ_C 168.7, 52.8; δ_H 3.97), and a phenolic hydroxy group signal (δ_H 11.29 s) in **1**. These indicated that the aldehyde group at C-8 and methyl group at C-6 in **1** were substituted by a methoxycarbonyl and a phenolic hydroxy group in **3**. In HMBC spectrum, the correlations of one phenolic hydroxy signal (δ_H 11.29 s) with C-5, C-6, and C-7, and the correlations of the other phenolic hydroxy signal (δ_H 11.07) with C-3′,5′ and C-4′ indicated that two phenolic hydroxy groups were located at C-6 and C-4′ respectively. A methoxy group located at C-7 was supported by the HMBC correlation of methoxy proton signal (δ_H 3.97 s) with C-7. The observation of a HMBC correlation of H-5 with C-4 suggested the methoxycarbonyl group should be located at C-8. Accordingly, the structure of 4',6-dihydroxy-7-methoxy-8-methoxycarbonylflavone (**3**) was established.

Since certain of the flavonoid derivatives exhibit potential anti-tobacco mosaic virus (anti-TMV) activities,[18-21] compounds **1** and **2** were tested for their anti-tobacco mosaic virus activity. The anti-TMV activities were tested using the half-leaf method.[22] Ningnanmycin (2% water solution), a commercial product for plant disease in China, was used as a positive control. The results showed that compounds **1**-**3** exhibited inhibition rates (%) of 18.4, 22.1 and 16.4, respectively.

EXPERIMENTAL

General. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. A Tenor 27 spectrophotometer was used for scanning IR spectroscopy with KBr pellets. 1D and 2D NMR spectra were recorded on DRX-500 spectrometers with TMS as internal standard, and the chemical shifts (δ) were expressed in ppm. HRESIMS was performed on an API QSTAR time-of-flight spectrometer and a VG Autospec-3000 spectrometer, respectively. Preparative HPLC was performed on a Shimadzu LC-8A preparative liquid chromatograph with a ZORBAX PrepHT GF (21.2 mm × 25 cm, 7 μm) column or a Venusil MP C_{18} (20 mm × 25 cm, 5 μm) column. Column chromatography was performed with Si gel (200–300 mesh, Qing-dao Marine Chemical, Inc., Qingdao, China). The fractions were monitored by TLC, and spots were visualized by heating Si gel plates sprayed with 5% H_2SO_4 in EtOH.

Plant material. The tobacco leaves were collected in Yuxi Prefecture, Yunnan Province, People's Republic of China, in September 2013. The identification of the plant material was verified by Prof. Chen Y. J (Yunnan University of Nationalities).

Extraction and Isolation. The air-dried and powdered tobacco leaves (4.2 kg) were extracted three times with 70% aqueous MeOH (3 × 5.0 L) at room temperature and filtered to yield a filtrate, which was successively evaporated under reduced pressure to obtained a crude extract (410 g). This crude extract was applied to Si gel (200-300 mesh) column chromatography eluting with a $CHCl_3$-MeOH gradient system (20:1, 9:1, 8:2, 7:3, 6:4, 5:5) to give six fractions A–F. The separation of fraction B (28.3 g) was subjected to Si gel column chromatography eluting with $CHCl_3$-$(Me)_2CO$ and then run on preparative HPLC (50-55% MeOH-H_2O, flow rate 12 mL/min) to yield compounds **1** (11.8 mg), **2** (12.6 mg), and **3** (14.5 mg). Fraction C (24.6 g) was subjected to Si gel column chromatography eluting with $CHCl_3$-$(Me)_2CO$ and then run on preparative HPLC (40-45% MeOH-H_2O, flow rate 12 mL/min) to yield compounds **4** (11.7 mg) and **5** (15.0 mg).

Anti-TMV Assays. The anti-TMV activities were tested using the half-leaf method,[22] and Ningnanmycin (2% water solution), a commercial product for plant disease in China, was used as a positive control.

8-Formyl-4'-hydroxy-7-methoxy-6-methylflavone (1): $C_{18}H_{14}O_5$, orange-yellow gum; UV (MeOH), λ_{max} (log ε) 210 (4.28), 255 (3.89), 287 (3.48), 368 (3.67) nm; IR (KBr) ν_{max} 3460, 1685, 1655, 1618, 1534, 1472, 1455, 1126, 1074, 985, 862 cm^{-1}; ^1H NMR and ^{13}C NMR data (C_5D_5N, 500 and 125 MHz) see Table 1; HRESIMS *m/z* 333.0732 $[M+Na]^+$ (calcd for $C_{18}H_{14}NaO_5$, 333.0739).

8-Formyl-4',7-dimethoxy-6-methylflavone (2): $C_{19}H_{16}O_5$, orange-yellow gum; UV (MeOH), λ_{max} (log ε) 210 (4.28), 255 (3.89), 287 (3.48), 368 (3.67) nm; IR (KBr) ν_{max} 3452, 1689, 1658, 1615, 1546, 1489, 1448, 1134, 1078, 986, 874 cm^{-1}; ^1H NMR and ^{13}C NMR data (C_5D_5N, 500 and 125 MHz) see Table 1; HRESIMS *m/z* 347.0888 $[M+Na]^+$ (calcd for $C_{19}H_{16}NaO_5$, 347.0895).

4',6-Dihydroxy-7-methoxy-8-methoxycarbonylflavone (3): $C_{18}H_{14}O_7$, orange gum; UV (MeOH), λ_{max} (log ε) 210 (4.28), 258 (3.86), 372 (3.74), nm; IR (KBr) ν_{max} 3418, 1708, 1659, 1612, 1547, 1483, 1446, 1292, 1165, 1058, 864, 785 cm^{-1}; ^{13}C NMR and ^1H NMR data (in C_5D_5N, 500 and 125 MHz) see Table 1; HRESIMS *m/z* 365.0637 $[M+Na]^+$ (calcd for $C_{18}H_{14}NaO_7$, 365.0637).

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