HETEROCYCLES, Vol. 91, No. 6, 2015, pp. 1198 - 1203. © 2015 The Japan Institute of Heterocyclic Chemistry
Received, 11th February, 2015, Accepted, 18th May, Published online, 28th May, 2015
DOI: 10.3987/COM-15-13190

FLAVONOIDS FROM THE LEAVES OF SUN CURED TOBACCO AND THEIR ANTI-TOBACCO MOSAIC VIRUS ACTIVITY

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Abstract — Two new flavonoids, 4',6-dihydroxy-7,8-dimethoxyisoflavone (**1**), 8-acetyl-4'-hydroxy-7-methoxy-6-methylflavone (**2**), together with four known flavonoids (**3**-**6**) were isolated from the leaves of Yunnan local sun cured tobacco. Their structures were determined by means of HRESIMS and extensive 1D and 2D NMR spectroscopic studies. Compounds **1**-**6** were tested for their anti-tobacco mosaic virus (anti-TMV) activities. Compound **2** showed moderate anti-TMV activity with inhibition rate of 35.4%. This rate is slightly stronger than the positive control. The other compounds also showed potential anti-TMV activity with inhibition rates in the ranges of 18.5-22.3%, 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 it contains 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,[13,14] phenylpropanoids,[15,16] chromanones,[17,18] biphenyls,[19] phenolic amides,[20] isocoumarins,[21] and the homologous, were isolated from this plant. Therefore, the multipurpose utilization of this palnts is an interesting topical and attracts more and more attentions. Motivated by search for more bioactive metabolites from this plant, an investigation on the chemical constituents of the leaves of Yunnan local sun cured tobacco (a variant of *N. tabacum*) was carried out. As a result, two new (**1** and **2**) and four known flavonoids (**3**-**6**) were isolated from this plant. In addition, the anti-tobacco mosaic virus (anti-TMV) activities of compounds **1**-**6** were

evaluated. This article deals with the isolation, structural elucidation and biological activities of these flavonoids.

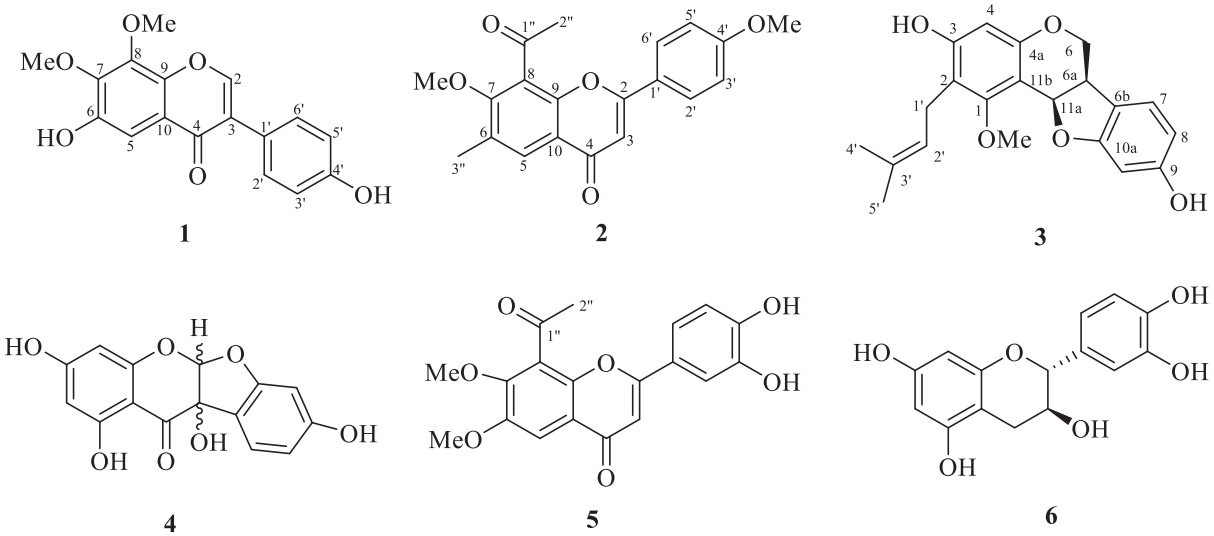

Figure 1. Isoflavones from the leaves of N. tabacum

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 two new flavonoids, 4',6-dihydroxy-7,8-dimethoxyisoflavone (**1**), 8-acetyl-4'-hydroxy-7-methoxy-6-methylflavone (**2**), and four known flavonoids (**3**-**6**). The structures of the compounds **1**-**6** were as shown in Figure 1, and the ^{1}H and ^{13}C NMR data of **1** and **2** were listed in Table 1. The known compounds, compared with literature, were identified as licoisoflavone (**3**),[22] 3,5,7,4'-tetrahydroxy-coumaronochromone (**4**),[23] rugosaflavonoid B (**5**),[24] and catechin (**6**).[25]

Compound **1** was obtained as an orange-yellow gum. The molecular formula of $C_{17}H_{14}O_6$ was determined from the HRESIMS spectra showing the sodiated molecular ion at *m/z* 337.0695 $[M+Na]^+$ (calcd 337.0688), suggesting 11 degrees of unsaturation. The UV spectrum showed absorption maxima at 210, 246, 315 and 350 nm, and the IR spectrum showed absorption bands at 3390, 1652, 1608, 1560, and 1432 cm^{-1}, indicating the presence of hydroxy group, carbonyl group, and aromatic ring. The ^{1}H and ^{13}C NMR spectrum of **1** (Table 1) along with analysis of the DEPT spectra displayed 17 carbon signals and 14 proton signals, respectively, corresponding to 4′,6,7,8-tetrasubstituted isoflavones nucleus (Table 1, C-1 – C-10 and C-1′–C-6′; H-5, H-8, H-2′, 6′, and H-2′, 6′),[25] two methoxy groups (δ_C 61.0, q and 56.0, q; δ_H 3.81, s and 3.86, s), and two phenolic hydroxy proton (δ_H 11.04, s and 10.85, s). The HMBC correlations (Figure 2) of H-2 (δ_H 7.94, s) with C-9 (δ_C 143.6), C-3 (δ_C 124.3), C-4 (δ_C 176.0), and C-1′ (δ_C 125.0), of H-2, 6 (δ_H 7.73) with C-3 (δ_C 124.3) also suggested that **1**

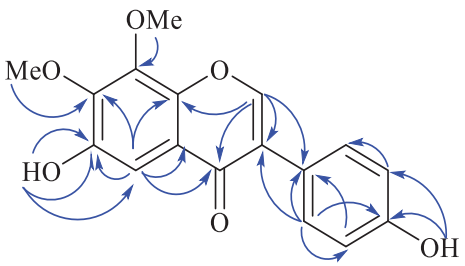

Figure 2. key HMBC (↷) correlations of **1**

should be an isoflavone.[25] The HMBC correlations of two methoxy protons (δ_H 3.81 and 3.86) with and C-7 (δ_C 148.8) and C-8 (δ_C 146.0) concluded the linkage of the two methoxy groups at C-7 and C-8, respectively. The locations of two phenolic hydroxy groups were assigned to C-6 and C-4′ positions on the basis of HMBC correlations of one phenolic hydroxy proton signal (δ_H 11.04) with C-5 (δ_C 109.8), C-6 (δ_C 142.2) and C-7 (δ_C 148.8), and of the other phenolic hydroxy proton signal (δ_H 10.85) with C-3′, 5′ (δ_C 116.7) and C-4′ (δ_C 157.5). The typical proton signals of H-5 (δ_H 6.75), H-2′, 6′ [δ_H 7.73 (d, J = 8.8 Hz)], and H-3′, 5′ [(δ_H 6.81 (d, J = 8.8Hz)) also supported this substituents pattern. Thus, the structure of **1** was established as 4',6-dihydroxy-7,8-dimethoxyisoflavone.

Compound **2** was obtained as an orange-yellow gum. A molecular formula $C_{19}H_{16}O_5$ was assigned from HRESIMS (*m/z*: 347.0890 $[M+Na]^+$, calcd 347.0895). The ^1H and ^{13}C NMR data of **2** (Table 1) displayed 19 carbon and 16 proton signals, corresponding to a flavonoid nucleus[24] (Table 1, C-1–C-10 and C-1′–C-6′; H-5, H-8, H-2′, 6′, and H-2′, 6′), one acetyl group (δ_C 198.6, 31.0; δ_H 2.56), one methoxy groups (δ_C 61.0, δ_H 3.82), one methyl group (δ_C 16.9 q; δ_H 2.35, s), and one phenolic hydroxy protons (δ_H 10.98). The typical protons signals at [δ_H 7.73, d (8.6 Hz) 2H; δ_H 6.82, d (8.8 Hz) 2H] revealed 4′-mono-substitution on ring B, and the HMBC correlation methoxy proton signal (δ_H 3.86) with C-7 (δ_C 165.9) suggested the methoxy group located at C-7. The methyl group located at C-6 was supported by the HMBC correlations of methyl proton signal (δ_H 2.35) with C-5 (δ_C 134.9), C-6 (δ_C 120.3), and C-7 (δ_C 165.9). The HMBC correlations of phenolic hydroxy proton (δ_H 10.98) with C-3', 5' (δ_C 116.9), C-4' (δ_C 157.4) suggested the location of this hydroxy group at C-4'. The HMBC correlation of the methyl proton signal (δ_H 2.56) with C-8 (δ_C 112.2) suggested the presence of an acetyl group at C-8. Thus, the structure of 8-acetyl-4'- hydroxy-7-methoxy-6-methylflavone (**2**) was established.

Table 1. ^1H NMR and ^{13}C NMR Data (in C_5D_5N) of compounds **1** and **2**

No.	Compound **1**		Compound **2**	
	δ_C (m)	δ_H (m, J, Hz)	δ_C (m)	δ_H (m, J, Hz)
2	122.0 d	7.94 s	163.8 s	
3	124.3 s		106.2 d	6.57 s
4	176.0 s		176.4 s	
5	109.8 d	6.75 s	134.9 d	7.38 s
6	142.2 s		120.3 s	
7	148.8 s		165.9 s	
8	146.0 s		112.2 s	
9	143.6 s		152.9 s	
10	118.9 s		119.0 s	
1′	125.0 s		122.8 s	
2′,6′	131.6 d	7.73 (d) 8.8	131.1 d	7.73 (d) 8.6
3′,5′	116.7 d	6.83 (d) 8.8	116.9 d	6.82 (d) 8.6
4′	157.5 s		157.4 s	
1″			198.6 s	
2″			31.0 q	2.56 s
3″			16.9 q	2.35 s
-OMe-7	61.0 q	3.81 s	61.0 q	3.86 s
-OMe-8	61.1 q	3.86 s		
Ar-OH-6		11.04 s		
Ar-OH-4′		10.85 s		10.98 s

Since certain of the flavonoids derivatives exhibit potential anti-tobacco mosaic virus (anti-TMV) activities,[13,14,26,27] compounds **1-6** were tested for their anti-TMV activity. The anti-TMV activity were tested using the half-leaf method.[28] Ningnanmycin (a commercial product for plant disease in China), was used as a positive control. Their antiviral inhibition rates at the concentration of 20 μM were listed in Table 2. Compound **2** showed moderate anti-TMV activity with inhibition rate of 35.4%. This rate is slightly stronger than the positive control. The other compounds also showed potential anti-TMV activity with inhibition rates in the ranges of 18.5-22.3%, respectively.

Table 2. TMV Infection Inhibition Activities of **1-6**

Compounds	Inhibition rate (%)	Compounds	Inhibition rates (%)
1	18.5 ± 3.2	**5**	19.3 ± 2.6
2	35.4 ± 3.5	**6**	22.3 ± 2.8
3	22.8 ± 2.8	ningnanmycin	33.4 ± 3.2
4	20.1 ± 2.9		

All results are expressed as mean ± SD; n = 3 for all groups.

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 sun cured tobacco leaves (Dali Leqiu tobacco, a variety of *Nicotiana tabacum*) were collected in Dali Prefecture, Yunnan Province, People's Republic of China, in September 2013. The identification of the plant material was verified by Prof. Yang. H. W (School of Tobacco, Yunnan Agriculture University).

Extraction and Isolation. The air-dried and powdered tobacco leaves (6.4 kg) were extracted three times with 70% aqueous MeOH (3 ×8.0 L) at room temperature and filtered to yield a filtrate, which was successively evaporated under reduced pressure to obtained a crude extract (522 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 C (8:2, 36.5 g) was subjected to Si gel column chromatography eluting with $CHCl_3$-$(Me)_2CO$ and then run on preparative HPLC (45-50% MeOH-H_2O, flow rate 12 mL/min) to yield compounds **1** (12.2 mg), **2** (10.9 mg), **3** (13.4 mg), and **5** (16.8 mg). Fraction C (18.2 g) was subjected to Si gel column chromatography eluting with $CHCl_3$- $(Me)_2CO$ and then run on preparative HPLC (38-42% MeOH-H_2O, flow rate 12 mL/min) to yield compounds **4** (16.3 mg) and **6** (15.5 mg).

Anti-TMV Assays. The anti-TMV activities were tested using the half-leaf method,[28] and Ningnanmycin (2% water solution), a commercial product for plant disease in China, was used as a positive control.

4',6-Dihydroxy-7,8-dimethoxyisoflavone (1): Obtained as orange-yellow gum; UV (MeOH), λ_{max} (log ε) 350 (3.62), 315 (3.31), 246 (3.78), 210 (4.26) nm; IR (KBr) ν_{max} 3390, 1652, 1608, 1560, 1506, 1432, 1280, 1128, 1064, 958, 832 cm^{-1}; ^1H NMR and ^{13}C NMR data (C_5D_5N, 500 and 125 MHz, respectively), Table 1; ESIMS *m/z* 337; HRESIMS (positive ion mode) *m/z* 337.0695 $[M+Na]^+$ (calcd 337.0688 for $C_{17}H_{14}NaO_6$).

8-Acetyl-4'-hydroxy-7-methoxy-6-methylflavone (2): Obtained as orange-yellow gum; UV (MeOH), λ_{max} (log ε) 364 (3.68), 268 (3.57), 210 (4.18) nm; IR (KBr) ν_{max} 3450, 1698, 1652, 1610, 1531, 1462, 1377, 1258, 1173, 1082, 1039, 956, 872 cm^{-1}; ^1H NMR and ^{13}C NMR data (C_5D_5N, 500 and 125 MHz, respectively), Table 1; ESIMS *m/z* 347 $[M+Ma]^+$; HRESIMS *m/z* 347.0890 $[M+Na]^+$ (347.0895 for $C_{19}H_{16}NaO_5$)

ACKNOWLEDGEMENTS

This research was supported by the National Natural Science Foundation of China (No. 31360081), the Applied Fundamental Foundation of Yunnan Province (No. 2013FB097), and the Basic Research Foundation of Yunnan Tobacco Industry Co. Ltd (2012JC01).

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