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THREE NEW FURAN-2-CARBOXYLIC ACID DERIVATIVES FROM THE STEM BARK OF *NICOTIANA TABACUM* AND THEIR BIOACTIVITY

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Abstract – Three new (1-3), together with three known (4-6) furan-2-carboxylic acid derivatives were isolated from the stem bark of *Nicotiana tabacum*. Their structures were determined by means of HRESIMS and extensive 1D and 2D NMR spectroscopic studies. Compounds 1~6 were tested for their anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) activity. The results revealed that compounds 1-6 showed good inhibition with IZD of 12.8±2.3, 13.5±1.8, 14.3 ±2.2, 15.1 ±2.0, and 14.7±2.2 mm. Compounds 1-6 were also tested for the antioxidant activity, and they showed notable antioxidant activity with an IC_{50} value of 3.86, 4.05, 3.62, 4.11, 3.57, and 3.64 μg/mL, respectively.

Nicotiana tabacum is a stout annual plant of the Nicotiana spices, Solanaceae family. As an important economic crop had been widely grown in world, its leaves are used as a raw material for the tobacco industry.[1] In addition, it also can be used as insecticide, anesthetic, diaphoretic, sedative, and emetic agents in Chinese folklore medicine.[2,3] The investigating its chemical constituents and their bioactivities is very important to provide valuable clues for photochemistry.[4,5] In previous literatures, many new bioactive compounds, diterpenoids[6,7] sesquiterpenoids,[8-10] flavonoids,[11-13] alkaloids,[14-16] coumarins,[15,16] furans,[17,18] and the like, had been identified from this plant.

Furan derivatives are an important class of secondary metabolites containing a five membered ring and holding oxygen as heteroatoms. This group of compounds can be found naturally in plants, algae and microbe.[19,20] The structure-activity relationships for furan derivatives have generated wide interest among

medicinal chemists, due to they possess a diverse set of properties that allow a wide range of applications in medicine.[21,22] In continuing efforts to utilize *N. tabacum* and identify bioactive natural products, the phytochemistry investigation of the stem barks of Yunyan-300 (a new variety of *N. tabacum* widely cultivated in China) led to the isolation of three new (**1**-**3**) and three known (**4**-**6**) furan-2-carboxylic acid derivatives. The structures of the isolated components were characterized by comprehensive spectroscopic analyses and by comparison with published data. Compounds **1**-**6** were also tested for their anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) and antioxidant activity.

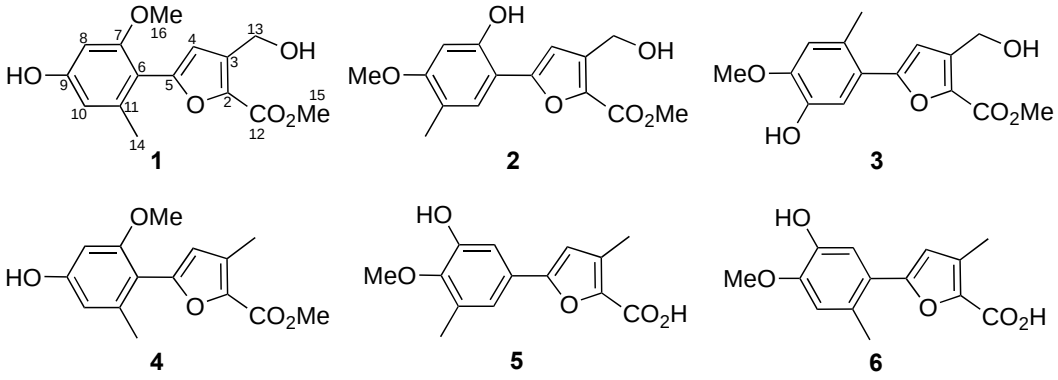

Figure 1. Furan-2-carboxylic acids from the stem bark of *Nicotiana tabacum*

The air-dried and powdered stem bark of *Nicotiana tabacum* (5.2 kg) was extracted with 70% aqueous Me_2CO (3×12 L) under reflux for three times (4 h each) and concentrated under reduced pressure to yield a crude extract, which was suspended in water and partitioned with EtOAc. The EtOAc extract (322 g) was subjected repeatedly to column chromatography on silica gel, Sephadex LH-20, RP-18 and preparative HPLC to afford three new furan-2-carboxylic acid derivatives, methyl 5-(4-hydroxy-2-methoxy-6-methylphenyl)-3-(hydroxymethyl)furan-2-carboxylate (**1**), methyl 5-(2-hydroxy-4-methoxy-5-methylphenyl)-3-(hydroxymethyl)furan-2-carboxylate (**2**), and methyl 5-(5-hydroxy-4-methoxy-2-methylphenyl)-3-(hydroxymethyl)furan-2-carboxylate (**3**), together with three known furan-2-carboxylic acid derivatives (**4**-**6**). The structures of the compounds **1**-**6** were shown in Figure 1, and the ^1H and ^{13}C NMR data of the compounds **1**-**3** were listed in Table 1. The known compounds, compared with literature data, were identified as: methyl 5-(4-hydroxy-2-methoxy-6-methylphenyl)-3-methylfuran-2-carboxylate (**4**),[18] 5-(3-hydroxy-4-methoxy-5-methylphenyl)-3-methylfuran-2-carboxylic acid (**5**),[18] and 5-(4-hydroxy-5-methoxy-2-methylphenyl)-3-methylfuran-2-carboxylic acid (**6**).[17]

Compound **1** was obtained as a pale-yellow gum, and has slight ester flavour. Its (+)HRESIMS gave a quasimolecular ion at *m/z* 315.0840 $[M+Na]^+$. These data, established the molecular formula of **1** as $C_{15}H_{16}O_6$, with eight degree of unsaturation. The IR spectrum of **1** exhibited absorption bands for hydroxy (3398 cm^{-1}), ester carbonyl (1716 cm^{-1}) and aromatic functionality (1614, 1540, and 1465 cm^{-1}). Its UV spectrum showed the maximum absorption at 316 and 250 nm was also supported the existences of aromatic functionality. The ^1H and ^{13}C NMR spectra data of **1** (Table 1) displayed signals for all 15 carbons and 16 protons, including a 1,2,4,6-tetrasubstituted benzene ring (C-6~C-11, H-8 and H-10), a 2,3,5-trisubstituted furan ring (C-2~C-5, H-4),[18] one methoxycarbonyl group (C-12 and C-15, H_3-15),[18] one hydroxymethyl group (C-13, H_2-13), one methyl group (C-14, H_3-14), one methoxy group (C-16 and H_3-16), and one phenolic hydroxy group (δ_H 10.61).

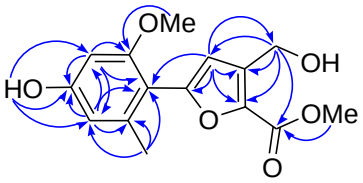

Figure 2. Key HMBC (↷) correlations of **1**

Table 1. ^1H NMR and ^{13}C NMR Data of compounds **1-3 (**in $CDCl_3$, 500 and 125 MHz**)**

No.	Compound **1**		Compound **2**		Compound **3**	
	δ_C (m)	δ_H (m, *J*, Hz)	δ_C (m)	δ_H (m, *J*, Hz)		
2	160.6 s		160.9 s		160.4 s	
3	105.9 s		106.6 s		106.5 s	
4	108.1 d	6.22 s	108.7 d	6.25 s	108.5 d	6.24 s
5	155.7 s		155.7 s		155.5 s	
6	112.1 s		110.5 s		125.6 s	
7	159.5 s		154.1 s		131.3 s	
8	100.9 d	6.34 s	103.3 d	6.47 s	116.6 d	6.67 s
9	156.8 s		161.8 s		153.1 s	
10	108.8 d	6.41	120.6 s		146.7 s	
11	137.5 s		128.7 d	7.17 s	114.5 d	6.98 s
12	162.0 s		162.2 s		162.6 s	
13	54.3 t	4.33 s	55.1 t	4.35 s	54.6 t	4.34 s
14	20.5 q	2.31 s	18.9 q	2.33 s	20.9 q	2.32 s
15	52.2 q	4.09 s	52.6 q	4.09 s	52.5 q	4.09 s
16	56.4 q	3.82 s	56.4 q	3.80 s	56.3 q	3.81 s
Ar-OH		10.61 s		10.78 s		10.83 s
13-OH		4.97 s		4.99 s		4.98 s

By analysis of its HMBC correlations, the existence of the 1,2,4,6-tetrasubstituted benzene ring was supported by the HMBC correlations from H-8 to C-6, C-7, C-9, and C-10, from H-10 to C-6, C-8, C-9, and C-11. The existence of 2,3,5-trisubstituted furan ring was supported by HMBC correlations from H-4 to C-2, C-3, and C-5, and the existence of methoxycarbonyl group was also supported by the HMBC

correlations from CH_3-15 to C-12. In addition, the HMBC correlation from H-4 to C-6 supported that the benzene ring and furan ring was connected from C-6 and C-5. The above evidences suggested that compounds **1** should be a methyl 5-phenylfuran-2-carboxylate.[17,18]

Since the skeleton was determined, the positions of substituents (one hydroxymethyl, one methyl, one methoxy, and one phenolic hydroxy group) also can be determined by further analysis of its HMBC data (Figure. 2). The hydroxymethyl group located at C-3 was supported by the HMBC correlations from H_2-13 to C-2, C-3, and C-4, and from H-4 to C-13. The methyl group located at C-11 was supported by the HMBC correlations from H_3-14 to C-6, C-10, and C-11, from H-10 to C-14. The methoxy group located at C-7 was supported by the HMBC correlation from the H_3-16 to C-7. Finally, the HMBC correlations from the phenolic hydroxy proton (δ_H 10.61) to C-8, C-9, and C-10 indicated that the phenolic hydroxy group located at C-9. Thus, the structure of **1** was established, and gave the systematic name of methyl 5-(4-hydroxy-2-methoxy-6-methylphenyl)-3-(hydroxymethyl)furan-2-carboxylate.

Methyl 5-(2-hydroxy-4-methoxy-5-methylphenyl)-3-(hydroxymethyl)furan-2-carboxylate (**2**) was also obtained as a pale-yellow gum, and has slight ester flavour. It showed a quasimolecular ion at *m/z* 315.0853 $[M+Na]^+$ in the HRESIMS (calcd *m/z* 315.0845), corresponding to the molecular formula $C_{15}H_{16}O_6$. The ^1H and ^{13}C NMR spectra of **2** were highly similar to those of **1**. The chemical shift differences resulted from the substituents position variation in compound **2**. By further analysis of its HMBC correlations, the HMBC correlations from H_3-14 to C-9, C-10, and C-11, from H-11 to C-14 supported the methyl group located at C-10, the HMBC correlations from H_2-13 to C-2, C-3, and C-4, and from H-4 to C-13 supported the hydroxymethyl group located at C-3, the HMBC correlation from the H_3-16 to C-9 supported the methoxy group located at C-9. Finally, the phenolic hydroxy group located at C-7 was supported by the HMBC correlations from the phenolic hydroxy proton (δ_H 10.78) to C-6, C-7, and C-8. The structure of **2** was therefore defined.

Compound **3**, a pale-yellow gum with slight ester flavour, gave a $[M+Na]^+$ peak at *m/z* 315.0849 in its HRESIMS spectrum, was assigned with a molecular formula of $C_{15}H_{16}O_6$. In addition to the substituents position variation on benzene ring, the NMR data (Table 1) of **3** were also highly similar to those of **1**. By further analysis of its HMBC correlations, the hydroxymethyl group located at C-3 was supported by the HMBC correlations from H_2-13 to C-2, C-3, and C-4, from H-4 to C-13, the methyl group located at C-7 was supported by the HMBC correlations from the H_3-14 to C-6, C-7, and C-8, from H-8 to C-14, the methoxy group located at C-9 was supported by the HMBC correlation of the methoxy resonance (δ_H 3.81) to C-9. Finally, the HMBC correlations from the phenolic hydroxy proton (δ_H 10.83) to C-9, C-10,

and C-11 indicated that the phenolic hydroxy group located at C-10. Therefore, the structure of methyl 5-(5-hydroxy-4-methoxy-2-methylphenyl)-3-(hydroxymethyl)furan-2-carboxylate (**3**) was established.

Since certain of the furan derivatives exhibit potential anti-TMV activity,[23,24] compounds **1**-**6** were tested for their anti-MRSA and antioxidant activities.

The anti-MRSA activity were screened for according to arbitrary criterion [25] with diameter of inhibition zone (IZD) as follow: very weak inhibition (with IZD of 6-8 mm), weak inhibition (with IZD of 8-12 mm), good inhibition (with IZD of 12-16 mm), and strong inhibition (with IZD of >16 mm) activities respectively. The IZD of the positive control was 32 mm and the negative control to zero. The results revealed that compounds **1**-**6** showed good inhibition with IZD of 12.8±2.3, 13.5±1.8, 14.3 ±2.2, 15.1 ±2.0, and 14.7±2.2 mm, respectively.

The antioxidant activity was also tested by the detection of the oxidative products with the 2',7'-dichlorofluorescin diacetate (DCFH) method reported previously.[26] The results revealed that compounds **1**-**6** shows notable antioxidant activity with an IC_{50} value of 3.86, 4.05, 3.62, 4.11, 3.57, and 3.64 μg/mL, respectively.

EXPERIMENTAL

General Experimental Procedures. UV spectra were obtained using a Shimadzu UV-1900 spectrophotometer. A Bio-Rad FTS185 spectrophotometer was used for scanning IR spectra. ^{1}H, ^{13}C, and 2D-NMR spectroscopic data were recorded on a DRX-500 NMR spectrometer with TMS as internal standard. ESIMS and HRESIMS analyses were measured on Agilent 1290 UPLC/6540 Q-TOF mass spectrometer. Chemical shifts (δ) are expressed in ppm with reference to the TMS signal. Semi-preparative HPLC was performed on an Agilent 1260 preparative liquid chromatograph with Zorbax PrepHT GF (2.12 mm × 25 cm) or Venusil MP C_{18} (2.0 mm × 25 cm) columns. Column chromatography was performed using silica gel (200-300 mesh, Qing-dao Marine Chemical, Inc., Qingdao, China), Lichroprep RP-18 gel (40 - 63 μm, Merck, Darmstadt, Germany), Sephadex LH-20 (Sigma-Aldrich, Inc, USA), or MCI gel (75 - 150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan). Column fractions were monitored by TLC visualized by spraying with 5% H_2SO_4 in EtOH and heating.

Plant Material. Stem barks of Yunyan-300 (a new variety of *N. tabacum* with high resistance to black shank disease widely cultivated in China) were in collected in Yuxi prefecture, Yunnan Province, P. R.

China, in September 2017. The identification of the plant material was verified by Prof. Chen Y. J. (Yunnan University of Nationalities). A voucher specimen (Ynni-17-09-120) has been deposited in Key Laboratory of Chemistry in Ethnic Medicinal Resources, Yunnan Minzu University, P. R. China.

Extraction and Isolation. The air-dried and powdered roots and stems of sun cured tobacco (5.2 kg) was extracted with 70% aqueous Me_2CO (3×12 L) under reflux for three times (4 h each), and concentrated under reduced pressure to yield a crude extract, which was suspended in water and partitioned with EtOAc. The EtOAc extract (322 g) was applied to silica gel (200–300 mesh) column chromatography, eluting with $CHCl_3$/MeOH gradient system (10:0, 9:1, 8:2, 7:3, 6:4, 5:5) to give six fractions A-F. Further separation of fraction C (8:2, 48.6 g) by silica gel column chromatography, eluted with $CHCl_3$/Me_2CO (9:1-2:1), yielded mixtures C1–C6. Subfraction C3 (7:3, 6.35 g) was subjected to silica gel column chromatography using petroleum ether/Me_2CO, and then semi-preparative HPLC (44% MeOH/H_2O, flow rate 20 mL/min) to give **1** (15.4 mg), **2** (16.2 mg), **3** (14.8 mg), and **4** (17.5 mg). Subfraction C5 (4:6, 6.82 g) was loaded on another silica gel column using $CHCl_3$/MeOH elution, and then separated semi-preparative HPLC (34% MeOH-H_2O, flow rate 20 mL/min) to afford **5** (18.6 mg), and **6** (16.9 mg).

Anti-MRSA Agar Disc Diffusion Assay. The MRSA strain ZR11 was clinical isolated from infectious samples of critically ill patients in the Clinical Laboratory of the First People's Hospital of Yunnan Province, and confirmed by standard cefoxitin disk diffusion test following CLSI standard procedures.[25] The anti-MRSA activity of the compounds was evaluated via the disc diffusion method. The ZR11 strain was inoculated in Müeller Hinton Broth and were incubated at 37 ºC for 24 h. The turbidity of bacterial suspension was adjusted to 0.5 McFarland standard which equals to 1.5×10^8 colony-forming units (CFU)/mL. Sterile filter paper discs (6 mm) were impregnated with 20 μL (50 μg) of each compound and placed on inoculated Müeller Hinton agar containing bacterial suspension which adjusted to 0.5 McFarland standard .The commercial available discs containing 30 μg Vancomycin were used as positive control whereas discs without samples (5% DMSO) acted as negative control. The inhibition zones including the diameter of the disc (mm) were measured and compared after incubation at 37 ºC for 24 h. The tests were carried out in triplicate for each sample.

Antioxidant Assay. The antioxidant activity was tested by 2',7'-dichlorofluorescin diacetate (DCFH) method reported previously.[26] Myelomonocytic HL-60 cells (1×10^6 cells/mL, ATCC) were suspended in RPMI 1640 medium with 10% FBS and antibiotics at 37 °C in 5% CO_2: 95% air. 125 μL of the cell

suspension was added to each well of a 96-well plate. After treatment with a different concentration of the test material for 30 min, the cells were stimulated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma) for 30 min. Then the cells were incubated for 15 min after the addition of 5 μg/mL DCFH-DA (Molecular Probes). DCFH-DA is a non-fluorescent probe that diffuses into cells. Cytoplasmic esterases hydrolyse DCFH-DA to 2',7'-dichlorofluorescin (DCFH), and the reactive oxygen species (ROS) generated within HL-60 cells oxidize DCFH to the fluorescent dye 2',7'-dichlorofluorescein (DCF). The ability of the test materials to inhibit exogenous cytoplasmic ROS-catalysed oxidation of DCFH in HL-60 cells was measured by PMA tred control incubations with and without the test materials. The levels of DCF were measured using a CytoFluor 2350, fluorescence measurement system (Millipore) with an excitation wavelength at 485 nm (bandwidth 20 nm) and emission at 530 nm (band width 25 nm).

Methyl 5-(4-hydroxy-2-methoxy-6-methylphenyl)-3-(hydroxymethyl)furan-2-carboxylate (1): $C_{15}H_{16}O_6$, obtained as pale-yellow gum, and has slight ester flavour; UV (MeOH), λ_{max} (log ε) 316 (2.85), 250 (3.46), 210 (3.72) nm; IR (KBr) λ_{max} 3398, 2941, 1716, 1614, 1540, 1465, 1246, 1163, 1052, 952, 853 cm^{-1}; ^{1}H NMR and ^{13}C NMR data ($CDCl_3$, 500 and 125 MHz, respectively), Table 1; ESIMS (positive ion mode) *m/z* 315 $[M+Na]^+$; HRESIMS (postive ion mode) *m/z* 315.0840 $[M+Na]^+$ (calcd 315.0846 for $C_{15}H_{16}NaO_6$).

Methyl 5-(2-hydroxy-4-methoxy-5-methylphenyl)-3-(hydroxymethyl)furan-2-carboxylate (2): $C_{15}H_{16}O_6$, obtained as pale-yellow gum, and has slight ester flavour; UV (MeOH), λ_{max} (log ε) 313 (2.89), 248 (3.49), 210 (3.69) nm; IR (KBr) λ_{max} 3392, 2946, 1714, 1612, 1548, 1469, 1242, 1157, 1056, 936, 864 cm^{-1}; ^{1}H NMR and ^{13}C NMR data ($CDCl_3$, 500 and 125 MHz, respectively), Table 1; ESIMS (positive ion mode) *m/z* 315 $[M+Na]^+$; HRESIMS (positive ion mode) *m/z* 315.0853 $[M+Na]^+$ (calcd 315.0845 for $C_{15}H_{16}NaO_6$).

Methyl 5-(5-hydroxy-4-methoxy-2-methylphenyl)-3-(hydroxymethyl)furan-2-carboxylate (3): $C_{15}H_{16}O_6$, obtained as pale-yellow gum, and has slight ester flavour; UV (MeOH), λ_{max} (log ε) 316 (2.82), 252 (3.43), 210 (3.65) nm; IR (KBr) λ_{max} 3395, 2950, 1715, 1615, 1562, 1457, 1248, 1155, 1062, 920, 842 cm^{-1}; ^{1}H NMR and ^{13}C NMR data ($CDCl_3$, 500 and 125 MHz, respectively), Table 1; ESIMS (positive ion mode) *m/z* 315 $[M+Na]^+$; HRESIMS (positive ion mode) *m/z* 315.0849 $[M+Na]^+$ (calcd 315.0845 for $C_{15}H_{16}NaO_6$).

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