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A New Alkaloid Glycoside from the Stems of *Zanthoxylum dissitum* Hemsl.

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Abstract: A new alkaloid glycoside (**1**) and six known alkaloids (**2**–**7**) were isolated from the stems of *Zanthoxylum dissitum* Hemsl. The structure of compound **1** was elucidated by UV, IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopic analyses. All compounds obtained in this research were evaluated for their inhibitions against NO release from LPS-activated RAW264.7 macrophages. Compounds **1**-**3**, **6** and **7** showed significant inhibition activities with IC_{50} values of 26.12±0.81, 8.41±0.23, 13.75±0.54, 6.97±0.77, and 5.78±0.42 μM, respectively.

Keywords: *Zanthoxylum dissitum* Hemsl.; alkaloid; macrophages; NO.

1. Introduction

The genus *Zanthoxylum* belongs to the Rutaceae family, including more than 250 species worldwide, ranges from the subtropical to the tropic zones. Among all suchspecies, 45 are distributed in China. Some fruits of these species are well known spices, while some species are widely used as folk medicine [1,2]. In addition, part of plants belong to *Zanthoxylum* are economically important for food, timber industries and wood working [3,4].

Zanthoxy lumdissitum Hemsl. (*Z. dissitum*), a climbing or sprawling shrub woody vine in the forest and mountains, is a member of *Zanthoxylum* plants, which are found in the southwest of Shanxi, Guangxi and Hunan provinces [5]. The roots, stems and leaves of *Z. dissitum* have been used as Chinese folk medicine for women's disorders, pains in the loin, limbs and arthritis. Previous phytochemical studies have repoted that the stem extracts from *Z. dissitum* exhibit powerful anti-microbial and anti-tumor activities [6,7]. Some interesting secondary metabolites such as alkaloids with anti-inflammatory, anti-microbial, and anti-tumor activities have also beenfound in this genus [8,9]. To search for structurally unique and biologically natural components [10], we have isolated and

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identified compounds from the stems of *Z. dissitum*. During this process, a new alkaloid glycoside (**1**), along with six known alkaloids (**2**−**7**), were obtained (Figure 1). Herein, the process of isolation, structural determination, and biological detection of these compounds are described.

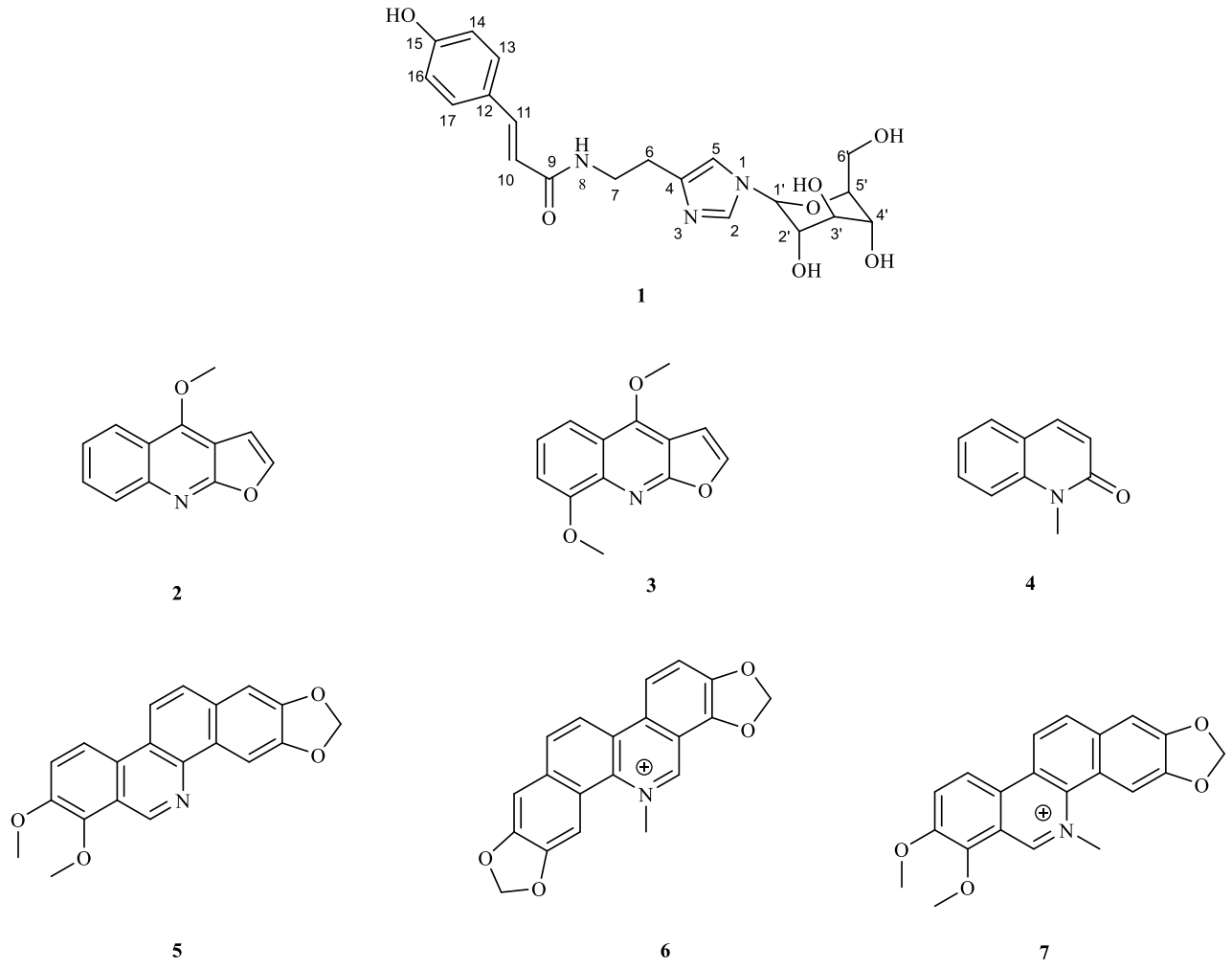

Figure 1. The structures of compounds **1**-**7**

2. Materials and Methods

2.1. General

A Bruker Avance III-600 spectrometer was used for NMR detections (Bruker, Shanghai, China). An Agilent Cary 3500 UV-visible spectrometer (Agilant, Santa Clara, CA, USA) and Bruker VERTEX 80/80v Fourier transform infrared spectrometer were used to detect UV and IR spectra of compound **1**, respectively. A Bruker Impact II mass spectrometer was used to obtain ESI-MS spectra. The MCI gel was purchased from Mitsubishi Chemical Holdings (Tokyo, Japan). Macroporous adsorption resin was purchased from Dalian Meilun Biotechnology (Dalian, China). Both column chromatography (CC) silica gel and thin layer chromatography (TLC) silica gel were purchased from Qingdao Haiyang Chemical (Qingdao, China). Sephadex LH-20 was purchased from Pharmacia (Rockville, MD, USA). All organic solvents used in this research were purchased from Tianjin Henxing Chemical Reagent (Tianjin, China).

Mouse mononuclear RAW264.7 macrophages were kindly provided by the Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). Consumables for cell experiments were purchased from NEST Biotech (Wuxi, China). Both high-glucose Dulbecco's-Modified eagle medium (DMEM)

and fetal calf serum were purchased from Gibco by Thermo Fisher Scientific (Waltham, MA, USA). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). NO detection kit was purchased from Beyotime Biotechnology (Shanghai, China).

2.2. Plant Material

The stems of *Zanthoxylum* dissitum were collected in September 2018 from Zhangjiajie, Hunan Province, China, and authenticated by Professor Bingmei Xiao, School of Pharmacy, Hunan University of Chinese Medicine. A voucher specimen (No. DMZ201809) was deposited with Hunan Provincial Engineering Technology Center of Standardization and Function of Chinese Herbal Medicine.

2.3. Extraction and Isolation

The air-dried stems of *Z. dissitum* Hemsl. (10 kg) were cut into small pieces and extracted three times with 70% ethanol by refluxing for 2 hours each time. After evaporation under reduced pressure, a dark-brown fluid extract (1.2 kg) was obtained. The extracts were suspended in H_2O and then successively extracted with $CHCl_3$, EtOAc, and n-BuOH.

The $CHCl_3$ portion (40.3 g) was subjected to silica CC and eluted with a gradient system from C_6H_{12}/$CHCl_3$ (10:1–0:1, V: V) to 100% MeOH with 10% increments. Based on the TLC detection, similar fractions were combined to obtain 11 fractions (C1–11). Fraction C8 (3.8 g) was applied to silica CC using a [C_6H_{12}/$CHCl_3$ (8:1) to $CHCl_3$/MeOH (1:1), v: v] wash to obtain six fractions (C8-1–C8-6). Fraction 8-4 (2.1 g) was loaded on a MCI gel and eluted with H_2O and 30%, 50%, 70%, 90% and 100% EtOH to obtain four parts (C8-4-1 to C8-4-4) and compound **2** (8.4 mg). Fraction C8-4-2 (1.1 g) was further repeatedly purified by Sephadex LH-20 CC [$CHCl_3$/MeOH (1:1), v:v] and preparative TLC [$CHCl_3$/MeOH (5:1), v: v] to obtain compounds **3** (8.5 mg) and **4** (9.6 mg). Fraction C10 (8.1 g) was also successively separated and purified by a MCI gel [MeOH/H_2O (0:100-100:0), v:v], then further purified by CC (C_6H_{12}/$CHCl_3$ 5:1, V:V) with 5% increment and Sephadex LH-20 [$CHCl_3$/MeOH (1:1), v:v], to obtain compound **5** (10.4 mg).

The *n*-BuOH portion (65.2 g) was suspended with H_2O to obtain two parts. The water soluble part was subjected to macroporous adsorption resin (AB-8) with a step-gradient of $H_2O$and 30%, 50%, 70%, 90% and 100% MeOH to yield four subfractions (B-1 to B-6). B-2 (35.3 g) was further purified by a silica gel column, using the gradient systems of $CHCl_3$/EtoAc (2:1, v:v) to 100% MeOH with a final MeOH/H_2O (75:15, v:v) wash to obtain compound **1** (36.8 mg). Compounds **6** (33.7 mg) and **7** (22.0mg) were obtained from B-4 (6.2 g) and B-5 (10.0 g), respectively.

2.4. Spectroscopic Data

Colorless needle crystal; UV (H_2O) λmax: 223, 298 nm; IR (KBr) max 3300, 2964, 1692, 1603, 1513, 1445, 832.14, 982.96 cm^{-1}; 1H NMR ($CDCl_3$, 600 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 150 MHz), see Table 1; HR-ESI-TOF-MS (positive ion model) *m/z* 420.1774 $[M+H]^+$, (calculated for $C_{20}H_{26}N_3O_7$, *m/z* 419.1693).

2.5. Determination of Inhibition NO Release

RAW264.7 cells were cultured in DMEM with 10% fetal calf serum in a humidified atmosphere of 5% CO_2 at 37 °C. Cells under logarithmic phase were suspended, and then plated in 96-well plates at 1×10^5 cells/well. The old culture medium was removed after the cells were incubated for 24h at 37°C. A total of 200 ng/mL LPS, combined with various concentrations of test samples, was added for intervening RAW264.7 cells. At 24 h after drug intervention, the cell culture medium in each well was collectedcentrifuged at 300×g for 10 min, and the liquid supernatant was use to test the concentrations of NO using an assay kit. Dexamethasone was used as a positive control. Each experiment was run in triplicate and averaged [11].

3. Results and Discussion

3.1. Structure Elucidation

Compound **1** had a colorless needle crystal form. The molecular composition was determined as $C_{20}H_{25}N_3O_7$, from the ESI-MS with a molecular ion peak found *m/z* 420.1774 $[M+H]^+$, (calculated for $C_{20}H_{26}N_3O_7$, *m/z* 419.1693). This molecule had 10 degrees of unsaturation. The UV spectrum showed the existences of a conjugated system (223 nm) and a carbonyl group (298 nm). The IR spectrum showed a functional hydroxyl group l (3300 cm^{-1}), para-substituted and conjugated benzene ring (2964, 1603, 1513, 1445, and 832 cm^{-1}), carbonyl (1692 cm^{-1}), and trans-double bond (982 cm^{-1}). In the ^{13}C NMR spectrum, it had resonated 20 carbons, which were classified as three methylenes, thirteen methines, and four quarternary carbons. A phenolic proton signal of δ: 9.82 (1H, s, H-1) was observed in the ^1H NMR spectrum. By reviewing the literature, this data was proved to be consistent with the hydroxyl signal in compounds containing 4-hydroxycinnamic acid amide [12]. There is a set of adjacently coupled proton signals of δ: 7.37 (2H, d, *J*=8.6 Hz) and 6.77 in the aromatic region (2H, d, *J*=8.5 Hz), combined with two strong carbon signals in the ^{13}C NMR spectrum (δ: 129.2 and 115.7), which indicated that the compound had para-disubstituted benzene ring. Peaks at δ: 7.31 (1H, d, *J*=15.7) and δ: 6.39 (1H, d, *J*=15.7 Hz) were related in the ^1H-^1H COSY spectrum, which showed a trans-double bond exist in this compound. The connection between the benzene ring and the double bond was determined by the correlation of CH-13, 17 (δ_H: 7.37) to C-11 (δ_C: 138.6) in the HMBC spectrum (Figure 2). In addition, the HMBC spectrum also showed the proton CH-11 (δ_H: 7.30) connected with the double bond coupled with carbonyl C-9 (δ_C: 163.5).The hydrogen atom NH-8 (δ_H: 8.04) was correlated with C-9 (δ_C: 163.5) and C-7 (δ_C: 38.8). The correlation of CH_2-6 (δ_H: 2.61) to CH_2-7 (δ_H: 3.38) was observed in the ^1H-^1H COSY spectrum. In the HMBC spectrum, CH_2-6 (δ_H: 2.61) to C-7 (δ_C: 38.8), C-4 (δ_C: 138.6) and C-5 (114.2) in the imidazole ring showed the connection of CH_2CH_2 and the imidazole ring. The NMR spectrum of the compound had a similar structure a aglycone. disolved in $CDCl_3$, the chemical shifts of C-2, 4 and 5, which maked up the imidazole ring combine with two nitrogen atoms, were 136.1, 136.1, and 117.9, respectively [13]. Combined with the data of the HMBC spectrum, we speculated that the imidazole ring exists in the structure of compound **1**. In the ^{13}C NMR spectrum, δ_C: 85.3, 72.5, 77.2, 69.3, 79.6, and 60.9 belonged to glycosyl signals. The correlation of CH-1' (δ_H: 5.03) to C-4 (δ_C: 138.9) and C-5 (δ_C: 114.2) indicated the part where the glycosyl connected with the aglycone in the imidazole ring. The signal of the anomeric carbon in the glycosyl was δ_C: 85.3, which implied that the glycosyl was connected with the nitrogen atom in the heterocyclic ring. In the spectral data of the compound with similar structure, the chemical shift of carbon at the same position was 87.2, which also supported our speculation [14] (See supporting infromationTable S1). The chemical shifts of the carbon and protons of the glycosyl in compound **1** were close to the relevant data of casimiroedine [13]. The coupling constant of the proton connecting with the anomeric carbon inthe glycosyl was 9.06 Hz, indicating that the glycosyl belonged to a β configuration. The structure of compound **1** was determined and named as dissitumine.

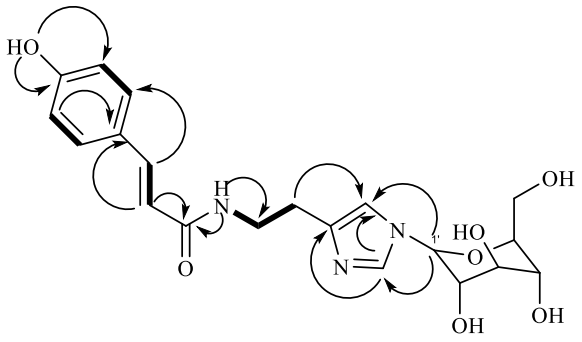

Figure 2. Key HMBC and ^1H-^1H COSY correlations of compound **1** **(HMBC, ^1H-^1H COSY)**.

By querying the SciFinder database, we found there are two compounds with a similar structure to compound **1**. The chemical shifts of their benzene ring and the double bond connected to the benzene ring were different from that of dissitumine, and there was an additional signal of a methyl group connected to the nitrogen atom in those known compounds. Thus, we speculate that compound **1** was a new alkaloid glycoside. Additionally, known compounds were identified as dictamnine (**2**), γ-fagarine (**3**), 4-methoxy-1methyl-2-quinolone (**4**), norchelerythrine (**5**), sanguinarine (**6**), and nitidine (**7**). The structures of the above mentioned compounds are shown in Figure 1.

3.2. The Assay of NO Release Inhibition

All compounds (**1**−**7**) were assessed for their activities of inhibiting NO release from LPS-stimulated RAW264.7 mouse macrophage cells [11]. Compounds **4** and **5** did not show significant results at high concentrations (50 μM). Compounds **1**–**3**, **6**, and **7** were evaluated for their activities to the inhibit macrophage release of inflammatory cytokines, and showed IC_{50} values of 26.12±0.81, 8.41±0.23, 13.75±0.54, 6.97±0.77, and 5.78±0.42 μM, respectively. Among those isolated compounds, dictamnine (**2**), sanguinarine (**6**), and nitidine(**7**) showed strong activity with IC_{50} values lower than 10.00 μM. Moreover, the activity of γ-fagarine (**3**) is weaker than dictamnine (**2**), which may imply that adding a methoxy group to the benzene ring of a uinolone alkaloid will down-regulate its activity to inhibit NO release from LPS-stimulated macrophages.

Table 1. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data of compound 1 (δ in ppm, *J* in Hz) in DMSO-d_6

Position	δ_H	δ_C	HMBC	COSY
2	7.60 (1H, s)	136.5	C-2 and 5	-
4	-	138.6	-	-
5	7.03 (1H, s)	114.2	C-2 and 4	-
6	2.61 (2H, t, 7.6)	28.4	C-4, 5 and 7	-
7	3.38 (2H, m)	38.8	C-4, 6 and 9	H-6 and 8
8	8.04 (1H, t, 5.6)	-	C-7 and 9	H-7
9	-	163.5	-	-
10	6.39 (1H, d, 15.7)	118.8	C-9 and 12	H-11
11	7.30 (1H, d, 15.7)	138.9	C-9,10, 13 and 17	H-10
12	-	125.9	163.5, 118.8, 129.2	-
13, 17	7.37 (2H, d, 8.6)	129.2	C-11, 13, 14, 16 and 17	H-14 and 16
14,16	6.78(2H, d, 8.5)	115.7	C-12, 14, 15 and 16	H-13 and 17
15	-	158.8		-
1'	5.03 (1H, d, 9.1)	85.3	C-2, 5, 2', 3' and 5'	H-2'
2'	3.46 (1H, m)	72.5	C-3' and 1'	H-1' and OH-2'
3'	3.28(1H, m)	77.2	C-3'	OH-3'
4'	3.16(1H, m)	69.3	C-2'	OH-4'
5'	3.32(1H,m)	79.6	C-4' and 6'	-
6'	3.68, 3.42 (each 1H, m)	60.9	C-4' and 5'	H-6' and OH-6'

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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