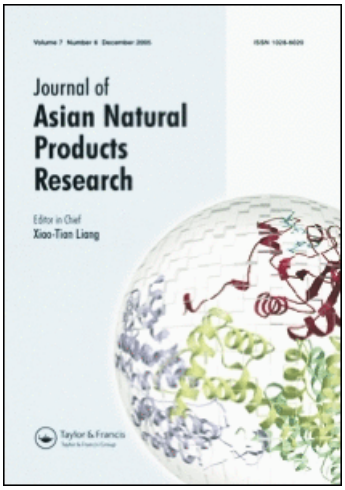

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A new acetyl flavonol, named 3-*O*-acetyl-7-*O*-methyl kaemferol (**1**), together with the five known compounds rhamnocitrin (**2**), rhamnocitrin-3-*O*-β-D-glucoside (**3**), rhamnocitrin-4′-β-D-glucoside (**4**), rhamnazin (**5**), and *p*-hydroxyl benzoic acid (**6**) was isolated from *Nervilia fordii* (Hance) Schltr. The structures of the compounds were determined by spectroscopic analysis. All the compounds were evaluated against nitric oxide (NO) release, based on the production of NO in mice RAW264.7 stimulated by lipopolysaccharide (LPS). This new compound (**1**) showed potent inhibitory activity against the production of NO in RAW264.7 stimulated by LPS with the IC_{50} value of 16.79 μM.

Keywords: *Nervilia fordii* (Hance) Schltr; 3-*O*-acetyl-7-*O*-methyl kaemferol; NO release

1. Introduction

Herbal plant *Nervilia fordii* (Hance) Schltr (Orchidaceae) is regionally distributed in Guangxi, Yunnan, Guangdong, and Sichuan Provinces of China. The whole plant (including rhizome) or aerial part of the plant is a traditional Chinese medicine "Qing Tian Kui." It is frequently used for the therapy of various diseases, such as bronchitis, stomatitis, acute pneumonia, and laryngitis [1]. A previous report has showed the antibacterial and antitumor activities of its extract [2]. Our research further evaluated the anti-inflammatory activities, cytotoxicity, antivirus, and antibacterial activities of the alcoholic extract from the medicine and its fractions partitioned with different polarities of solvents. The ethyl acetate (EtOAc) fraction indicated significant inhibitory activity on nitric oxide (NO) release in mice RAW264.7 cell line, an important index related to anti-inflammatory activity [3]. In this paper, we reported the isolation and identification of chemical constituents in the bioactive fraction from the alcoholic extract of the title plant, and the NO release activities of the isolated compounds in mice RAW264.7 stimulated by lipopolysaccharide (LPS).

2. Results and discussion

Compound **1** was isolated as a yellowish amorphous powder. The molecular formula was deduced to be $C_{18}H_{14}O_7$ from the quasi-molecular ion peak at *m*/*z* 341.0667 $[M-H]^-$ in HR-ESI-MS. In the ^{13}C NMR spectrum, the compound

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Table 1. ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz) spectral data and HMBC correlations of compound **1** in DMSO-d_6 (J in Hertz).

Position	δ_H (multiplicity, J)	δ_C	HMBC correlation (H → C)
2		156.3	
3		129.8	
4		175.0	
5		160.7	
6	6.42 (d, $J = 2.0$)	98.2	C-7, C-8, C-10, C-4′
7		165.5	
8	6.79 (d, $J = 2.0$)	92.8	C-6, C-7, C-9, C-10
9		156.5	
10		104.4	
1′		119.2	
2′	7.80 (d, $J = 8.8$)	130.1	C-1′, C-3′, C-4′
3′	6.96 (d, $J = 8.8$)	115.9	C-2, C-2′, C-4′
4′		160.9	
5′	6.96 (d, $J = 8.8$)	115.9	C-2, C-2′, C-4′
6′	7.80 (d, $J = 8.8$)	130.1	C-1′, C-4′, C-5′
7-*O*-Me	3.88 (s)	56.3	C-7
3′-*O*-Ac	2.36 (s)	20.2	Carboxyl carbon from *O*Ac
		167.9	
5-OH	12.18 (s)		C-5
4′-OH	10.80 (brs)		C-4′

Note: The assignments were completed with the aid of HSQC and HMBC.

exhibited 16 signals, including 2 carboxyl carbons (δ 175.0 and 167.9), 12 aromatic or ethylenic carbon signals, and 1 methoxyl carbon signal (δ 56.1). A DEPT experiment revealed that the 16 carbon signals comprised of 4 methines, 2 methyls, and 10 quaternary carbons. ^{1}H and ^{13}C NMR spectra indicated the presence of an acetoxyl group due to the signals at δ_C 20.2 and 167.9 and δ_H 2.36 (3H, s). The methoxyl group was confirmed by the signals at δ_C 56.1 and δ_H 3.88 (3H, s). The remaining 13 carbon signals belonged to the basic skeleton of the flavonoid with the consideration of the signal overlap (δ_C 130.1 and 115.9) between C-2′ and C-6′, C-3′ and C-5′ at 1′,4′-disubstituted B-ring. The B-ring was substantiated by the signals at δ_H 7.80 (2H, d, $J = 8.8$ Hz) and 6.96 (2H, d, $J = 8.8$ Hz) in the ^{1}H NMR spectrum. The remaining signals at δ_H 6.79 (1H, d, $J = 2.0$ Hz) and 5.63 (1H, s, $h_{1/2} = 2.0$ Hz) suggested the 5,7-disubstitution in A-ring. The signal at δ_H 12.18 (1H, s) indicated the presence of a 5-OH in A-ring. The methoxyl group was connected at C-7 due to the long-range correlation between the proton at δ_H 3.86 and the carbon δ_C 165.5. The acetoxyl group was attached at either C-3 or C-4′. Although HMBC experiments did not show any correlation of NMR signal between the acetyl group and the flavonoid skeleton, we can deduce that the acetoxyl group was at C-3 with the consideration of the obvious downfield shift (from δ 135.9 to 156.3) of C-2 and upfield shift (from δ 147.2 to 129.8) of C-3 in comparison with the data of the known compound rhamnocitrin. The UV spectrum exhibited absorption maxima at λ_{max} 205.5, 265.0, and 337.5 nm. The maximum at λ_{max} 337.5 nm suggested that there are no free hydroxyl groups at C-3, substituted by acetoxyl group, because the flavonol with 3-OH usually had absorption maximum at λ_{max} 352–385 nm, as those seen in rhamnocitrin (**2**), rhamnocitrin-4′-β-D-glucoside (**4**), and rhamnazin (**5**). The assignment of NMR spectral data for the

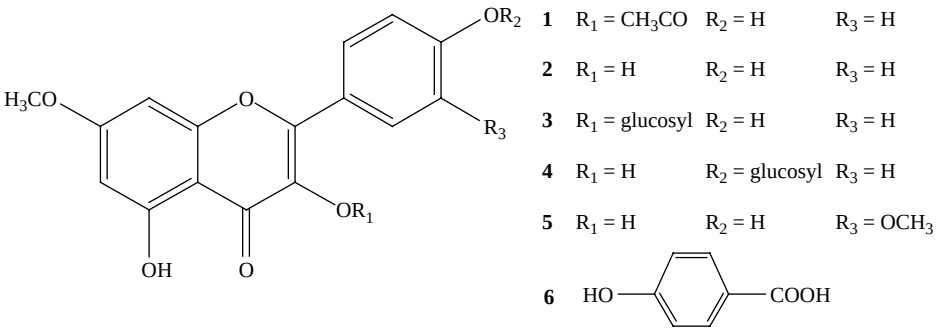

Figure 1. The structures of compounds **1**–**6**.

molecule was listed in Table 1 on the basis of HSQC and HMBC analyses. Thus, the structure of compound **1** was elucidated to be 3-*O*-acetyl-7-*O*-methyl kaemferol, a new acetyl flavonol.

The other five compounds (**2**–**6**) were identified with their NMR spectral data, ESI-MS, and some physical–chemical properties. They were the four known flavonoids: rhamnocitrin (**2**), rhamnocitrin-3-*O*-β-D-glucoside (**3**), rhamnocitrin-4′-β-D-glucoside (**4**), and rhamnazin (**5**), and *p*-hydroxyl benzoic acid (**6**). Compounds **3** and **4** were isolated from the plant for the first time (Figure 1).

NO plays an important role in the inflammatory process and an inhibitor of NO release may be considered as a therapeutic agent in the inflammatory diseases [4]. Therefore, compounds **1**–**6** were tested for the inhibitory activity against the production of NO in RAW264.7 stimulated by LPS, as described previously [5]. Because NO is unstable and rapidly metabolized into nitrite ion in the solution for cell culture, NO production on macrophage RAW264.7 cell lines was quantified by measuring nitrite levels present in cellular supernatant using Griess reagent. Compound **1** exhibited a remarkable inhibitory effect against the production of NO, with an IC_{50} value of 16.79 μM. However, compounds **2**–**6** did not show significant inhibitory activity ($IC_{50} > 100$ μM) on NO release at the test concentration of 100 μM.

3. Experimental

3.1 General experimental procedures

Melting points were determined on a micromelting point apparatus and are uncorrected. UV spectra were recorded on a JASCO V-550 UV/VIS spectrometer. IR spectra were obtained using a JASCO FT/IR-480 plus spectrometer. LR-ESI-MS spectra were taken on a FINIGAN LCQ Advantage MAX mass spectrometer. HR-ESI-MS spectra were acquired using a Micromass Q-TOF mass spectrometer. Measurement of 1D and 2D NMR spectra was done with a Bruker AV-400 spectrometer using a residual DMSO-d_6 as an internal reference.

3.2 Plant material

The experimental material was purchased at the Qingping market for Chinese Medicinal Material of Guangzhou, China in August 2007. The material was collected in Guangxi and traded to Guangzhou. The original plant of the aerial material was authenticated as *N. fordii* (Hance) Schltr by Prof. Guangxiong Zhou of Pharmacognosy, College of Pharmacy, Jinan University. A voucher specimen (GX-QTK-1) was deposited in the Department of Pharmacognosy in the college.

3.3 Extraction and isolation

The air-dried aerial parts (2.0 kg) of *N. fordii* (Hance) Schltr were powdered and soaked in 10 l of 95% alcohol for 24 h. Then, it was filtered to collect the alcoholic solution. The residue was re-soaked twice with 95% alcohol (5 l) for 24 h each time. After filtration, the filtrate was combined and evaporated to dryness *in vacuo*. The non-alcoholic extract was suspended in water, and partitioned with petroleum ether, chloroform ($CHCl_3$), and EtOAc, successively, to afford 47.1, 23.5, and 9.1 g of extracts, respectively, and 78.6 g water-layer residue. All the partitioned extract and the original alcoholic extract were tested for the inhibitory activities of NO release, cytotoxicities against cancer cell lines, COX-2 inhibitory activities, and antivirus and antibacterial activities. From the EtOAc extract with NO release-inhibiting activity, six fractions (E1–E6) were obtained by fractionation with a polyamide column chromatography eluting with gradient aqueous methanol. An E4 fraction from 80% methanol eluate was subjected to Sephadex LH-20 column chromatography to yield compounds **2** (200 mg) and **5** (6 mg). The E3 from 60% methanol eluate was repeatedly chromatographed on Sephadex LH 20 which led to the isolation of compounds **3** (50 mg), **4** (17 mg), and **1** (9 mg) with aids of preparative HPLC. The E2 fraction from 40% methanol eluate was subjected to silica gel chromatography to afford compound **6** (40 mg).

3.3.1 Compound *1*

It was obtained as an amorphous powder; mp 202–204°C; UV (MeOH) λ_{max} 205.5, 265, 337.5 nm; IR (KBr) ν_{max} 3309.3, 1775.2, 1663.3, 1602.5, 1497.5, 1342.2, 1281.5, 1209.2, 1188.9, 1136.8, 975.8, 800.3 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz) spectral data, see Table 1; LR-ESI-MS (negative-ion mode) *m/z* 341.2 $[M-H]^-$, 299.3; HR-ESI-MS (negative-ion mode) *m/z* 341.0667 $[M-H]^-$ (calcd for $C_{18}H_{13}O_7$, 341.0672).

The known compounds **2**–**6** were identified on the basis of ^1H, ^{13}C NMR, and DEPT spectra, ESI-MS, and the comparison of their spectroscopic data with those reported previously.

3.4 RAW264.7 cell culture

Mice mononuclei macrophages cell line RAW264.7 cells (purchased from the cell banks of Chinese Academy of Science) were cultured in RPMI 1640 medium containing 25 mM HEPES buffer, 4.5 g/l D-glucose, 0.2% sodium bicarbonate, and 2 mM L-glutamine. Both culture media were supplemented with 10% fetal bovine serum, and 1% of antibiotic solution containing penicillin (100 U/ml) and streptomycin (10 μg/ml; all purchased from Gibco Co., Carlsbad, CA, USA). All cultures were maintained in a humidified incubator (Thermo Co., Waltham, MA, USA) in 5% CO_2 at 37°C and the cells were split two times a week.

3.5 Determination of nitrite production

Nitrite accumulation was determined as an indicator of NO production in the culture media, as previously described [6]. Briefly, 200 μl of diluted RAW264.7 cells culture at 5×10^5 cells/ml was seeded in 96-well plates and allowed cell number at 1×10^5 cells/well. After 1-h incubation, each well was added with some volume of appropriate media containing LPS and 0.4 μl of sample concentrations, leading to the final LPS concentration of 1 μg/ml and a series of sample concentrations. Meanwhile, the ranks only with LPS and only with DMSO (without both LPS and sample) were set in the same plate as reference. Each sample had four parallel wells in a rank. After 24-h incubation at 37°C in a CO_2 incubator, samples (100 μl) of cell culture supernatants were taken and incubated with 100 μl of

Griess reagent (1:1; 1% sulfanilic amide, 0.1% *N*-1-naphthyl-ethylenediamine dihydrochloride in 3% phosphoric acid solution) at room temperature for 10 min in 96-well plates. The absorbance at 540 nm was read using an ELISA plate reader (Biotek, Winooski, VT, USA). Standard calibration curves were prepared using sodium nitrite as a standard in a series of concentrations (1, 5, 10, and 50 μmol/l) of $NaNO_2$. The NO_2^- concentrations in cell culture supernatants and the inhibitory rates of NO release were calculated on the standard curve. The calculation of NO release used the following equation: inhibitory rate of NO release (%) $= 100 \times ([NO_2^-]_{LPS} - [NO_2^-]_{LPS+sample})/([NO_2^-]_{LPS} - [NO_2^-]_{blank})$.

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