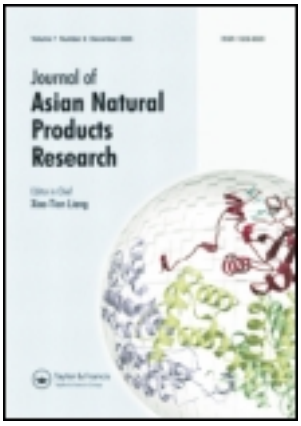

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Anti-inflammatory flavonolignans from *Hydnocarpus anthelminthica* seeds

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A new flavonolignan, anthelminthicol A (**1**), together with four known compounds, was isolated from the EtOAc extracts of the seeds of *Hydnocarpus anthelminthica*. Their structures were elucidated using extensive spectroscopic techniques. Bioassay showed that compounds **3–5** could inhibit nitric oxide production in LPS-stimulated RAW 264.7 macrophage cell lines, with IC_{50} values of 7.81, 9.38, and 10.55 μM, respectively.

Keywords: *Hydnocarpus anthelminthica*; Flacourtiaceae; flavonolignan; anthelminthicol A; nitric oxide (NO) production

1. Introduction

Hydnocarpus anthelminthica Pierre ex Laness (Flacourtiaceae) is a tall evergreen tree, mainly distributed in Southeast Asia [1]. Its seed has been used as a famous folk medicine for lepra, acariasis, and gonorrhea [2]. Our previous paper has reported the isolation of some chaulmoogric acid analogs from this plant [3]. Continuous study of the chemical constituents led to the isolation of a new flavonolignan, named anthelminthicol A (**1**), together with four known flavonolignans (Figure 1). Their structures were determined by spectroscopic analysis, especially 2D NMR experiments. Increasing evidences showed that inflammation is implicated in many diseases such as cancer, infection, and rheumarthritis. Since NO and PGE_2 are considered to be the major inflammation mediators, in correlation with the traditional medical applications of this herb, the isolates were therefore evaluated for their inhibitory effects on NO production in LPS-stimulated RAW 264.7 macrophage cells. This report deals with the isolation, structural elucidation, and biological evaluation of these compounds.

2. Results and discussion

Compound **1** was obtained as amorphous yellow powder. Its molecular formula was established as $C_{25}H_{20}O_9$ from its positive HR-ESI-MS at *m/z* 487.1012 $[M + Na]^+$. The UV spectrum of **1** exhibited absorption maxima at 281 and 339 nm characteristic of a flavone [4]. The ^{1}H- and ^{13}C-NMR spectral data of **1** (Table 1) were similar to those of isohydnocarpin

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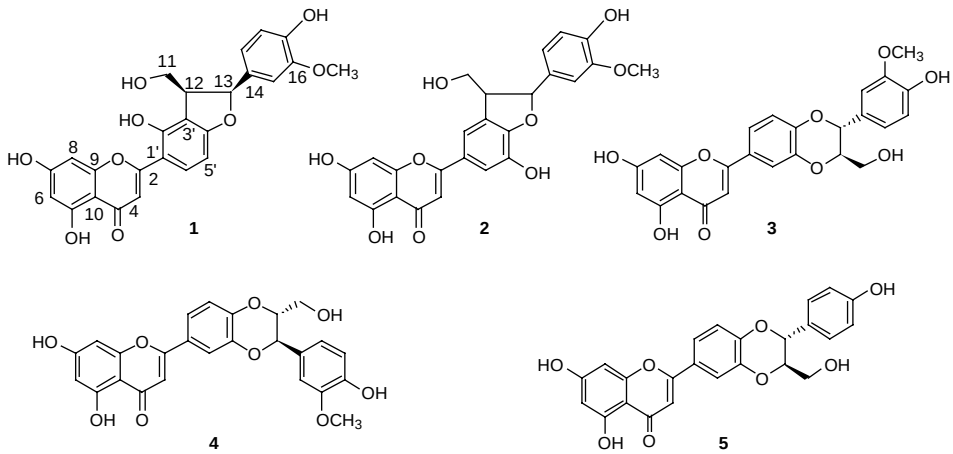

Figure 1. Structures of compounds **1–5**.

[5], implying **1** to be a flavonolignan analog. The difference was mainly at the substitution pattern of one phenyl group. Two vicinal protons were observed for **1** with $J_{H\text{-}5',H\text{-}6'}$ value of 8.4 Hz, in correspondence with distinct $^1H-^1H$ COSY correlation of H-5′ and H-6′. The HMBC spectrum of **1** (Figure 2) showed the following key correlations: H-3 with C-2, C-4, C-10, and C-1′; H-6 with C-5, C-7, and C-10; H-13 with C-11, C-12, C-14, C-15, C-19, C-3′, and C-4′; H-15 with C-13, C-16, and C-19; H-20 with C-16; H-5′ with C-3′ and C-4′; H-6′ with C-1′, C-2′, and C-4′, which confirmed the structure of **1**. Complete assignments (Table 1) of ^{1}H-NMR signals were unambiguously performed by careful analysis of 2D NMR experiments. The relative configurations of two chiral centers in the structure of **1** were mainly determined by the NOESY experiment (Figure 3), which showed correlations of H-11/H-15 and H-19, suggesting a *cis*-relationship of H-12 and H-13, in accordance with a small coupling constant of 2.8 Hz for H-13. Accordingly, the structure of **1** was assigned as 5,7-dihydroxy-2-((2β,3β)-4-hydroxy-2-(hydroxyl-3-methoxyphenyl)-3-(hydroxymethyl)-2,3-dihydrobenzofuran-5-yl)-4*H*-

Table 1. ^{1}H- and ^{13}C-NMR spectral data of compound **1** in DMSO-d_6.

Position	δ_H (J in Hz)	δ_C	Position	δ_H (J in Hz)	δ_C
1			14		132.9 s
2		164.7 s	15	6.93 (1H, br s)	110.1 d
3	6.61 (1H, s)	105.1 d	16		147.7 s
4		181.7 s	17		146.4 s
5		157.2 s	18	6.76 (1H, overlap)	115.6 d
6	6.19 (1H, d, 1.7)	99.1 d	19	6.76 (1H, overlap)	117.9 d
7		161.5 s	20	3.74 (3H, s)	55.8 q
8	6.38 (1H, d, 1.7)	94.0 d	1′		119.1 s
9		164.3 s	2′		145.3 s
10		103.8 s	3′		126.9 s
11	3.66 (1H, m) 3.35 (1H, m)	62.9 t	4′		147.7 s
12	3.97 (1H, m)	54.6 d	5′	6.84 (1H, d, 8.4)	116.6 d
13	5.71 (1H, d, 2.8)	86.1 d	6′	7.31 (1H, d, 8.4)	122.9 d

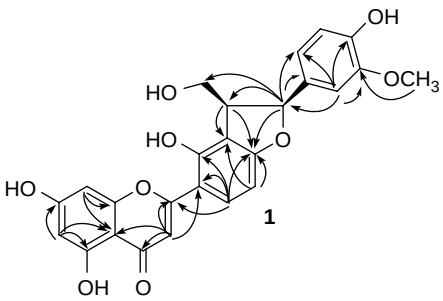

Figure 2. Important HMBC correlations of **1**.

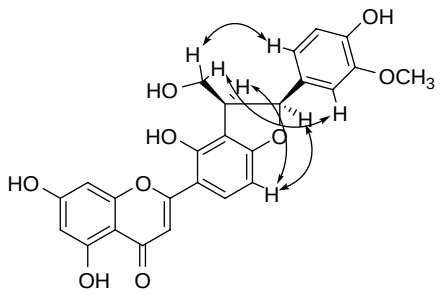

Figure 3. Significant ROESY correlations of **1**.

chromen-4-one and named anthelminthicol A.

In comparison with the literature data, four known compounds were determined as isohydnocarpin (**2**) [4], hydnocarpin-D (**3**) [6], hydnocarpin (**4**) [6], and sinaiticin (**5**) [7], respectively, which were isolated from the *Hydnocarpus* genus for the first time to the best of our knowledge.

Considering the traditional medicinal uses of *H. anthelminthica* seeds, the isolated compounds were evaluated for their inhibitory effects on NO release in LPS-stimulated RAW 264.7 macrophage cell line. The results showed that compounds **3–5** could inhibit NO production with IC_{50} values of 7.81, 9.38, and 10.55 μM (Table 2), respectively. This study not only revealed the medicinal basis of this herb, but also suggested that *H. anthelminthica* seeds could be a potential source for searching anti-inflammatory agents.

Table 2. Inhibition of NO production (IC_{50} μM).

1	2	3	4	5	MG132
>25	>25	7.81	9.38	10.55	0.085

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a Horiba SEPA-300 polarimeter. The UV spectrum was recorded on a Shimadzu UV-2401PC spectrophotometer. The NMR spectra were recorded on Bruker AV-400 and Bruker DRX-500 spectrometers in DMSO-d_6 solution with TMS as an internal standard. FAB-MS were recorded with a VG Autospec-3000 spectrometer. ESI-MS and HR-ESI-MS were recorded with an API QSTAR Pulsar 1 spectrometer. Silica gel (200–300 mesh, Qingdao Marine Chemical, Inc., Qingdao, China), RP-18 gel (40–63 μm, Daiso Co., Osaka, Japan), and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) were used for column chromatography.

3.2 Plant material

The dried seeds of *H. anthelminthica* were purchased from Yunnan Company of Materia Medica (YCMM), Yunnan Province, China, in December 2007, and identified by Prof. H.Y. Sun at YCMM, Yunnan Province, China. A voucher specimen (CHYX-0321) is deposited in the State Key Laboratory of Photochemistry and Plant Resources of our institute in West China.

3.3 Extraction and isolation

The dried and powdered seeds of *H. anthelminthica* (26 kg) were extracted with 95% EtOH under reflux (4× 30 l). After evaporation of ethanol *in vacuo*, the concentrated extract was suspended in water and extracted successively with petroleum ether, EtOAc, and *n*-BuOH,

respectively. The EtOAc fraction (30 g) was subjected to silica gel column chromatography eluted with gradient $CHCl_3$–MeOH (99:1–70:30) to obtain four fractions. Fraction I (6.8 g) was repeatedly subjected to column chromatography over silica gel, using gradient EtOAc/MeOH as eluent, Sephadex LH-20 eluted with $CHCl_3$–MeOH (60:40), and RP-18 eluted with MeOH–H_2O (85:15) to afford compound **5** (3 mg). Compounds **3** (8 mg) and **4** (11 mg) were obtained by semi-preparative, reversed phase HPLC with MeOH–H_2O (70:30) from Fraction II (4.3 g). In the same manner, compounds **1** (4 mg) and **2** (8 mg) were isolated from fraction IV (5.9 g).

*3.3.1 5,7-Dihydroxy-2-((2β,3β)-4-hydroxy-2-(hydroxyl-3-methoxyphenyl)-3-(hydroxymethyl)-2,3-dihydrobenzofuran-5-yl)-4*H*-chromen-4-one (**1**)*

Amorphous yellow powder; $R_f = 0.56$, silica gel GF_{254}, $CHCl_3$–MeOH (12:1); $[\alpha]_D^{20.4} + 0.00$ ($c = 0.60$, MeOH). UV (MeOH) λ_{max} (log ε): 385 (5.05), 339 (5.01), 281 (5.23) nm. IR (KBr) ν_{max}: 3424, 2925, 1656, 1611, 1514, 1501, 1441, 1384, 1357, 1306, 1273, 1165, 1123, 1024, 995 cm^{-1}. ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) spectral data, see Table 1. FAB-MS (positive): *m/z* 465 $[M + H]^+$. HR-ESI-MS (positive): *m/z* 487.1012 $[M + Na]^+$ (calcd for $C_{25}H_{20}O_9Na$, 487.1005).

3.4 Inhibition of NO production

The murine monocytic RAW 264.7 macrophages were dispensed into 96-well plates (2×10^5 cells/well) containing RPMI 1640 medium (Hyclone, Logan, USA) with 10% FBS under a humidified atmosphere of 5% CO_2 at 37°C. After 24 h of preincubation, cells were treated with serial dilutions of the compounds **1–5** with the maximum concentration of 25 μM in the presence of 1 μg/ml LPS for 18 h. Each compound was dissolved in DMSO and further diluted in the medium to produce different concentrations. NO production in each well was assessed by adding 100 μl of Griess reagents A and B to 100 μl of each supernant from LPS or the compound-treated cells in triplicate. After 5 min of incubation, the absorbance was measured at 570 nm with 2104 Envision Multilabel Plate Reader (Perkin-Elmer Life Sciences, Inc., Boston, MA, USA). Cytotoxicity was determined by the MTT assay. MG-132 was used as a positive control.

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