Flavonoids from the leaves of *Apocynum venetum* and their anti-inflammatory activity

Hong-Min Fu¹, Chun-Ling Yin², Zhi-Yong Shen³ and Ming-Hua Yang¹

**Abstract**

Chemical investigation of the EtOAc-soluble extract of the leaves of *Apocynum venetum* allowed the isolation of seven flavonoids including a new compound named 4′-hydroxy-7-O-(4-hydroxybenzyl)-3-methoxy-6-prenylflavone (1) and six known compounds (2–7). The structures of these compounds are elucidated by spectroscopic and physico-chemical analyses. All the isolates are evaluated for in vitro anti-inflammatory activity by measuring their inhibitory activities on nitric oxide and tumor necrosis factor-α production in lipopolysaccharide-induced mouse peritoneal macrophages (RAW 264.7). Among them, 5 exhibits significant inhibitory activity toward nitric oxide and tumor necrosis factor-α production with IC₅₀ values of 9.0 ± 0.7 and 42.1 ± 0.8 μM, respectively. In addition, 1 also shows moderate inhibitory activity toward nitric oxide production with an IC₅₀ value of 17.2 ± 0.9 μM.

**Keywords**

anti-inflammatory activity, Apocynaceae, *Apocynum venetum*, flavonoid, nitric oxide, tumor necrosis factor-α

**Introduction**

*Apocynum venetum* L. (Apocynaceae) is a small perennial shrub that is widely distributed in the temperate regions of Europe, North America, and Asia.¹ In China, *A. venetum* commonly grows in the salt marshes of the Yellow River delta, and its flowers and leaves are used as a medicine and as tea.² As a traditional Chinese medicine, the leaves of *A. venetum* are used to treat neurasthenia, hypertension, nephritis, and heart disease.³ Pharmacological studies showed that the extract of *A. venetum* possesses significant antioxidant, anti-hyperlipidemic, anti-hyperglycemic effect and reverses the effects of depressive-like behaviors.⁴⁻⁷ Phytochemical investigations on *A. venetum* revealed that flavonoids were the major active constituents which exhibited various pharmacological activities including anti-hypertensive, antioxidant, anti-depressant, anti-anxiety, hepatoprotective, and cardiotonic effects.¹²⁻¹⁰ During our studies on finding novel natural products with potent anti-inflammatory activity, we found that the EtOAc-soluble extract of the

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leaves of *A. venetum* showed moderate inhibitory activity on nitric oxide (NO) production in lipopolysaccharide (LPS)-induced mouse peritoneal macrophages (RAW264.7). Thus, a series of studies focused on the leaves of *A. venetum* were carried out. As a result, a new flavonoid named 4’-hydroxy-7-O-(4-hydroxybenzyl)-3-methoxy-6-prenylflavone (1), together with six known compounds (2–7) were isolated. Herein, we report the isolation and structural elucidation of these seven compounds. In addition, their in vitro anti-inflammatory activities were also evaluated.

### Results and discussion

**Structural elucidation**

Compound 1 was obtained as a yellow amorphous powder, and the molecular formula was established as C_{35}H_{26}O_{6} based on the [M + H]^+ HRESIMS mass ion at m/z 459.1652 (Supplemental Figure S4). The IR spectrum showed typical absorption bands due to hydroxy and carbonyl groups at 3408 and 1732 cm⁻¹, respectively. The ¹H NMR spectrum (Table 1, Supplemental Figure S1) revealed the presence of two sets of AA’XX’-type aromatic protons at δ_H 7.60 (2H, d, J=8.5 Hz, H-2′/6′), 7.26 (2H, d, J=8.5 Hz, H-3′/5′), 7.51 (2H, d, J=8.5 Hz, H-3″/7″), and 7.22 (2H, d, J=8.5 Hz, H-4″/6″), a pair of singlet aromatic protons at δ_H 8.04 (1H, s, H-5) and 6.63 (1H, s, H-8), a prenyl group at δ_H 3.39 (1H, d, J=7.5 Hz, H-1″), 5.44 (1H, t, J=7.5 Hz, H-2″), 1.67 (3H, s, H-5″), and 1.68 (3H, s, H-5′), a methoxy group at δ_H 3.78 (3H, s) and an oxygenated methylene at δ_C 5.14 (2H, s, H-1‴). The ¹³C NMR spectrum of 1 (Supplemental Figure S2) revealed 28 carbons including a carbonyl carbon at δ_C 178.3; 18 aromatic carbons at δ_C 164.5-100.0; a pair of typical oxygenated olefinic carbons at δ_C 156.5 and 138.1; a set of prenyl carbons at δ_C 133.2, 127.5, 26.1, 28.5, and 18.0; a methoxy carbon at δ_C 56.2; and an oxygenated methylene carbon at δ_C 70.5. The key HMBC correlations of δ_H 5.14 (H-1‴) with δ_C 164.5 (C-7), 125.6 (C-2″), and 129.5 (C-3‴/C-7″); δ_H 7.51 (H-3‴/7″) with δ_C 70.5 (C-1‴), 125.6 (C-2″), 116.1 (C-4‴/6″), and 160.3 (C-5‴); δ_H 7.22 (H-4″/6″) with δ_C 125.6 (C-2″), 129.5 (C-3‴/7″), and 160.3 (C-5‴) revealed the presence of an oxygenated 4-hydroxybenzyl moiety which linked with C-7 (Figure 1, Supplemental Figure S3). Furthermore, the locations of the prenyl and methoxy groups could be deduced by the key HMBC correlations of δ_H 3.39 (H-1″) with δ_C 123.0 (C-5) and 124.9 (C-6), and δ_H 3.78 (OCH₃) with δ_C 138.1 (C-3). Thus, the structure of compound 1 was assigned as 4’-hydroxy-7-O-(4-hydroxybenzyl)-3-methoxy-6-prenylflavone.

Moreover, six known compounds 2–7 were also obtained from the leaves of *A. venetum* and identified as bavachin (2), chrysoeriol (3), 6,7-dimethoxy-4’-hydroxy-8-formylflavone (4), 4’,7-dihydoxy-8-formyl-6-methoxyflavone (5), quercetin (6), and quercetin-3-0-beta-D-glycosides (7) based on the NMR data and by comparison with literature data (Figure 2).

### Analysis of the biological activity results

Compounds 1–7 were evaluated for their anti-inflammatory in vitro. Based on NO and tumor necrosis factor-α (TNF-α) are two key mediators for the pathogenesis of inflammatory diseases such as psoriasis, ulcerative colitis, and osteoarthritis, thus, the anti-inflammatory activities of 1–7 were evaluated by measuring the production of NO and TNF-α in LPS-induced RAW264.7 cells. As shown in Table 2, compounds 1 and 3–7 showed different levels of anti-inflammatory activities with IC₅₀ values ranging from 9.0 ± 0.7 to 81.2 ± 1.2 μM. Among these compounds, 4’,7-dihydroxy-8-formyl-6-methoxyflavone (5) exhibited significant inhibitory activity toward NO and TNF-α production with IC₅₀ values of 9.0 ± 0.7 and 42.1 ± 0.8 μM, respectively. Compound 1 also exhibited moderate inhibitory activity toward NO production with an IC₅₀ value of 17.2 ± 0.9 μM. In addition, natural product 5 showed higher anti-inflammatory activity compared with compound 4, which suggested that the hydroxy group linked at C-7 in 5 might be responsible for the increased inhibitory activity toward NO and TNF-α production. Furthermore, a comparison of the IC₅₀ data between compounds 6 and 7 indicated that the sugar moiety located at C-3 of 7 might play a negative role on the inhibitory activity toward NO production. Based on the significant inhibitory activity of compound 5, in-depth pharmacological studies on 5 should be further explored.

### Table 1. ¹H and ¹³C NMR spectral data of compound 1 in pyridine-d₅ (¹H: 500 MHz, ¹³C: 125 MHz).

| Carbon no. | δ_H (mult, J in Hz) | δ_C (mult) |
|------------|---------------------|------------|
| 2          | –                   | 156.5      |
| 3          | –                   | 138.1      |
| 4          | –                   | 178.3      |
| 5          | 8.04, s             | 123.0      |
| 6          | –                   | 124.9      |
| 7          | –                   | 164.5      |
| 8          | 6.63, s             | 100.0      |
| 9          | –                   | 163.2      |
| 10         | –                   | 115.1      |
| 1‴         | –                   | 122.1      |
| 2‴         | 7.60, d (8.5)       | 129.1      |
| 3‴         | 7.26, d (8.5)       | 116.8      |
| 4‴         | –                   | 159.8      |
| 5‴         | 7.26, d (8.5)       | 116.8      |
| 6‴         | 7.60, d (8.5)       | 129.1      |
| 1‴         | 3.39, d (7.5)       | 28.5       |
| 2‴         | 5.44, t (7.5)       | 127.5      |
| 3‴         | –                   | 133.2      |
| 4‴         | 1.67, s             | 26.1       |
| 5‴         | 1.68, s             | 18.0       |
| 1‴         | 5.14, s             | 70.5       |
| 2‴         | –                   | 125.6      |
| 3‴         | 7.51, d (8.5)       | 129.5      |
| 4‴         | 7.22, d (8.5)       | 116.1      |
| 5‴         | –                   | 160.3      |
| 6‴         | 7.22, d (8.5)       | 116.1      |
| 7‴         | 7.51, d (8.5)       | 129.5      |
| OCH₃       | 3.78, s             | 56.2       |
Conclusion

In this study, a new flavonoid named 4-hydroxy-7-O-(4-hydroxybenzyl)-3-methoxy-6-prenylflavone (1), together with six known compounds (2–7), was isolated from the EtOAc-soluble extract of the leaves of A. venetum. All the compounds were evaluated for their in vitro inhibitory activities toward NO and TNF-α production in mouse macrophage RAW264.7 cells. It is worth noting that compound 5 exhibited significant inhibitory activities on NO and TNF-α production with IC50 values of 9.0±0.7 and 42.1±0.8 μM, respectively. In addition, flavone 1 also showed moderate inhibitory activity toward NO production with an IC50 value of 17.2±0.9 μM. Based on the potent anti-inflammatory activity and the few phytochemical investigations of A. venetum, efforts directed toward finding novel compounds with potential anti-inflammatory activity should be intensified.

Experimental

General

UV spectra were obtained on a Hitachi U-3310 UV/vis spectrometer (Hitachi, Tokyo, Japan). IR spectra were recorded with a Nicolet Avatar 370 FTIR spectrophotometer (Nicolet, Wisconsin, USA). Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker AV-500 MHz spectrometer with tetramethylsilane (TMS) as an internal standard (Bruker, Karlsruhe, Germany). Mass spectra were obtained on a QTOF2 high-resolution mass spectrometer (Micromass, Wythenshawe, UK). Column chromatography was conducted using silica gel 60 (100 and 200 μm particle size, Merck, Darmstadt, Germany). Thin-layer chromatography (TLC) was performed with precoated silica gel GF254 glass plates (Qingdao Marine Chemical Co., Ltd, Qingdao, China) and RP-18 (150-63 μm particle size, Merck, Darmstadt, Germany). High-performance liquid chromatography (HPLC) was carried out using a Shimadzu System LC-20AT pump equipped with a SPD-10Avp UV detector (Shimadzu, Tokyo, Japan), and a YMC ODS-A column (250 mm × 4.6 mm, 5 μm).

Plant material

The leaves of A. venetum were collected in Tengchong, Yunnan Province, P.R. China, and authenticated by Professor Qiaofeng Wu (College of Pharmacy, Zhejiang Chinese Medical University). A voucher specimen of the plant (no. 20200724) was deposited at the College of

Table 2. Anti-inflammatory activities of compounds 1–7.

| Compound | IC50 (μM)a | NO     | TNF-α   |
|----------|------------|--------|---------|
| 1        | 17.2±0.9   | 71.4±1.7 |
| 2        | >100       | >100   |
| 3        | 81.2±1.2   | >100   |
| 4        | 23.6±0.9   | 57.4±1.2 |
| 5        | 9.0±0.7    | 42.1±0.8 |
| 6        | 31.7±1.0   | >100   |
| 7        | 52.4±1.3   | >100   |
| AHb      | 8.3±0.4    | -      |
| Silybinb  | -          | 65.2±0.6 |

AH: aminoguanidine hydrochloride.

aIC50 values represent the means ± SEM of three parallel measurements.
bPositive control.
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**Extraction and isolation**

The dried leaves of *A. venetum* (10.0 kg) were extracted three times with 75% MeOH under reflux, and the solution was concentrated in vacuo to yield the extract (1.8 kg). This extract was suspended in H₂O, partitioned successively with petroleum ether (PE), CH₃Cl₂, EtOAc, and n-BuOH. The EtOAc extract of *A. venetum* showed moderate inhibitory activity toward NO production with an IC₅₀ value of 64.3 ± 0.2 μg/mL. Thus, the EtOAc fraction (161.5 g) was subjected to silica gel column chromatography eluting with a gradient of CH₂Cl₂-MeOH (from 100:0 to 0:1) to give 12 fractions (Fr.1-Fr.12). Fr.6 (13.9 g) was applied to silica gel column chromatography eluting with a gradient of CH₂Cl₂-MeOH (from 100:0 to 0:1) to give 12 fractions (Fr.6.1-Fr.6.12). Furthermore, Fr.6.7 (1.8 g) was subjected to the RP-18 column and eluted using a gradient of PE-EtOAc (from 100:0 to 0:1) and was separated into 12 fractions (Fr.6.1-Fr.6.12). Fr.6.7.5 (121.2 mg) was further purified by HPLC and eluted with a gradient of 55%–65% MeOH in H₂O at a flow rate of 3.0 mL/min over 70 min. This resulted in twelve compounds (enzyme-linked immunosorbent assay) kit (Solarbio, Beijing, P.R. China) according to the manufacturer’s instructions. TNF-α was determined from a standard curve and silybin was used as the positive control. Experiments were performed at least three times.

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**Supplemental material**

Supplemental material for this article is available online.

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