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# Flavone *C*-glycosides from the flowers of *Trollius chinensis* and their anti-complementary activity

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Phytochemical investigation of ethanol extract from the flowers of *Trollius chinensis* Bunge resulted in the isolation of two new flavone *C*-glycosides (**1**–**2**), along with 10 known compounds (**3**–**12**). The structures of the new compounds were established as 6‴-(3-hydroxy-3-methylglutaroyl)-2″-*O*-β-D-galactopyranosyl orientin (**1**) and 6‴-(3-hydroxy-3-methylglutaroyl)-2″-*O*-β-D-galactopyranosyl vitexin (**2**) on the basis of various spectroscopic analysis (including different 1D and 2D NMR spectroscopies, high-resolution electrospray ionization mass spectrometry) and chemical evidences. Bioassay showed that eight flavonoids inhibited complement activation on the classic pathway *in vitro*, with their $IC_{50}$ values ranging from 0.88 to 4.02 mM, which may contribute to the applications of the herb in treatment of acute respiratory distress syndrome, etc.

**Keywords:** *Trollius chinensis*; flavanoid; *C*-glycoside; anti-complementary activity



## 1. Introduction

*Trollius chinensis* Bunge is a traditional Chinese medicinal herb widely cultivated in northern China. Its flowers have been used as medicinal drugs and tea drinks to prevent or treat upperrespiratory infections, pharyngitis, tonsillitis, and bronchitis [1]. Chemical researches have revealed that orientin, vitexin, and other flavone *C*-glycosides are rich in *Trollius* genus [2–5]. These flavonoids were responsible in major for diverse bioactivities such as antiviral, antimicrobial, antioxidant, radioprotection activities, etc. [2,6]. As part of our ongoing search of natural ingredients for functional foods or medicinal drugs, we carried out phytochemical investigations on this herb. As a result, two new flavonoids (**1**–**2**, Figure 1) have been obtained from the ethanol extract, together with 10 known compounds (**3**–**12**). This paper reported the isolation and identification work of these compounds. Anti-complementary activities of these compounds on the classic pathways were also undertaken, which helps to evaluate the pharmacological functions of the *Trollius* flowers.

## 2. Results and discussion

Compound **1** was obtained as a yellow powder. The positive HRESIMS exhibited a pseudomolecular ion peak at *m*/*z* 777.1859 $[M + Na]^+$, corresponding to the molecular formula of $C_{33}H_{38}O_{20}$, and the degrees of unsaturation were 15. Its UV spectrum showed a typical absorption of flavonoids at 276 and 338 nm. In the $^1H$ NMR spectrum of **1**, the occurrence of an ABX spin system [$\delta_H$ 7.42 (s), 6.84 (1H, d, $J = 8.5$ Hz) and 7.52 (1H, d, $J = 8.5$ Hz)] characterized an *ortho*-di-substituted B ring of the flavone aglycon. Signals at $\delta_H$ 6.58 (1H, s) and 6.24 (1H, s) were attributed to the aromatic protons at H-3 and H-6

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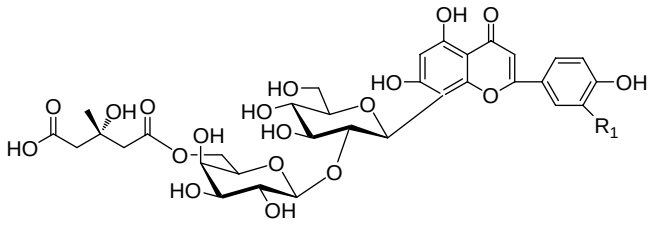

| | $R_1$ |
|---|---|
| **1** | OH |
| **2** | H |

Figure 1. Chemical structures of compounds **1–2**.



of the A-ring, respectively. Additionally, the anomeric proton at $\delta_H$ 4.81 (1H, d, $J = 10.0$ Hz) and other signals between $\delta_H$ 3.0 and 4.1 can be assumed as the protons of sugars. Signals at $\delta_H$ 2.42 (2H, s) and 1.20 (3H, s) at high field indicated that there are protons of some other substitute groups. The $^{13}$C NMR (DEPT) spectral data of **1** revealed the presence of 33 carbons, including 1 methyl ($\delta$ 27.3), 4 methylenes ($\delta$ 45.2, 45.6, 61.4 × 2), 10 methines of sugars ($\delta$67.4–81.9, and $\delta$ 105.5), 15 olefinic carbons of flavones ($\delta$ 98.1–181.8), 1 quaternary carbon ($\delta$ 45.6), as well as 2 carbonyl carbons at $\delta$ 169.9 and 172.8. $^1$H and $^{13}$C NMR signals of **1** were further assigned by extensive analysis of HSQC, $^1$H-$^1$H COSY and HMBC spectra (Table 1). The spectral data of **1** showed rather similarity to those of orientin-2″-*O*-β-D-galactopyranoside (**3**) [4], with some differences at C-5‴/6‴ of galactosyl (Gal) group. Moreover, the remaining signals in the $^1$H and $^{13}$C NMR spectra of **1** indicated the presence of a 3-hydroxy-3-methylglutaroyl (HMG) group (Table 1) [7–9], which was coincident with its molecular weight. *S*-configuration for the C-3⁗ stereogenic carbon of HMG was deduced because HMG esters are formed via the acylation of the hydroxyl group with (*S*)-HMG-CoA in natural products. In HMBC spectrum of **1** (Figure 2), the correlations from Glc-1″-H at $\delta_H$ 4.81 to C-7 at $\delta_C$ 162.5/C-8 at $\delta_C$ 103.8/C-9 at $\delta_C$ 156.2, from Glc-2″-H at $\delta_H$ 4.04 to Gal-C-1‴ at $\delta_C$ 105.5, from Gal-1‴-H at $\delta_H$ 3.91 to Glc-C-2″ at $\delta_C$ 81.3, from both Gal-6‴-H at $\delta_H$ 3.27 and 3.48 to HMG-C-1⁗ at $\delta_C$ 169.9, from HMG-2⁗-2H at $\delta_H$ 2.42 to HMG-C-1⁗ at $\delta_C$ 169.9, from HMG-6⁗-3H at $\delta_H$ 1.20 to HMG-C-2⁗ at $\delta_C$ 45.2/HMG-C-4⁗ at 45.6, and HMG-4⁗-2H at $\delta_H$ 2.50 to HMG-C-5⁗ at $\delta_C$ 172.8 could be observed, which confirmed the structural fragments' connection. On acid hydrolysis, **1** afforded one sugar moiety that identified as D-galacose based on the gas chromatography (GC) analysis of its L-cysteine derivate [10]. On partial acid hydrolysis, **1** gave orientin (**5**) and orientin-2″-*O*-β-D-galactopyranoside (**3**) in HPLC analysis, which further convinced the absolute configuration of two monosugar moieties. Thus, the structure of **1** was established as 6‴-(3-hydroxy-3-methylglutaroyl)-2″-*O*-β-D-galactopyranosyl orientin.

Compound **2** was obtained as a yellow powder. UV spectrum of **2** also showed a typical absorption of flavonoids at 270 and 330 nm. The molecular formula of **2** was determined to be $C_{33}H_{38}O_{19}$ by HR-ESI-MS at *m/z* 761.1911 $[M + Na]^+$. In the $^1$H-NMR spectrum of **2**, six aromatic signals corresponding to H-3 at $\delta_H$ 6.77 (s) and H-6 at $\delta_H$ 6.27 (s), and to an AA′BB′ spin system

Table 1. $^{1}$H- (400 MHz) and $^{13}$C- (100 MHz) NMR spectral data for compounds **1** and **2** (in DMSO-$d_6$, $\delta$ in ppm, $J$ in Hz).[a]

| | **1** | | **2** | | | **1** | | **2** | |
|---|---|---|---|---|---|---|---|---|---|
| No. | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ | No. | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 2 | | 163.8 | | 163.6 | Glc-1″ | 4.81 (d, 10.0) | 71.5 | 4.78 (d, 10.0) | 71.3 |
| 3 | 6.58 (s) | 102.7 | 6.77 (s) | 102.7 | 2″ | 4.04 (dd, 10.0/10.0) | 81.3 | 4.03 (m) | 81.3 |
| 4 | | 181.8 | | 181.8 | 3″ | 3.51 (overlap) | 78.7 | 3.49 (overlap) | 78.6 |
| 5 | | 160.5 | | 161.1 | 4″ | 3.10 (overlap) | 71.5 | 3.13 (overlap) | 71.5 |
| 6 | 6.24 (s) | 98.1 | 6.27 (s) | 98.2 | 5″ | 3.27 (overlap) | 81.9 | 3.27(overlap) | 81.7 |
| 7 | | 162.5 | | 162.6 | 6″ | 3.76 (m), 3.54 (overlap) | 61.4 | 3.78 (m), 3.57 (overlap) | 61.3 |
| 8 | | 103.8 | | 104.8 | Gal-1‴ | 3.91 (br d) | 105.5 | 3.90 (br d) | 105.5 |
| 9 | | 156.2 | | 156.2 | 2‴ | 3.10 (overlap) | 71.5 | 3.13 (overlap) | 71.5 |
| 10 | | 103.6 | | 103.7 | 3‴ | 3.07 (m) | 72.8 | 3.06 (m) | 72.8 |
| 1′ | | 122.1 | | 121.7 | 4‴ | 3.47 (overlap) | 70.2 | 3.45 (overlap) | 69.4 |
| 2′ | 7.42 (s) | 114.0 | 8.02 (d, 8.0) | 128.8 | 5‴ | 3.38 (overlap) | 67.4 | 3.37 (overlap) | 67.4 |
| 3′ | | 145.9 | 6.89(d, 8.0) | 115.8 | 6‴ | 3.48 (overlap), 3.27 (overlap) | 61.4 | 3.45 (overlap), 3.25 (overlap) | 61.0 |
| 4′ | | 149.6 | | 160.5 | HMG-1⁗ | | 169.9 | | 169.9 |
| 5′ | 6.84 (d, 8.5) | 115.7 | 6.89(d, 8.0) | 115.8 | 2⁗ | 2.42 (s) | 45.2 | 2.22 (s) | 45.9 |
| 6′ | 7.52 (d, 8.5) | 119.1 | 8.02 (d, 8.0) | 128.8 | 3⁗ | | 68.9 | | 68.9 |
| | | | | | 4⁗ | 2.50 (overlap) | 45.6 | 2.42 (overlap) | 46.2 |
| | | | | | 5⁗ | | 172.8 | | 172.8 |
| | | | | | 6⁗ | 1.20 (s) | 27.3 | 1.22 (s) | 27.3 |

[a] The assignments were based on a combination of 1D and 2D NMR ($^{1}$H–$^{1}$H COSY, HSQC, and HMBC) experiments.

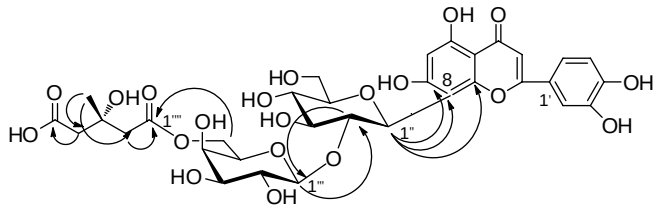

Figure 2. Key HMBC correlations (H → C) for compound **1**.

of H-2′/6′ at $\delta_H$ 8.02 (1H × 2, d, $J = 8.0$ Hz) and H-3′/5′ at $\delta_H$ 6.89 (1H × 2, d, $J = 8.0$ Hz) on the B ring of the aglycone were found. The $^1$H and $^{13}$C NMR spectral data of **2** resembled those of **1**, and the only difference between **2** and **1** (Table 1) was that the hydroxyl group at C-3′ in **1** was replaced by a proton in **2**. Compound **2** afforded D-galacose in GC analysis [10], and vitexin (**6**) in HPLC analysis by partial acid hydrolysis. Accordingly, the structure of **2** was established as 6‴-(3-hydroxy-3-methylglutaroyl)-2″-*O*-β-D- galactopyranosyl vitexin.

In addition, 10 known compounds were also isolated and identified by comparing their NMR and MS spectral data with those reported in the literature. They are assigned to be orientin-2″-*O*-*β*-D-galactopyranoside (**3**) [4], orientin-2″-*O*-β-D-glucopyranoside (**4**) [11], orientin (**5**) [12], vitexin (**6**) [11], 7-methoxyl-2″-*O*-(2‴-methylbutyryl) orientin (**7**) [5], 7-methoxyl-2″-*O*-(2‴-methylbutyryl) vitexin (**8**) [5], 7-methoxyl-2″-*O*-(3‴,4‴-dimethoxybenzoyl) vitexin (**9**) [3], 2″-*O*-(2‴-methylbutyryl) isoswertisin (**10**) [4], trollioside (**11**) [12], and 4-(*β*-D-glucopyranosyloxy)-3-(3-methyl-2-butenyl) benzoic acid (**12**) [12], respectively. Among them, compounds **4**, **9** and **12** were isolated from *Trollius* genus for the first time.

Seven flavonoids (Table 2) together with trollioside (**11**), were evaluated for their anti-complementary activity on the classic pathways *in vitro* [13], while compounds **4** and **8** were not tested due to limited amount. Bioassay showed that all flavonoids inhibited complement activation on the classic pathway apparently, with their $IC_{50}$ data ranging from 0.88 to 4.02 mM, and trollioside (**11**) showed no effect in this assay. By comparison with orientin (**5**), vitexin (**6**) with less hydroxyl group in B ring showed higher activity; 7-methoxylation in A ring was not favored by comparison of **7** and **10**; esterification of sugars strengthened anti-complementary activity apparently, as observed by comparison of **7** and **5**. Increasing a Gal group to orientin lowered this activity, as indicated by **3**; however, adding an additional HMG to **3** increased the activity, as shown by **1**, and **2** showed highest activity with $IC_{50}$ value to be 0.88 mM. The results indicated that esterification of sugar moiety of these flavone *C*-glycosides played important roles for their anti-complementary activity, while aglycon substitution also affected their bioactivity.

Table 2. Anticomplementary activity through classical passway of the samples ($n = 3$, $\bar{x} \pm$ SD).

| Compound | $IC_{50}$ (mg/ml) | $IC_{50}$ (mM) | Compound | $IC_{50}$ (mg/ml) | $IC_{50}$ (mM) |
|---|---|---|---|---|---|
| **1** | 0.87 ± 0.059 | 1.16 ± 0.08 | **6** | 1.35 ± 0.141 | 3.12 ± 0.33 |
| **2** | 0.65 ± 0.018 | 0.88 ± 0.02 | **7** | 0.56 ± 0.099 | 1.01 ± 0.18 |
| **3** | 2.45 ± 0.125 | 4.02 ± 0.20 | **9** | 1.28 ± 0.238 | 2.04 ± 0.38 |
| **5** | 1.51 ± 0.081 | 3.36 ± 0.18 | **10** | 1.43 ± 0.041 | 2.70 ± 0.08 |
| **Heparin** | 0.096 ± 0.025 | | | | |

However, further anti-complementary assays should be undertaken to test this result. Accumulating data have suggested that excessive activation of complement is involved in the pathogenesis of many autoimmune disorders, inflammatory diseases, and inflammation responses, including acute respiratory distress syndrome [13,14]. The results suggested that these flavone *C*-glycosides should be main representative constituents for the applications of the herb in treatments of acute respiratory distress syndrome, etc.

## 3. Experimental

### 3.1 General experimental procedures

Optical rotations were measured on Perkin-Elmer PL341 polarimeter. UV spectra were measured on Perkin-Elmer Lambda 25 uv/vis spectrometer instrument (Perkin-Elmer, Inc., Waltham, MA, USA). NMR spectra were recorded on a Varian 400M NMR System (Varian, Inc., Palo Alto, CA, USA). HRESI mass spectra were performed with a Finnigan LCQ-Deca high-resolution mass spectrometer (Thermo-Finnigan, Inc., San Jose, CA, USA). GC analysis was carried out on a Shimadzu GC-14C gas chromatograph with FID detection, using a Rtx-1 capillary column (30 m × 0.25 mm, i.d.; Shimadzu Corporation, Tokyo, Japan). HPLC separations were performed on Shimadzu LC-20A series apparatus with a SPD-20A UV detector (Shimadzu Corporation, Tokyo, Japan), equipped with a 250 × 20 mm i.d. preparative Cosmosil AR-II $C_{18}$ column (Nacalai Tesque, Inc., Kyoto, Japan); Medium pressure liquid chromatography (MPLC) was performed on a Lisure HP Purifier apparatus with a UV detector (Lisure science Corporation, Suzhou, China). Polyamide chromatography (100–200 mesh) was manufactured by Taizhou Luqiao Biochemical Co., Taizhou, China. Silica gel for column chromatography and precoated silica gel plates 60 $F_{254}$ for thin layer chromatography were applied by Qingdao Haiyang Chemical, Ltd., Qingdao, China. Cosmosil $C_{18}$ reversed-phase silica gel (75 μm, Nacalai tesque, Inc., Kyoto, Japan) was used for column chromatography.

### 3.2 Plant material

The flowers of *T. chinensis* Bunge were collected from Chengde, Hebei Province, China, in August 2006, and the botanical origin of material was identified by Doctor Jinna Cai (Shanghai Mediclon Inc., Shanghai, China). The voucher specimens (No. TC060901) are deposited at the herbarium of College of Pharmaceutical Sciences, Soochow University, Suzhou, China.

### 3.3 Extraction and isolation

The flowers (5 kg) were extracted with 70% aqueous ethanol (v/v) for two times (50 liters, 1 h each) under reflux. After evaporation, the residue was suspended in $H_2O$ (10 liters) and filtered. The liquor solution was subjected to AB-8 macroporous resin column, eluting with $H_2O$, 10%, 70%, and 95% ethanol (each 30 liters) successively. The 70 and 95% eluants were combined and concentrated under reduced pressure to give a residue, which was suspended in $H_2O$ and partitioned using EtOAc (5 × 4 l) to give EtOAc fraction (TE, 126 g) and water fraction (TW, 165 g). TW was applied to vacuum silica gel column eluting with EtOAc–EtOH (7:3, 5:5, 1:9) to afford three fractions (JW-1 ~ 3). JW-1 (8.2 g) was subjected to silica MPLC using a stepwise gradient elution of $CHCl_3$–MeOH (0.1% $CH_3COOH$) (7:3, 5:5, 2:8), purified by Sephadex LH-20 eluting with 90% MeOH to give **5** (70 mg) and **6** (50 mg). JW-2 (21.0 g) was subjected to polyamide MPLC eluting with EtOAc–EtOH (7:3, 5:5, 2:8) to afford three fractions (JW-2-1 ~ 3). JW-2-2 (4.8 g) was subjected to ODS column eluting with $CH_3CN$–$H_2O$ (0.1% $CH_3COOH$)

(16:84) to afford three subfractions (JW-2-2-1 ~ 3). JW-2-2-3 (0.6 g) was subjected to preparative HPLC, using a mobile phase of $CH_3CN-H_2O$ (0.1% $CH_3COOH$) (16:84) to give **1** (21 mg) and **2** (19 mg). By similar separation procedures, compound **3** (20 mg) from JW-2-2-1 (0.9 g), and **4** (15 mg) from JW-2-3 (1.2 g) were also obtained. Part of the EtOAc fraction (20.0 g) was applied to polyamide MPLC using a stepwise gradient elution of $CHCl_3$–EtOAc (8:2, 5:5, 4:6, 2:8) to afford five fractions (JE-1 ~ 5). JE-3 (3.8 g) was subjected to reverse MPLC eluting with MeOH–$H_2O$ (0.1% HAc) (35:65, 40:60) to give **10** (70 mg), **11** (45 mg), and **9** (34 mg). By similar separation procedures, compounds **12** (25 mg), **8** (28 mg), and **7** (24 mg) were also separated from JE-4 (4.5 g).

#### 3.3.1 Compound *1*

Yellow amorphous powder, $[\alpha]_D^{20}$ −12 (*c* 0.042,MeOH). UV $\lambda_{max}$ (MeOH) nm: 276, 338. For $^1H$ (400 MHz, DMSO-$d_6$) and $^{13}C$ NMR (100 MHz, DMSO-$d_6$) spectral data, see Table 1. HRESIMS: *m/z* 777.1859 $[M + Na]^+$ (calcd for $C_{33}H_{38}O_{20}$ Na, 777.1854).

#### 3.3.2 Compound *2*

Yellow amorphous powder, $[\alpha]_D^{20}$ −25 (*c* 0.056,MeOH). UV $\lambda_{max}$ (MeOH) nm: 270, 330. For $^1H$ (400 MHz, DMSO-$d_6$) and $^{13}C$ NMR (100 MHz, DMSO-$d_6$) spectral data, see Table 1. HRESIMS: *m/z* 761.1911 $[M + Na]^+$ (calcd for $C_{33}H_{38}O_{19}Na$, 761.1905).

### 3.4 Acid hydrolysis and GC and HPLC analysis of 1–2

The GC analysis was carried out according to the method described [10]. The absolute configuration of the monosaccharide was confirmed to be D-galacose by comparison of the retention time of its L-cysteine derivative with that of D-galacose (13.4 min) standard sample. As for partial acid hydrolysis, compounds **1** and **2** (3 mg each) were solved separately in 2 M HCl (3 ml) and heated at 60°C on a water bath for 2 h, neutralized with 2 M NaOH to get sample solutions. The two samples were analyzed on HPLC by comparison of the retention times with those of reference compounds, with acetonitrile–water (contained 1% acetic acid) as eluant using a gradient elution of 13–15% acetonitrile in 25 min, and the detection wavelength was set at 340 nm. The mobile phase flow rate was 1.0 ml/min. Orientin-2″-*O*-β-D-galactopyranoside ($t_R$ 16.9 min) and orientin ($t_R$ 20.9 min) were detected from hydrolyte of **1**, and vitexin ($t_R$ 28.5 min) was detected from hydrolyte of **2**, respectively.

### 3.5 Anti-complementary activity assay through the classical pathway

The bioassay was carried out according to the method described [13]. Each compound (3 mg, dissolved with DMSO) was applied for this assay, with heparin (sodium salt, 160 IU/mg) used as positive control. The 50% percent inhibition was calculated as shown in Table 2. Trollioside showed no effect in this assay.

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