Chemical constituents from the leaves of *Tripterygium wilfordii* and their cytotoxic activity

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Abstract
A new flavonol glycoside derivate named kaempferol 3-O-trans-p-coumaroyl-7-O-α-L-rhamnoside (1), together with five known compounds (2–6), was isolated from the EtOAc-soluble extract of the leaves of *Tripterygium wilfordii*. The structure of new compound was elucidated with spectroscopic and physico-chemical analyses. All the isolates were evaluated for in vitro cytotoxic activity against four human cancer cells including HepG2, Hep3B, A549 and MCF-7. Among them, compound 1 exhibited the significant cytotoxic activities against HepG2 and Hep3B cells with IC₅₀ values of 6.8 ± 1.6 and 4.2 ± 1.3 μM, respectively. The above data demonstrated the potential of 1 for the treatment of liver cancer. In addition, 4 also showed moderate inhibitory activity on HepG2 and Hep3B cells with IC₅₀ values of 16.1 ± 2.3 and 10.7 ± 1.8 μM, respectively.

Keywords
Celastraceae, cytotoxic activity, flavonol glycoside, Hep3B, *Tripterygium wilfordii*

Introduction
*Tripterygium wilfordii* Hook. f. (Celastraceae) is a vine shrub, which is widely distributed in China, Korea and Japan.¹ The roots of *T. wilfordii*, also known as ‘Lei-Gong-Teng’ in China, were used to treat inflammatory and auto-immune diseases as a traditional Chinese medicine.² Pharmacological studies confirmed that *T. wilfordii* possessed anti-inflammatory,³ immunosuppressive, anti-tumour⁴ and neuroprotective activities.⁵ And a large number of phytochemical investigations on the roots of *T. wilfordii* revealed the presence of dihydroagarofuran sesquiterpenoids,⁶ sesquiterpene alkaloids,⁷ abietane diterpenoids,⁸ triterpenoids,⁹ megastigmane glycosides¹⁰ and a small amount of flavonoids and lignans.¹¹,¹² However, there are less chemical studies on the leaves of *T. wilfordii* compared with its roots. Thus, a series of studies focused on the leaves of *T. wilfordii* were carried out. As a result, a new flavonol glycoside derivate named kaempferol 3-O-trans-p-coumaroyl-7-O-α-L-rhamnoside (1), together with five known compounds (2–6), was isolated. Herein, we report the isolation and structural elucidation of these compounds.
nmr data at δ_H 5.30 (1H, d, J = 1.2 Hz, H-1″), 3.98 (1H, m, H-2″), 3.48 (1H, dd, J = 9.0, 3.0 Hz, H-3″), 3.16 (1H, m, H-4″), 3.30 (1H, dd, J = 9.0, 6.0 Hz, H-5″) and 0.80 (3H, d, J = 6.0 Hz, H-6″) combined with 1^3^C NMR data at δ_C 101.8 (C-1″), 70.3 (C-2″), 70.6 (C-3″), 71.1 (C-4″), 70.0 (C-5″) and 17.5 (C-6″) (Supplemental Figure S2). And this sugar moiety could be further confirmed by acid hydrolysis assay. The low-field region of 1^3^C NMR spectrum of 1 revealed 24 carbons including 2 carbonyl carbons at δ_C 177.7 (C-4) and 167.7 (C-9″), 18 aromatic carbons and 4 olefinic carbons at δ_C 164.2–93.7. The comparison of the 1D NMR data of 1 and kaempferol 7-O-α-L-rhamnopyranoside revealed that the skeleton of 1 was flavonol glycoside. Furthermore, the structure of trans-coumaroyl moiety could be determined by the key HMBC correlations of δ_H 7.49 (H-2″/6″) with δ_C 125.1 (C-1″), 115.0 (C-3″), 159.2 (C-4″) and 144.6 (C-7″), δ_H 6.81 (H-3″/5″) with δ_C 125.1 (C-1″), 115.0 (C-2″) and 159.2 (C-4″), δ_H 7.51 (H-7″) with δ_C 125.1 (C-1″), 115.0 (C-2″/6″), 114.5 (C-8″) and 167.7 (C-9″), and δ_C 6.32 (H-8″) with δ_C 125.1 (C-1″), 144.6 (C-7″) and 167.7 (C-9″) (Figure 1, Supplemental Figure S3 and S4). Although the position of trans-p-coumaroyl moiety could not be determined by the HMBC correlations, two hydroxy signals with larger chemical shifts at δ_H 11.1 and 10.9 could indirectly reveal that the trans-p-coumaroyl moiety was linked with C-3. In addition, the aglycone isolated from the acid hydrolysis assay of 1 was identified as p-coumaroylkaempferol (2) by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) methods, and it further demonstrated the structure of 1. Thus, the structure of compound 1 was assigned as kaempferol 3-O-trans-p-coumaroyl-7-O-α-L-rhamnopyranoside.

Moreover, five known compounds (2–6) were obtained from the leaves of T. wilfordii and identified as p-coumaroylkaempferol (2),^14^ tripteryg B (3),^15^ 3β-O-trans-coumaroylbutelanic acid (4),^16^ camaldulenic acid (5)^17^ and crategic acid (6)^18^ based on the NMR data and comparison with literature data (Figure 2).

### Analysis of biological activity results

The in vitro cytotoxic activity of compounds 1–6 against HepG2, Hep3B, A549 and MCF-7 cells was evaluated by
the MTT method, and doxorubicin was used as the positive control. As shown in Table 2, compounds 1–6 showed different levels of cytotoxic activity with IC\textsubscript{50} values ranging from 4.2 ± 1.3 to 47.8 ± 2.0 μM. Among these compounds, 1 showed the significant cytotoxic activity on HepG2 and Hep3B cells with IC\textsubscript{50} values of 6.8 ± 1.6 and 4.2 ± 1.3 μM, respectively. In addition, 4 also exhibited moderate cytotoxic activity towards Hep3B cells with IC\textsubscript{50} value of 10.7 ± 1.8 μM. In general, the potential structure–activity relationships could be deduced. The comparison of cytotoxic activity on four cancer cells between 1 and 2 indicated that the increasing cytotoxic activity might be associated with the α-l-rhamnoside moiety which linked with C-7 of 1. Based on the significant cytotoxic activity of 1 on Hep3B cells, the deeply pharmacological investigations on 1 should be further carried out.

**Conclusion**

In this study, a new flavonol glycoside derivate named kaempferol 3-O-trans-p-coumaroyl-7-O-α-l-rhamnoside (1), together with five known compounds (2–6), was isolated from the EtOAc-soluble extract of the leaves of *T. wilfordii*. All compounds were tested for their in vitro cytotoxic activity against HepG2, Hep3B, A549 and MCF-7 cells. It was noted that compound 1 showed the significant cytotoxic activity on HepG2 and Hep3B cells with IC\textsubscript{50} values of 6.8 ± 1.6 and 4.2 ± 1.3 μM, respectively. This investigation confirmed that p-coumaroyl and caffeoyl moieties could highly increase the cytotoxic effect of alternariol on cancer cells. The previous studies mainly reported the presence of various dihydroagarofuran sesquiterpenoids with potent cytotoxic activity from the roots of *T. wilfordii*. This study provided a prediction that this kind of phenylpropanoid-conjugated flavonol glycoside might be considered as lead compounds to treat liver cancer. Thus, the in-depth pharmacological investigations on 1 should be carried out.

**Experimental**

**General**

Optical rotations were determined on a Perkin-Elmer 241MC automatic digital polarimeter (PerkinElmer, Waltham, USA). UV spectra were conducted on a JASCO V-650 spectrometer (JASCO, Tokyo, Japan). IR spectra were recorded with a JASCO FT-IR 620 spectrophotometer (JASCO, Tokyo, Japan). Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker AV-600 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 (200 μm particle size, Yantai Xinde Chemical Co., Ltd, Yantai, China) and RP-18 (150-63 μm particle size, Merck, Darmstadt, Germany). TLC was performed with precoated silica gel GF\textsubscript{254} glass plates (Qingdao Marine Chemical Co., Ltd). A Shimadzu LC-6AD HPLC with a SPD-20A detector (Shimadzu, Tokyo, Japan) and an YMC Pack C\textsubscript{18} column (250 mm ×
10 mm, i.d., 5 μM, YMC Co. Ltd., Japan) were used for the semi-preparative separations.

Plant material

The leaves of *T. wilfordii* were collected in Maanshan, Anhui province, China, and authenticated by Professor Hongxiang Lou (College of Pharmacy, Shandong University). A voucher specimen of the plant (No. 202000910) was deposited at the Cheeleeo College of Medicine, Shandong University, Shandong, China.

Extraction and isolation

The dried leaves of *T. wilfordii* (10.0 kg) were extracted three times with 75% MeOH (80.0 L × 2 h × 3) under reflux, and the solution was concentrated in vacuo to yield a black extract (1.2 kg). This extract was suspended in H2O (10.0 L), partitioned successively with petroleum ether (PE), CH2Cl2, EtOAc and n-BuOH (each 8.0 L × 3). The EtOAc extract of *T. wilfordii* displayed the moderate in vitro cytotoxicity on four human cancer cell lines. Therefore, the EtOAc fraction (123.4 g) was chromatographed over a silica gel column using a gradient of CH2Cl2-MeOH (from 100:0 to 0:1) and was separated into 12 fractions (Fr.11.1–Fr.11.12). Furthermore, Fr.11.4 (8.5 g) was fractionated by column chromatography on silica gel using a gradient of CH2Cl2-MeOH (from 100:1 to 1:1), and was separated into 17 fractions (Fr.1–Fr.17). Fr.11 (8.5 g) was chromatographed over a silica gel column using a gradient of CH2Cl2-MeOH (from 100:0 to 0:1) and was separated into 12 fractions (Fr.11.1–Fr.11.12). Furthermore, Fr.11.4 (1.1 g) was subjected to the RP-18 column and using the elution of MeOH-H2O (7:3:0.5) to afford 13 fractions (Fr.11.4.1–Fr.11.4.13). Fr.11.4.4 (143.1 mg) was eventually separated via preparative HPLC using an isocratic solvent system 50%–55% MeOH in H2O over 60 min yielded com-

Acid hydrolysis of compound 1

The sample of compound 1 (2.0 mg) was added to MeOH solution (3.0 mL) and refluxed with 2 M HCl (3.0 mL) at 100 °C for 4 h. After removal of MeOH under reduced pressure, the reaction mixture was diluted with H2O (5.0 mL) and neutralized with NaHCO3 solution, followed by extraction with EtOAc (3 × 10 mL). Next, the collected organic phase was evaporated and subjected to PTLC using CHCl3-MeOH (5:1) as an eluent to yield the aglycone. The water layer was concentrated and subjected to PTLC (CHCl3-MeOH/H2O = 7:3:0.5) to yield the sugar. The identification of l-rhamnose was carried out by the comparison of Rf values in TLC (CHCl3/MeOH/H2O = 7:3:0.5) between the obtained sugar and authentic sample. Spot was detected by spraying with vanillin-sulfuric acid followed by heating. In addition, their similar optical rotation could also confirm the type of sugar (obtained sugar sample: [α]D20 + 8.4; authentic standard: [α]D20 + 8.2).13,19

Cytotoxicity assay

The MTT assay was used to determine the cytotoxicity of the compounds against human cancer cell lines according to the method previously described.20 Briefly, the cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% foetal bovine serum, 100 U mL−1 penicillin and 100 μg mL−1 streptomycin in a humidified 37 °C incubator supplied with 5% CO2. Then, cells were seeded at 5 × 103 cells mL−1 per well onto 96-well flat bottom plate and incubated for 12 h at 37 °C with 5% CO2. Compounds 1–6 were dissolved in DMSO to obtain a initial concentration (10 mM) and diluted to obtain a series of concentra-

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Supplemental material

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