A new flavonol glycoside from the florets of *Carthamus tinctorius* L.

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One new flavonol glycoside, 6-hydroxykaempferol-3-O-\(\beta\)-d-glucoside-7-O-\(\beta\)-d-glucuronide (1), together with eight known flavonoids and three known quinochalcones, was isolated from the florets of *Carthamus tinctorius* L. Their structures were determined by extensive spectroscopic analyses. Their cardioprotective effects against \(\text{H}_2\text{O}_2\)-induced apoptosis in H9c2 cells were also evaluated; compounds 1, 2, 4–5, 7–10 and 12 provided significant protective effects on \(\text{H}_2\text{O}_2\)-induced H9c2 cells at the concentration of 25 \(\mu\)g/mL.

**Keywords:** *Carthamus tinctorius* L; cardioprotective activity; 6-hydroxykaempferol-3-O-\(\beta\)-d-glucoside-7-O-\(\beta\)-d-glucuronide

1. Introduction

*Carthamus tinctorius* L. has been used as a Chinese folk medicine for over 1000 years (Ge et al. 1996). The florets have the function of promoting blood circulation by removing blood stasis (Yin & He 2000). It showed therapeutic potential for coronary heart disease, stroke, gynaecological disease, angina and hypertension (Jiang et al. 2008; Fan et al. 2009). The extract of *C. tinctorius* L. has been developed as an intravenous injection, and has been extensively applied in hospitals to treat cardiovascular diseases (Fan et al. 2009).

Chemical components include flavonoids, alkaloids, lignans and fatty acids, which were isolated and identified from safflower (Yin et al. 2000). Then flavonoids with potent antioxidative effects are the major effective components in traditional herbal medicine used in treating cardiovascular diseases (Sun et al. 2011). Meanwhile, the H9c2 cell line has been widely used in studies investigating cardiomyocyte cellular mechanisms (Hescheler et al. 1991). \(\text{H}_2\text{O}_2\) has been extensively used as an inducer of oxidative stress *in vitro* (Ryter et al. 2007). Thus, this

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study aimed to research the isolated flavonoids against H$_2$O$_2$-induced cytotoxicity in cultured H9c2 cells.

Based on our investigation, nine flavonoids and three quinocochalcones were obtained, including a new flavonoid glycoside namely 6-hydroxykaempferol-3-O-β-D-glucoside-7-O-β-D-glucuronide (1) and 11 known compounds Quercetin-3-O-β-D-glucoside-7-O-β-D-glucuronide (2) (Budzianowski et al. 1991), 6-hydroxykaempferol-3, 6-di-O-β-D-glucoside-7-O-β-D-glucuronide (3), Quercetin-3-O-β-D-rutinoside (4), kaempferol-3-O-β-D-glucoside (5) (Kazuma et al. 2000), kaempferol-7-O-β-D-glucoside (6) (Yoshida et al. 1987), Kaempferol (7) (Markham et al. 1978), Quercetin (8) (Kim et al. 1992), Apigenin (9) (Markham et al. 1978), Hydroxysafflor yellow A (10), Safflor yellow A (11) (Meselhy et al. 1993) and Saffloquinoside A (12) (Jiang et al. 2010) (Figure 1). Among them, compounds 1, 2, 4–5, 7–10 and 12 provided significant protective effects on H$_2$O$_2$-induced H9c2 cells at the concentration of 25 μg/mL.

2. Results and discussion

Compound 1 was obtained as a yellow amorphous powder. Its molecular formula was assigned as C$_{27}$H$_{28}$O$_{18}$ from its quasimolecular ion peak at m/z 639.1201 [M – H]$^-$ (calcd for 639.1203) in the HR-ESI-Q-TOF-MS. In the $^1$H NMR spectrum, three signals in the aromatic region were observed as one singlet at δ 6.96 (1H, s, H-8), as well as an AA'BB'-coupled system at δ 8.03 (2H, d, $J=8.4$ Hz, H-2', H-6') and 6.89 (2H, d, $J=8.4$ Hz, H-3', H-5'), and three hydroxy groups at δ 12.39 (1H, s, OH-5), 10.20 (1H, s, OH-4') and 8.63 (1H, s, OH-6), suggested it to be a

Figure 1. Structure of compounds 1–12.
6-hydroxykaempferol (Hattori et al. 1992). Moreover, two anomic proton signals were observed at δ 5.47 (1H, d, J = 7.2 Hz, 3-O-Glc-1) and 5.23 (1H, d, J = 7.2 Hz, 7-O-GlcA-1) which indicated that both sugar residues had β-pyranosyl configurations. The 13C NMR spectrum displayed signals for 25 carbons, which included the signals of 6-hydroxykaempferol 3-O-glycoside with two sugar moieties (Markham et al. 1978).

Furthermore, signals of each sugar unit were assigned by HSQC, HMBC, 1H–1HCOSY and NOESY analyses. The NOESY spectrum showed correlations between 3-O-Glc H-1 and H-3, H-1 and H-5, H-3 and H-5, H-2 and H-4, H-4 and H-6, and 7-O-GlcA H-1 and H-3, H-1 and H-5, H-3 and H-5. From the above deduction, two sugar moieties were detected as β-D-glucose and β-D-glucuronic acid when the literature indicating most of 6-hydroxykaempferol glycosides from C. tinctorius L. with alduronic acid was attached to glucuronic acid in which 7-O-GlcA OH-2 and OH-4 were equatorial (Kazuma et al. 2000; Fan et al. 2009). In the HMBC spectrum, appreciable shift correlations were observed between the anomic proton signal at δ 5.47 (1H, d, J = 7.2 Hz, 3-O-Glc-1) and a C-atom signal at δ 133.2 (C-3), and between the anomic proton signal at δ 5.23 (1H, d, J = 7.2 Hz, 7-O-GlcA-1) and a C-atom signal at δ 151.1 (C-7). Furthermore, a significant NOE was observed between the anomeric proton at δ 5.23 (1H, d, J = 7.2 Hz, 7-O-GlcA-1) and δ 6.96 (1H, s, H-8), which confirmed that glucuronic acid was directly linked to OH-7. Thus, the structure of compound 1 was elucidated to be 6-hydroxykaempferol-3-β-D-glucoside-7-β-D-glucuronide, and it was a new compound.

All the nine flavonoids and three quinochalcones were evaluated for their anti-oxidative effects against H2O2-induced apoptosis in cultured H9c2 cells. Among them, compounds 1, 2, 4–5, 7–10 and 12 provided significant protective effects on H2O2-induced H9c2 cells at the concentration of 25 μg/mL (Figure 2). The cardioprotective effects of compounds 7 and 8 were more effective than those of compounds 1–6 which indicated that glycosylation of flavonoids diminished their activity when compared to the corresponding aglycones (Cai et al. 2006). Compounds 1 and 2 exhibited significant cardioprotective effects due to diOH-7, 8 or diOH-3’, 4’ groups (Seyoum et al. 2006). The structure of compound 9 was similar to that of compound 7 (it possesses an additional OH-3 group), which had stronger activity (Amaral et al. 2009). Compound 10 was more active than the other two quinochalcones (compounds 11 and 12) due to the more phenolic hydroxyl groups (Modak et al. 2005).

The results suggested that flavonol aglycones with diOH-7, 8 or diOH-3’, 4’ groups displayed important roles for their protective effects against H2O2-induced cardiotoxicity. Moreover, the number of the phenolic hydroxyl groups contributed to the activity of quinochalcones. However, further investigations were needed to explain the detailed structure–activity relationships.

3. Experimental

3.1 General experimental procedures

NMR spectra were recorded on a Bruker AV-400 instrument using TMS as internal standard (Bruker Scientific Technology Co., Ltd., Zurich, Switzerland). HR-ESI-MS spectra were measured on an LCT Premier XE (Waters) mass spectrometer (Waters Technology Ltd., Milford, USA). Preparative HPLC was performed on an Agilent 1260 system using an Agilent XDB-C18 column (250 mm × 21.2 mm, 7 μm, Agilent Technologies, Santa Clara, CA, USA). Column chromatography (CC) was performed on silica gel (100–200 mesh, 200–300 mesh; Qingdao Marine Chemical Ltd., Qingdao, China), Diaion HP-20 (Mitsubishi-Chemical, Tokyo Met, Japan), Sephadex LH-20 (Pharmacia, Uppsala, Sweden) and C18 reverse-phased silica gel (60 μm, Acchrom Technologies Co., Ltd., Dalian, China). Precoated silica gel GF254 plates (Qingdao Marine Chemical Plant, Qingdao, China) were used for thin-layer chromatography. All solvents were of analytical grade (Shanghai Chemical Reagents Company, Ltd., Shanghai,
China), and the solvents used in HPLC were of HPLC grade (J&K Scientific Ltd., Beijing, China).

### 3.2 Plant material

The florets of *C. tinctorius* L. were collected from Changji Hui tribe autonomous prefecture of the Xinjiang Uygur Autonomous Region of China in July 2013. The specimen was identified by Zhaoqing Meng, Jiangsu Kanion Pharmaceutical Co. Ltd., Lianyungang, People’s Republic of China, where a voucher specimen (No. CKG 20140503G) has been deposited.

### 3.3 Extraction and isolation

The florets of *C. tinctorius* L. (3 kg) were exhaustively extracted twice with 70% (v/v) EtOH under refluxed conditions. The EtOH extracts were concentrated under reduced pressure to give a residue (320 g), which was suspended in H2O and partitioned with EtOAc and *n*-BuOH successively. The *n*-BuOH extract was subjected to column chromatography (CC) over HP-20 macroporous adsorptive resins, eluted with water, 5%, 30% and 95% EtOH in successive. The 5% (v/v) EtOH eluate was separated by using C18 reversed-phase silica (MeOH/H2O, 5–50%) to give 11 fractions (Fr.A1–Fr.A11). Fr.A5 was further purified by preparative HPLC (MeCN/0.01% TFA-H2O, 12%) to yield compounds 1 (5 mg) and 2 (10 mg). Fr.A6 was subjected to preparative HPLC (MeCN/0.01% TFA-H2O, 12%) to obtain compound 3 (98 mg). Fr.A7 was
chromatographed over Sephadex LH-20 (MeOH/H₂O, 50%) to give compound 10 (985 mg). Fr. A8 was separated by preparative HPLC (MeCN/0.01% TFA-H₂O, 15%) to yield compounds 11 (30 mg) and 12 (24 mg). The 30% (v/v) EtOH eluate was fractionated by CC on silica gel (CHCl₃/MeOH, 30:1–3:1) to yield Fr.B1–Fr.B8. Fr.B3 was purified by preparative HPLC (MeOH/H₂O, 55%) to yield compounds 7 (16 mg), 8 (18 mg) and 9 (8 mg). Fr.B5 was subjected to preparative HPLC (MeCN/0.01% TFA-H₂O, 15%) to give compounds 5 (15 mg) and 6 (21 mg). Fr.B7 was further purified by preparative HPLC (MeOH/H₂O, 31%) to afford compound 4 (18 mg).

3.4 Spectroscopic data
6-hydroxykaempferol-3-Ö-b-D-glucoside-7-Ö-b-D-glucuronide (1): yellow amorphous powder; HR-ESI-Q-TOF-MS m/z: 639.1201 [M – H]⁻, calcd for C₂₇H₂₇O₁₈: 639.1203. ¹H NMR (400 MHz, DMSO-d₆): δ 12.39 (1H, s, OH-5), 8.63 (1H, s, OH-6), 6.96 (1H, s, H-8), 8.03 (2H, d, J = 8.4 Hz, H-2', H-6'), 10.20 (1H, s, OH-4'), 6.89 (2H, d, J = 8.4 Hz, H-3', H-5'); 3-O-Glc, 5.47 (1H, d, J = 7.2 Hz, H-1), 3.17 (1H, t, J = 7.2 Hz, H-2), 3.21 (1H, m, H-3), 3.07 (1H, m, H-4), 3.08 (1H, m, H-5), 3.54 (1H, brd, J = 11.7 Hz, H-6a), 3.33 (1H, brd, J = 11.7 Hz, H-6b); 7-O-GlcA, 5.23 (1H, d, J = 7.2 Hz, H-1), 3.39 (1H, m, H-2), 3.35 (1H, m, H-3), 3.42 (1H, m, H-4), 4.04 (1H, d, J = 9.4 Hz, H-5). ¹³C NMR (100 MHz, DMSO-d₆): δ 157.0 (C-2), 133.2 (C-3), 177.9 (C-4), 146.1 (C-5), 130.1 (C-6), 151.1 (C-7), 93.5 (C-8), 148.4 (C-9), 106.3 (C-10), 121.2 (C-1'), 131.0 (C-2', C-6'), 115.1 (C-3', C-5'), 159.9 (C-4'); 3-O-Glc 100.8 (C-1), 72.7 (C-2), 75.1 (C-3), 71.3 (C-4), 75.4 (C-5), 170.0 (C-6).

3.5 Biological assay
The anti-oxidative effects of the isolated flavonoids were evaluated against H₂O₂-induced cytotoxicity in cultured H9c2 cells.

3.5.1 Cell culture
H9c2 cardiomyocytes were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum at 37°C in a water-saturated 5% CO₂ incubator. Reagents for cell cultures were purchased from Sigma (St. Louis, MO, USA).

3.5.2 Cell viability test
H9c2 cells (3000 cells/well) were seeded into 96-well plates after incubation with 50 µM H₂O₂ for 3 h followed by treatment with compounds 1–12 (25 µg/mL) for 3 h. At the end of the incubation, CCK-8 reagent was added into each well followed by further incubation for 4 h. The optical density was measured at 450 nm using a multiscan microplate reader (Teken, Beijing, China). Each determination represented the average mean of six replicates.

3.5.3 Statistical analysis
Data were expressed as mean ± standard deviation (SD). A post hoc Dunnett’s test was used to obtain corrected p-values in group comparisons. Statistical analyses were performed with one-way ANOVA (SPSS version 17.0: Chicago, IL, USA). A p-value of 0.05 or less was considered significant.
4. Conclusions

One new flavonol glycoside, 6-hydroxykaempferol-3-O-β-D-glucoside-7-O-β-D-glucuronide (1), was isolated from the florets of *Carthamus tinctoriu* L., together with eight known flavonoids and three known quinochalcones. Their structures were determined by extensive spectroscopic analyses. Their cardioprotective effects against H$_2$O$_2$-induced apoptosis in H9c2 cells were also evaluated; compounds 1, 2, 4–5, 7–10 and 12 provided significant protective effects on H$_2$O$_2$-induced H9c2 cells at the concentration of 25 μg/mL.

Supplementary material

Supplementary figures relating to this article are available online at http://dx.doi.org/10.1080/14786419.2015.1045905.

Disclosure statement

No potential conflict of interest was reported by the authors.

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