A new anthraquinone from seed of *Cassia obtusifolia*

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Seeds of *Cassia obtusifolia* L. are known as homology of medicine and food material, which is a commonly consumed beverage in China. One new compound, 8-hydroxy-1,7-dimethoxy-3-methylanthracene-9,10-dione-2-O-\(\beta\)-D-glucoside (1), together with 11 known compounds, including seven anthraquinones (2–8), was isolated from the seeds. The 2D NMR data of compound 2 are reported for the first time. The structures of the compounds were established on the basis of 1D and 2D NMR, IR and HR-ESI-MS spectra. The cytotoxic activities of all the compounds against five cell lines (LO2, HCT-116, A549, HepG2 and SGC7901) were evaluated by using CCK8 methods. Compounds 1, 3 and 7 show moderate cytotoxicity towards HCT-116 cells compared with oxaliplatin.

**Keywords:** *Cassia obtusifolia*; anthraquinone; antineoplastic activity

1. Introduction

*Cassia obtusifolia* Linn., a member of the genus *Cassia* (Leguminosae), is a well-known traditional Chinese medicinal plant. It belongs to the medically and economically important family Leguminosae (syn. Fabaceae), subfamily Caesalpinioideae (Joshi & Kapoor 2003). *Semen Cassiae* is the dry ripe seed of *C. obtusifolia* (Leguminosae). This herb is distributed mainly in China, Korea, India and the west tropical regions (Vadivel et al. 2012). The seeds are reported to have the effects of improving eyesight, alleviating constipation and lowering hypertension and hyperlipidemia (Hao et al. 2001). In previous investigations of this plant, a number of constituents were isolated, including anthraquinones, anthrones, flavonoids and triterpenoids (Guo et al. 1998; Zhang et al. 2009; Sob et al. 2010). Many studies have indicated that anthraquinones have antitumour or antimetastasis effects (Zhang et al. 1998; Lee 2001; Kuo

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et al. 2004). In previous research, we had isolated cytotoxic anthraquinone glycosides from marine Streptomyces sp. strain (Lu et al. 2012). With the aim of finding new cytotoxic anthraquinone, we investigated the chemical constituents of Semen Cassiae. In our ongoing efforts towards identifying new bioactive components from C. obtusifolia, we have conducted an investigation of the ethanol extract from the seeds of C. obtusifolia and isolated one new anthraquinone (see Figure 1), as well as nine known compounds and evaluated their anticancer activities in several different cancer cell lines. Herein, we describe the experimental details of the separation process as well as information pertaining to the structural elucidation of these compounds based on their spectroscopic properties. In addition, we examined the anti-tumour activities of the anthraquinones.

2. Results and discussion
2.1. Structural elucidation of the isolated compounds
Compound 1 was obtained as an orange amorphous powder. It was positive to Borntrager’s reaction, revealing that it was a hydroxyl anthraquinone compound (Wagner & Bladt 1996). High-resolution mass spectroscopy (HR-MS) analysis of the pseudomolecular peak at m/z 475.1452 [M – H]⁻ (calculated for 475.1447) in the ESI-MS, in combination with the ¹H and ¹³C NMR data (Table S1), supported the molecular formula of C₂₃H₂₄O₁₁ and indicated 12 degrees of unsaturation. The ¹H NMR spectrum showed a set of two ortho-coupled aromatic hydrogens at δ 7.68 (d, J = 8.4 Hz, H-5) and δ 7.40 (d, J = 8.4 Hz, H-6), suggesting a tetra-substituted aromatic ring B, one singlet aromatic proton at δ 7.86 due to H-4 showed the presence of penta-substituted ring A in addition to the singlet for methyl group at δ 2.43. Also, ¹H NMR spectrum revealed a signal at δ 13.11, assigned to a hydroxyl group H-bonded to a carbonyl. The spectrum also showed a distinct signal for anomeric proton, a hexose anomeric proton resonance at δ 5.01 (1 H, d, J = 7.4 Hz).

¹³C NMR spectra displayed 12 aromatic carbon signals, 4 of them with oxygen substitution (δ 154.3, 153.7, 153.3 and 151.8). The signals at δ 188.3 and 180.2 were assigned to the two carbonyl carbons. The ¹³C NMR spectrum also reveals two methoxy carbon signals at δ 61.4 and 56.2, and one methyl at δ 17.6. In addition, assignments of the aglycone carbons were aided by comparison with the reported chemical shifts of emodin 2-O-β-glucoside (Choi et al. 1996). In the HMBC spectrum, the signals at δ 2.43 (H-11) had correlations with carbons C-2, C-3 and

Figure 1. Structures of isolated compounds (1 and 2).
C-4, indicating that the methyl group was located at C-3. The methoxyl at C-1 and C-7 was confirmed by the HMBC correlation signals (see Figure S1 and Table S1), H-12/C-1 and H-13/C-7, respectively. Long-range correlation between H-1 and C-1 confirmed the position of the aglycone substituent on C-1 of anthraquinone parent skeleton. The spectrum also showed a distinct signal for anomeric proton resonance at δ 5.01 (1H, d, J = 7.4 Hz), indicating glucosyl with β-configuration. Based on the above-mentioned data, compound 1 is deduced to be 8-hydroxy-1,7-dimethoxy-3-methylanthracene-9,10-dione-2-O-β-D-glucoside.

Compound 2 was obtained as an orange amorphous powder. It was positive to Bornträger’s reaction, revealing that it was a hydroxyl anthraquinone compound. HR-MS analysis of the pseudomolecular peak at m/z 491.1354 [M–H]− (calculated for 491.1346) in the ESI-MS, in combination with the 1H and 13C NMR data (Table S1), supported the molecular formula of C23H24O12. Based on the detailed analysis of its NMR spectra as well as the comparison of the NMR data with those of compound 1, it was found that they were very similar, except that compound 2 has the substitution of a hydroxyl at C-6 position. The substitution positions of ring B were confirmed by the HMBC spectra (see Figure S1 and Table S1). The aromatic proton at δH 7.18 correlations with the carbon C-6, -7, -10, -8a and -10a, indicating that aromatic proton was located at C-5. The correlations between δH 13.25 (—O—H) and C-7, -8, -8a confirmed hydroxyl at C-8. The correlation between δH 3.85 (—OC—H3) with δC 139.8 (C-7) suggested that the methoxyl is substituting at C-7 position. Thus, the position of the last one hydroxyl was unambiguously at C-6. Thus, compound 2 was elucidated as 6,8-dihydroxy-1,7-dimethoxy-3-methylanthracene-9,10-dione-2-O-β-D-glucoside.

The other 10 known compounds (3–12) were identified as 1-desmethylobtusin (3) (Zhu et al. 2008), chryso-obtusin (4) (Zhu et al. 2008), obtusin (5) (Zhu et al. 2008), aurantio-obtusin (6) (Zhu et al. 2008), chryso-obtusin-2-O-β-D-glucopyranoside (7) (Choi et al. 1996), aurantio-obtusin-6-O-β-D-glucopyranoside (8) (Tang et al. 2009), rubrofusarin-6-O-β-D-glucopyranoside (9) (Messana et al. 1991), casside (10) (Messana et al. 1991), torachrysone glucosides (11) (El-Halawany et al. 2007) and torachrysone apioglucoside (12) (Hatano et al. 1999).

2.2. Cytotoxic activity

The isolated compounds were evaluated for their cytotoxic activity against LO2, HCT-116, A549, HepG2 and SGC7901 cells (Table 1). Many anthraquinone compounds showed moderate cytotoxicity towards HCT-116 and A549 cell lines, compared with the control, emodin and oxaliplatin. In contrast, compounds 9–12 did not exhibit apparent cytotoxicity even at a sample concentration of 50 μg/mL.

Among the anthraquinone compounds, 1, 3 and 7 showed equal cytotoxicity towards the HCT-116 cell line as compared with oxaliplatin, and their cytotoxicity towards LO2 is lower than the control.

The above-mentioned results indicated that the C-6 hydroxy group might significantly reduce the cytotoxic activity in these compounds. And the difference in the cytotoxicity value between the aglycons and glycosides is not obvious.

3. Experiment

3.1. General method

Thin layer chromatography was performed on silica gel GF254 (Qingdao Haiyang Chemical Co. Ltd, Qingdao, China) plates. Octadecysilanised silica gel (ODS; 40–63 μm, YMC, Tokyo, Japan) was used for column chromatography. HPLC was performed on Agilent 1100 couple with Shim-pack ODS-C18 (20 × 250 mm) column. UV–vis absorption spectra were acquired using a Shimadzu UV-1650PC spectrophotometer; NMR spectra were obtained using a Bruker DRX-500...
Table 1. Cytotoxic activity of the isolated compounds 1–12 on LO2, HCT-116, A549, HepG2 and SGC7901 cells.

| Compounds | HCT-116 (μg/mL) | A549 (μg/mL) | HepG2 (μg/mL) | SGC7901 (μg/mL) | LO2 (μg/mL) |
|-----------|-----------------|--------------|---------------|-----------------|-------------|
| 1         | 4.5 ± 2.1       | 7.6 ± 1.3    | 22.8 ± 3.0    | 20.7 ± 2.4      | 18.1 ± 2.5  |
| 2         | 43 ± 4.7        | –            | –             | –               | –           |
| 3         | 5.1 ± 1.5       | 10.0 ± 2.6   | –             | 25.4 ± 4.1      | –           |
| 4         | 10.5 ± 1.4      | 14.6 ± 3.2   | –             | 12.0 ± 2.3      | 15.8 ± 3.0  |
| 5         | 13.1 ± 3.7      | 29.2 ± 5.0   | –             | 15.2 ± 1.3      | –           |
| 6         | 18.9 ± 1.5      | 20.1 ± 1.7   | –             | 22.0 ± 2.4      | 23.1 ± 1.8  |
| 7         | 5.8 ± 0.8       | 8.8 ± 1.8    | 14.0 ± 3.4    | 14.6 ± 3.9      | 13.35 ± 2.32|
| 8         | 31.1 ± 3.7      | –            | –             | 23.3 ± 3.3      | –           |
| 9         | –               | –            | –             | –               | –           |
| 10        | –               | –            | –             | –               | –           |
| 11        | –               | –            | –             | –               | –           |
| 12        | –               | –            | –             | –               | –           |
| Emodin    | 20.1 ± 1.5      | 18.5 ± 2.4   | 30.6 ± 1.2    | 38.1 ± 3.5      | –           |
| Oxaliplatin| 6.1 ± 0.8      | 7.8 ± 1.0    | 6.4 ± 1.4     | 9.2 ± 1.5       | 8.2 ± 1.1   |

*Data are the mean of three independent experiments.

*The data shown represent means ± SEM of three replicates.

*Mean IC₅₀ > 50 μg/mL.

spectrometers using dimethyl sulphoxide-d₆ (DMSO-d₆) as solvent with tetramethylsilane as internal reference, the chemical shifts were given in δ and coupling constants in Hz. ESI-MS and HR-ESI-MS experiments were recorded on an Agilent 1100 Series MSD Trap mass spectrometer and Mariner ESI-TOF spectrometer, respectively.

3.2. Materials and chemicals

Plant material of *C. obtusifolia* was collected from Fuyang (Anhui, China) in 2011. The plant materials were authenticated by Professor X.J. Wang. The voucher specimen (CO201101) has been deposited at the Nanjing Institute for Comprehensive Utilization of Wild Plants. DMSO-d₆ was obtained from Merck (Darmstadt, Germany). All the other chemicals and solvents used in this study were purchased from Merck and were of analytical grade.

3.3. Extraction and isolation

*Semen Cassiae* (the seeds of *C. obtusifolia*, 5 kg) was extracted three times with 95% ethanol at ambient temperature for 24 h. The resulting ethanol mixture was then filtered, and the filtrate was collected and concentrated under vacuum at 50°C until approximately 90% of the solvent had been evaporated. The ethanol extract (60 g) was isolated by using silica gel chromatography yielding five fractions (Fr. 1–Fr. 5). Then, Fr. 1 (5 g) was separated by ODS column chromatography affording 3 (60 mg), 4 (88 mg), 5 (23 mg) and 6 (35 mg). Fr. 2.1 (2 g) and Fr. 2.2 (4 g) were obtained from Fr. 2 (8 g) by ODS column chromatography. Compound 7 [retention time (RT): 15 min, 10 mg] was purified by HPLC on an ODS column with MeOH–H₂O (1:1) from Fr. 2.1. Compounds 1 (RT: 22 min, 30 mg) and 10 (RT: 30 min, 20 mg) were purified by HPLC on an ODS column with MeOH–H₂O (1:1, v/v). Fr. 3 (11 g) was separated on an ODS column to give three fractions (Fr. 3.1–Fr. 3.3). Compounds 2 (RT: 15 min, 20 mg) and 9 (RT: 20 min, 30 mg) were isolated from Fr. 3.2 (0.5 g) by HPLC on an ODS column with MeOH–H₂O (1.2:1, v/v). Compound 8 (50 mg) was purified from Fr. 2.3 (1 g) by ODS column.
chromatography. Compounds 11 (1.6 g) and 12 (2.4 g) were isolated from Fr. 4 (15 g) and Fr. 5 (12 g) by ODS column chromatography, respectively.

3.4. Structure identification

8-Hydroxy-1,7-dimethoxy-3-methylantracene-9,10-dione-2-O-β-D-glucoside (1), orange amorphous powder, HR-ESI-MS m/z: 475.1452 [M − H]− (calculated for C_{23}H_{23}O_{11}, 475.1447). 1H NMR (DMSO-d_{6}, 500 MHz): δH 7.86 (1H, s, H-4), 7.68 (1H, d, J = 8.4 Hz, H-5), 7.40 (1H, d, J = 8.4 Hz, H-6), 13.11 (1H, Br s, 8-OH), 2.43 (3H, s, 3-CH3), 3.89 (3H, s, 1-OCH3), 3.93 (3H, s, 7-OCH3), 5.01 (1H, d, J = 7.4 Hz, H-1′), 3.30 (1H, m, H-2′), 3.09 (1H, t, J = 9.0 Hz, H-3′), 3.16 (1H, t, J = 9.0 Hz, H-4′), 3.25 (1H, t, J = 9.0 Hz, H-5′), 3.42 (1H, dd, J = 9.0 Hz, J = 12.2 Hz, H-6′), 3.61 (1H, d, J = 12.2 Hz, H-6′). 13C NMR (DMSO-d_{6}, 125 MHz): δC 153.3 (C-1), 154.3 (C-2), 125.0 (C-4), 107.9 (C-5), 156.7 (C-6), 124.6 (C-7), 141.5 (C-3), 139.8 (C-7), 151.8 (C-8), 125.3 (C-2′), 74.0 (C-2′), 103.7 (C-1′), 77.3 (C-3′), 69.8 (C-4′), 76.4 (C-5′), 60.9 (C-6′). Identification and NMR signal assignment of 6,8-dihydroxy-1,7-dimethoxy-3-methylantracene-9,10-dione-2-O-β-D-glucoside (1) were supported by the analysis of the HMHC and HMBC data.

6,8-Dihydroxy-1,7-dimethoxy-3-methylantracene-9,10-dione-2-O-β-D-glucoside (2), orange amorphous powder, HR-ESI-MS m/z: 491.1354 [M − H]− (calculated for C_{23}H_{22}O_{12}, 491.1346). 1H NMR (DMSO-d_{6}, 500 MHz): δH 7.82 (1H, s, H-4), 7.18 (1H, d, J = 8.4 Hz, H-5), 13.25 (1H, Br s, 8-OH), 2.41 (3H, s, 3-CH3), 3.87 (3H, s, 1-OCH3), 3.85 (3H, s, 7-OCH3), 5.01 (1H, d, J = 7.5 Hz, H-1′), 3.28 (1H, m, H-2′), 3.09 (1H, t, J = 9.0 Hz, H-3′), 3.15 (1H, t, J = 9.0 Hz, H-4′), 3.25 (1H, t, J = 9.0 Hz, H-5′), 3.42 (1H, dd, J = 9.0 Hz, J = 12.2 Hz, H-6′), 3.61 (1H, d, J = 12.2 Hz, H-6′). 13C NMR (DMSO-d_{6}, 125 MHz): δC 153.2 (C-1), 154.6 (C-2), 124.6 (C-7), 140.7 (C-3), 139.8 (C-2′), 77.3 (C-3′), 69.8 (C-4′), 76.4 (C-5′), 60.9 (C-6′). Identification and NMR signal assignment of 6,8-dihydroxy-1,7-dimethoxy-3-methylantracene-9,10-dione-2-O-β-D-glucoside (2) were supported by the analysis of the HMHC and HMBC data.

3.5. Cell culture

Five human cell lines were used in this study which were purchased from the Cell Bank of the Shanghai Institute of Cell Biology, a normal human liver cell line (LO2) and four cancer cell lines: human colorectal cancer cell line (HCT-116), human lung adenocarcinoma epithelial cell line (A549), human hepatocellular carcinoma cell line (HepG2) and human gastric carcinoma cell line (SGC7901). These cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS; TBD Science, China), 100 U/ml penicillin and 100 μg/ml streptomycin (Yuanmu, Shanghai, China) at 37°C with 5% CO2.

3.6. Cytotoxicity and proliferation assay

A CCK8 assay was used to measure cell proliferation and viability. Briefly, cells were plated in 96-well plates (1 × 10^4 cells/well) and routinely cultured for 24 h. Then, cells were treated with various concentrations (50, 10, 2 and 0.4 μg/mL) of compounds 1–12 or 1% DMSO (vehicle) in DMEM supplemented with 3% FBS. After 44 h of treatment, 10 μl CCK8 solution (2.5 mg/mL) was added to each well, followed by incubation for 4 h at 37°C with 5% CO2. Absorbance was measured at 450 nm using a microplate reader. The reference compounds emodin and oxaliplatin were treated at the same concentration and condition like compounds 1–12.
Results were obtained from more than three independent experiments. Statistical analysis of data was performed using one-way analysis of variance followed by Student’s t-test with GraphPad PRISM 5 (GraphPad Software Inc., San Diego, CA, USA). Error bars denoted the standard deviation.

4. Conclusion
In summary, phytochemical analysis of the ethanol extract of Semen Cassiae resulted in the isolation and characterisation of one new compound and together with 10 known compounds, including six anthraquinones. Their structures were elucidated by using chemical and spectroscopic analyses, including 1D, 2D NMR and HR-ESI-MS. Biological studies disclosed that anthraquinone compounds (1, 3 and 7) show equal cytotoxicity towards HCT-116 cells compared with oxaliplatin, and their cytotoxicity towards LO2 is lower than the control.

The results of our chemical investigation further revealed the chemical composition of C. obtusifolia L., and the biological investigation of these compounds also can help us to find new application of traditional Chinese herb.

Supplementary data
Supplementary data associated with this article can be found in the online version. It includes 1D, 2D NMR spectra and data of compound 1.

Disclosure statement
No potential conflict of interest was reported by the authors.

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Note
1. Authors Bao-jun Shi and Wei-dong Zhang contributed equally to this work.

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