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The constituents from the stems of *Garcinia cowa* Roxb. and their cytotoxic activities

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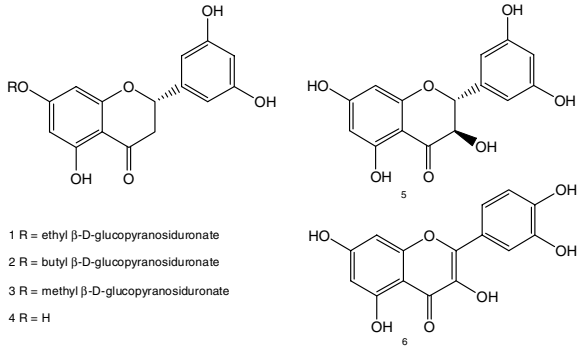
Three new flavanone glycosides named garccowaside A, garccowaside B, garccowaside C, and three other known compounds were isolated from the ethanol extract of the stems of *Garcinia cowa*. These structures were established on the basis of spectroscopic evidence. Twelve compounds isolated from the stems of *Garcinia cowa* were tested for cytotoxic activities.

1. Introduction

In a previous paper (Shen and Yang 2006), we described the isolation and structure elucidation of two new xanthones and six known xanthones from the stems of *Garcinia cowa* Roxb. (Guttiferae). In this paper, we report the isolation and structural determination of three new compounds and three known compounds (**1–6**). The cytotoxic activities of compounds **1–6** and six xanthones were investigated since many other xanthones from this plant were evaluated for antimalarial, antimicrobial and antioxidative activities.



2. Investigations, results and discussion

An ethanolic extract of the stems of *G. cowa* was concentrated and partitioned further into petroleum ether, ethyl acetate, *n*-butanol, water soluble fractions. The ethyl acetate and *n*-butanol fractions were subjected to chromatographic purification to afford three new compounds, garccowaside A–C (**1–3**) along with three known compounds *S*-(−)-5,7,3′,5′-tetrahydroxyflavanone (**4**) (Yi et al. 2002), (+)-3,5,7,3′,5′-pentahydroxyflavanone (**5**) (Ding et al. 1997), quercetin (**6**) (Ding et al. 1997).

Compound **1** was obtained as a pale yellow powder, m.p. 146–147 °C. Its molecular formula was elucidated as $C_{23}H_{24}O_{12}$ by analysis of its HR-FAB-MS. The UV spectrum of **1** showed the characteristic absorptions of flavanone derivative at 204, 225, 283 nm, and its IR spectrum contained some absorption bands due to an ester carbon (1732 cm^{-1}) and a γ-pyrone (1641 cm^{-1}). The ^1H NMR spectrum of **1** showed signals at δ 12.00, 9.10, 9.00, indication of three aromatic hydroxyl group substitutions, whereas a singlet at δ 12.00 assigned to the hydroxyl group maybe linked to the C-5 of the flavanone aglycone. In the ^1H NMR spectrum, an AMX-system δ 5.44 (1 H, dd, J = 13, 2.5 Hz), δ 2.72 (1 H, dd, J = 16, 2.5 Hz) and δ 3.22 (1 H, m) was observed, which also confirmed **1** as flavanone derivative; two signals δ 6.17 (1 H, d, J = 2 Hz) and δ 6.13 (1 H, d, J = 2 Hz) indicated 5,7 substitutions in ring A. Two singlets δ 6.87 (1 H) and δ 6.74 (2 H) suggested unusual 3′,5′ substitutions in ring B of **1**. In addition, resonances at δ 5.20 (1 H, d, J = 7.5 Hz), δ 4.13 (1 H, m) were present for the sugar moiety and δ 4.13 (2 H, m) and δ 1.18 (3 H, t, J = 7 Hz) suggested an ethyl group. In the ^{13}C NMR spectrum (see Table 1), 23 carbon signals were revealed, which the structure of **1** contained a flavanone moiety, a saccharide moiety and an ethyl group. Compared with the resonances of the known compound **4**, characterized as *S*-(−)-5,7,3′,5′-tetrahydroxyflavanone, the data indicated the presence of the skeleton of 5,7,3′,5′-tetrahydroxyflavanone in **1**. The saccharide moiety was confirmed as β-D-glucuronic acid according to acid hydrolysis by TLC analysis with an authentic sample. Furthermore, the ^1H and ^{13}C NMR spectroscopic assignments for **1** were determined by the HSQC and HMBC spectrum (see Table 1). In the HMBC spectrum, correlations between H-1‴ and C-6″ (δ 168.7) showed that an ethyl group was attached to C-6″; cross-peak between the proton H-1″ and C-7 (δ 164.7) was present for the position of ethyl β-D-glucopyranosiduronate in **1**. The absolute configuration carbon 2 was assigned as 2*S* from the positive and negative Cotton effects in the CD spectra at 336 nm (n → π* transition) and 288 nm (π → π* transition) (Gaffield 1970). The structure of compound **1** was determined to be *S*-(−)-5,7,3′,5′-tetrahydroxyflavanone 7-*O*-(ethyl-β-D-glucopyranosiduronate) and given the trivial name garccowaside A.

Table 1: ^{1}H NMR and ^{13}C NMR spectroscopic data of compounds 1–3 in DMSO-d_6

Position	1[a]		2[a]		3[a]	
	H	C	H	C	H	C
2	5.44 dd (13, 2.5)	78.7	5.43 dd (13, 3)	78.8	5.44 dd (13, 2.5)	78.7
3	3.22 m	42.2	3.22 m	42.2	3.22 m	42.2
	2.72 dd (16, 2.5)		2.72 dd (17.5, 3)		2.71 dd (17.5, 3)	
4		197.2		197.2		197.2
5		162.9		162.9		162.9
6	6.13 d (2)	96.2	6.14 d (2)	96.3	6.13 d (2)	96.3
7		164.7		164.7		164.7
8	6.17 d (2)	95.2	6.18 d (2)	95.2	6.16 d (2)	95.2
9		162.8		162.7		162.8
10		103.4		103.4		103.4
1′		129.2		129.2		129.2
2′	6.74 s	118.0	6.74 s	118.0	6.74 s	118.0
3′		145.8		145.8		145.8
4′	6.87 s	114.4	6.86 s	114.4	6.87 s	114.4
5′		145.2		145.2		145.2
6′	6.74 s	115.3	6.74 s	115.3	6.87 s	115.3
1″	5.20 d (7.5)	98.8	5.18 d (7)	98.9	5.19 d (7.5)	98.7
2″		72.7		72.7		72.6
3″		75.3		75.4		75.3
4″		71.2		71.2		71.3
5″	4.13 m	75.1	4.10 m	75.1	4.10 m	75.0
6″		168.7		168.7		169.2
1‴	4.13 m	60.1	4.10 m	64.3	3.65 s	52.0
2‴	1.18 t (7)	13.9	1.54 m	30.0		
3‴			1.32 m	18.4		
4‴			1.83 t (7.5)	13.5		
5-OH	12.00 s		12.00 s		12.00 s	
3′-OH	9.10 s		9.10 s		9.10 s	
5′-OH	9.00 s		9.00 s		9.00 s	

[a] Assignments based on HMQC and HMBC

Compound **2** was also obtained as a pale yellow powder. The molecular formula was deduced as $C_{25}H_{18}O_{12}$ by HR-FAB-MS. The UV and IR spectrum absorptions were similar to **1**, which suggested **2** to be a flavanone derivative. A comparison of the NMR spectra of **2** with those of garccowaside A indicated **2** owning the same flavanone skeleton and a glucuronic acid group. Compound **2** mainly differed from **1** in the ethyl group by the ^{1}H and ^{13}C NMR spectroscopic analyses (see Table 1). A butyl group was confirmed and attached to C-6″ by the HSQC and HMBC spectrum. In the HMBC spectrum, a correlation H-1″/C-7 (δ 164.7) was observed, which showed the presence of butyl β-D-glucopyranosiduronate in **2**. The absolute configuration of carbon 2 was determined by the same method as for compound **1**. From these findings, compound **2** was identified as *S*-(−)-5,7,3′,5′-tetrahydroxyflavanone 7-*O*-(butyl-β-D-glucopyrano-siduronate) (named as garccowaside B). However, the presence of a butyl ester side chain in **2** may be artifactual since the compound was separated from *n*-butanol fractionation.

The positve FAB-MS spectrum of flavanone glycoside **3** gave a quasi molecular ion at m/*z* 501 $[M + Na]^+$, which differed from that of flavanone glycoside **1** by 14 mass units. Compound **3** showed strong similarities to **1** and **2** in EI-MS, UV, IR, NMR spectrum, which suggested the presence of a 5,7,3′,5′-tetrahydroxyflavanone group and a β-D-glucopyranosiduronate group. In the ^{1}H NMR spectrum, a singlet for three protons was observed at δ 3.61, which indicated the presence of a methoxyl group substituted on a saccharide group. Analysis of the HMQC and HMBC spectra permitted the assignment of carbon signals for **3**. The correlation of H-1‴/C-6″ (δ 169.2) in the HMBC spectrum confirmed the position of a methoxyl group. The methyl β-D-glucopyranosiduronate group was also attached to C-7 for the cross peak of H-1″/C-7 (δ 164.7). The absolute configuration carbon 2 was also identified by CD spectrum as **1** and **2**. Thus, the structure of 3 was established as *S*-(−)-5,7,3′,5′-tetrahydroxyflavanone 7-*O*-(methyl-β-D-glucopyranosiduronate) (named as garccowaside C).

Compounds **1–4** all contained rare 3′,5′ substitutions of ring B, which the flavanone having the ring B was only found in the plant *Pseudotsuga sinensis*. *S*-(−)-5,7,3′,5′-tetrahydroxyflavanone was determined by the comparison of the MS and NMR data, the absolute configuration carbon 2 was elucidated by CD, which it may be obtained as racemic modification in the previous paper.

Plants of the genus *Garcinia* contain a lot of bioactive phenolic compounds, which are mainly xanthones, biflavonoids and benzophenones.

Six compounds (**1–6**) and formerly reported six xanthones: 1,5,6-trihydroxy-3-methoxy-4-(3-hydroxyl-3-methylbutyl)-xanthone (**7**) and 1,5-dihydroxy-3-methoxy-6′,6′-dimethyl-2*H*-pyrano (2′,3′ : 6,7)-4-(3-methylbut-2-enyl) xanthone (**8**), 1,3,5-trihydroxy-6′,6′-dimethyl-2*H*-pyrano (2′,3′ : 6,7)-xanthone (**9**), dulxanthone A (**10**), 1,5,6-trihydroxy-3,7-dimethoxyxanthone (**11**), 1,3,6,7-tetrahydroxyxanthone (**12**) were tested for cytotoxic activities. Seven phenolic compounds including five xanthones showed moderate cytotoxicity on three cancerous cell lines tested (Table 2). Among them, dulxanthone A was the best one. Compounds **1–3** exhibited almost no remarkable growth inhibitory effect within the tested concentrations, which the aglycone owned some cytotoxic activities. Only compound **11** was different with other 1,3,5,6 oxygenation patterns of xanthones, which had no activities in three tests.

Table 2: Cytotoxicity of the compounds on different cancerous cell lines

Cell lines	IC_{50} (μg/mL)							
	Betulinic acid	**4**	**5**	**7**	**8**	**10**	**11**	**12**
HepG2	13	15	55	20	13	8	10	11
MCF-7	8	50	53	94	56	15	35	48
SF-268	10	85	38	45	15	17	28	53

3. Experimental

3.1. Apparatus

Melting points were determined on an X4 micro-melting point apparatus and are uncorrected. The CD spectra were recorded on a JASCO J-810 spectropolarimeter. UV spectra were measured with a Hitachi UV-2201 spectrophotometer and IR spectra with an Impact 400 FTIR spectrometer. ^1H and ^{13}C NMR spectra were recorded in DMSO-d_6 with an INOVA spectrometer at 500 MHz (^1H) and 125 MHz (^{13}C), using visual DMSO-d_6 resonances for internal reference. Mass spectra were recorded on an Auto-Spec Ultima-TOF spectrometer.

3.2. Plant material

The stems of *Garcinia cowa* were collected from Yunnan Province P.R. China and identified by Prof. ShaoRong Guo. A voucher specimen is deposited at the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, P.R. China.

3.3. Extraction and isolation

The dried stems of *Garcinia cowa* (20 kg) were extracted three times with 95% EtOH for 2 h under reflux, and then extracted two times with 70% EtOH for 2 h under reflux. After combination and removal of solvent, the residue (2.1 kg) was suspended in water and successively partitioned with petroleum ether, EtOAc, and *n*-BuOH. The EtOAc residue (272 g) was chromatographed over silica gel (200–300 mesh) and eluted with $CHCl_3$–MeOH (10 : 0–1 : 1) to give 16 fractions. Fraction 7 (1.2 g) was chromatographed over silica gel (200–300 mesh) column eluting with $CHCl_3$–MeOH (10 : 0–0 : 10) and gave **4** (38 mg), **5** (140 mg) and **6** (70 mg). Fractions 13 (840 mg) were also separated by silica gel (200–300 mesh) column elution with $CHCl_3$–MeOH (10 : 0–1 : 1) to yield **1** (110 mg). The *n*-BuOH-soluble fraction (130 g) was also chromatographed over silica gel (200–300 mesh) and eluted with $CHCl_3$–MeOH (10 : 0–0 : 10) to give 8 fractions. Fractions 3 (17.5 g) were separated by a silica gel (200–300 mesh) column eluting with $CHCl_3$–MeOH (8.5 : 1.5) to yield **2** (200 mg) and **3** (23 mg). Each compound was purified by Sephadex LH-20 CC with MeOH as eluent.

3.4. Garccowaside A (**1**)

Pale yellow powder, m.p. 146–147 °C. $[\alpha]_D^{20}$ −41.09° (c 0.036, MeOH); UV λ_{max}^{MeOH} nm: 204 (4.55), 225 (4.37), 283 (4.23). CD (MeOH): $\Delta\,\varepsilon_{235} - 5.04$, $\Delta\,\varepsilon_{248} + 1.88$, $\Delta\,\varepsilon_{288} - 22.89$, $\Delta\,\varepsilon_{336} + 4.52$. IR (KBr, ν_{max}, cm^{-1}): 3402, 2908, 1732, 1641, 1579, 1523, 1446, 1383, 1296, 1173, 1100, 1088, 1036. FAB-MS m/z: 493 $[M + H]^+$; EI-MS m/z: 492 $[M^+]$, 288, 205, 166, 153, 136, 123; HR-FAB-MS m/z: 515.1158 $[M + Na]^+$ (calcd for $C_{23}H_{24}O_{12}Na$, 515.1165); For ^1H NMR and ^{13}C NMR spectra, see Table 1.

3.5. Garccowaside B (**2**)

Pale yellow powder, m.p. 122–123 °C. $[\alpha]_D^{20}$ −44.94 (c 0.044, MeOH); UV λ_{max}^{MeOH} nm: 204 (4.61), 225 (4.40), 283 (4.28). CD (MeOH): $\Delta\,\varepsilon_{234} - 2.45$, $\Delta\,\varepsilon_{249} + 0.45$, $\Delta\,\varepsilon_{289} - 14.80$, $\Delta\,\varepsilon_{334} + 2.54$. IR (KBr, ν_{max}, cm^{-1}): 3402, 2962, 2875, 1736, 1643, 1579, 1523, 1448, 1298, 1171, 1100, 1066, 1036. FAB-MS m/z: 543 $[M + Na]^+$; EI-MS m/z: 288, 271, 179, 153, 136, 123; HR-FAB-MS m/z: 543.1509 $[M + Na]^+$ (calcd for $C_{25}H_{28}O_{12}Na$, 543.1478); For ^1H NMR and ^{13}C NMR spectra, see Table 1.

3.6. Garccowaside C (**3**)

Pale yellow powder, m.p. 151–152 °C. $[\alpha]_D^{20}$ −41.56 (c 0.039, MeOH). UV λ_{max}^{MeOH} nm: 204 (4.61), 225 (4.40), 283 (4.28). CD (MeOH): $\Delta\,\varepsilon_{235} - 5.65$, $\Delta\,\varepsilon_{249} + 1.29$, $\Delta\,\varepsilon_{288} - 20.13$, $\Delta\,\varepsilon_{335} + 3.43$. IR (KBr, ν_{max}, cm^{-1}): 3402, 2918, 1739, 1643, 1579, 1523, 1444, 1294, 1171, 1100, 1088. FAB-MS m/z: 501 $[M + Na]^+$; EI-MS m/z: 288, 179, 166, 153, 136, 123; HR-FAB-MS m/z: 501.1035 $[M + Na]^+$ (calcd for $C_{22}H_{22}O_{12}Na$, 501.1009); For ^1H NMR and ^{13}C NMR spectra, see Table 1.

3.7. Cytotoxicity assay

HepG2, MCF-7 and SF-268 (ATCC) cells were maintained in RPMI 1640 (Gibco) containing 10% FBS (Gibco), 2 mg/ml sodium bicarbonate, 100 μg/ml penicillin sodium salt and 100 μg/ml streptomycin sulfate. Cells were grown to 70% confluence, trypsinized with 0.05% trypsin-2 mM EDTA, and plated for experimental use. In all experiments, cells were grown in RPMI-1640 medium with 10% FBS for 24 h prior to treatment. All compounds were dissolved in DMSO at a concentration of 100 mM, then diluted in tissue culture medium and filtered before use.

1.5×10^4 HepG2, MCF-7 and SF-268 cells were seeded in 96 well tissue culture plates and treated with the tested compounds (100–3.125 μg/mL) or vehicle (0.1% DMSO) at various concentrations and incubated for 48 h followed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay at 570 nm. Betulinic acid was used as positive control. Briefly, IC_{50} values of the test compounds on different cell lines were obtained from the concentration-effect curves.

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