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CYTOTOXIC XANTHONE GLYCOSIDES FROM AERIAL PART OF *CENTAURIUM SPICATUM*

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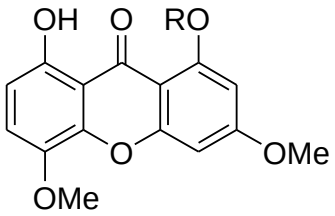
Abstract – A new xanthone glycoside, 8-hydroxy-3,5-dimethoxy-1-*O*-[α-L-arabinofuranosyl-(1→6)-β-D-glucopyranosyl]-xanthone (**1**) in addition to two known xanthones named 8-hydroxy-3,5-dimethoxy-1-*O*-β-D-glucopyranosyl-xanthone (**2**) and 1,8-dihydroxy-3,5-dimethoxyxanthone (methylbellidifolin) (**3**) were isolated from the methanol extract of aerial part of *Centaurium spicatum*, and tested for their cytotoxic activities against three different types of cell lines HeLa, THP-1and HL-60 cell lines. All compounds showed cytotoxicities to all cell lines, whereas showed a moderate activity against human monocytic cell line HL-60. Among three tested xanthones, the cytotoxic activity of compound 1 was stronger than those of other xanthones and the IC50 values for compound 1 were 3.22 (against THP-1), 8.67 (against HeLa) and 74.6 (against HL-60) μM, respectively.

Centaurium spicatum L. Fritsch (Gentianaceae) is an annual herb occurring in Southern Europe and Northern Africa where it is used together with other *Centaurium* species such as *C. pulchellum* in traditional medicines treated for abdominal pain, hypertension, gallstones, kidney and ureter stones, renal colic, wounds and diabetes. A survey of the current literatures revealed that the isolation and identification of secoiridoids, sweroside, swertiamarin and gentiopicrin, as well as polyoxygenated xanthones from the plant.[1-4] Alkaloids of the pyridine type (*e. g*. gentianine), spicatine and the series of amides derived from the secoiridoid glucoside swertiamarin and kantaurin were also shown to be present.[3] The study of xanthones is

noticed not only from the chemosystematic investigation but also from the pharmacological point of view. Xanthone aglycones possessed antidepressant and antitubercular activities, while xanthone glycosides showed a depressant effect.[5] A choleretic, diuretic, antimicrobial, antiviral and cardiotonic activities by some xanthones have also been established.[5-8] The pharmacological interest in xanthones is based on their inhibitory effect on monoamine oxidase as well as their cytotoxic and antitumour activity.[9,10] In our course study, it was found that a classification of xanthones called xanthone alpha mangostin can suppress development of malignant tumors. It was also revealed that one xanthone analogue, garcicone E, can also prevent liver, lung and colon cancers.[11] In the present work, we reported the isolation and structural elucidation of a new xanthone glycoside in addition to two known xanthones from *C. spicatum* and were tested for their cytotoxic activities against three different tumor cell lines *in vitro*.

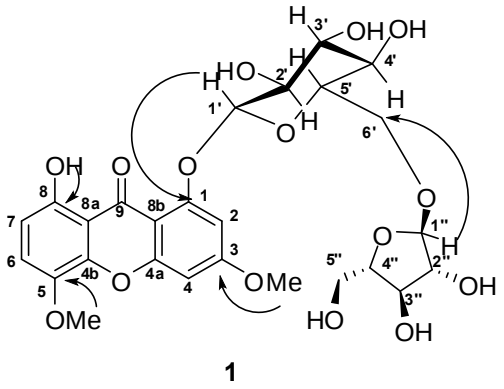


1 R = Glu-Ara
2 R = Glu
3 R = H

Figure 1. Chemical structures of xanthone glycosides isolated from *C. spicatum*

A methanol extract of *C. spicatum* revealed inhibitory activity of cell proliferation in cultured HeLa, THP-1 and HL-60, respectively. The *n*-butanol fraction from the methanol extract decreased cell viability in cultured HeLa, THP-1 and HL-60 cells with IC_{50} = 42 ± 8, 55 ± 7 and 180 ± 5 μg/mL respectively**.** The *n*-butanol fraction was then separated by chromatography on Diaion HP-20 resin. Fractions were successively eluted with water, 100% methanol, and 100% acetone, and biological activities were estimated. The fraction-eluted with 100% methanol showed the strongest cytotoxic activities. IC_{50} values for inhibitory activity in these cells were 18 ± 9 (HeLa), 22 ± 4 (THP-1) and 120 ± 6 μg/mL (HL-60), respectively. The inhibitory activities of methanol extract were 2.5- to 1.5-times higher than those of the original *n*-butanol extract. The 100% methanol-eluted fraction was further separated into five fractions by silica gel column chromatography using chloroform-methanol as eluents. These fractions 2 and 4, which showed a strongest activities, were then separated by HPLC equipped with ODS column, yielding three active xanthones. Two xanthones, **2** and **3**, were known compounds, and identified as 8-hydroxy-3,5-dimethoxy-1-*O*-β-D-glucopyranosyl-xanthone **2** (18 mg) and 1,8-dihydroxy-3,5-

dimethoxyxanthone or methylbellidifolin **3** (20 mg) by comparison of their spectral data with those reported in the literature (**Figure 1**).[12,13]



1

Figure 2. HMBC key correlations for **1**

A new xanthone **1**, 8-hydroxy-3,5-dimethoxy-1-*O*-[α-L-arabinofuranosyl-(1→6)-β-D-glucopyranosyl]-xanthone, was obtained as yellow needles (20 mg) with a molecular formula of $C_{26}H_{30}O_{15}$ as determined by HRFABMS *m*/*z* 583.1673. The UV spectrum of **1** in methanol suggested a xanthone derivative.[14] The ^{1}H and ^{13}C NMR spectrum showed the typical pattern of a xanthone glycoside skeleton where a pair of meta-coupled aromatic protons at (δ_H 6.74 and 6.77 ppm) assignable to H-2 and H-4, respectively in addition to a pair of ortho-coupled aromatic protons at (δ_H 7.36 and 6.65 ppm) assignable to H-6 and H-7 respectively were observed. Further, ^{1}H NMR spectrum revealed presence of two methoxy (δ_H 3.91 and 3.85 ppm) and one hydroxyl group at δ_H 12.45 ppm (8-OH) (**Table 1**). The spectrum also showed presence of two sugar moieties where there were two doublet signals at δ_H 4.17 and 5.07 confirmed the presence of two sugars in the molecule. The terminal sugar moiety was suggested to be arabinose which was confirmed from ^{1}H-NMR spectrum where the presence of a proton at δ_H 3.78, d, *J*= 3.7 (i.e. equatorial coupling) assigned for H-4 of arabinose (*c.f.* xylose where H-4 is of an axial coupling).[15] In ^{13}C NMR spectrum, the presence of 11 of both methin (δ_C 100.8, 73.2, 75.7, 69.5, 76.5, 69.6, 104.2, 73.3 and 76.3) and methylene (δ_C 68.6 and 65.6) signals confirmed the glycosylation of the molecule. A long-range correlation observed in HMBC experiment, between the downfield shifted C-1 of the xanthone moiety (δ_C 158.6) and the anomeric proton of glucose (δ_H 5.07) confirmed that this was the site of glycosylation and that glucose was the first sugar in turn, HMBC long range coupling between the downfield shifted C-6 of glucose (δ_C 68.6) and the anomeric proton of arabinose (δ_H 4.17) was indicative of a 1–6 linkage between the two glycosidic moieties.[5] Another important long-range correlation observed in HMBC experiment between the downfield shifted C-3 (δ_C 165.6) of the xanthone moiety and the methoxy singlet protons (δ_H 3.91) from one side and between C-5 (δ_C 139.1) of the xanthone moiety and the methoxy singlet protons (δ_H 3.85) from the other side confirming the site of attachments of the two methoxy groups to C-3 and C-5 of

the xanthone moiety (**Figure 2**). The other carbons were assigned from the combination of ^{1}H–^{1}H correlated spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) data and by comparison with the literature.[16] The identification of a glucopyranosyl and an arabinofuranosyl moiety was done by means of comparison with literature data.[17] In addition, acid hydrolysis of the glycosides was carried out and the sugars were identified as glucose and arabinose. Also optical rotation values of L-arabinofuranosyl tetrabenzoate derivatives of the acid hydrolysis products of **1** were $[\alpha]_D^{25}$ –87.5 (*c* 0.825, $CHCl_3$) for β-D-glucopyranosyl tetrabenzoate and $[\alpha]_D^{25}$ +112.0 (*c* 0.825, $CHCl_3$) for α-L-arabinofuranosyl tetrabenzoate which were identical with the synthetic models for both β-D–glucopyranose tetrabenzoate with $[\alpha]_D^{31}$ –88.0 (*c* 1.0, $CHCl_3$) and α-L-arabinofuranose tetrabenzoate with $[\alpha]_D^{29}$ +112.5 (*c* 0.848, $CHCl_3$) respectively.[13,18-20]

Table 1. ^{13}C and ^{1}H NMR spectroscopic data for **1**[a]

Position	^{13}C NMR (δ, mult.)	^{1}H NMR [δ, mult., *J* (Hz)]	Position	^{13}C NMR (δ, mult.)	^{1}H NMR [δ, mult., *J* (Hz)]
1	158.6,s	-	Glu		
2	99.3,d	6.74,d,2.4	1'	100.8,d	5.07,d,7.9
3	165.6,s	-	2'	73.2,d	3.30,t,7.8
4	94.9,d	6.77,d,2.4	3'	75.7,d	3.63,m
4a	159.0,s	-	4'	69.5,d	3.24,m
4b	143.8,s	-	5'	76.5,d	3.41,m
5	139.1,s	-	6'	68.6,t	2.96,m
6	119.6,d	7.36,d,8.9			3.65,m
7	108.7,d	6.65,d,8.9	Ara		
8	153.5,s	-	1"	104.2,d	4.17,d,7.5
8a	108.9,s	-	2"	73.3,d	2.98,dd,8.6,7.5
9	180.5,s	-	3"	76.3,d	3.08,m
9a	105.2,s	-	4"	69.6,d	3.78,d,3.7
3-OMe	56.5,q	3.91,s	5"	65.6,t	3.63,m
5-OMe	56.3,q	3.85,s			3.95,m
8-OH		12.45,s			

a) Spectral data were recorded in DMSO-d_6.

Also, the retention time of each of the benzoyl derivatives of the hydrolysis products L-arabinofuranosyl tetrabenzoate and D-xylopyranosyl tetrabenzoate was found to be 8.2 and 9.1 min, respectively which was identical with the retention times of synthetic models of tetrabenzoate derivatives of both L-arabinofuranosyl and D-xylopyranosyl tetrabenzoate where it was found to be 8.5 and 9.1 min, respectively[19,20] which confirming both sugar moieties to be β-D-xylopyranose and α-L-arabinofuranose.

NOE correlation was also noted between H-3" (δ_H 3.08) and H-5" (δ_H 3.63, 3.95) protons of the L-arabinose confirmed the presence of α-L-arabinofuranosyl moiety. The combination of these data led to the identification of **1** as 8-hydroxy-3,5-dimethoxy-1-*O*-[α-L-arabinofuranosyl-(1→6)-β-D-glucopyranosyl]-xanthone and considered as a new compound. In addition, two known xanthones named 8-hydroxy-3, 5-dimethoxy-1-*O*-β-D-glucopyranosyl-xanthone (**2**) and 1,8-dihydroxy-3,5-dimethoxy-xanthone (methylbellidifolin) (**3**) were isolated from *C. spicatum* aerial part and the data were compared by those of the previously published.[17,18] (**Figure 1**).

Table 2. Cytotoxic activities of isolated three xanthone glycosides against three different cell lines [a]

	Inhibitory acitivities (IC_{50}) [a] (μM)			
Compounds	HeLa	THP-1	HL-60	*n*
1	3.22 ± 0.84	8.67 ± 0.25	74.6 ± 0.11	4
2	6.42 ± 0.47	17.3 ± 0.14	163 ± 0.19	4
3	5.28 ± 0.69	5.02 ± 0.18	308 ± 0.12	4
mitomycin-c	10 ± 0.19	10 ± 0.42	10 ± 0.32	4

[a)] IC_{50} values were expressed as the mean ± S.D. of four experiments

After establishing their structures, we investigated their cytotoxicities against cultured HeLa, THP-1 and HL-60 cells lines. Cells were treated with compounds **1-3** at various concentrations (1-100 μM) for 48 h, and determined cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. The cell viabilities of HeLa or THP-1 cells at 48 h were significantly decreased by treatment of these compounds in concentration-dependent manners. The IC_{50} values for **1, 2** or **3** against HeLa cell were 3.22 ± 0.84, 6.42 ± 0.47 and 5.28 ± 0.69 μM respectively. Cell viability in THP-1 cell treated with compounds **1**, **2** or **3** also decreased, and their IC_{50} values were 8.67 ± 0.25 (for **1**), 17.3 ± 0.14 (for **2**) and 5.02 ± 0.18 (for **3**) μM. However, these compounds showed moderate cytotoxicity in cultured human leukemic HL-60 in contrast to the results obtained in HeLa and THP-1 cells (**Table 2**). A positive control agent, mitomycin C, was also showed a cytotoxic activity against three cell lines, and its average of IC_{50} values in three different cell lines was approximately 10 μM (**Table 2**). Although an aglycone of xanthone glycoside (compound **3**) still has a selective cytotoxicity against three different tumor cell lines, whose activity in the case of xanthone glycoside (*i.e.* compound **1**) is higher than that of **3**. It could therefore be discussed that the presence of sugar moieties of the tetraoxygenated xanthones would be important to show the enhancement of cytotoxicity by aglycone xanthone. In this study, we isolated a new xanthone

glycoside **1** accompanying two known compounds **2** and **3**, which showed a selective cytotoxicity, from the areal part of *C. spicatum* L.

EXPERIMENTAL

General Optical rotations were determined with a Horiba SEPA-3000 high-sensitivity polarimeter (Horiba). We determined UV spectra on a Shimadzu UV-1600 UV-visible spectrometer, IR spectra on a Shimadzu FTIR-8400 IR spectrophotometer and NMR spectra on a JEOL GSX-600 spectrometer in DMSO and $CDCl_3$. Chemical shifts were referenced to the residual solvent peaks DMSO (δ_H 2.49 and δ_C 39.7) and $CDCl_3$ (δ_H 7.2 and δ_C 77). Fast atom bombardment (FABMS) and high-resolution fast atom bombardment (HRFABMS) were carried out on a JEOL JMS SX-102 mass spectrometer. Reversed-phase high-performance chromatography (HPLC) was undertaken on an ODS column (particle size: 5 μm, TOSO, 18 × 250 mm) RP-23 (5 μm; Waters). Diaion HP-20 (Mitsubishi) (Tokyo, Japan), silica gel (63–210 μm; Kanto Kagaku) and ODS (63–212 μm; Wako Pure Chemical) (Tokyo, Japan) were used for open column chromatography. Thin-layer chromatography (TLC) was carried out on silica gel (SiO_2, 60-100 mesh; Wako Pure Chemical) 60 F_{254} and RP-18 F_{254S} (Merck).

Plant Material Aerial part of *C. spicatum* L. (Gentianaceae) was collected in May 2009 from New Valley, 200 km southwest of Assiut City, Egypt. The plant was identified and authenticated by Prof. Dr. A. Fayed, Professor of Plant Taxonomy, Faculty of Science, Assiut University. A voucher specimen (T-318) was deposited at the Department of Pharmacognosy, Faculty of Pharmacy Assiut University, Assiut, Egypt and at the Department of Pharmacognosy and Chemistry of Natural Products, School of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Japan.

Extraction and isolation Air-dried *C. spicatum* aerial parts (2 kg) were extracted thrice with MeOH (5 L) at room temperature. The solvents were combined and filtered through filter paper (Advantec MFS Incorporated). The solvent was removed under reduced pressure at 40 °C to yield the MeOH extract (550 g), which was partitioned between distilled water and EtOAc (1 L of each) to give the aqueous fraction (300 g) and the EtOAc fraction (80 g). The aqueous fraction was further partitioned by *n*-BuOH to give the *n*-BuOH fraction (100 g) and the rest aqueous fraction (140 g). The EtOAc fraction was in turn partitioned between 90% MeOH and *n*-hexane to give 90% MeOH fraction (50 g) and *n*-hexane fraction (20 g). The *n*-BuOH fraction (100 g) was separated on a Diaion HP-20 column using water (2 L), MeOH (25%, 50%, 75%, and 100%) (2 L of each) and acetone (2 L) to give three crude fractions. Individual fractions water fraction (40 g), MeOH fraction (15.4 g) and the acetone fraction (5 g) were obtained after removal and evaporation of the respective solvents. The MeOH-eluted fraction (25 g) was further separated by chromatography on an ODS column (80 × 200 mm) using five concentrations of MeOH -H_2O (10, 30, 50, 60, and 90% v/v; elution volume: 1.5 L each) to give six corresponding fractions. The

biologically active fraction eluted with 50% MeOH (15.4 g) was further separated by chromatography on an silica gel column (80 × 200 mm) with a step-wise gradient of $CHCl_3$ and MeOH (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 0:1) to give seven corresponding fractions. The fraction eluted with 9:1 gives compound **3** (20 mg). The fraction eluted with 4:6 (280 mg) was further separated by chromatography on an ODS column (80 × 200 mm) (Cosmosil 140 C_{18} PREP, Nacalai Tesque, Tokyo, Japan) using seven concentrations of MeOH-H_2O (10, 25, 40, 50, 60, 70, and 90% v/v; elution volume: 200 mL of each) to give seven corresponding fractions. A 150-mg portion of the fraction eluted with 25% v/v MeOH-H_2O was further separated by preparative HPLC, ODS column: C30 UG-5, 20 mm× 250 mm. particle size: 5 μm, flow rate: 6 mL/min. (Develosil, Nacalai Tesque, Tokyo, Japan) equipped with a UV detector (210 nm). The mobile phase was 25% v/v MeOH-H_2O which resulted in elution of compound **2** (18 mg). These preparative HPLC conditions were also used after gradually increasing the mobile phase to 50% v/v MeOH-H_2O giving compound **1** (20 mg). Two of the three isolated compounds were identified as 8-hydroxy-3,5-dimethoxy-1-*O*-β-D-glucopyranosyl-xanthone (**2**) and 1,8-dihydroxy-3,5-dimethoxy-xanthone or methylbellidifolin (**3**) by comparison of their spectral data with those reported in the literature.[17,18] The elution profile of the fraction eluted with 4:6 $CHCl_3$-MeOH of *C. spicatum* aerial part was obtained by HPLC using the mobile phase of 25%, 35%, 50% MeOH. The two main peaks originating from compound **2** (t_R, 35.5 min, mobile phase: 25% MeOH) and compound **1** (t_R, 46.4 min, mobile phase: 50% MeOH) were found under the analytical condition. Each peak at the individual retention time was compared with purified samples.

Compound 1 8-Hydroxy-3,5-dimethoxy-1-*O*-[α-L-arabinofuranosyl-(1→6)-β-D-glucopyranosyl]-xanthone, yellow needles (20 mg); $[\alpha]_D^{25}$ -109.9° (*c* 0.333, MeOH). UV (CH_3OH) λ_{max} (log ε) 234, 264, 318, and 375 nm; IR ν_{max} (KBr) 3445, 1783, 2987, 1645, 1533 cm^{-1}; for ^1H NMR spectroscopic data (600 MHz, CD_3OD) and ^{13}C NMR spectroscopic data (125 MHz, CD_3OD). HRFABMS *m/z* 583.1673 $[M+H]^+$ (calcd for $C_{26}H_{30}O_{15}$). FAB^+ MS *m/z* 289 [aglycone] $^+$, 583 $[M+H]^+$ and 605 $[M+Na]^+$.

Acid hydrolysis Acid hydrolysis of the glycosides was carried out by refluxing 5 mg of compound in 5 mL of 6% HCl in MeOH for 3 h. The reaction mixture was partitioned against EtOAc (3 × 10 mL). The aglycone was obtained from the EtOAc layer and the aqueous layer was evaporated and developed crystal needles with EtOAc–H_2O–MeOH–HOAc (13:3:3:4). Identification of glucose, arabinose and xylose present in the sugar fraction was carried out by comparison with authentic samples of glucose (R_f 0.48), arabinose (R_f 0.50) and xylose (R_f 0.55) (Sigma) (Tokyo, Japan) in TLC over silica gel ($CHCl_3$-MeOH-H_2O 8:5:1) using 5% H_2SO_4 in MeOH as spraying reagent followed by heating the plates at 120 °C for 15-20 min.

Secondary sugar units Benzoyl chloride (0.5 mL) was added to each ice-cooled solution of either D-glucopyranose or L-arabinofuranose in dry pyridine (1.0 mL) and each mixture was stirred at room

temperature for 15 h. MeOH (1.0 mL) was added dropwise to the reaction mixture, stirred for 30 min and then diluted with EtOAc and aqueous Na_2CO_3 and the layers were separated. Each organic layer was washed with brine and the combined aqueous layers for each were extracted with EtOAc. Each combined organic extracts were dried over $MgSO_4$ and concentrated. Each residual dark brown oil was purified by silica gel cc (eluting with hexane/EtOAc 5:1) to give both corresponding tetrabenzoyl derivatives. The tetrabenzoyl derivatives of each hydrolysis products of **1** were subjected to chiral HPLC analyses using CHIRALPAK IB (Daisel Chemical Industries, 4.6 × 250 mm; MeOH, 1.0 mL/min; UV detection at 254 nm).

Cytotoxic assay To determine the cytotoxic activities of the tested samples by HeLa, THP-1 or HL-60 cell lines, individual cells (Dainippon Pharmaceutical Company, Tokyo, Japan) were cultured and maintained in appropriate medium such as D-MEM (Sigma, St. Louis, MO, USA) (for HeLa) or RPMI-1640 (Sigma, St. Louis, MO, USA) (for THP-1 and HL-60) media. Cell suspended in medium were seeded at 1.0×10^5 cells/mL into 96-well tissue culture plates and then incubated at 37 °C in a humidified CO_2 incubator.[21] The tested samples (purity > 93%) (20 μL in DMSO/PBS) at various concentrations were added in each well. After 48-h cultivation, supernatants were removed and adherent cells (HeLa) incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 20 μL, 5 mg/mL in PBS) for 4 h, and then solubilized with 10% (w/v) sodium dodecyl sulfate (SDS; in 60% [v/v] dimethylformamide) solution (50 μL) for 18 h. The plate was then centrifuged at 1,200 rpm for 5 min to precipitate wells and formazan. The absorbance was measured at 570 nm using a microplate reader and the cytotoxicity calculated by comparing absorbance with that of the non treated control culture. Cell growth curve was graphed using statistical analysis software (Kaleida Graph version 4.00; Synergy Software) and IC_{50} values calculated using simple linear regression. IC_{50} values were calculated from dose response curves for a 48 h drug exposure. Data were obtained through independent measurements of cell density and cell viability by the MTT assays.[22] A dose– response curve was plotted for each compound and the IC_{50} value was calculated as the concentration of the test compound resulting in 50% reduction of optical density compared with the control. The IC_{50} values of three experiments and correlation coefficient values for the tested samples in each experiment were calculated using the same software. Differences were considered significant at $p < 0.005$.

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