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Cytotoxic Sesquiterpene Coumarins from the Roots of *Heptaptera cilicica*

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Abstract: Preliminary 2-day cancer cell screening assays indicated that the dichloromethane extract of the roots of *Heptaptera cilicica* showed relatively strong cytotoxic activity. Eleven sesquiterpene coumarin ethers with potent and selective cytotoxic activities were isolated from the dichloromethane extract of the roots of *H. cilicica*. Furthermore, revised spectroscopic data for 14′-acetoxybadrakemone (**4**) and complete NMR spectroscopic assignments for colladonin (**6**) and colladin (**7**) are reported.

Keywords: *Heptaptera cilicica*; Apiaceae; sesquiterpene coumarins; cytotoxic activity.

1. Introduction

Cancer remains one of the leading causes of death worldwide [1]. The discovery of new compounds from natural sources to fight cancer has become a matter of great interest among researchers [2]. As part of our continuing studies on potential anticancer phytochemicals from the genus *Heptaptera* (Apiaceae), we report here the isolation and structure elucidation of potent cytotoxic sesquiterpene coumarins from the dichloromethane root extract of *Heptaptera cilicica* (Boiss. & Bal.) Tutin. *H. cilicica* is an endemic plant growing in Turkey and its distribution is restricted to the small area of the eastern Taurus Mountains in Mersin Province of the Mediterranean Region of Turkey [3].

The genus *Heptaptera* Marg. & Reuter (Apiaceae) is represented by 10 species worldwide; four of them: *H. cilicica* (Boiss. &Bal.) Tutin, *H. anisoptera* (DC.) Tutin, *H. anatolica* (Boiss.) Tutin and *H. triquetra* (Vent.) Tutin grow in Turkey [3,4]. *Heptaptera* species are known to contain sesquiterpene coumarin derivatives [5-7].These compounds have various biological activities such as; cytotoxicity, P-glycoprotein inhibitory, cancer chemopreventive, anti-inflammatory, antibacterial,

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antileishmanial, antiviral, and antidiabetic [7-13]. The sesquiterpene coumarin ethers with an umbelliferone nucleus are commonly found in the genera *Ferula* and *Heptaptera* of the Apiaceae family. Dioscorides and Avicenna described the use of sesquiterpene coumarin-containing *Ferula* gum-resins for the treatment of tumors [14,15].

Initial screening of the root extracts of *H. cilicica* confirmed the presence of cytotoxic compounds in the dichloromethane (DCM) extract, and isolation carried out on the DCM extract led to structure elucidation and identification of eleven sesquiterpene coumarin ethers with potent and selective cytotoxic activities.

2. Materials and Methods

2.1. General Experimental Procedures

UV spectra were recorded on the Shimadzu UV-Vis Spectrophotometer, UV-1700 (Kyoto, Japan). IR spectra (neat) were recorded on the Perkin-Elmer FT-IR Spectrometer, SPECTRUM 2000 (Waltham, MA, USA). NMR spectra were acquired on a Bruker Avance III spectrometer (Rheinstetten, Germany) operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C and equipped with a 5 mm indirect observation probe. HRESIMS data were acquired on a Thermo Q-ExactiveOrbitrap MS instrument (San Jose, CA, USA). Initial purification of the dichloromethane extract was carried out on a Sephadex LH-20 (GE Healthcare, Chicago, IL, USA) column. Further purification of column fractions was performed using silica gel F254 PLC plates (1 mm thickness) (Merck KGaA, Darmstadt, Germany) or combination of PLC and silica gel column chromatography (Silica gel 60, 0.063-0.2 mm particle size, Merck KGaA, Darmstadt, Germany).

2.2.Plant Material

The roots of *H. cilicica* were collected from Mersin province of Turkey, between Gülek-Tarsus near Karakütük village in June 2013 (13.06.2013) and identified by Prof. Ahmet Duran. A voucher specimen [A. Duran 9591, C. Sağlam & Y. Gürbüz (KNYA)] was deposited in the Herbarium of Selçuk University. The root material was cut into narrow slices and dried in a well-ventilated area protected from sunlight.

2.3. Extraction and Isolation

Air-dried and coarsely powdered root (100 g) of *H. cilicica* was extracted sequentially with dichloromethane (2 L) and methanol (3 L) at room temperature and concentrated, in vacuo, to dryness (yields; 4.99 g dichloromethane extract and 14.01 g methanol extract). The methanol extract was redissolved in a mixture of methanol/water (10:90) and then partitioned with ethyl acetate (EtOAc), the resulting extracts were separately concentrated in vacuo to dryness. Ethyl acetate and aqueous-methanol extracts of the roots were 0.88 g and 12.95 g respectively.

The dichloromethane extract (4 g) was separated on a Sephadex LH-20 column (4.5x100 cm) using a hexane/dichloromethane/methanol (14:9:1) mixture and 81 fractions were collected. The fractions which had the same spots on TLC were combined.

The combined fractions 4–44 sample (1.770 g) was chromatographed on a silica gel column (2.5x40 cm) with hexane gradually enriched with ethyl acetate. Compound **1** (500 mg) was crystallized from subfractions 10-22. The subfractions 30–34 were subjected to prep. TLC (1 mm thickness, silica gel F254 developed with hexane/ethyl acetate, 9:1) and compound **7**(47 mg) was obtained. The subfraction 35 and 36-37 were separately further purified by prep. TLC using hexane/ethyl acetate 9:1 as solvent system and yielded compounds **3** (22 mg) and **4 (**17 mg) respectively.

The combined fractions 56–60 (251.9 mg) of Sephadex LH-20 column were chromatographed on a silica gel column (1.5x25 cm) using a gradient solvent hexane/ethyl acetate (99:1-90:10, % V:V).

Compound **9** (25 mg) was obtained from subfractions 17-18 by prep. TLC using hexane/ethyl acetate (7:3) as solvent system.

Compound **5** (22 mg) and 6 (18 mg) were isolated from the combined fractions 61-68 (247.1 mg) and 69 (65.6 mg) of Sephadex LH-20 column, respectively, by prep. TLC using hexane/ethyl acetate (7:3) as solvent system.

The fractions 80-81 (194.1 mg) of the Sephadex LH-20 column were merged and separated by prep. TLC using hexane/ethyl acetate (1:1) to yield compounds **2** (6 mg), 8 (2 mg) and **11** (15 mg), respectively.

2.4. Cytotoxicity Assay on Renal Cancer Cell Lines

The assay used was a two-day, two cell line XTT bioassay [14], an in vitro antitumor colorimetric assay developed by the MTP Assay Development and Screening Section. The renal cancer cell lines used were UO31 and A498. Colon cancer cell lines were COLO205 and KM12, and A673 and TC32 were Ewing sarcoma cell lines. Cells were harvested and plated (45 µL) at a seeding density of 3.0×10^5 cells per well for the UO31 cell line, 2.5×10^5 cells per well for the A498 cell line, 3.5×10^5 cells per well for the COLO205 and KM12 colon cancer cell lines, and 3.0×10^5 cells per well for the MCF7 breast cancer cell line. The respective cell lines were separately plated into 384-well assay plates and then incubated for 24 h. DMSO solutions of the test materials (8 µL) were diluted 1:25 with medium (192 µL) and then subjected to five 2:1 serial dilutions (100 µL each) on a 96-well plate. Duplicate 40 µL aliquots of each sample concentration were then transferred to a 384- well "dilution plate", which could accommodate the duplicate samples from two 96-well plates. A 5 µL portion of each solution in the dilution plate was transferred to the cell cultures in the 384-well assay plates to give a final volume of 50 µL and a DMSO concentration of 0.4%. Control wells included 8 wells with the positive control sanguinarine chloride at 20 µM, as well as DMSO only controls and no cell controls. The Z' factors for the individual plates were calculated and were >0.5 in all cases. The cells were incubated for 48 h at 37 °C in the presence of the test samples and then treated with the tetrazolium salt XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide). Viable cells reduced XTT to a colored formazan product, and after an additional 4 h incubation period the amount of formazan produced was quantified by absorption at 450 nm, using a 650 nm reference. Plates were read on a PerkinElmer EnVision (model # 2104) reader. Similar methodology and seeding densities were used for COLO205, KM12 (colon), A673, and TC32 (Ewing sarcoma) cell lines.

3. Results and Discussion

3.1. Structure Elucidation

The fractionation of the dichloromethane extracts of the roots of *H. cilicica* yielded eleven known sesquiterpene coumarin ethers; umbelliprenin (**1**), karatavicinol (**2**), badrakemone (**3**), 14′-acetoxybadrakemone (**4**) [6], badrakemin (**5**), colladonin (**6**), colladin (colladonin acetate) (**7**) [17-19], 14′-hydroxycolladonin (**8**), 14′-acetoxycolladonin (**9**) [6], 14′-acetoxybadrakemin (**10**) and anatolicin (**11**) [7] (Figure 1).

Although these compounds were previously isolated and their structures reported, earlier reports of 14′-acetoxybadrakemone (**4**) spectroscopic data was based on the analytical data generated from the mixture of badrakemone (**3**)/14′-acetoxybadrakemone (**4**) or semi-synthetically generated oxidation products of 14′-acetoxycolladonin (**9**) and 14′acetoxybadrakemin (**10**) [6]. Since the ^{1}H NMR and ^{13}C-NMR data of **4** reported earlier included several misassigned signals, here we report the complete ^{1}H-NMR and ^{13}C-NMR data of purified natural 14′-acetoxybadrakemone (**4**) (Table 1) using 1D and 2D-NMR spectroscopic data including 2D-COSY, HSQC, HMBC and NOESY spectra. In addition, complete ^{1}H-NMR and ^{13}C-NMR assignments of colladonin (**6**) and colladin (colladonin acetate) (**7**) are also reported in Table 1.

14′-Acetoxybadrakemone (**4**) was isolated as colorless gum; IR (NaCl) ν_{max}: 3084, 2919, 2850, 1736, 1711, 1613, 1463, 1397, 1350, 1230, 1123, 1034, 836 cm^{-1}; ^{1}H and ^{13}C-NMR (see Table 1); HRESIMS *m/z*439.2102 $[M + H]^{+}$(calcd. 439.2121, err. -3.06 ppm).

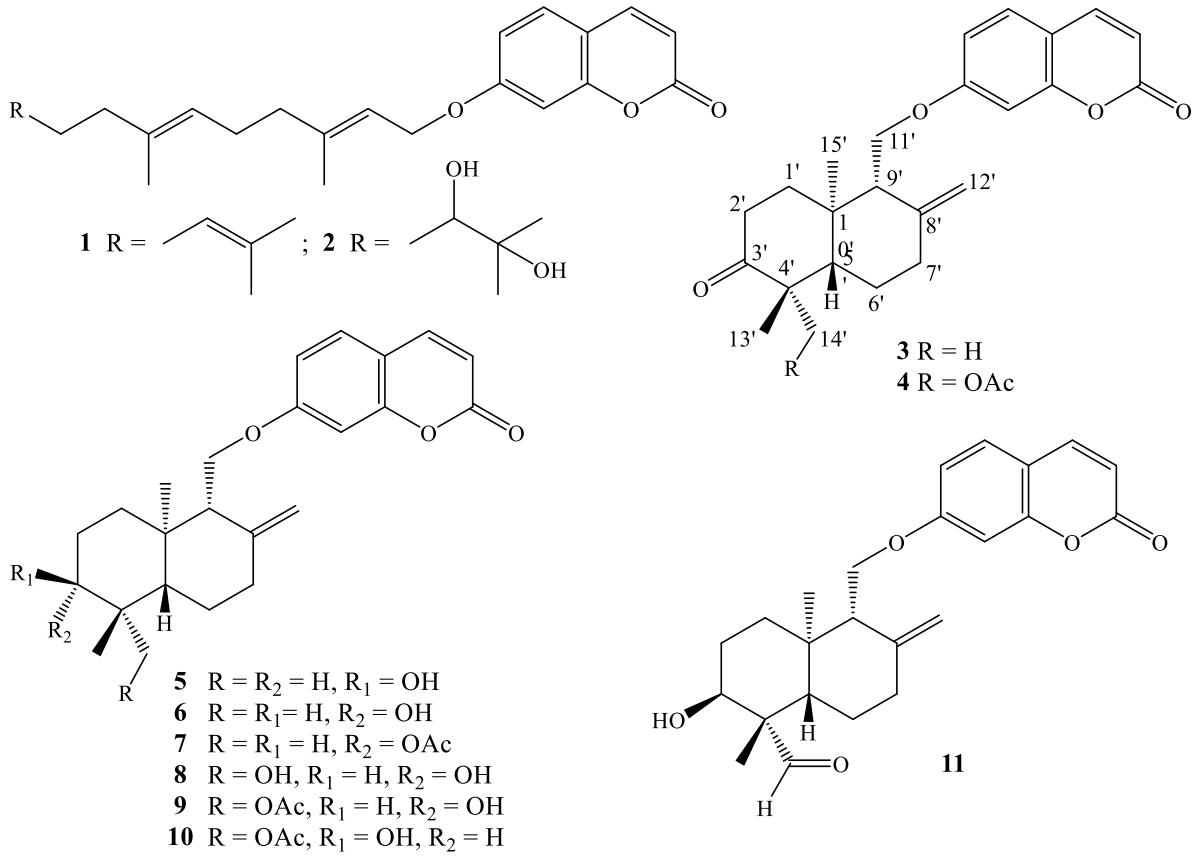

Figure 1. Structures of the sesquiterpene coumarin ethers isolated from the dichloromethane extract of the roots of *Heptaptera cilicica*

3.2. Cytotoxic Activity

The dichloromethane extract of the roots displays high cytotoxic activity against KM12 and COLO205cancer cell lines with IC_{50} values 7.9 and 15.8 μg/mL, respectively. Whereas, both ethyl acetate and aqueous methanol extracts did not show cytotoxic activities against KM12 and COLO205 cancer cell lines up to 50 μg/mL concentrations (Table 2).

The cytotoxic activities of sesquiterpene coumarins isolated from the dichloromethane extract of the roots of *H. cilicica* were tested against COLO205 (colon), KM12 (colon), A498 (renal), UO31 (renal), A673 (Ewing sarcoma) and TC32 (Ewing sarcoma) cancer cell lines (Table 3). The purity of compounds were assessed by their ^{1}H-NMR spectra (please see the supporting information) and found that are > 95%. While most sesquiterpene coumarin derivatives isolated from the root of *H. cilicica* showed no or weak inhibitory activity against these cell lines, colladonin (**6**) displayed strong cytotoxic activity against KM12, moderate cytotoxic activity against COLO205, A498 and weak cytotoxic activity against TC32 cancer cell lines. While badrakemin (**5**) showed relatively strong cytotoxic activity against KM12 but moderate cytotoxic activity against A498 cancer cell lines, colladin (**7**) displayed only a moderate activity against KM12 cancer cell line.

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Table 1. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of compounds **4**, **6** and **7** (δ in ppm, *J* in Hz)

Position	14′-Acetoxybadrakemone (4)		Colladonin (6)		Colladin (7)	
	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR
2	-	161.24	-	161.42	-	161.31
3	6.25; d; 9.7; 1H	113.39	6.24; d; 9.5; 1H	113.13	6.23; d; 9.5; 1H	113.17
4	7.63; d; 9.7; 1H	143.46	7.62; d; 9.5; 1H	143.59	7.62; d; 9.5; 1H	143.52
5	7.36; d; 9.2; 1H	128.97	7.35; d; 9.3; 1H	128.87	7.35; dd; 1.0, 9.3; 1H	128.86
6	6.80; dd; 2.4, 9.2; 1H*	113.15	6.82; dd; 2.4, 9.3; 1H	113.25	6.81; dd; 2.4, 9.3; 1H	113.26
7	-	161.97	-	162.32	-	162.27
8	6.80; d; 2.4; 1H*	101.46	6.81; d; 2.4; 1H	101.42	6.80; bs; 1H	101.43
9	-	156.06	-	156.02	-	156.05
10	-	112.81	-	112.58	-	112.62
1′α 1′β	2.15; ddd; 3.0, 6.0, 13.4; 1H** 1.83; m; 1H***	37.39	1.80; m; 1H* 1.45; m; 1H**	37.29	1.76; m; 1H* 1.53; m; 1H**	36.99
2′α 2′β	2.80; dt; 6.0, 14.6; 1H 2.42; ddd; 3.0, 4.6, 14.8; 1H	35.44	1.63; dddd; 3.4, 11.7, 12.5, 13.2; 1H 1.74; m; 1H*	27.80	1.64; m; 1H** 1.74; m; 1H*	24.24
3′	-	212.33	3.30; dd; 4.4, 11.5; 1H	78.66	4.54; dd; 4.3, 11.6; 1H*	80.47
4′	-	52.23	-	39.30	-	38.81
5′	1.75; dd; 2.6, 12.7; 1H	54.21	1.18; dd; 2.7, 12.5; 1H	54.38	1.26; dd; 2.6, 12.5; 1H	54.54
6′α 6′β	1.57; m; 1H 1.82; m; 1H***	24.66	1.44; m; 1H** 1.77; m; 1H*	23.53	1.45; dq; 4.2, 13.0; 1H** 1.76; m; 1H*	23.42
7′α 7′β	2.51; ddd; 2.4, 5.1, 13.4; 1H 2.11; bdt; 5.6, 13.4; 1H**	38.16	2.46; ddd; 2.4, 4.2, 13.2; 1H 2.09; bdt; 5.0, 13.2; 1H	37.54	2.45; ddd; 2.4, 4.2, 13.2; 1H 2.10; bdt; 5.4, 13.2; 1H***	37.43
8′	-	145.14	-	146.38	-	146.25
9′	2.29; bt; 5.5; 1H	57.01	2.21; bdd; 5.2, 6.3; 1H	54.85	2.21; bt; 5.6; 1H	54.81
10′	-	38.80	-	38.93	-	38.19
11′a 11′b	4.24; dd; 6.6, 10.0; 1H**** 4.20; dd; 5.2, 10.0; 1H****	65.86	4.21; dd; 4.3, 9.8; 1H*** 4.17; dd; 7.6, 9.8; 1H***	65.75	4.19; dd; 4.9, 9.7; 1H**** 4.16; dd; 7.0, 9.7; 1H****	65.81
12′a 12′b	4.98; bs; 1H 4.61; bs; 1H	109.23	4.91; bd; 1.0; 1H 4.54; bd; 1.0; 1H	107.98	4.91; bs; 1H 4.52; bs; 1H*	108.07
13′	1.19; s; 3H	21.07	1.02; s; 3H	28.46	0.90; s; 3H	28.41
14′a 14′b	3.95; d; 11.5; 1H 4.59; d; 11.5; 1H	66.33	0.81; s; 3H	15.49	0.88; s; 3H	16.76
15′	1.11; s; 3H	15.66	0.84; s; 3H	15.65	0.86; s; 3H	15.51
OAc	1.99; s; 3H	170.95, 20.91	-	-	2.05; s; 3H***	171.04, 21.41

*, **, ***, **** Overlapped or partially-overlapped signals

Table 2. Cytotoxic activities (IC_{50}, μg/mL)of extracts obtained from *H. cilicica*

Extracts	CH_2Cl_2	EtOAc	Aq. MeOH
COLO205	15.8	> 50	>50
KM12	7.9	> 50	> 50

Nevertheless, with the exception of 14′-acetoxybadrakemone (**4**) and 14′-hydroxycolladonin (**8**), all sesquiterpene coumarin ethers isolated from the dichloromethane extract of the root of *H. cilicica* showed strong cytotoxic activity against the UO31 renal cancer cell line. 14′-acetoxybadrakemin (**10**) and anatolicin (**8**) displayed the most significant cytotoxic activity against UO31 cancer cell line with 17 and 24 nM IC_{50} values, respectively.

Table 3. Cytotoxic activities of sesquiterpene coumarins isolated from *Heptaptera cilicica*

Compounds	Cytotoxic Activity (IC_{50} values in μM)					
	COLO205	KM12	A498	UO31	A673	TC32
1	>50	>50	>50	1.8	>50	>50
2	>50	>50	>50	7.6	>50	>50
3	>50	>50	>50	11	>50	>50
4	>50	>50	>50	>50	>50	>50
5	>50	9.1	20	0.38	>50	>50
6	19	2.5	21	0.75	>50	45
7	>50	29	>50	0.39	>50	>50
8	>50	>50	>50	>50	>50	>50
9	>50	>50	>50	0.37	>50	>50
10	>50	>50	>50	0.017	>50	>50
11	>50	>50	>50	0.024	>50	>50

4. Conclusion

Previously, Özbek *et al*. reported four sesquiterpene coumarin ethers from *H. cilicica*; umbelliprenin, badrakemone, badrakemin, badrakemin acetate; a simple prenylated coumarin ester: Prunate and a furanocoumarin derivative: Trichoclin angelate from the chloroform extract of the roots [20]. Güvenalp *et al.* reported five sesquiterpene coumarin ethers; umbelliprenin, umbelliprenin-10',11'-monoepoxide, conferone, mogoltacin and feselol from the chloroform extract of the fruits of *H. cilicica* [21].

In this study karatavicinol (**2**), 14′-acetoxybadrakemone (**4**), colladonin (**6**), colladin (**7**),14′-hydroxycolladonin (**8**), 14′-acetoxycolladonin (**9**), 14′-acetoxybadrakemin (**10**), and anatolicin **(11**) were isolated for the first time from *H.cilicica*.

Although badrakemin (**5**), colladonin (**6**) and colladin (**7**) appear to be responsible for the cytotoxic activities observed on the preliminary extract testing on COLO205 and KM12 colon cancer cell lines, the sesquiterpene coumarin ethers isolated from the roots of *H. cilicica* display selective and potent cytotoxic activity against UO31 renal cancer cell line. This is the first report of the cytotoxic activities of colladin (**7**) and 14′-acetoxycolladonin (**9**) against the UO31 renal cancer cell line.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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