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COUMARINS FROM *CACHRYS SICULA* L.

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Abstract

One new coumarin, 8-hydroxymethylpsoralen (**1**), was isolated from the dried leaves of *Cachrys sicula* L. along with the known coumarins sprengelianin (**2**) and (*RS*)-oxypeucedanin (**3**). The structure of the new compound was elucidated on the basis of NMR and ESI-MS spectral analysis.

Keywords: *Cachrys sicula; Umbelliferae; Coumarins; Cytotoxic activity*

Introduction

The genus Cachrys, belonging to the Apiaceae family, is widely distributed in Europe, Asia and Mediterranean region, but few studies are reported in the literature on chemical constituents and their biological properties (1).

Cachrys sicula L. (synonymous Hippomarathrum pterochlaenum Boiss.) is an endemic species of the South-western Mediterranean region (2,3), which causes skin irritation and blistering probably due to the presence of furanocoumarins (4). Furanocoumarins are widespread within the species of Apiaceae family as the major secondary metabolites (5). Previous studies on the C. sicula whole plant resulted in the isolation and characterization of furocoumarins, together with ferulol esters, and N,N'-di-o-tolylethylendiamine (4,6).

This paper deals with the structure determination of one new and two known coumarins, isolated from the ethyl acetate extract of the plant, together with their cytotoxic evaluation on HeLa (cervix carcinoma) and Jurkat (T-cell leukemia) cell lines.

Materials and Methods

General

Briefly, An Atago AP-300 digital polarimeter with a sodium lamp (589 nm) and 1 dm microcell was used to measure optical rotations. UV spectra were registered on a Perkin-Elmer Lambda spectrophotometer. NMR experiments were recorded on a Bruker DRX-600 spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a Bruker 5 mm TCI CryoProbe, acquiring the spectra in methanol-d_4. (7). ESI-MS (positive mode) were obtained from a Finningan LC-Q Advantage Termoquest spectrometer (ThermoFinnigan, USA). Thin Layer Chromatography (TLC) was performed on precoated Kieselgel 60 F_{254} plates (Merck, Darmstadt, Germany); compounds were detected by cerium disulfate/sulfuric acid (Sigma-Aldrich, Milan, Italy). Column chromatographies were performed over Silica gel 60 (Merck, Darmstadt, Germany), followed by reverse phase - high performance liquid chromatography (RP-HPLC) performed on Shimadzu LC-8A series pumping system with Shimadzu RID-10A refractive index detector, C_{18} µ-Bondapak column (30 cm × 7.8 mm, 10 µm, Waters, Milford, MA, USA), using mixtures of methanol/water at flow 2.0 mL/min) (8).

Plant material

C. sicula was collected in Fosse di San Gandolfo, Madonie, Palermo, Italy, in 2012. A voucher specimen was deposited at the Botanical Garden of Palermo, Italy.

Extraction and isolation

Dried leaves of the plant (200 g) were defatted with petroleum ether at room temperature, and then extracted with ethyl acetate to obtain the respective residue. Part of the ethyl acetate residue (5 g) was chromatographed on silica gel column (80 cm x 3.5 cm) eluting with *n*-hexane-chloroform (1:1), followed by chloroform and increasing concentrations of methanol in chloroform (between 1% and 100%). Fractions of 20 mL were collected, analyzed by TLC, and grouped into 12 major fractions (A-L). Fraction C yielded compound **2** (10.0 mg). Fraction G (95.0 mg) was purified by RP-HPLC with methanol-water (4:1) as eluent to obtain compounds **1** (3.0 mg, t_R 10 min) and **3** (15.0 mg, t_R 12 min).

8-hydroxymethylpsoralen (**1**). Pale yellow crystals; mp 120-127 °C; UV max (MeOH): 303 (log ε 3.98), 268 (4.05), 253 (3.72), 235 (3.65) nm; ^{1}H- and ^{13}C-NMR: see Table 1; ESI-MS *m/z*: 217 $[M+H]^+$; (Calc. for $C_{12}H_8O_4$).

Chemicals

All solvents used for extraction and separation processes were purchased from Sigma-Aldrich (Milan, Italy). Fetal Bovine Serum (FBS) was obtained from GIBCO (Life Technologies, Grand Island, NY, USA). RPMI medium, DMEM medium, L-glutamine, and sodium pyruvate were purchased from Invitrogen (Life Technologies, Grand Island, NY, USA). Streptomycin and penicillin, etoposide, MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) and propidium iodide were from Sigma-Aldrich (Milan, Italy).

Cytotoxicity assay

Cells and treatment

Jurkat (T-cell leukemia) and HeLa (cervical carcinoma) cell lines were obtained from the American Type Cell Culture (ATCC) (Rockville, MD, USA). Cells were maintained in DMEM (HeLa, A2780, MCF-7, RKO) or RPMI 1640 (Jurkat), supplemented with 10% FBS, 100 mg/L streptomycin and penicillin 100 IU/mL at 37 °C in a humidified atmosphere of 5% CO_2 (9). To ensure logarithmic growth, cells were subcultured every two days. All plant extracts were initially dissolved in dimethyl sulfoxide (Sigma-Aldrich, Milan, Italy) at 200 mg/mL stock concentration and stored at -20 °C until required. Stock solutions (50 mM) of purified compounds in dimethyl sulfoxide were stored in the dark at 4 °C. Appropriate dilutions were prepared in culture medium immediately prior to use. In all experiments, the final concentration of dimethyl sulfoxide did not exceed 0.15% (v/v).

Cell viability

Cells were seeded in 96-well plates and incubated for the established times in the absence (vehicle only) and in the presence of different concentrations of compounds **1-3** and etoposide as positive control. The day before treatments, cells were seeded at a cell density of 2 x 10^3 cells/well. After the time course is completed, MTT assay was performed as described previously (10). The supernatant was washed out and replaced with fresh medium containing MTT (0.5 mg/mL, Sigma-Aldrich, Milan, Italy) and incubated in the dark for 4 h at 37 °C in a CO_2 enriched atmosphere (5%) to allow the formation of formazan salt. In order to dissolve the reduced formazan salt crystals, the supernatant was removed carefully and 150 µL of dimethyl sulfoxide were added and mixed. Absorption at 540 nm for each well was assessed using a microplate reader (LabSystems, Vienna, VA, USA). Background absorbance (average absorbance of wells containing dimethyl sulfoxide but no cells) was subtracted from the average absorbance of test wells. In some experiments cell viability was also checked by Trypan Blue exclusion assay using a Bürker counting chamber. IC_{50} values were calculated from cell viability dose – response curves and defined as the concentration resulting in 50% inhibition in cell survival as compared to controls.

Statistical analysis

Data reported are the mean values ± SD from three independent experiments. Differences between treatment groups were analyzed by Student's *t*-test. Differences were considered significant when $p \leq 0.05$.

Results and Discussion

The ethyl acetate extract from *C. sicula* leaves was firstly chromatographed on a dry silica gel column and pure components **1-3** were isolated after recrystallization and/or chromatography.

Compound **1** was isolated as pale yellow solid. Its molecular formula $C_{12}H_8O_4$ was deduced from the ESI-MS analysis (*m/z* 217 $[M+H]^+$), ^{13}C-, ^{13}C-DEPT NMR spectra, and elemental analysis. The ^1H-NMR (Table 1) showed H-3 and H-4 occurring as a pair of doublets (*J* 9.7 Hz) at δ 6.14 and 7.78, respectively. In the ^1H-NMR spectrum a singlet at δ 7.34 ascribable to H-5 and two doublets at δ 6.83 and 7.69 (*J* 2.5 Hz) assigned to H-3' and H-2', respectively, were also evident. Proton and carbon signals of compound **1** were superimposable with those of psoralen (11,12), except for the presence in **1** of a singlet at δ 4.33 due to a hydroxymethyl group linked at C-8 instead of a singlet due to H-8. All the ^1H- and ^{13}C-NMR signals (Table 1) of **1** were assigned using DQF-COSY, HSQC, and HMBC experiments. The positional assignment of the hydroxymethyl group was unambigously confirmed by key HMBC correlations. Consequently, the structure of **1** was determined to be 8-hydroxymethylpsoralen.

Beside this new coumarin, sprengelianin (**2**) and (*RS*)-oxypeucedanin (**3**) were identified and their structures were established on the basis of spectral evidences and comparison of physical and spectral data with literature value (4,12). The presence of compounds **2** and **3** in *C. sicula* was already mentioned (4).

The antiproliferative activity of the isolated compounds was evaluated in HeLa (cervix carcinoma) and Jurkat (T-cell leukemia) cancer cell lines. Cells were exposed to increased concentrations of all isolates and cell viability was evaluated after 48 h exposure time by MTT assay. Only compound **1** ($IC_{50=}$ 52±0.86) inhibited moderately the growth in a dose-dependent manner for both Jurkat and HeLa cells.

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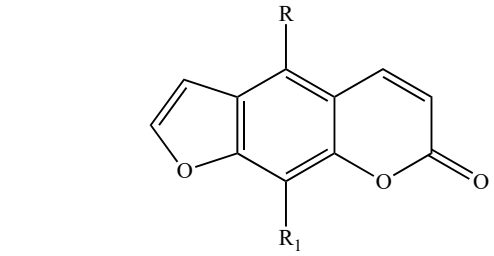
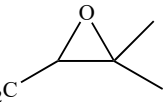
Table 1: ^{1}H- and ^{13}C-NMR data of compound **1** in CD_3OD, δ ppm, *J* (Hz)[a]

		1
	δ_H	δ_C
2		163.0
3	6.14 d (9.7)	116.5
4	7.78 d (9.7)	145.5
4a		113.8
5	7.34 s	114.0
6		126.5
7		149.0
8		110.5
8a		149.0
CH_2OH	4.33 s	62.7
2'	7.69 d (2.5)	147.5
3'	6.83 d (2.5)	108.4

[a]Data assignments were confirmed by DQF-COSY, HSQC and HMBC experiments.



1 R = H R 1 = CH_2OH



3 R = OH_2C R 1 = H

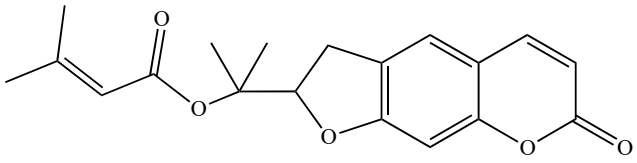


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Figure 1: Structures of compound **1-3**