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Induction of Apoptosis and Cell Cycle Arrest in Human Colon Carcinoma Cells by *Corema album* Leaves

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The leaves of *Corema album* (Ericaceae), an endemic shrub which grows in Atlantic coastal areas of the Iberian Peninsula, are rich in flavonoids and other secondary metabolites. Silica gel column chromatography of the ethyl acetate extract from dried leaves was performed and a flavonic active fraction was obtained. The cytotoxic activity of this fraction was assessed using the colon cancer cell lines HCT116 and HT29. After 48 hours of treatment, cell viability was determined with luminescence-based ATPLite assay, showing IC_{50} values of 7.2 ± 0.7 and 6.8 ± 1.2 µg/mL, respectively. The study by flow cytometry revealed that the cytotoxicity of this fraction was mediated, at least in part, by induction of apoptosis and G_2/M cell cycle arrest. The active fraction was then subjected to Sephadex LH-20 chromatography and two flavonoids were separated and identified as the flavanone pinocembrin and 2',4'-dihydroxychalcone after UV, MS and NMR analysis.

Keywords: *Corema album*, Ericaceae, Cytotoxicity, HCT116 colon carcinoma cell line, HT29 colon carcinoma cell line, 2',4'-Dihydroxychalcone, Pinocembrin.

Colorectal cancer is one of the leading causes of death worldwide, and the most common cancer in the European Union, taking into account incidences in both sexes [1]. Due to the narrow therapeutic range of 5-fluoruracil (5-FU), irinotecan and platinum-based drugs, currently used for colorectal cancer, research into new drugs for alternative treatments is growing, with a special interest in natural products as sources of new anticancer lead molecules.

Corema album (L.) D. Don (Ericaceae), is an endemic shrub native of the Atlantic coast of the Iberian Peninsula, growing in sandy coastal areas from La Coruña to Cadiz. Their acidic, tasty fruits are spherical, white berries which are consumed as appetizers, juices or used in folk medicine. We have recently reported a high content of phenolic compounds, mainly phenolic acids, and *in vitro* antioxidant properties of *C. album* berries [2,3], and we have recently isolated cytotoxic dihydrochalcones from the EtOAc extract of the leaves [4]. Therefore, continuing the study of this plant, we have looked for other cytotoxic compounds from the leaves of this species and their mechanism of action on HCT116 and HT29 colon cancer cell lines.

Subjection to column chromatography of the EtOAc extract obtained from *C. album* leaves gave a flavonic fraction (F_3), which presented cytotoxic activity against HCT116 and HT29 human colon cancer cell lines. Treatment with F_3 significantly reduced cellular viability in a time and dose-dependent manner ($p < 0.05$). The IC_{50} values after 48 h of treatment, determined using ATPLite® luminescence-based assay were 7.2 ± 0.7 µg/mL in HCT116 and 6.8 ± 1.2 µg/mL in HT29.

Annexin V-FITC/PI staining and flow cytometry were used to determine the effects of F_3 on the induction of apoptosis in HCT116 and HT29 colon cancer cells (Figure 1). The percentage of HCT116 cells in apoptosis was increased in a dose-dependent way after 48 h of F_3 treatment, but it was not significantly affected after 24 h

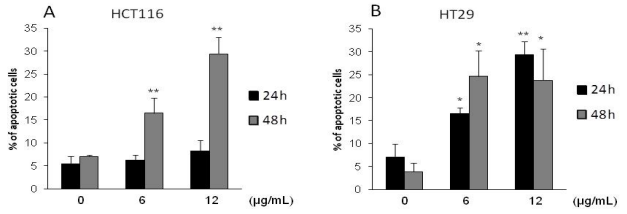

Figure 1: Proapoptotic effect on HCT116 and HT29 cell lines after 24 h and 48 h treatments with 0, 6 and 12 µg/mL of the active fraction (F_3) of *C. album* leaves. Percentage of cells in apoptosis was analyzed by flow cytometry using the Annexin V-FITC assay. Compared with DMSO control: * $p < 0.05$, ** $p < 0.01$.

(Figure 1A). Otherwise, all tested concentrations raised the percentage of HT29 cells in apoptosis, reaching a five-fold increase at higher doses (Figure 1B).

To determine whether a cell cycle modification was produced by F_3, treated cells were stained with propidium iodide and analyzed by flow cytometry. As shown in Figures 2A and 2B, a 12 µg/mL F_3 treatment for either 24 h or 48 h produced a significant G_2/M arrest on HCT116 cells accompanied by a reduction in the proportion in S phase. The same effect was observed in HT29 cells after 24 or 48 h of incubation with 6 µg/mL F_3 (Figures 2C and 2D). However, increasing concentrations produced a decrease of this effect on HT29. The active fraction was then subjected to Sephadex LH-20 chromatography using CH_2Cl_2/MeOH (1:1) for elution and two flavonoids were separated and identified as the flavanone, pinocembrin, and 2',4'-dihydroxychalcone by comparing their UV, ^{1}H NMR, and ^{13}C NMR spectroscopic and MS data, with that in the literature [5,6].

In vitro studies have demonstrated that chalcones present in some medicinal plants (*e.g.*, isoliquiritigenin, from liquorice) inhibit the proliferation of cancer cells by inducing apoptosis and blocking cell cycle progression [7,8].

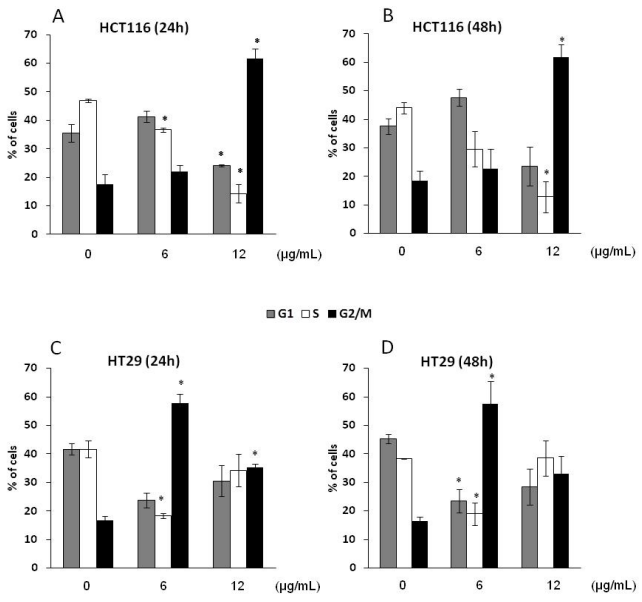

Figure 2: Cell cycle distribution of HCT116 and HT29 cell lines after 24 h and 48 h treatments with 0, 6 and 12 µg/mL of the active fraction (F_3) of *C. album* leaves. Percentage of cells in G_0/G_1, S and G_2/M phases was analyzed after propidium iodide staining by flow cytometry. Compared with DMSO control: * $p < 0.05$.

In conclusion, a crystallized fraction of the EtOAc extract from *Corema album* yielded pinocembrin and 2',4'-dihydroxychalcone. These isolates showed a significant effect on the viability of two different colon cancer cell lines in a low micromolar concentration range. The observed cytotoxicity may be due to an induction of apoptosis and an accumulation of cells on G_2/M cell cycle phases.

Experimental

General: 1D and 2D NMR, Bruker AVANCE-500; EI and FAB MS, Micromass AUTOSPECQ; UV, Shimadzu UV-1800 UV–vis; TLC, Merck silica gel 60 F254. EtOAc and *n*-hexane were purchased from Panreac.

Plant material: Aerial parts of *Corema album* were collected in September 2008 in Huelva (SW Spain, 37° 04´ 10.15" N - 6° 41´ 15.45" W) and identified by Dr Díaz Barradas. A voucher specimen was deposited at the herbarium under no. SEV277798.

Extraction and isolation: Dried and uncrushed leaves (250 g) were extracted in an ultrasound bath with EtOAc (500 mL) at room temperature for 45 min. After filtration and evaporation of the solvent under reduced pressure, part of this crude extract (3 g) was fractionated by CC over silica gel, using *n*-hexane with a EtOAc gradient of increasing polarity (from 99:1 to 90:10), to afford 20 fractions of 100 mL each. Fraction 3 (F_3, 200 mg) was obtained as yellow needles and selected for cell cytotoxicity assays. Active fraction F_3 was subjected to Sephadex LH-20 CC, using CH_2Cl_2/MeOH (1:1) for elution. Two compounds were isolated and identified as pinocembrin (12 mg) and 2',4'-dihydroxychalcone (30 mg), on the basis of their UV, EIMS and NMR data.

Cell culture: HCT116 (p53 wt adenocarcinoma) and HT29 (p53 mutant adenocarcinoma) colon carcinoma cell lines were maintained at 37°C (5% CO_2) in DMEM with 10%, v/v, fetal bovine serum (Invitrogen). A 20 mg/mL F_3 stock solution was prepared in DMSO. Cells were treated with 0-12 µg/mL F_3 for times up to 48 h. DMSO did not exceed 0.1% in any treatment.

Cell viability: Cell viability was assessed via measurement of cellular ATP levels using ATPLite® kit (Perkin Elmer) according to the manufacturer's instructions. Briefly, HCT116 and HT29 cells were seeded in white Packard Viewplates-96 (1 x 10^4 cells per well) and incubated overnight to allow cell attachment, after which the cells were treated with 0-12 µg/mL F_3 for 48 h. Luminescence was directly proportional to cell viability expressed as fold change from DMSO control.

Flow cytometry: For flow cytometry assays, 1 x 10^5 or 2.5 x 10^5 cells per well were seeded on 6-wells plates, left to adhere overnight and then treated with appropriate concentrations of F_3 for 48 h. Apoptosis was determined by using the Annexin V-FITC kit (Bender Medsystems), as described previously [9]. The percentage of live, apoptotic or necrotic cells in the whole population was determined using a FACScan flow cytometer (Becton Dickinson). Otherwise, the effect of this fraction on the cell cycle was studied by analysis of DNA content using FACScan, according to a previously described method [10].

Statistical analysis: All samples were assayed in 3 independent experiments and results are reported as the mean ± SEM. Statistical comparisons with DMSO treated cells were made by means of Student's *t*-test; *p* values < 0.05 were regarded as significant.

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