

Extracts from *Viscum* and *Crataegus* are Cytotoxic against Larynx Cancer Cells

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Viscum cruciatum Sieber, *Crataegus monogyna* Jacq., Cultured HEP-2 Cells,
Triterpenes-Enriched Fractions

The effects of hexanoic extracts of *Viscum cruciatum* Sieber parasitic on *Crataegus monogyna* Jacq. (I), *Crataegus monogyna* Jacq. parasitized with *Viscum cruciatum* Sieber (II), and *Crataegus monogyna* Jacq. non-parasitized (III), and of a triterpenes enriched fractions isolated from I, II and III (CFI, CFII, CFIII respectively), on the growth of HEP-2 cells have been evaluated. All the samples demonstrated significant cytotoxic activity against cultured HEP-2 cells, and all of them showed a stronger *in vitro* activity than 6-mercaptopurine solution used as a positive control. With the hexanoic extracts I, II and III almost similar activity was obtained, but the hexanoic extract I showed comparably better results. Almost complete inhibition was observed with triterpenes-enriched fractions CFI, CFII and CFIII, at the dose 6 µg/ml, after 72 h of treatment. The most intense response was obtained with the triterpenes-enriched fraction CFIII (from *Crataegus monogyna* non-parasitized), where the inhibition was 93%, but the fraction CFI and CFII showed similar inhibition (92% and 83%).

Introduction

In this paper, we studied the cytostatic activity against cultured HEP-2 cells of three hexanoic extracts obtained of *Viscum cruciatum* Sieber (Viscaceae) parasitic on *Crataegus monogyna* Jacq. (I), *Crataegus monogyna* Jacq. parasitized (II) and *Crataegus monogyna* Jacq. (Rosaceae) non-parasitized (III), and of triterpenes enriched fractions from hexanoic extracts (CFI, CFII, CFIII).

In our laboratory the composition of the hexanoic extracts from I, II and III was investigated (Ahumada, 1995), and was found to contain a fractions where were identified several triterpenes and aliphatic alcohols. We called them "triterpenes enriched fractions" (CFI, CFII and CFIII). The main component of the fractions CFII and CFIII was cycloartenol (79.5 and 80.9% respectively). However, in the fraction CFI, the aliphatic alcohols were the main components (74.4%), and cycloartenol was found 7.6%.

Other triterpenoids and steroids were obtained in the hexanoic extracts: β -amyirin acetate (in hexanoic extract I), ursolic acid and β -sitosterol (in hexanoic extracts I, II and III).

A large number of triterpenic from different families have demonstrated cytostatic activity in several cell cultures (Bezanguer-Beauquesne, 1982; Zhang *et al.*, 1986; Lee *et al.*, 1987; Kaneda *et al.*, 1992). And *Viscum cruciatum* Sieber have been studied in our laboratory due its cytostatic and antimitotic activities, when it was parasitizing several hosts (Ayuso *et al.*, 1987; Ahumada *et al.*, 1995).

For these reasons, we have studied and compared cytostatic activity against larynx carcinoma HEP-2 cell of three hexanoic extracts (I, II and III), and of triterpenes-enriched fractions (CFI, CFII and CFIII) isolated from them.

Material and Methods

Plant material

Aerial parts of *Viscum cruciatum* Sieber and *Crataegus monogyna* Jacq. parasitized and non-parasitized were collected in Puerto de los Vientos, Serranía de Ronda, Málaga (Spain) in February. A voucher specimen of both species was deposited at the herbarium of the Department of Vegetal Biology (University of Sevilla) (SEV-F and SEV 137261 respectively), and were identified by Prof. Silvestre.

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Extraction and isolation of triterpenes-enriched fractions

The plant material (500 g of each sample) was extracted with hexane (Panreac, 95.0%, Barcelona) in a soxhlet extractor. The hexanoic extracts were concentrated under reduced pressure. 2 g of the residues thus obtained were chromatographed by silica gel column (0.063–0.200 mm and 0.2–0.5 mm, Merck) and successively eluted with hexane and CHCl_3 (Panreac, 99.0%, Barcelona). From these columns some fractions were obtained that were rechromatographed using a hexane/ethyl ether (Panreac, 99.0%, Barcelona) gradient (200 ml hexane, 100 ml hexane/ethyl ether, 90:10 v/v; 100 ml hexane/ethyl ether 80:20 v/v and 100 ml hexane/ethyl ether, 70:30 v/v). Triterpenes-enriched fractions CFII and CFIII crystallized from fraction corresponding to hexane/ethyl ether (70:30) eluate which yielded to 20–25 °C of temperature whitish needles of CFII and CFIII. Fraction CFI was obtained by preparative silica gel chromatography using hexane/ethyl ether (70:30 v/v) as solvent system. (Tlc silica gel gave a blue purple spot with oleum reagent ($\text{H}_2\text{SO}_4/\text{CH}_3\text{COOH}/\text{H}_2\text{O}$, 2:40:80 v/v), R_f 0.40).

The composition of these fractions was determined by gas chromatography (GC) and identification of each component was carried out by combined gas chromatography-mass spectrometry (GC-MS) (70 eV, capillary column 25mx25 mm, column 230 °C (6 min) + 4 °C/min to 300 °C).

The analyses showed the predominance of cycloartenol in the crystalline fractions CFII and CFIII. Cycloartenol accounted 79.5% of CFII and 80.9% of CFIII. Aliphatic alcohols were main compounds in CFI (accounted 74.4%) and cycloartenol was only 7.6%.

Cytostatic Test Procedure

The cytostatic activity was determined by measuring the inhibition of the development of a single-layer culture of HEP-2 cells. This cellular line, derived from a human epidermoid carcinoma of the larynx (Moore *et al.*, 1955), was cultivated in Eagle's essential minimum medium (MEM), according to the method described by Geran *et al.* (1972). Cells were grown in MEM, supplemented with 5% of bovine fetal serum and 2% solution of penicillin and streptomycin (5000 IU/ml-5000 µg/

ml) to pH 7.2 and 36 °C. After distribution in the nutrient medium and when a continuous monolayer culture had been obtained, the samples in the study were sterilized through a filter Millipore 0.22 µm and then inoculated. 1 ml of hexanoic extracts I, II, and III, and the triterpenes-enriched fractions (CFI, CFII and CFIII) respectively, were inoculated in 10 ml of nutrient medium; the concentrations obtained were 30 µg/ml of each extract and 6 µg/ml of the triterpenes-enriched fractions (ID_{50} values recommended by the National Cancer Institute of Health Bethesda, Maryland, USA for plant extracts and pure compounds, respectively (Geran *et al.*, 1972)). A solution of 6-mercaptopurine as positive control (Darias *et al.*, 1990) was used under identical conditions. A control test was carried out simultaneously. 72 h after inoculation of the samples, incubated at 36 °C, the cellular protein concentration was determined to evaluate the inhibitory effect on growth. The method of Bradford (1976) was used, calibration was done with different concentrations of a standard solution of human albumin. Each assay was carried out in triplicate and the average of the readings was recorded.

Statistical analysis

Student's t-test was used to compare results against the control group. The values are expressed as mean ± SE.

Results and Discussion

The results obtained on cytostatic activity are summarized in Table I. The samples of hexanoic extracts assayed exhibited a higher degree of growth inhibition than 6-mercaptopurine solution used as a positive control. Hexanoic extract of *Viscum cruciatum* Sieber, parasitic on *Crataegus monogyna* Jacq. (I), was the most active (63.1%), although the hexanoic extracts of *Crataegus monogyna* parasitized (II) and non-parasitized (III) showed almost similar activity (61.5% and 57.5%, respectively).

Triterpenes-enriched fractions isolated from the extracts (CFI, CFII and CFIII), clearly showed significant cytotoxicity against the growth of human epidermoid larynx carcinoma. In all cases the percentage of inhibition was >80%. The data obtained show that the ID_{50} for these samples are

Table I. *In vitro* cytostatic activity against HEp-2 cells of the hexanoic extracts I, II and III, and of triterpenic fractions (CFI, CFII, CFIII) measured as percent inhibition of cell growth.

Additions	Protein concentration [µg/ml]	Cellular inhibition % of control
Control	9.04±0.15	—
Hexanoic extracts (30 µg/ml)		
I	3.15±0.24***	65.1
II	3.48±0.27***	61.5
III	3.85±0.33***	57.5
Triterpenic fractions (6 µg/ml)		
CFI	0.72±0.20***	92.0
CFII	1.51±0.23***	83.3
CFIII	0.61±0.27***	93.2
6-Mercaptopurine (0.5 µg/ml)	4.26±0.083***	52.9

*** $p < 0.001$ compared with control group (Tween 80, 0.8% in water).

lower than those recommended by the protocols of the National Cancer Institute of USA (Geran *et al.*, 1972). For this reason more assays are being performed with these triterpenic fractions in order to confirm its activity against experimental tumors *in vivo*. Although there are no significant differences in the results with the triterpenic fractions assayed, a slight but greater inhibition was observed when fraction CFIII was tested (93.2%).

This inhibition in cellular growth could be due to the high content of triterpenic alcohols that is present in the hexanoic extracts and the fractions, although this action of the triterpenic fractions could be influenced in part to aliphatic alcohols, which were also found in the fractions in different percentages. Therefore, we are planning to perform other experiments, both *in vivo* and *in vitro*, to analyse more precisely their mode of action and to establish what compound of these mixture are mainly responsible for this action.

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